Screening of medicinal plant extracts and isolated compounds against sensitive and resistant strains of *Plasmodium falciparum* for antimalarial activity

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By

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STATEMENT UNDER O. Ph. D 8/ (iii) OF
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This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of
Prof. Sarita S. Gupta.

Vadodara **Soni Sanketkumar Chandrakant.**

Date: 4th January, 2012

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph.D. degree of The Maharaja Sayajirao University of Baroda by **Mr. Sanket Soni** is his original work. The entire research work and the thesis have been built up under my supervision.

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Dedicated to my parents

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CHAPTER 1

1.1 History of Traditional Medicine and Drug Discovery from Natural Products

Plants have been existed on earth even before human and are the soul of whole ecosystem of our planet. Human have relied on nature for their basic needs like food, shelter, clothing, medicine since ages (Cragg, G. M. and Newman, D. J., 2005). History of herbal medicine is as old as human civilization. The documents of many plants revealed that plants have been used medicinally in China, India, Egypt and Greece long before beginnings of Christian era. One of the most famous surviving remnants is *Papyrus Ebers*, a scroll about of 60 feet long and a foot wide, dating back to 16th century before Christ, containing more than 800 formulae and 700 different drugs such as acacia, castor oil, fennel, along with minerals like iron oxide, sodium chloride, sodium carbonate and sulphur. In China, many of medicinal plants have been in use since 5000 BC. The oldest known script is *Pen t' sao* written by emperor Shen Nung narormd 3000 BC, contained 365 drugs, one for each day of the year. Indians meticulously worked to examine and classify herbs which they came across, into groups called *Gunas* (Property of drug). Charaka made fifty groups of ten herbs each. Similarly, he arranged 760 herbs in 7 distinct sets based on their common properties. A large portion of the Indian population even the present time depends on Indian system of medicine called Ayurveda, an ancient science of life. Charaka Samhita and Sushruta Samhita are well known treaties in Ayurveda.

Today, a vast store of knowledge concerning therapeutic properties of medicinal plants has accumulated through either experiences or knowledge evolved and transferred to the generation of tribal people, traditional healers or practitioners. The herbs can provide starting material for isolation or synthesis of conventional drugs. In the modern world, finding of cinchona in 17th century, followed by digitalis, morphine, and so on, and then introduction of synthetic aspirin, a derivative of a plant-based drug, compelled human beings to believe in the wonders of the diverse floristic wealth (Ruskin, I. and Ripoll, C., 2004). A large number of plants used in the traditional medicine have now become a part of the modern world health care system (Fabricant, D.S. and Farnsworth, N.R., 2001).

Natural product chemistry actually began with the work of Fredrick Surterner (1800 AD), who first isolated morphine from opium. This, in turn, was obtained from opium poppy (*Papaver somniferum*) by processes that have been used for over 5,000 years. Many such similar developments followed. Indian fever bark form cinchona tree (*Cinchona officinalis*), was used as an infusion by native people of the Andes and Amazon highlands to treat fevers; the leaves of the coca tree have been primarily chewed to obtain perceived benefits in Andean cultures and later on in 1860, cocaine was isolated as the chemical responsible for local anesthetic activity; tubocurarine used in modern medicine is from arrow head poison used by East Amazonians; alkaloid pilocarpine isolated from jaborandi tree (*Pilocarpus jaborandi*) is weapon against the blinding disease, glaucoma; traditionally pineapple juice was used by American Indians to reduce inflammation in wounds and other skin injuries, later on, an enzyme that broke down proteins (bromelain) was isolated from the fresh juice of pineapple and was found to break down blood clots. In 1891, other pharmaceuticals that have their origin in botanicals include atropine, hyoscine, digoxin, colchicines, reserpine, emetine etc. It is pertinent to note that most of these early discoveries are mainly based on traditional medicines.

World Health Organization defines traditional medicine as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. While, traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed alternative or complementary medicine. Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients.

WHO mentions some of important key facts on traditional medicines.

- In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care.

- Herbal medicines are the most lucrative form of traditional medicine, generating billions of dollars in revenue.
- Traditional medicine can treat various infectious and chronic conditions: new antimalarial drugs were developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years.
- Counterfeit, poor quality or adulterated herbal products in international markets are serious patient safety threats.
- More than 100 countries have regulations for herbal medicines.

1.2 Drug discovery process from medicinal plants

In pharmaceutical sector, drug discovery process begins with target identification, target prioritization, lead identification and lead optimization. This cycle involves various aspects like preclinical technologies which covers preliminary *in vitro* and *in vivo* assays for selection of therapeutically active compounds, chemistry manufacturing and controls (CMC)/pharmaceutics to formulate the drugs by complete studies of its property and intended for clinical use, pharmacology/toxicology to determine efficacy and safety and clinical investigation of three stages mentioning effect on humans and ultimately approval as NDA (New Drug Application) as well as MAA (Marketing Authorization Application). The drug development process is becoming more and more complex and capital intensive, and companies remain 'target rich' but 'lead poor', with lead discovery as a greater bottleneck. It is estimated that, to develop one successful drug, 12 – 15 years and US $ 900 million required.

Lag phase for botanical medicine is now rapidly changing for a number of reasons. Problems with drug-resistant microorganisms, side effects of modern drugs, and emerging diseases where no medicines are available, have stimulated renewed interest in plants as a significant source of new medicines and the process of drug discovery from plants (Fig. 1.1) begins with a botanist, ethno botanist, ethno pharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (*i.e.* traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plant(s) of interest are collected (Baker, J. T. *et al*., 1995). Phytochemists (natural product chemists) prepare extracts from the plant material, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets. Pharmacognosy encapsulates all of these fields into a distinct interdisciplinary science.

Numerous methods used to acquire compounds for drug discovery include: isolation from plants and other natural sources; synthetic chemistry; combinatorial chemistry, and molecular modelling (Ley, S.V. and Baxendale, I.R., 2002; Geysen, S. M., *et al.,* 2003; Lombardino, J. G. and Lowe Ill, J. A., 2004). Despite the recent interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, the natural products, and particularly that of medicinal plants, remain an important source of new drugs, drug leads, and chemical entities (Newma, D. J. *et al.,* 2000; Newman, D. J., *et al*., 2003; Butler, M. S., 2004). An example is Arteether, a potent antimalarial drug. It is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* (Asteraceae), a plant used in traditional Chinese medicine (TCM) (van Agtmael, M. A. *et al.,* 1999; Graul, A. L., 2001).

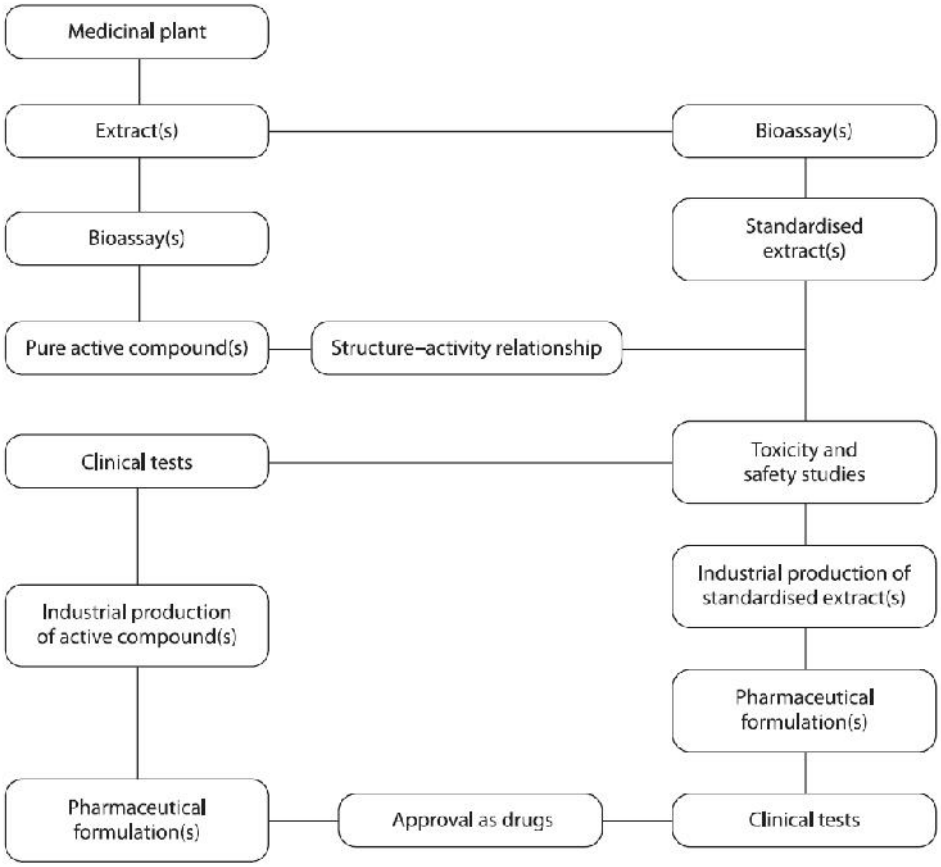

Fig. 1.1 Drug Discovery from Medicinal Plants

(Source: Houghton, P. J. and Mukharjee and P. K., Evaluation of Herbal Medicinal Products- perspectives on quality, safety and efficacy)

1.3 Reverse Pharmacology Approach (Fig.1.2 & 1.3)

Traditional Indian medicinal system covers huge documentaries of health and medicines through practices of ancient scholars of Ayurveda. Many drugs are proven potential therapeutically and phytoactives isolated from them are as successful therapeutic agents. Reverse pharmacology, as an academic discipline, can be perceived to consist of three phases:

- Robust documentation of clinical observations of the biodynamic effects of standardized Ayurvedic drugs by meticulous record keeping

- Exploratory studies for tolerability, drug interactions, dose-range findings in ambulant patients with defined subsets of the disease and paraclinical studies in relevant *in vitro* and *in vivo* models to evaluate the target activity
- Experimental studies, basic and clinical, at several levels of biological organisation, to identify and validate the reverse pharmacological correlates of Ayurvedic drug safety and efficacy (von Bertalanffy, L., 1968)

This approach save a lot of time and cost of drug discovery and chances of getting success are more as the drugs are already proven in traditional system of medicine and documented to be used by people. Some Ayurvedic products, mainly herbs used for phytotherapy, have been tested with promising results. Studies suggest that Turmeric and its derivative curcumin are antioxidants (Tilak, J. C., *et al*., 2004, Aggarwal, B. B. *et al*., 2007). *Tinspora cordifolia* has been tested (Panchabhai, T. S. *et al*., 2008). Among the medhya *rasayanas* (intellect rejuvenation), two varieties of *Salvia* have been tested in small trials; one trial provided evidence that *Salvia lavandulifolia* (Spanish sage) may improve word recall in young adults (Tildesley, N. T. *et al*., 2003) and another provided evidence that *Salvia officinalis* (Common sage) may improve symptoms in Alzheimer's patients (Akhondzadeh, S. *et al*., 2003). In some cases, Ayurvedic medicine may provide clues to therapeutic compounds. For example, derivatives of snake venom have various therapeutic properties (Koh, D. C. *et al*., 2006). Many plants used as *rasayana* (rejuvenation) medications are potent antioxidants (Govindrajan, R. *et al*., 2005). Neem appears to have beneficial pharmacological properties (Subapriya, R. and Nagini, S., 2005).

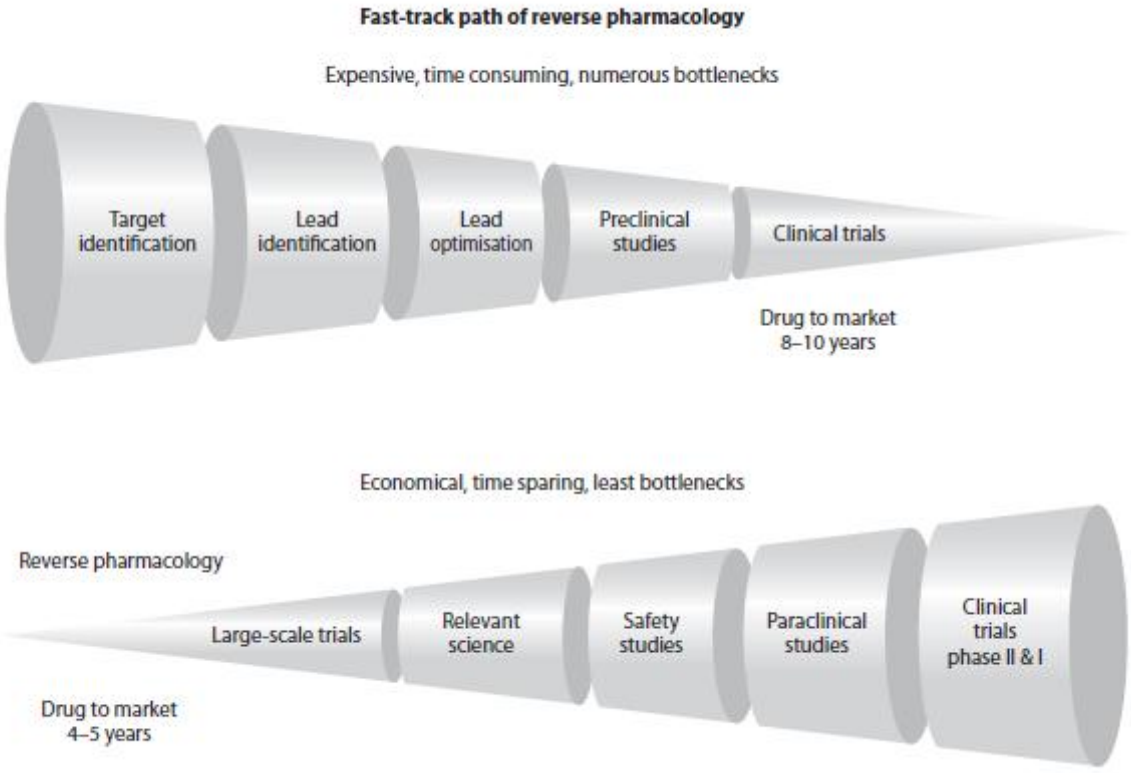

Fig. 1.2: Phases of drug discovery; Reverse pharmacology

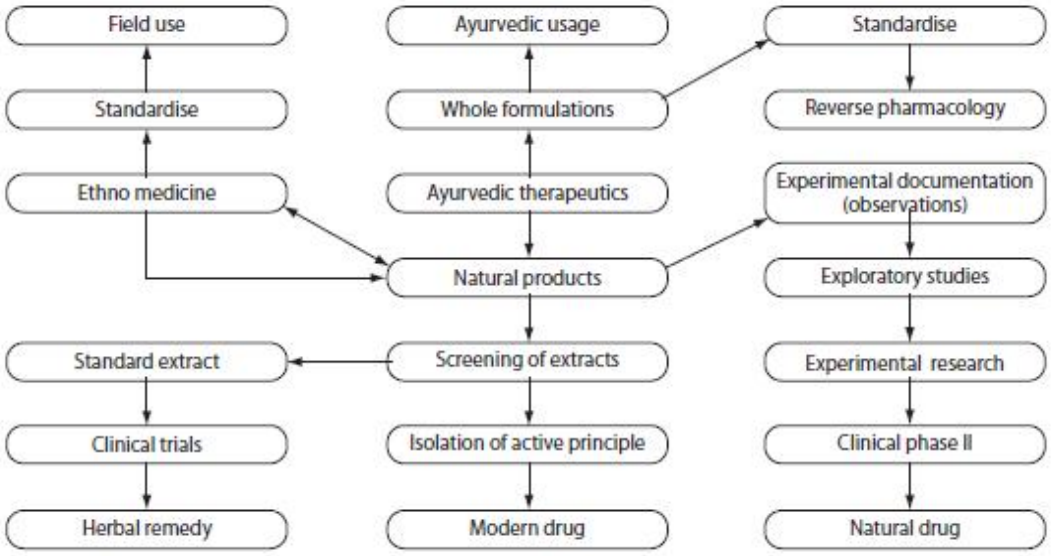

Fig. 1.3: Drug discovery from Traditional medicine

(Source: Houghton, P. J. and Mukharjee and P. K., Evaluation of Herbal Medicinal Products- perspectives on quality, safety and efficacy)

1.4 Infectious diseases

Despite the tremendous progress in medicine, infectious diseases caused by bacteria, fungi, viruses and parasites continue to pose a threatening challenge to public health (Cos, P. *et al*., 2006). The burden of these diseases is felt the most in developing countries due to poverty, unavailability of medicines and the emergence of widespread resistance of pathogens to the available drugs (Okeke, L. N., *et al*., 2005). The World Health Organisation in 2002 has also reported that infectious and parasitic diseases account for 26.2% of the global cause of death, the vast majority of which occurred in the developing countries (WHO, 2003).

Every year, more than half of the deaths associated with infectious diseases continue to be attributed to three illnesses: HIV/AIDS, tuberculosis and malaria. These diseases are present in epidemic proportion, profoundly affecting and serving as major obstacles to the economic growth and development in many of the poorest countries in the world (Mandell, G. L. *et al.,* 2005). Urgent solutions are required if the poorest regions in the world is to develop.

1.5 Malaria

According to World Health Organization, there were an estimated 243 million cases worldwide by 2008. The vast majority of cases (85%) were in the African Region, followed by the South-East Asia (10%) and Eastern Mediterranean Regions (4%). The number of cases in the South-East Asia Region is higher, owing to updated household survey information for Bangladesh and Indonesia, where patients seek treatment for fever. Malaria accounted for an estimated 863 000 deaths in 2008, of which 89% were in the African Region, followed by the Eastern Mediterranean (6%) and the South- East Asia Regions (5%).

In 2007, the Indian National Vector-borne Disease Control Programme (NVBDCP) reported 1,502,742 malaria cases and 1,274 deaths. The updated documents at WHO by NVBDCP represents seriousness of disease as malaria endemic states are *Andhra Pradesh, Chhattisgarh, Gujarat, Jharkhand, Madhya Pradesh,*

Maharashtra, Orissa, Rajasthan where 77% of *Plasmodium faciparum* cases and 43% of deaths are reported.

1.5.1 Human Malaria Parasites

Parasitic infections are still one of the major causes of mortality in the poor or underdeveloped countries. Parasitic protozoan belonging to genus *Plasmodium* causes malaria, one of the most severe tropical diseases. The four identified species of the parasite responsible for inflicting human malaria are *Plasmodium falciparum, P. vivax, P. ovale, and P. malariae*. Of these, *P. falciparum* and *P. vivax* account for more than 95% of malaria cases in the world. Malaria infections caused by *P. falciparum* are prevalent in the major parts of Africa, sub-Saharan Africa and East Asian countries, whereas *P. vivax* is the causative species primarily of Indian sub-Continent. Interestingly, the disease can be treated in just 48 hr, yet it can cause fatal complications, if the diagnosis and treatment are delayed. The tropical regions provide ideal breeding and living conditions for the anopheles mosquito. Malaria is re emerging as the biggest infectious killer and is currently the first priority tropical disease of theWorld Health Organization (WHO).

1.5.2 Life cycle of *Plasmodium falciparum*

Plasmodium species, as members of *Apicomplexa*, share many common morphological features. Each of the developmental stages in the life cycle of malaria parasites exhibits a remarkable conservation and distinct patterns of structural organization (Aikawa, M., 1988; Sinden, R. E., 1978).

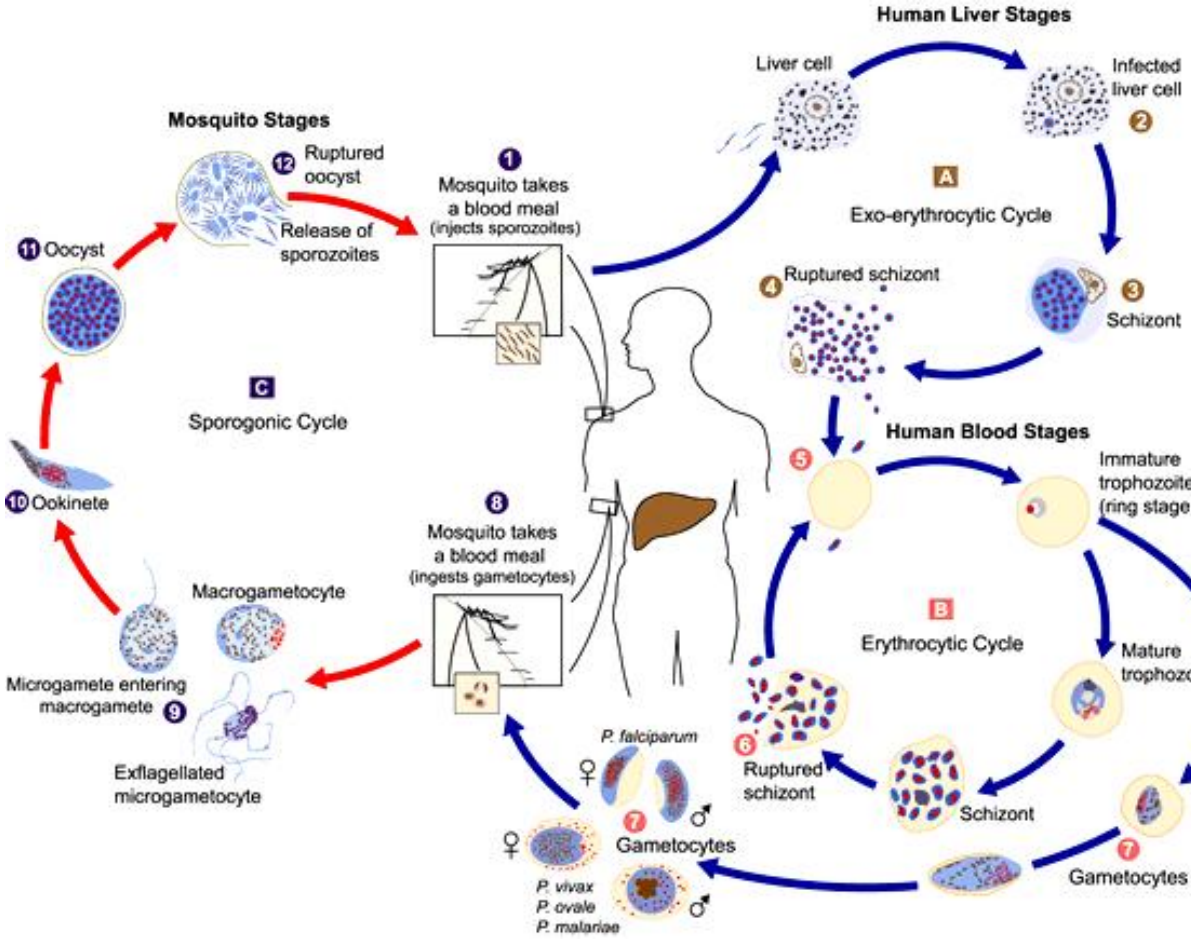

Fig. 1.4: Life cycle of *Plasmodium falciparum*

The life cycle of malaria parasite (Fig. 1.4) begins with the invasion of sporozoits by byte of female infected mosquitoes of genus Anopheles. The sporozoits enter in the blood circulation of host and invade in hepatocytes shortly after few time of infection. This process leads to interactions of parasite surface adhesive proteins and host cell surface molecules. Sporozoites infected in the hepatocytes develop into preerythrocytic (exoerythrocytic) schizonts during the next 5–15 days depending on the *Plasmodium* species. *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium cynomolgi* have a dormant stage, named hypnozoite (Krotoski, W. A. *et al.*, 1982) that may remain in the liver for weeks to many years before the development of preerythrocytic schizogony. This results in relapses of malaria infection. *Plasmodium falciparum* and *Plasmodium malariae* have no persistent phase.

A preerythrocytic schizont contains 10,000 to 30,000 merozoites, which are released into the blood circulation and invade the red blood cells. The merozoite develops within the erythrocyte through ring, trophozoite and schizont stages (erythrocytic schizogony). The parasite modifies its host cell in several ways to enhance its survival. The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes. Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. The entire invasion process takes about 30 s. Concomitantly, a small portion of the parasites differentiate from newly invaded merozoites into sexual forms, which are macrogametocyte (female) and microgametocyte (male). What triggers this alternative developmental pathway leading to gametocyte formation is unknown. Mature macrogametocytes, taken into the midgut of the *Anopheles* mosquito, escape from the erythrocyte to form macrogametes. Microgametocytes exflagellate, each forming eight haploid motile microgametes after a few minutes in the mosquito midgut. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Within 18–24 h, the non-motile zygotes transform into motile ookinetes. The ookinetes have to cross two barriers: the peritrophic matrix (PM) and midgut epithelium. After traversing the midgut epithelium, the ookinete reaches the extracellular space between the midgut epithelium and the overlaying basal lamina, the salivary gland epithelium. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again.

1.5.3 Clinical manifestations

Malaria is a febrile illness characterized by fever and related symptoms. The clinical manifestations of malaria are due to the erythrocytic phase in the development of the parasite. The first symptoms of malaria after the prepatent period (period between inoculation and symptoms that is the time when the sporozoites undergo schizogony in the liver) are called the ''primary attack.'' It is usually atypical and may resemble any febrile illness. As the disease gets established, the patient starts getting relapse of symptoms at regular intervals of 48–72 hr. These are called as ''short term relapses.'' Some patients will get ''long term relapses'' after a gap of 20–60 days or more. In *vivax* and *ovale* malaria, these are due to reactivation of the hypnozoites in the liver. In *falciparum* and *malariae* infections, these are called as ''recrudescence'' and are due to persistent infection in the blood.

1.5.4 Pathophysiology of malaria

During prepatent period, the infected individual may not have any symptoms and rarely suffer from a prodromal illness characterized by vague aches and pains, headache, nausea, etc. At the end of erythrocytic schizogony, the mature schizonts rupture the RBCs and release merozoites into the blood, which in turn infect more RBC's. The growing parasite progressively consumes and degrades intracellular proteins, principally haemoglobin, resulting in formation of the 'malarial pigment' and haemolysis of the infected red cell. This also alters the transport properties of the red cell membrane, and the red cell becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins, (such as, red cell membrane lipid, glycosyl phosphatidyl inositol anchor of a parasite membrane protein), which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages, resulting in chills and high-grade fever. This occurs once in 48/72 hr, corresponding to the erythrocytic cycle.

In *P. falciparum* malaria, the infected RBC's develop protrusions on their cell membranes, which are sticky. This results in increased adhesion of red blood cells that clump together. The red blood cells may also adhere to the wall of the minute capillaries, venules, and arterioles, thereby blocking the blood flow. This helps the

parasite to multiply faster. This increased cytoadherence and clumping, resulting in occlusion of blood-flow culminates in damage to vital organs like the brain, kidneys, lungs, liver, and gastrointestinal tract which leads to the various fatally serious complications of *P. falciparum* malaria. Cerebral malaria is the most serious complication of falciparum malaria. However, its pathophysiology is not completely understood. The basic underlying defect seems to be the clogging of the cerebral microcirculation by the parasitized red blood cells. These cells develop knobs on their surface and develop increased cytoadherance properties, as a result of which they tend to adhere to the endothelium of capillaries and venules. This results in sequestration of the parasites in these deeper blood vessels. Also, rosetting of the parasitized and non-parasitized red cells and decreased deformability of the infected red cells further increases the clogging of the microcirculation. Obstruction to the cerebral microcirculation results in hypoxia and increased lactate production due to anaerobic glycolysis. In patients with cerebral malaria, cerebrospinal fluid lactate, the adherent erythrocytes may also interfere with gas and substrate exchange throughout the brain. However, complete obstruction to blood flow is unlikely, since the survivors rarely have any permanent neurological deficit. *P. vivax* and *P. ovale* infections are generally benign and complications leading to significant morbidity and mortality are uncommon. Some of the sporozoites of *P. vivax* and *P. ovale* in the liver go into hibernation. These hypnozoites can get reactivated and cause relapses of the clinical illness once in 2–3 months. In the cases of *P. falciparum* and *P. malariae* infections, relapses from the liver do not occur; however, the blood infection may remain chronic and, if untreated, may remain chronic for years in case of *P. falciparum* and decades in case of *P. malariae*.

1.6 Malaria according to Ayurveda

From the period of *Atharva veda* (1500 B.C.), there are descriptions of malarial types of fever. *Takman*, for instance, is the term used for a type of fever attended with trembling, rigor, headache, debility, and cough ending in pallor and yellowness. It was endemic to particular regions in the Indian subcontinent like *Munjvan, Mahavrsa, Gandhara, Anga,* and *Magandha*. The epidemics mostly occurred in summer, the rainy season, and autumn. There were types such as *anyedyu*

(quotidian), *tritiyaka* (tertian), and *sadandi* (remittent). In severe types of the patient often was reported to have suffered from delirium and died. From the descriptions it is assumed that the mortality rate was high.

Classical texts of Ayurveda describe what seems to be malaria in following way:

"Sukshma Sukshma tarasyesu doso raktadi margesu sanairapla cirena yat kramoyam tena vicchhina santapo laksyate jvara visamo visamarabha kriya anusangavan"

Astanga samgraha, Jvara nidana, 69

Vitiated dosha spreads slowly in minute channels of blood and other orifices and does not spread to all parts of body simultaneously but only to predominant parts. This is the manner in which fever is seen in interrupted or irregular onset and relief. It is called *visama jvara* because of irregular onset, action, time and reappearance.

Of the eight main types of fever explained in Ayurvedic medical texts, there is a certain category of fever referred to as *visama jvara* (Caraka samhita, *cikitsa sthana*, 3/55-75; Susruta samhita, *Uttara tantra*, 39/51-58; Astanga hrdya, *nidana sthana*, 1/56-57). This type of fever is characterized by irregular onset, action, and recurrence. It is this category of fever, which is correlated to malaria. The symptoms exhibited by patients suffering from this kind of fever vary according to its stages. Ayurvedic theory describes these stages as being due to the involvement of particular body tissues, viz., *rasa, rakta, mamsa, meda, asthi, majja,* and *sukra*.

1.7 Drugs resistance and Urgent need of antimalarials

For several decades, the gold standard for the treatment of malaria was chloroquine (CQ), a 4- minoquinoline that was previously characterized by its efficacy, low toxicity and affordability (less than US $0.2 for a three-day adult treatment course) (Marsh, K. *et al*., 1995) CQ acts by binding to haem moieties produced from proteolytically processed haemoglobin inside infected erythrocytes, thereby interfering with haem detoxification (Taylor, T. E. *et al*, 1993, English, M. *et*

al., 1996) Massive worldwide use of CQ, beginning in the late 1940s, was followed a decade later by the first reports of CQ-resistant strains of *P. falciparum* (English, M. *et al*., 1997). Today, CQ resistance has spread to the vast majority of malaria-endemic areas, rendering this drug increasingly ineffective. However, in spite of the prevalence of CQ-resistant *P. falciparum*, this drug continues to be widely used. This is particularly problematic in sub- Saharan Africa, where resource limitations are profound and where highly immune populations often seem to respond — at least partially — to CQ therapy, and therefore somewhat mask the spread of resistance. CQ resistance almost certainly contributes to the recent finding that malaria-associated mortality is on the increase in Africa (Miller, L. H. *et al*, 1971). SP, a combination antifolate drug is the only other widely used inexpensive antimalarial, but resistance is also leading to unacceptable levels of therapeutic failure in many areas in Asia, South America and now Africa (Dondorp, A. M. *et al*,. 2000). Despite some optimism about new drug development for the future, as noted above, the malaria endemic regions of the world are faced with an unprecedented situation in which the only affordable treatment options are rapidly losing therapeutic efficacy. New antimalarial drugs must meet the requirements of rapid efficacy, minimal toxicity and low cost. Immediate prospects for drugs to replace CQ and sulfadoxine and pyrimethamine (SP) include amodiaquine (a CQ-like quinoline) and chlorproguanil dapsone (LapDap, another antifolate combination that inhibits the same enzymes as SP). These replacements will probably provide a few years of efficacy, particularly in Africa, but they already suffer from some cross resistance with CQ and SP, which increases the likelihood that full-blown resistance to these drugs will emerge rapidly (English, M. C. *et al*., 1996, Newton, C. R. *et al*., 1997, Berkley, J. *et al*, 1999). High on the list of mid-term replacements are artemisinin derivatives. However, these drugs have very short half-lives, which necessitate their use in combination with a longer-acting drug. Clearly, additional new drugs are needed. If we are to avoid an ever- increasing toll of malaria on tropical areas, it is imperative to rapidly put into action strategic plans for the discovery and development of novel antimalarial compounds that are not encumbered by pre-existing mechanisms of drug resistance.

In light of this focus, present investigation is to identify lead compounds from medicinal plant by applying traditional and chemorational approaches.

1.8 Objectives of thesis

1. Isolation and identification of compounds from potential medicinal plants
2. Screening of medicinal plant extracts and isolated compounds against chloroquine sensitive strain of *Plasmodium falciparum* for antimalarial activity
3. Screening of medicinal plant extracts and isolated compounds showing anti malarial activity against chloroquine resistant culture of *Plasmodium falciparum*
4. To study combinatorial effects (synergism) of potential anti malarial candidates
5. Study of possible mechanism of action

1.9 Structure of thesis

Chapter 1 Introduction

Various aspects including history of traditional medicines, drug discovery from natural products and traditional medicine, malaria pathology and its impact, malaria according to Ayurveda are discussed.

Chapter 2 Primary evaluation of medicinal plant extracts and isolated compounds against chloroquine sensitive strain of *Plasmodium falciparum*

In vitro screening for antimalarial activity of selection of plants by traditional and chemorational approach was carried out to choose a lead for further detailed investigation.

Chapter 3 Evaluation of *Enicostemma littorale* against chloroquine sensitive strain of *Plasmodium falciparum*

Activity guided fractionation of the plant to identify potential lead, method development of isolation of the selected lead and probable conversions of the same were carried out and evaluated for antimalarial activity.

Chapter 4 Screening of selected compounds against chloroquine resistant strain of *Plasmodium falciparum*

Best active compounds and bio enhancers like hesperidin, curcumin and quercetin were screened to evaluate further their potency against resistant culture.

Chapter 5 An *in vitro* assay for β hematin formation inhibitory action

The compounds were studied for hemazoin formation inhibition, a potential target through which majority of antimalarials act.

Chapter 6 Evaluation of synergy between antimalarial compounds against chloroquine resistant strain of *Plasmodium falciparum*

Synergy amongst potential antimalarial compounds was studied to achieve best active therapeutic potency. The activity was further studied for combination of best proven bio enhancer compound along with chloroquine and compared with standard. Logical evaluation of best acting flavonoid like hesperidin was also carried out to support calcium channel blocking potential phenomenon of the same.

Chapter 7 Summary and conclusion

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2.1 Introduction

Malaria is common in poor and less developed countries of the world. According to an estimate, about 350-500 million people are infected from malarial parasite *Plasmodium falciparum* and approximately one to three million deaths recorded annually, which represents at least one death every 30 sec (Breman, J., 2001, Trafford, H., 2005). The vast majority of cases occur in children under the age of 5 years (Greenwood, B .M. et. al., 2005, Winstanley, P. A., 2000). Countries like Africa faces its hard impact (Bull. WHO, 1993; Ekthawatchai, S. *et al*., 1999), while other hit hard hit tropical areas are South East Asia, India and China. Experts estimate that around 40 % of the cases of malarial fever in India are of *Plasmodium falciparum* (Kumar, S., 1994).

The first gold standard drug for treatment of malaria was quinine, a compound isolated from the bark of a Rubiaceae tree called Cinchona and the drug is still in use. Later on, its derivative was synthesized having more improvised action and lesser side effects like nausea, vomiting and gastric refluxes. The drug is in therapeutic use from around 40 years and is still the cheapest and most effective therapy for malaria. However, due to massive use, resistance with gold standard chloroquine has been widely spread and mortality is on increase in Africa. Treatment of chloroquine-resistant malaria is with alternative drugs or drug combinations, which are rather expensive and sometimes toxic. Furthermore, these combinations are not always based on pharmacokinetic principles due to inadequate knowledge of metabolism and mechanism of action of most antimalarial drugs. Resistance and viability are still problems with another choices of treatment like sulfadoxine pyrimethamine and artemisinins (Boland, P., 2001, Fidock, D. A., 2004, Ridley, R. G., 2002).

Chinese herb "Quinghao" solved the problem of resistance. The latin name of this herb is *Artemisia annua* and artemisinin, a sesquiterpene lactone isolated from the same became block buster anti malarial drug. Unfortunately, the therapy had drawbacks like drug resistance and economical viability.

There is a consensus among the scientific community that natural products have been playing a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases (Newman, D. J., 2002). Indeed, the vast majority of the existing antimalarial chemotherapeutic agents are based on natural products, and this fact anticipates that new leads may certainly emerge from the tropical plant sources, since biological chemo diversity continues to be an important source of molecular templates in the search for antimalarial drugs (Ziegler, H. L. *et al*., 2002**,** Kalauni, S. K. *et al.,* 2006**,** Portet, B. *et al.,* 2007**)**.

2.1.1 Selection of medicinal plants and compounds

Two approaches we used for selection of plants: Traditional and chemorational. In traditional approach, we reviewed many plants mentioned for treatment of *visham jvara* (malarial fever) in Ayurvedic literature. Majority of the plants have been reported for *visham* and *chaturthaka jvara* (symptoms are more as malarial fever). Names of some selected plants are mentioned in table 2.1. We also correlated the traditional approach to chemo rational approach, phyto constitutes of plants should be logically related to the activity. We also aimed to identify the lead which should comply with traditional and chemo rational criteria as well as should be more promising and safe and unique.

Table 2.1: Antimalarial plants reported in traditional literature

Name of the plant	Common name	Details mentioning association with malaria	Classical References	Scientific Report status (References)
Adhatoda zeylenica	Vasaka, Ardusi	Used in *visham jvara* formulations like Vatsakadi kwatha, Vasadighritam	Charaka Samhita, Cikitsa, 3/223-224	Reported (Henrik Toft Simonsen *et al.*, 2001)
Embelia ribes	Vidanga	Used as *krimigna* in Vidanga churna	Bhavapraksha, Krimirogadhikara, 7-21	Not reported
Enicostemma littorale	Mamejavaka, Katu chirata, Chhota chirata	Mentioned specifically for *visham jvara*	Dravyaguna vigyana, Part II , 704	Not reported
Picrorhiza kurrao	Katuka	Used in *visham jvar nashak pancha kwatha* for *santata* and *satata jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Singh, V and Banyal, HS, 2011)
Holorhenna antidysentrica	Indrayava	Used in *visham jvar nashak pancha kwatha* for *santata* and *anyedyushka jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Verma, G. *et al.*, 2011)
Azadirachta indica	Neem	Used in *visham jvar nashak pancha kwatha* for *satata jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Iwu, MM *et al.*, 1986)
Cyprus rotundens	Motha	Used in *visham jvar nashak pancha kwatha* for *satata, anyesyushka* and *chaturthaka jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Thebtaranonth, C. *et al*., 1995)
Cissumpalos parrera	Patha	Used in *visham jvar nashak pancha kwatha* for *santata jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Singh, V and Banyal, HS, 2011)
Santalum album	Chandana	Used in *visham jvar nashak pancha kwatha* for *chaturthaka jvara*	Charaka Samhita, Cikitsa, 3/200-203	Reported (Henrik Toft Simonsen *et al.*, 2001)

From review of traditional literature, we identified three plants reported for treatment of fever and related symptoms of malaria: *Embelia ribes, Adhatoda vasica* and *Enicostemma littorale*. *Adhatoda vasica* was reported for treatment of *visham jvara*

in *Charaka Samhita* but *Enicostemma littorale* and *Embelia ribes* are not mentioned in *Brihat Trayi* – Three main books of Ayurveda *viz. Charaka Samhita*, *Sushruta Samhita* and *Astanga hridayam*. Laghu Trayi mentions their uses in *jvara* and *visham jvara*. We also found no records mentioning antimalarial activities of these plants in scientific literature except *Adhatoda vasica* which was previously reported by few authors (Henrik Toft Simonsen *et al.,* 2001).

We also applied a chemo rational approach on the selected plants and reviewed correlations between main compounds of selected plants and their probable role for antimalarial activities. Parasite requires electron transfer for energy. Existence of coenzyme Q_8 redox entity, in *Plasmodium* is responsible for electron transfer of the parasite. Quinines possess antimalarial action by inhibiting this enzyme (Yieh-Ping Wan *et al*., 1974). Quinones are also highly active against *P. falciparum in vitro* (Carvalho, L. H. *et al*., 1992). From these, we hypothesized probable antimalarial action of benzoqinone; embelin, a major phytochemical of *Embelia ribes*.

Vasicine, a quinazoline alkaloid of *Adhatoda vasica* resembles with phenomenon, that weak bases play a major role in antimalarial action by raising intravesicular pH (Yayon, A. *et al*., 1984; Paul, H. Schlesinger *et al*., 1988) and thereby affecting transport of macromolecules as well as membranes and phospholipase activity (Stahl, P. And Schwart, A. L., 1986).

Flavonoids are the largest group of phytochemicals. Earlier studies have reported that flavonoids possess antimalarial activity and demonstrate marked and selective potentiating effect on the antiplasmodial activity of artemisinin (Liu, K. C. C., 1992). We selected a flavonoid hesperidin as it is nowhere reported for antimalarial activity and can be easily obtained by sources like waste orange peel.

Irridoids are mono or sequi terpenoidal glycosidal compounds from the plants and also called bitter glycosides. One of the best examples of similar class of compound is artemisinin from *Artemisia annua*. Many irridoids has been identified for antimalarial activity of many plants. Swertiamarin from *Enicostemma littorale* is a

compound of similar category; hence we selected the same for study (Cláudio R. *et al*., 2011).

These selected plants and their relevant compounds were screened to define their antimalarial activity on chloroquine sensitive strain of *Plasmodium falciparum*. We also used alkaloids isolated from cinchona bark and quinine dihydrochloride as respective standards. Viable methods of isolation were established by us to achieve proposed compounds (particularly embelin, hesperidin and cinchona alkaloids; where working standards to be used).

2.2 Materials and methods

Part A: Preparation of extracts and isolation of compounds

2.2.1 Chemicals

All the chemicals used were of analytical grade.

2.2.2 Extracts / compounds selected for study

Extracts: Methanolic extract of *Enicostemma littorale, Embelia ribes* and *Adhatoda vasica*

Compounds: Embelin (benzoquinone), vasicine (isoquinoline alkaloid), swertiamarin (secoirridoid glucoside), hesperidin (a flavonol glucoside) and mixture of alkaloids isolated from cinchona bark.

Embelin was isolated from *Embelia ribes*, Hesperidin was isolated from orange peel and cinchona alkaloids were isolated from *Cinchona officinalis* bark, while, another compounds were procured as gift from VED Pharmaceuticals Pvt Ltd., Baroda.

2.2.3 Plant material and preparation of extracts

The whole herb of *Enicostemma littorale* was collected from Bhavnagar, the coastal area of Gujarat in the month of August; *Adhatoda vasica* leaves were collected from our campus in month of August. While, *Embelia ribes* fruits were purchased from a local supplier in Baroda. The specimen were sent for identification to Botany

department of M S University of Baroda and identified as an authentic material. The plant material collected were dried in oven at 50 °C, crushed in a mixer to yield powder of 40 – 60 mesh which was further used for isolation. The dried leaf powder (1 kg) was extracted with methanol (1 litre x 3). The resulting methanol soluble fractions were pooled together and concentrated under reduced pressure. The extracts were stored in air tight vial.

2.2.4 Isolation of compounds

Embelin, hesperidin and cinchona alkaloids were used as working standards, after confirming purity and proper characterization. We developed rapid method of isolation of these compounds in a way to make them commercially viable. Brief procedures of isolation of respective compounds are mentioned below.

2.2.4.1 *Embelin from Embelia ribes*

Hundred grams of coarsely powdered berries of *Embelia ribes* were extracted with chloroform (500 ml x 3). The chloroform soluble fractions were pooled together and evaporated to reduce volume up to 500 ml. This was covered and kept in freeze (4 – 8 ^{o}C) for overnight. The golden colored needles so obtained were filtered by watmann no. 1 filter paper. The needles were purified by washing them with petroleum ether (60 – 80 ^{o}C) and dried at room temperature. The resulting compound was immediately transferred to air tight vial and stored in refrigerator.

2.2.4.2 *Hesperidin from orange peel*

Twenty five gm of Orange peel powder was defatted with petroleum ether (60-80 ^{o}C) (500 ml x 3). The resulting marc was extracted with methanol (500 ml x 3) repetitively and concentrated to a syrupy mass. Glacial acetic acid (25 ml) was added to the concentrated extract, and diluted with 250 ml of water. The white fluffy precipitates so obtained were mixture of hesperidin and rutin. This mixture was washed repetitively with methanol and pure product so obtained was dried under vacuum.

2.2.4.3 *Cinchona alkaloids*

Bark of cinchona was received from a supplier from Amazon. Powder of cinchona bark 20 gm was defatted with petroleum ether (60-80^{o}C) (3 x 100 ml) and the marc was extracted with methanol repetitively until gives negative dragendorff's test. The methanol soluble portion was concentrated and dissolved in 10% orthophosphoric acid. This orthophosphoric acid solution was again basified with ammonia up to pH 9 and extracted with dichloromethane and concentrated to dryness. The dried portion was rinsed with cyclohexane and the solvent was evaporated. The compound was obtained as pale yellowish white and off white amorphous crystal form.

2.2.5 Characterization of extracts/compounds

2.2.5.1 Characterization of *Enicostemma littorale*/swertiamarin; *Adhatoda vasica*/vasicine and *Embelia ribes*/embelin

The extracts and their relevant compounds were evaluated by using High Performance Thin Layer Chromatography (HPTLC). We used optimized chromatographic conditions as reported by authors previously. For *Enicostemma littorale*/swertiamarin, method mentioned by Vishwakarma, S.L. (Vishwakarma, S.L. *et al*., 2004); *Adhatoda vasica*/vasicine by S. Soni (Soni, S. *et al*., 2009) and Embelia ribes/embelin by Kukkar, R. (Kukkar, R. *et al*, 2010) were used. The detailed HPTLC conditions are as mentioned below.

TLC plates

Pre coated silica gel 60 F_{254} (E. Merck) of uniform thickness (0.2 mm) were used.

Preparation of test solution

Twenty five mg of each extract of drug was dissolved in methanol. The methanol soluble portions were pooled together and concentrated to make up the volume of 50 ml in a volumetric flask. Same way, 2 mg of each standard was dissolved in 1 ml of methanol.

Procedure

Ten µl of each solution was applied on TLC plate with help of Linomat V spotter. The plated were allowed to run in respective optimized solvent system up to 8 cm height and scanned at 254 nm by using TLC scanner 3, attached with winCATS software to obtain densitogram. Optimized solvent system conditions are mentioned as below.

Name of extract and relevant compound	**Solvent system**
Enicostemma littorale and swertiamamrin	Ethyl acetate : methanol (8:2)
Embelia ribes and embelin	*n* propanol: *n* butanol: ammonia (7:1:2)
Adhatoda vasica and vasicine	Ethyl acetate: methanol: ammonia (8:2:0.2)

2.2.5.2 ***Characterization of hesperidin***

Hesperidin was confirmed by various methods as mentioned below.

Evaluation by Ultraviolet spectrometry:

The solution 1 was drawn to a quartz cuvette of 1 ml and scanned for the wavelength ranging between 200 – 800 nm on Helios UV spectrophotometer. The wavelength at maxima peak was recorded.

Evaluation by melting point

One mg of compound was taken in a capillary tube and placed in silicon oil. The temperature was continuously recorded and the point at which the compound melts was recorded.

Evaluation of the compound by mass fragmentation pattern

For mass spectral identification by ESI-MS, 1 mg of compound was dissolved in methanol and was introduced for direct fragmentation in ionization chamber in positive and negative ionization mode. The base peak was recorded and compared with the molecular weight.

2.2.5.3 *Characterization of cinchona alkaloids*

The liquid chromatography of mixture of alkaloids was carried out by following a validated HPLC, a most convenient technique for mixture of isomers. Method reported by Ravishankara (Ravishankara, M. N. *et al.*, 2001) was followed for the same. In brief, the mixture containing alkaloids was dissolved in 10 percent sulphuric acid to obtain concentration of 1 mg/ml and chromatographed by using stationary phase of C_{18} column (Restek, 250 mm length, 4.6 mm internal diameter and 5 µm particle size) and mobile phase of 85 mM potassium dihydrogen ortho phosphate solution containing 40 mM of triethyl amine adjusted to pH 2.8 with orthophosphoric acid solution. The resulting solution was mixed with acetonitrile in ratio of 90:10 v/v, flow rate adjusted was of 1.2 ml/min in HPLC system (Perkin Elmer Series 200) with UV/VIS detector adjusted at wavelength of 226 nm. Quinine sulphate was used as a standard by dissolving quinine sulphate tablets in water to make 1 mg/ml of solution.

Part B: Antimalarial activity

2.3 Parasite culturing and antiplasmodial assay of extracts/compounds

2.3.1 Culturing *Plasmodium falciparum*

Cultivation of malaria parasites is an important tool for the understanding of parasite biology, biochemistry, molecular biology, immunology, and pharmacology. We followed technique of the erythrocytic stages of *P. falciparum* as that originally described by Trager and Jensen as well as Schuster (Trager, W. and Jensen, J. B., 1976; Schuster, F. L., 2002). Detailed methodologies for parasite culturing are mentioned as below.

2.3.1.1 Malarial parasites

Two strains of *Plasmodium falciaprum* namely MRC 20 and RKL 19 were used in the study. The MRC 20 strain was isolated from the patient infected with *Plasmodium falciparum* before any medication, known as sensitive to chloroquine while, RKL 19 was isolated and maintained from the patient with relapse of malaria

after treatment of chloroquine, was known as resistant to chloroquine. These both strains were procured from National Institute of Malaria Research (NIMR), New Delhi.

2.3.1.2 Culture medium

The RPMI medium was routinely used for *in vitro* culture of *Plasmodium falciparum* throughout this study. It was prepared by dissolving 10.4 gm of RPMI 1640 powder containing *L*- glutamine but without sodium bicarboanate (Hi Media) and 5.94 gms of HEPES buffer (SIGMA) in 960 ml of double distilled water. Two militres of gentamycin sulphate (40 mg/ml) was added to the medium solution before sterilization by filtration of millipore filter of 0.22 μm porosity. One hundred millilitres of the sterile medium was transferred in to sterile glass bottle as a stock medium, which was stored for a period up to one month at 4 °C.

Each stock medium (100 ml) was added with 4.2 ml of 5 % (w/v) $NaHCO_3$ (Hi Media) to give a pH of 7.4, 1 ml of 10 mM *D* – glucose (SIGMA), and 1 ml of 2 mM *L* – glutamine (SIGMA). This medium was referred to as complete medium without serum (C-RPMI without serum). The complete RPMI medium was supplimented with 10 % (v/v) human inactivated serum (group A, B, and O) before using for cultivation and this medium was referred to as complete medium with serum (C-RPMI with serum). This medium was stored at 4 °C and used within one month.

2.3.1.3 Normal human serum

Normal human serum of all blood groups was collected from healthy donors who had no history of malaria infection. Moreover, donors did not receive any drug within two weeks prior to blood collection. Sera group A, B and O were used for routine culture of *Plasmodium falciparum,* whereas group O serum was used for assessment of antimalarial activity.

Blood was collected intravenously through a bag (after removing anti coagulant solutions) and allowed to clot at room temperature for 30 min. The bag

was stored at 4 °C overnight to allow complete clotting. The clotted blood was centrifuged at 750g for 30 min (4 °C) to separate the fresh serum and the clotted blood. This fresh serum was transferred aseptically to a reservoir bag and inactivated at 56 °C for 30 min in a water bath. Approximately 10-12 ml of fresh serum was distributed aseptically into screwed-cap tubes, stored at -20 °C and used within 3 months.

2.3.1.4 Non infected erythrocytes

Human erythrocytes were collected from healthy donors with type "O" blood group who had no experience of malaria infection. Approximately 300 ml of blood was collected in sterile bag containing anti coagulant CPD solution. After thoroughly mixing, blood suspension was aseptically dispensed as aliquots in sterile vials, stored at 4 °C and used within one month.

For cultivation, 50 % non-infected erythrocytes suspension was prepared as follows. Ten millilitres of the whole erythrocytes was transferred aseptically to a 15 ml centrifuge tube and centrifuged at 750g for 15 min (4 °C). Supernatant and buffy coat were removed carefully, and then the packed erythrocytes were washed twice with C-RPMI without 10 % normal human serum. For the use in cultivation, the washed packed erythrocytes were re suspended with an equal volume of C-RPMI with 10 % normal human serum to make 50 % (v/v) non infected erythrocytes.

2.3.1.5 Cultivation of Plasmodium falciparum and maintenance of continuous culture

According to the continuous culturing technique as described by Trager and Jensen (Trager, W. and Jensen, J.B., 1976), the parasites were routinely maintained as continuous culture in culture flasks 25 cm^2 containing C-RPMI medium with 10 % normal human serum. The initial parasitemia was 0.5-1.1 % with 3 % cell suspension in a total volume of 5 ml. The inoculated flasks were placed in a candle jar (A vacuum desiccators equipped with a stopcock and candle). The candle was lit and the cover was put on with the stop cock opened. When the candle went out, the stop

cock was immediately closed. By this method, an atmosphere with low oxygen and high carbon dioxide content, i.e., 17 % O_2, 3 % CO_2 and 80 % N_2 was produced. The candle jar was incubated at 37 °C in an incubator.

The medium was changed daily; old medium was being removed with a pipette. The thin blood smear was made and stained with Giemsa stain. Percentage parasitemia was calculated by counting the infected erythrocytes in a total of 10,000 erythrocytes. Sub culturing was performed when parasitemia is higher than 5-6%. Briefly, the infected erythrocytes were collected in to a centrifuge tube and centrifuged at 500g for 7 min (4 °C). The packed erythrocytes were washed twice with C-RPMI without 10 % human serum. After the final wash, the packed erythrocytes were suspended to make 50 % (v/v) suspension in to flasks, percent cell suspension were reduced from 50 % to 3 % with C-RPMI medium with 10% normal human serum. Approximately 5 ml of this suspension was then placed in to culture flasks as mentioned above.

2.3.1.6 Synchronization of culture

Generally, continuous culture of *P. falciparum* results in a loss of synchronicity characteristic, which can complicate the evaluation of experimental results. Thus, it is important to start all experiments with a synchronous ring stage. Briefly, synchronous cultures of MRC 20 and RKL 19 strains were collected in to centrifuge tube and centrifuged at 500g for 10 min (4 °C). After the removal of supernatant, 5 % (w/v) sterile *D* – sorbitol was added to the packed erythrocytes at the ratio of 4:1 and gently mixed. The mixture was incubated at 37 °C for 20 min in the waterbath. During the period of incubation, only erythrocytes harbouring late trophozoites and schizonts were selectively lysed, leaving only the erythrocytes infected with ring stage among non-infected erythrocytes. Sorbitol was removed by centrifugation and the packed erythrocytes were then washed twice with C – RPMI without 10 % normal human serum. After final wash, the packed erythrocytes was adjusted to 3 % cell suspension and cultured in a candle jar as described above.

2.3.1.7 Freezing and storage of parasites

Plasmodium falciparum parasites in continuous culture were harvested whenever at least 5 % ring stage was obtained. The culture suspension was collected into a centrifuge tube and centrifuged at 500g for 7 min (4 °C). After the removal of supernatant, the packed erythrocytes were resuspended in an equal volume of freezing solution (sorbitol, 0.9 % NaCl and 99 % glycerine). An aliquot of 1-1.5 ml of the suspension was carefully added into a cryopreserved tube and frozen at -80 °C refrigerator. These frozen parasites were stored as stock culture.

2.3.1.8 Thawing of parasites

Frozen cryopreservative tubes were removed from -80 °C refrigerator and thawed at 37 °C in a waterbath. The suspension was transferred to a sterile centrifuge tube, to which an equal volume of sterile 2.5 % sodium chloride solution was added, and then centrifuged at 500g for 7 min (4°C). After the removal of supernatant, the packed erythrocytes were washed three times with C-RPMI without 10 % normal human serum. After the final wash, the packed erythrocytes was resuspended in C-RPMI with 10 % normal human serum to make 3 % cell suspension, and transferred in to sterile culture flask and cultured in a candle jar as described above.

2.3.1.9 Preparation of parasite inoculums

Up to 5 % ring stage *P. falciparum* obtained by several rounds of sorbitol lysis were pooled ascpetically to a 15 ml centrifuge tube and centrifuged at 500g for 7 min (4°C). After the removal of supernatant, the packed erythrocytes were resuspended in an equal volume of C-RPMI medium without 10 % normal human serum to make a 50 % (v/v) cell suspension. The 50 % non-infected erythrocytes were added to adjust percentage of parasitemia approximately 1 to 1.5 %. The erythrocyte suspension was diluted to 3 % cell suspension with C-RPMI medium with 20 % normal human serum. This mixture of suspension is referred to as parasite inoculums.

2.3.1.10 Giemsa staining of parasites

The stain preparation was optimized by us. In brief, 25 mg of giemsa powder was triturated in a mortar pastel with 5 ml of glycerol, 15 ml of methanol was added to it and filtered. The resulting solution was immediately transferred to a amber colored reagent bottle and stored in dark. For parasite staining, 5 ml of the solution was diluted with 25 ml of cold water. The smeared culture slides were stained by fixing with methanol first and then allowed to be dipped in prepared giemsa staining solution for 15 minutes. Then the slides were washed with water to remove excess stain, allowed to dry in air and observed under microscope by putting a drop of immersion oil.

2.3.1.11 Schizont Maturation Inhibition Assay

Various techniques for antiplasmodial activity of plant extracts/compounds were followed as previously reported by the authors ((Desjardins, R. E. *et al*, 1979; Ringwald, P. *et al*, 1996; Wernsdorfer, W. H., 1994).

We evaluated the extracts and compounds for antimalarial activity by a stage specific assay method called schizont maturation inhibition assay (SMI). In brief, culture *Plasmodium falciparum* was maintained as mentioned above and observed after 3, 6 and 24 h for regular development of parasite stages. The cultures were synchronized using sorbitol (4.2%) and parasitaemia was adjusted to 3-4 % by diluting with fresh human erythrocytes. The cells were diluted with complete media to make 8% haematocrit. Again the slides of culture were prepared and observed for the calculation of parasitemia, particularly for young trophozoites or ring stages.

One milligram of each extract/compound was dissolved in 100 µl of DMSO and then 900 µl of incomplete RPMI 1640 medium (without serum) was added. A series of eleven concentrations were prepared from the stock solutions by 2 fold dilutions in 96 well micro titre plates. After interval of 24 hr and 48 hr, thin films of the contents of each well were prepared and examined under the microscope. Parasite count for each blood film was made using a compound microscope under

oil immersion with X 100 objective after staining the film with giemsa solution. Each film was observed at 20 different visual fields and at three different parts of slide.

The number of schizonts per 200 parasites were noted and compared between control and test wells for the determination of the % inhibition. All doses were studied in cultures and the mean was observed for purposes of inferences. The inhibition of parasite growth in the drug-treated groups was calculated as follows: Parasitaemia in the control (non-treated) group minus parasitaemia in the drug treated group, divided by parasitaemia in the control (non-treated) group, expressed as percentages. All values are expressed as percentage growth inhibition. Dose response curves of the fractions were obtained by plotting percentage inhibition against log concentration. The values of the compounds provided a mid-point value where parasite growth would be 50%. Linear regression analysis was applied to the linear portion of the sigmoidal curve was plotted and IC_{50} values were derived for each extract/compound.

2.4 Results

2.4.1 *Characterization of extracts/compounds by HPTLC*

Comparative TLC pattern (figure 2.1 a) and densitograms (figure 2.1 b) of *Adhatoda vasica* leaf extract and vasicine represented a band at R_f 0.40 corresponding to vasicine in both reference and test solution when the plate was visualized under UV 254 nm. Presence of a single peak of vasicine (track 2 of figure 2.1 b) in densitogram showed its purity and corresponding peak in test sample represented the same as a major compound of the herb.

Figure 2.2 (a & b) mentions TLC pattern and densitograms of *Enicostemma littorale* extract and swertiamarin. Swertiamarin was observed at 0.55 R_f in both test and standard tracks when the plate was observed under UV254 nm. From TLC and densitograms, it was observed that swertiamarin was in pure form and was a major compound of the herb.

A band of R_f 0.12 corresponded to embelin in both test and standard solutions were compared by TLC and obtained densitograms as mentioned in figure 2.3 a, b & c. Figure 2.3 a represents TLC pattern when the plate was observed under UV 254 nm, while figure 2.3 b represents pink spots of embelin observed at natural light after derivatization of the same plate with 5 % methnolic solution of potassium hydroxide. The isolated compound was also found to be pure form recorded densitogram (figure 2.3 c, track 2).

2.4.2 *Characterization of hesperidin*

The hesperidin was isolated as a white amorphous powder with the yield of 8 percent. The melting point ranged between 258 ° to 262 °C. It was insoluble in water and sparingly soluble in methanol, clearly soluble in pyridine and dimethyl sulfoxide while insoluble in acetone, chloroform and petroleum ether. The UV max of compound was ranged between 286-289 nm. Further it was confirmed by Liquid chromatographic (figure 2.4 a) and mass pattern (figure 2.4 b) showing molecular weight of 610 m/z.

2.4.3 *Characterization of cinchona alkaloids*

The mixture was found to contain four distinct peaks corresponding to cinchonine, cinchonidine, quinidine and quinine at retention time (Rt) of 2.09, 4.39, 6.43 and 7.32 min. respectively. Quinine standard was also resolved at Rt of 7.32 min. From the relative reference (Ravishankara, M. N., 2001), we standardized this mixture and found that quinine was a major compound 59.75%, while, quinidine, cinchonidine and cinchonine were present in 14.28, 15.53 and 10.43 % respectively. Figure 2.5 a represents HPLC pattern of mixture, while figure 2.5 b represents that of standard quinine.

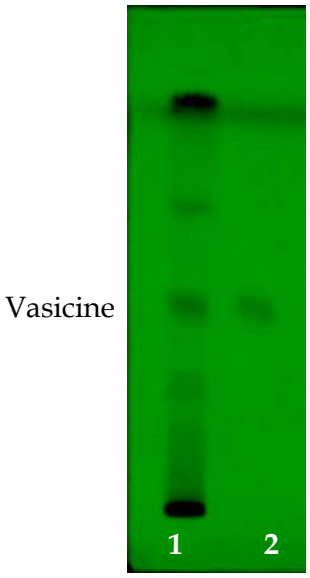


Fig. 2.1a

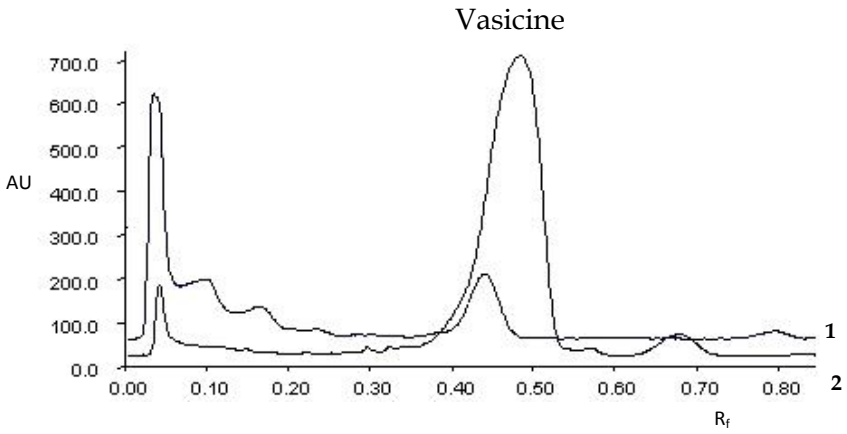


Fig. 2.1b

Figure: 2.1a TLC profile observed under UV 254 nm; 2.1 b densitograms of tracks 1*: Adhatoda vasica* extract, 2: Vasicine

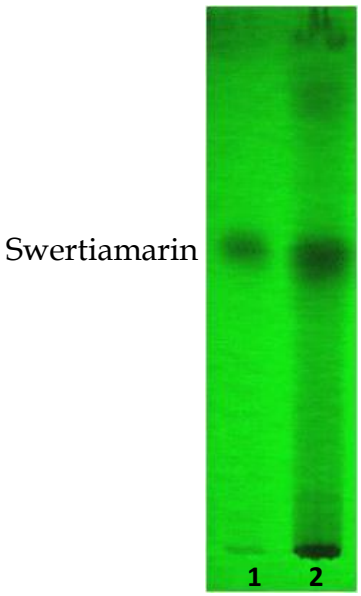


Fig. 2.2a

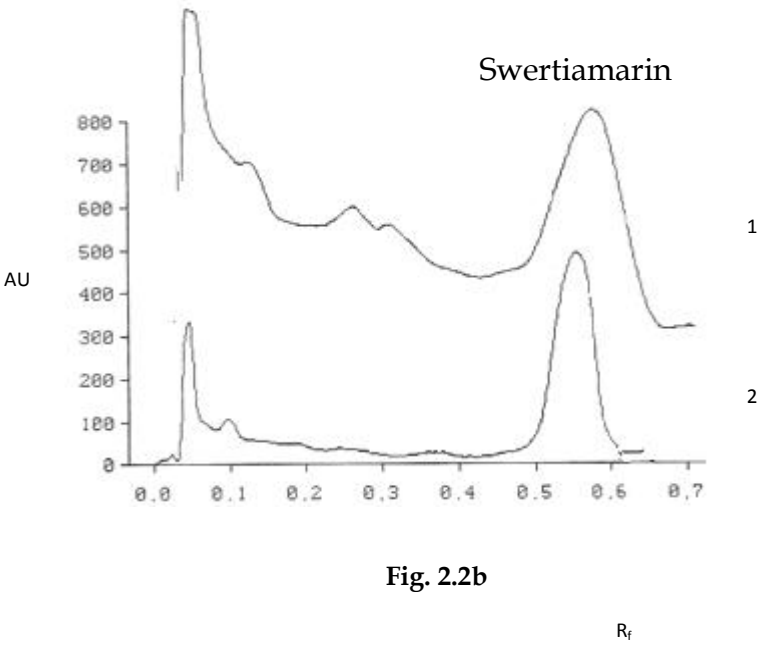


Fig. 2.2b

Figure: 2.2a TLC profile observed under UV 254 nm; 2.2 b densitograms of tracks 1*: Enicostemma littorale* extract, 2: swertiamarin

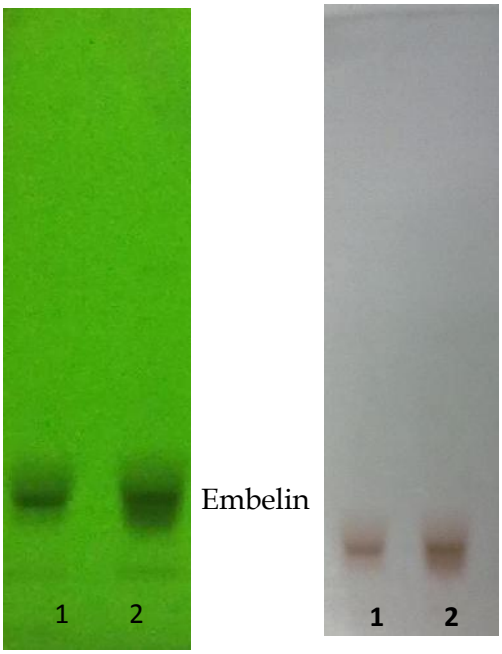


Fig. 2.3a **Fig. 2.3b**

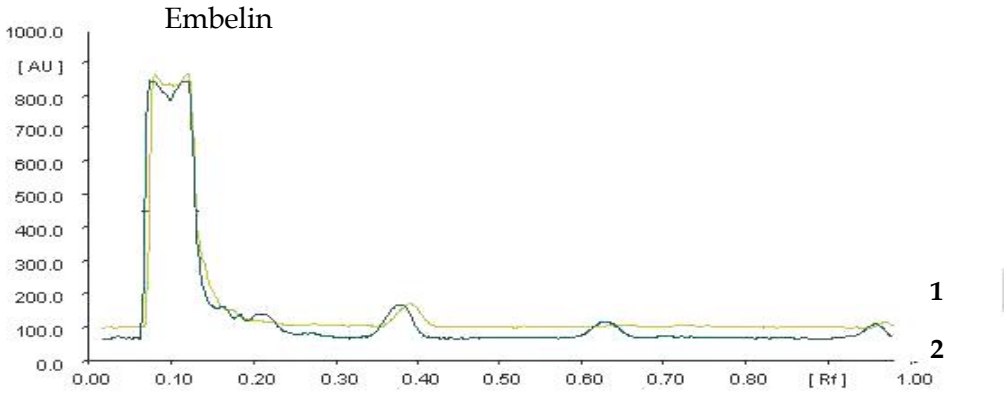


Figure: 2.3a TLC profile observed under UV 254 nm; 2.3b plate derivatized with KOH and observed under natural light; 2.3 c densitograms of tracks 1*:* *Embelia ribes* extract, 2: embelin

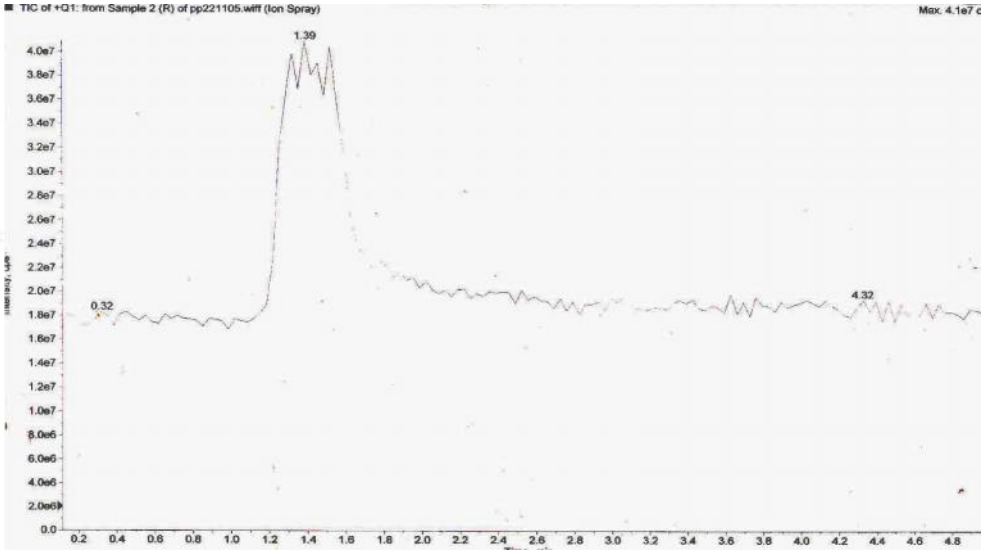


Fig. 2.4a

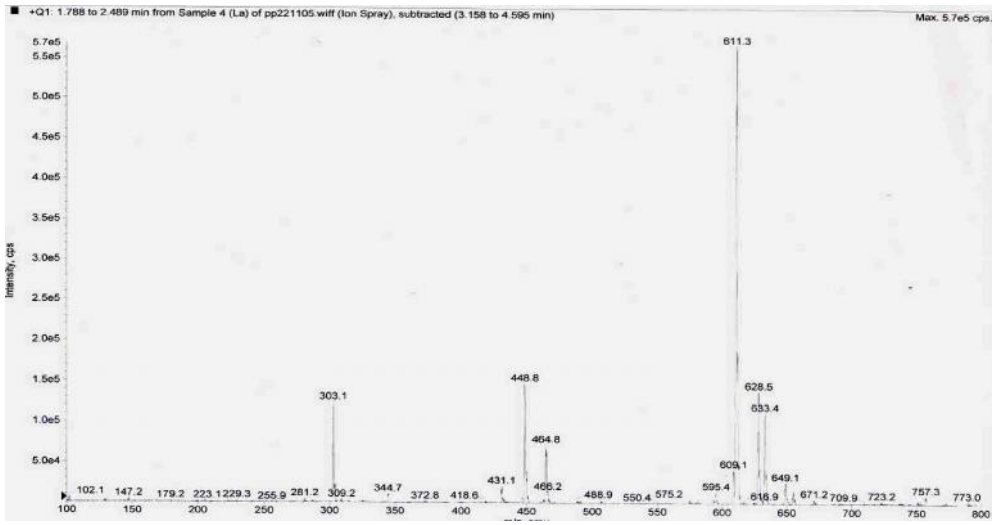


Fig. 2.4b

Figure 2.4a: Liquid chromatogram; 2.4 b: Mass spectra of of hesperidin

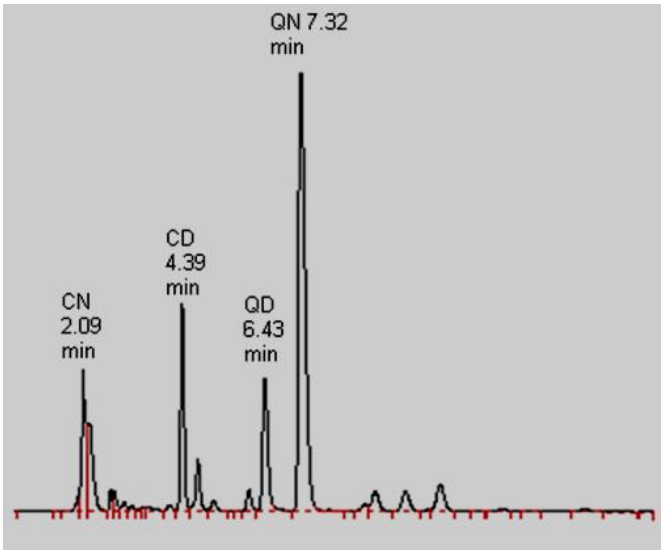


Fig. 2.5a

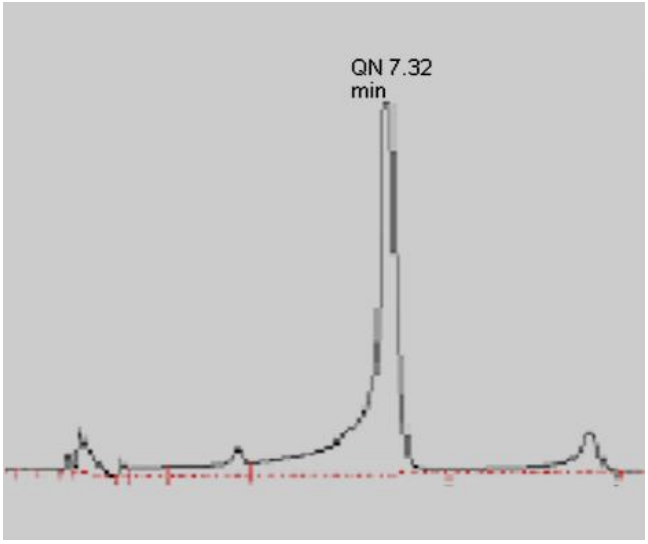


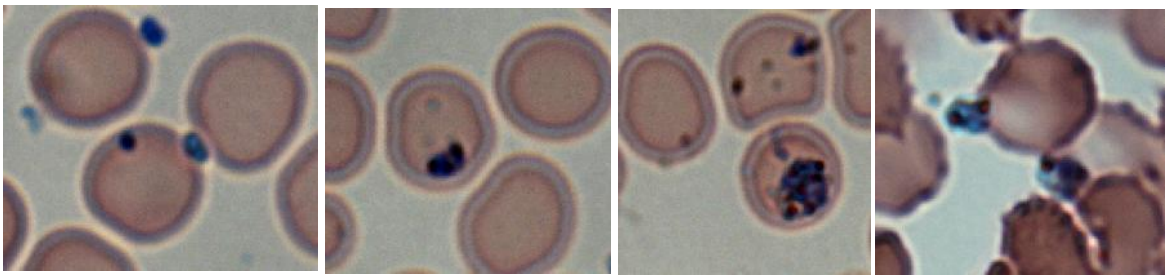
Fig. 2.5b

Fig. 2.5 a Liquid chromatogram of cinchona alkaloids; 2.5 b Liquid chromatogram of quinine

Cinchonine (CN); Cinchonidine (CD); Quinidine (QD) and Quinine (QN)

2.4.4 Parasite culture

All the stages of parasite development were found distinct from trophozoits to schizonts. Parasite stages were observed regularly after 48 hr intervals. Some distinct features of *Plasmodium falciparum* were seen like double chromatin (nucleus), multiple invasions (more than one parasite entry in RBC), maurer's dots (brown colored crystals of metabolized haemoglobin in to parasite food vacuole). The size and shape of RBCs were not altered. Figure 2.6 a, b, c and d represents stages like merozoit invasion, trophozoit, mature schizont and trophozoits with double chromatin.



Merozoit invasion to RBC
Fig. 2.5 a

Trophozoit in RBC
Fig. 2.5 b

Mature schizont
Fig. 2.5 c

Trophozoit with double chromatin

Fig. 2.5 d

Figure 2.6: Different stages of parasite development observed during culturing of *Plasmodium falciparum*

2.4.5 Antiplasmodial assay of extracts/compounds

Detailed antiplasmodial activity of medicinal plant extracts and relevant compounds are mentioned in table 2.2.

Table 2.2: Antiplasmodial activities of selected extracts and compounds

Name of extract and relevant compound	Inhibitory concentrations (IC) in µg/ml from Schizont maturation inhibition (SMI) assay	
	IC_{50}	IC_{90}
Adhatoda vasica	15.48 ± 0.34	38.21 ± 0.23
Vasicine	40.95 ± 2.11	73.01
Embelia ribes	42.19 ± 4.57	ND
Embelin	9.67 ± 0.0	31.19
Enicostemma littorale	529 ± 24.93	7390 ± 44.96
Swertiamarin	0.82 ± 0.0	1.86 ± 0.0
Cinchona alkaloids	7.54 ± 0.11	14.11 ± 0.13
Quinine dihydrochloride	0.03 ± 0.002	0.066 ± 0.006
Hesperidin	55.4 ± 0.24	140.86

n=3; Mean ± S.E.M; ND: Not detected

From results for antiplasmodial activity of extracts, we found that extract of *Adhatoda vasica* leaf inhibited 50 % of the schizont formation at concentration of 15.78 µg/ml, while, 90 % of the activity was achieved at concentration of 38.21 µg/ml (figure 2.7a). Above this range, the extract was found to lyse the culture cells. *Embelia ribes* extract also showed a significant activity having IC_{50} and IC_{75} values of 42.19 and 67 µg/ml respectively. We could not detect the IC_{90} value because there was lysis of culture cells at that range (figure 2.7b). *Enicostemma littorale* extract showed 75 % and 90 % inhibition of schizont formation from trophozoits at dose range of

1980 and 7390 µg/ml respectively (figure 2.7c). Dose dependent evaluations of all the extracts for inhibiting schizont formation from trophozoit stages are mentioned in figure 2.6. Cinchona alkaloid mixture was used as reference standard (figure 2.7d) exhibited antimalarial activity by exhibiting IC_{50} and IC_{90} of 7.54 and 14.11 µg/ml respectively.

In case of compounds, vasicine inhibited 50 % and 90 % inhibition of schizonts at dose range of 40.95 and 73.01 µg/ml respectively. Embelin was also effective at respective concentrations of 9.67 and 31.19 µg/ml. Swertiamarin exhibited highest antimalarial activity with respective concentrations of 0.82 and 1.86 µg/ml. While, hisperidin exhibited activity of 50 and 90 % at 55.4 and 140.86 µg/ml doses respectively. Quinine was used as a reference standard, also exhibited inhibition of schizont formation in dose dependent manner.

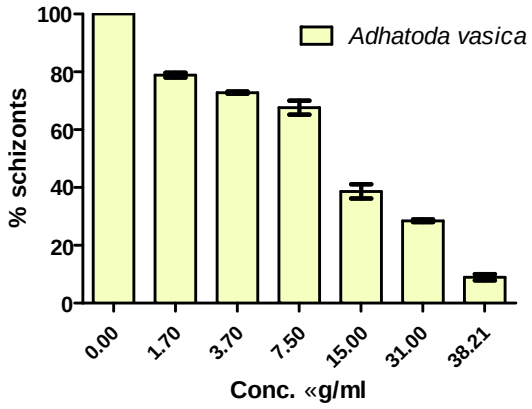


Figure 2.7a: Dose dependent inhibition of schizont formation by of *Adhatoda vasica* extract

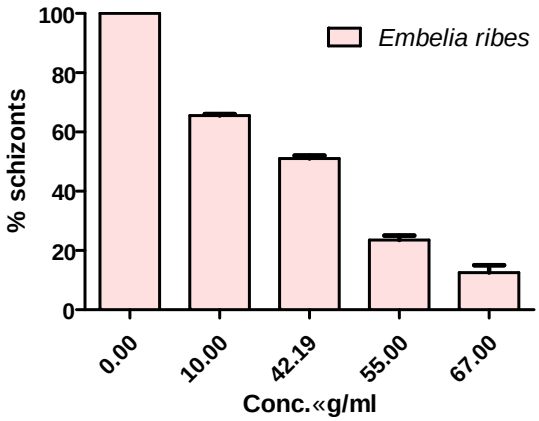


Figure 2.7b: Dose dependent inhibition of schizont formation by of *Embelia ribes* extract

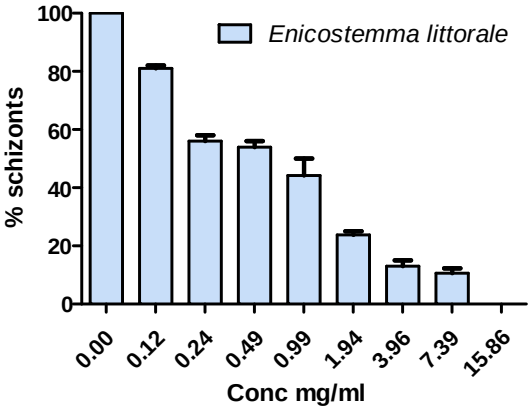


Figure 2.7c: Dose dependent inhibition of schizont formation by of *Enicostemma littorale* extract

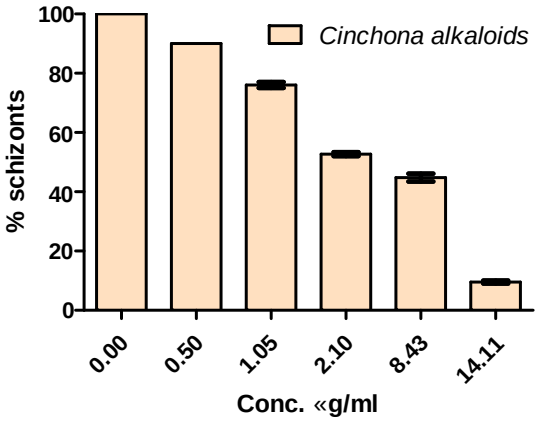


Figure 2.7d: Dose dependent inhibition of schizont formation by of *Cinchona alkaloids*

Figure 2.7: Antimalarial activity of extracts against chloroquine sensitive strain of *Plasmodium falciparum* MRC 20

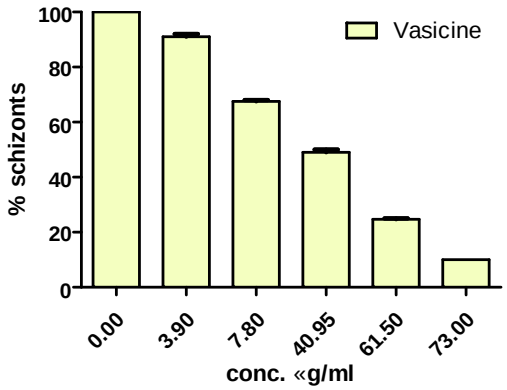


Figure 2.7a: Dose dependent inhibition of schizont formation by vasicine

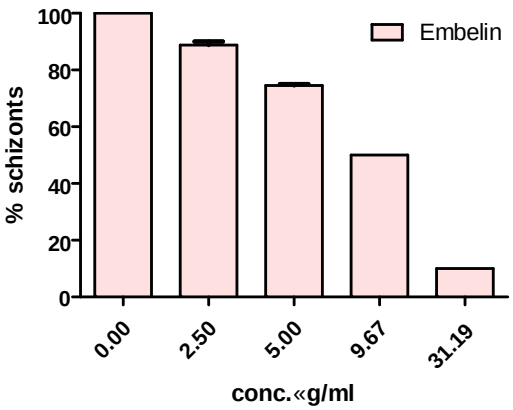


Figure 2.7b: Dose dependent inhibition of schizont formation by embelin

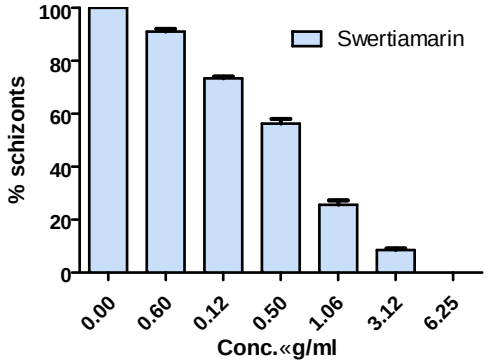


Figure 2.7c: Dose dependent inhibition of schizont formation by swertiamarin

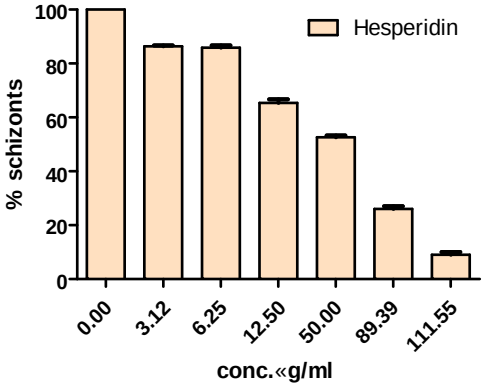


Figure 2.7d: Dose dependent inhibition of schizont formation by hesperidin

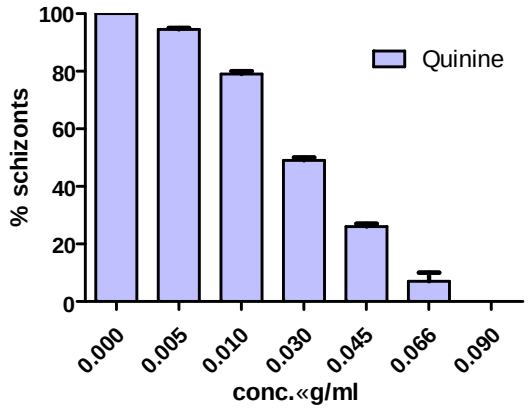


Figure 2.7e: Dose dependent inhibition of schizont formation by quinine

Figure 2.7: Comparative antimalarial activity of extracts against chloroquine sensitive strain of *Plasmodium falciparum* MRC 20

2.5 Discussion

Regarding the criteria for considering the *in vitro* antiplasmodial activity of extracts as "good", "moderate", "low" or "inactive", earlier studies by Basco and co-workers (Basco, L. *et al*., 1994; Dolabela, M. F. *et al*., 2008) had been adopted with the following criteria: IC_{50} < 10 µg/mL, good activity; IC_{50} of 10-50 µg/mL, moderate activity; IC_{50} of 50-100 µg/mL, low activity; and IC_{50} > 100 µg/mL, inactive. On the other hand, criteria for compounds were considered as having IC_{50} < 1 µM, excellent/potent activity; IC_{50} of 1-20 µM, good activity; IC_{50} of 20-100 µM, moderate activity; IC_{50} of 100-200 µM, low activity and IC_{50} > 200 µM, inactive (Batista, R. *et. al*., 2009).

Among all the tested extracts, we found extracts *Adhatoda vasica* and *Embelia ribes* were moderately active as per Basco criteria because their IC_{50} values were 15.48 and 42.19 µg/ml respectively. While, *Enicostemma littorale* extract was found as inactive. Order of antimalarial activity of extracts was *Adhatoda vasica* >*Embelia ribes* >*Enicostemma littorale.* We also found that *Adhatoda vasica* leaf extract was more effective than vasicine; a major phytoconstituent of the plant. Sometimes pure drugs that are industrially produced or isolated from plants may be chosen for their high activity against a human disease, but they have disadvantages. They rarely have the same degree of activity as the unrefined extract at comparable concentrations or dose of the active component (Wagner, H. and Ulrich-Merzenich, G. 2009). Extracts of *Adhatoda vasica* and *Embelia ribes* were also found to lyse plasmodium infected RBCs when tested further at subsequent higher concentrations than that of their respective IC_{75} and IC_{90}. So, it was the reason not to take those plants at further antiplasmodial study. Standardized mixture of cinchona alkaloids was used as a positive control in this study and represented higher activity.

While, in case of compounds, swertiamarin was found as most effective amongst all, as the IC_{50} of the compound was 2.19 µM which was in the defined range of good acting compounds as mentioned above. Embelin and vasicine were found to have IC_{50} values of 32.19 and 46.54 µM respectively, referred as moderately active. The order of activity of compounds was as swertiamarin > embelin > vasicine

> hesperidin. In case of infectious diseases, it is always advisable to prefer lowest concentration of therapeutic drug to avoid developing resistance. Swertiamarin was found to fulfil same phenomenon. Detailed microscopic observations of parasite morphology in swertiamarin treated groups also represented immature trophozoits. Hence, swertiamarin not only inhibited schizont formation but also affected maturity of young trophozoits and this was not observed with another tested compounds. Detailed literature search of embelin shows that the compound represents antifertility activity in both male and female rats (Prakash, A. O., 1981). Toxicity of the compound has also been reported as long term oral administration cause damage to liver and kindney (Prakash, A. O., 1994).

Traditional literature mentions plant *Enicostemma littorale* as "chota chirayata"; normally a substitute of chirayata (*Swertia chirata)* **(**Bamber, C.J., 1916) belonging to Gentianaceae family and is a well documented drug for treatment of malaria and also as a main ingredient of widely used polyherbal antimalarial formulations *viz.* Sudarshan ghanvati, AYUSH 64 etc. (Valecha, N. *et al.*, 2001). *Swertia chirata* contains swertiamarin as a major chemical constituent. The amount of swertiamarin reported in the plant is 0.94 % w/w (Yujoro, N. *et al*., 2006), while in *Enicostemma littorale* it is reported to be present in 7.7 % w/w (Vishwakarma, S. L. *et al*., 2004). Our present study postulated potential activity of the major compound swertiamarin, a secoirridoid glycoside of *Enicostemma littorale*. Generally irridoids are responsible for bitter taste of herbs, so also called as bitter glycosides. So, our present study also proves traditional usage of bitter plants for treatment of fever and having febrifuge properties. However, extract of *Enicostemma littorale* could not produce significant activity as compared to another samples tested in our study but its main compound produced potential activity reflects certain probabilities of antagonism by some another compounds as plant extracts are mixture of several compounds.

Lipinski rule of 5

Lipinski rule of 5 helps in distinguishing between drug like and non drug like molecules. It predicts high probability of success or failure due to drug likeness for molecules complying with 2 or more of the following rules:

- Molecular mass less than 500 Dalton
- High lipophilicity (expressed as LogP less than 5)
- Less than 5 hydrogen bond donors
- Less than 10 hydrogen bond acceptors
- Molar refractivity should be between 40-130

These filters help in early preclinical development and could help avoid costly late-stage preclinical and clinical failures.

In this context, isolated compounds used in study *viz.* swertiamarin, hesperidin, quercetin, curcumin, vasicine and embelin were also checked for their compliance to match above mentioned five criteria of Lipinski. Calculations were carried out with help of software designed and service provided by Supercomputing facility for Bioinformatics and Computational Biology – IIT Delhi. Results mentioning criteria of compounds for Lipinski rule of 5 are mentioned in below table:

Compound name	Molecular weight Dalton	H bond donor	H bond acceptor	Log P	Molar Refractivity
Swertiamarin	340	0	10	0.000	0.000
Hesperidin	572	0	14	0.000	0.000
Quercetin	292	0	7	1.866	63.329
Curcumin	384	0	3	0.000	0.000
Vasicine	176	0	1	0.000	0.000
Embelin	268	0	4	0.080	69.150

From results, it was found that all compounds were following criteria mentioned by Lipinski except hesperidin as it has more H bond acceptors and high molecular weight. Rest of the compounds were predicted for high probability of success.

2.6 Conclusion

The plant *Enicostemma littorale* had not been reported in any scientific literature for treatment of malaria. Traditional people used the same as a substitute of chirayata (*Swertia chirata*), swertiamarin is a major constituent reported in the same. We also referred that *Enicostemma littorale* is richest source of swertiamarin amongst all the Gentianaceae plants. Our results represented swertiamarin as best active antimalarial principle against chloroquine sensitive strain of *Plasmodium falciparum* (MRC 20) and these results are also supported by traditional backbone as usage of the plant in treatment of malarial fever; we decided to focus for detailed investigation of *Enicostemma littorale* (Chapter 3).

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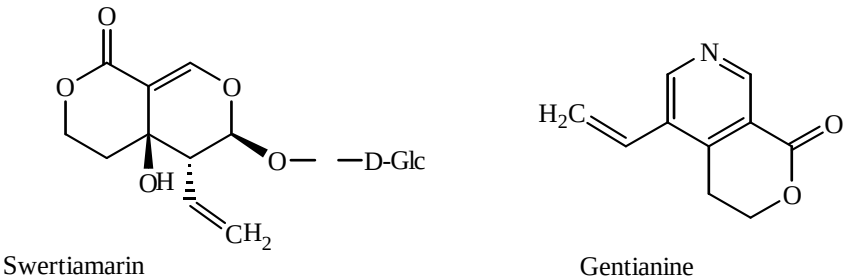
3.1 Introduction

Enicostemma littorale (Figure 3.1) is a glabrous, perennial herb, distributed throughout India including coastal region up to 450m in lower hills. Drug consists of dried whole plant (mostly root and vegetative parts) of *Enicostemma hyssopifolium* (Willd.) Verd. (syn. *Enicostemma littorale* Blume) belonging to family Gentianaceae.

A. Flowering twig **B.** Root and stem

Figure 3.1 flowering twig and root of plant

Major chemical constitutes of the plant are Swertiamarin and gentianine (Rai, J. and Thakar, K. A., 1966, Govindachari, T. R. *et al*., 1956, 1957, 1966) (Figure 3.2). While, Apigenin, genkwanin, isovitexin, swertisin, saponarin, 5-*O*-glucosylswertisin and 5-*O*- glucosylisoswertisin (Ghosal, S., Jaiswal, D.K., 1980), *n*-hexacosanol, heptacosane, nonacosane, myristic acid, stearic acid and oleic acid (Mehta, C. R. and Devani, M. B, 1959), gentiocrucine (Ghosal, S. *et al*., 1974) are also reported to be present in minor amount.



Swertiamarin Gentianine

Figure 3.2 Structures of swertiamarin and gentianine

Enicostemma littorale has been reported as a folk remedy for treatment of diabetes mellitus in Western and Southern regions of India. Various formulations

mentioned in Ayurveda – Indian system of medicine, containing *E. littorale* as one of the ingredients have been shown to produce antihyperglycemic activity (Gupta, S. *et al*., 1962). Earlier studies in our laboratory represented hypoglycaemic potential of aqueous extract in alloxan-induced diabetic rats (Vijayvargia, R. *et al*., 2000; Maroo, J. *et al*., 2002, 2003a, 2003b) and also reported it's hypoglycemic, antioxidant and hypolipidaemic potential in newly-diagnosed NIDDM patients (Vasu, V. T., *et al*., 2003). The antidiabetic effect was also reported by other workers (Murali, B. *et al*., 2002; Vishwakarma, S. L. et. al, 2003, Upadhyay, U. M. and Goyal, R. K. 2004; Srinivasan, M. *et al*., 2005). An active herbal compound SGL1 isolated from extract of the plant showed islet neogenic potential when screened for differentiation on two model cell lines PANC-1 and NIH3T3 (Gupta, S. *et al*., 2010).

Arial part of the herb had shown hypolipidemic potential in *p*-dimethylaminobenzene (*p*-DAB) induced hepatotoxic animals (Gopal, R. *et al*., 2004). The hypolipidemic and antioxidant effect of *Enicostemma littorale* aqueous extarct in cholesterol fed rats was reported first time by our laboratory (Vasu, V. T. *et al*., 2005). Later on it had been investigated that swertiamarin present in the plant possesed anti hyperlipidemic activity and can be a potential remedy for artherosclerosis (Vaidya, H. *et al*., 2009).

The herb is also known for its anti oxidant action in our laboratory (Vasu, V. T *et al*., 2005)) and others (Jaishree *et al*., 2008). Also anti anti-inflammatory (Sadique, J. *et al*., 1987) and anticancer property (Kavimani, S. and Manisenthlkumar, K. T., 2000) of the herb have been reported. The alkaloids isolated from the plant have produced hypotensive effect and depressant action on plain muscles of intestine, uterus of rat and isolated heart of frog (Sharma, S. N., 1960). The flavonoid glycosides *i.e*., swertisoside, isolated from plant potentiated the pressor responses of adrenaline and nor-adrenaline but depressed the depressor response of acetylcholine (Raj, T., 1976). The extracts of plant showed antibacterial activity against gram positive and gram negative oraganisms (Patel, R. P., 1957).

Bitter herbs from Gentianaceae family like *Swertia chirata* are reported traditionally useful in all types of fevers especially for chronic and intermittent

fevers. Ethanol extract of *Swertia chirata* has been reported for antimalarial activity (Bhat, G. P. and Surolia, N., 2001). Ayush-64 an Ayurvedic coded compound formulation consisting "*Kiratatikta-Swertia chirata*" was found to be effective in malaria (Valecha, N. *et al*, 2001). *Enicostemma littorlae* is also known as *chhota chirayata* and hot infusion of the plant is used in treatment of malarial fever in tribals of north Gujarat. In past, the plant was reported to be used as a substituent of cinchona bark and *Swertia chirata* (Bamber, C. J., 1916) for treatment of fevers. Swertiamarin is a major ingredient of *Enicostemma littorale* and it is reported that *Enicostemma littorale* is a richest source of swertiamarin (Anwar, M. *et al*., 1996). Swertiamarin is a representative of many crude drugs which are well marketed in Japan and other countries and these crude drugs are normally evaluated for higher swertiamarin content (Takei, H. *et al*., 2001). But amongst all of the plants of Gentianaceae, *Enicostemma littorale* is a richest source of swertiamarin. Swertiamarin is safe and effective with very low toxicity (Jaishree, V. *et al*., 2010).

Gentianine is another phytochemical present in plants of Gentianaceae and mainly *in Enicostemma littorale* (Valecha, N. *et al*., 2001, Rai, J. and Thakar, K. A., 1966, Govindachari, T. R., *et al*., 1956, 1957). Irridoid glucosides, when treated with ammonia, dialdehyde and ammonia react to form dihydropyridines, which are further converted to pyridines, a more stable structure. Swertiamarin and gentiopiocroside are more susceptible to this treatment since they provide good yield of stable pyridine alkaloid, gentianine (Govindachari, T. R. et.al., 1966).

Both swertiamarin and gentianine are interconversible. Previous pharmacological studies suggest that gentianine is a metabolite of swertiamarin in intestinal microflora (el-Sedawy, A. I., 1989). Gentianine was also reported for anti malarial activity (Natarajan, P. N., 974). From previous reports, it was a huge controversy on anti malarial profile of gentianine. We report that, the alkaloid is moderately effective in malaria when compared to swertiamarin. Gentianine was obtained as a product from chemical conversion of swertiamarin.

Previous studies also suggest that enzymatic hydrolysis of swertiamarin yields a low molecular weight compound, erythrocentaurin. The sugar component of known irridoid compounds isolated from plants of genus *Gentiana,* family gentianaceae, was investigated as D-glucose; however number and positions of its residue are varying (Jensen, S. R. and Schripsema, J., 2002).

In context with this, we developed rapid method for isolation and purification of swertiamarin from *Enicostemma littorale*. The compound was converted to gentianine and hydrolysed form by semi synthetic methods to achieve probable metabolites from reported studies as mentioned above. Compounds were also isolated from ethyl acetate fraction of the plant and .various isolated compounds were used for evaluation of antimalarial activity.

3.2 Materials and Methods

Part A: Preparation of extracts and compounds

3.2.1 Chemicals

All the chemicals used in this study were of analytical grade.

3.2.2 Extracts /fractions/ compounds used in study

Extracts: Methanolic extract of *Enicostemma littorale*

Fractions: Diethyl ether soluble fraction, successive ethyl acetate and butanol fractions obtained from methanolic extract of the plant**.**

Compounds: Swertiamarin, gentianine, hydrolysed form of swertiamarin, swertisin

3.2.3 Plant material and preparation of extracts

Methodology for preparation of extract is as mentioned in previous chapter (chapter 2, 2.2.3).

3.2.4 Preparation of various fractions from *Enicostemma littorale*

Various successive fractions were prepared from methanolic extract by using solvents ranging from non polar to polar. In brief, methanolic soluble fractions were pooled together and concentrated on a water bath at 60 – 80 ^{o}C. The dried mass was dissolved in 1 litre distilled water and resulting suspension was transferred to a separating funnel. The mixture was first fractionated with chloroform (1 lit. X 3). Chloroform soluble fractions were pooled together and discarded as they contained soluble plant pigments like chlorophyll and fats. The remaining solution was again fractionated with ethyl acetate (1 lit. X 3). Ethyl acetate soluble fractions were pooled together and concentrated in a beaker by regulating temperature of 70 ^{o}C. The remaining water soluble portion of methanol extract was fractionated with *n*-butanol (1 lit. X 3). *n* - Butanol soluble fractions were pooled together and dried on water bath at 90 – 100 ^{o}C to evaporate the solvent.

Fraction containing irridoids as main compounds was prepared by following method as reported by Vishvakarma (Vishvakarma, S. L., *et al*., 2004). In brief, methanol soluble dried extract (100 gm) was again dissolved in methanol and diethyl ether was slowly added to it, the precipitates so formed were filtered by watt man no. 1 filter paper. The precipitates were dried and transferred to air tight amber colour vial.

3.2.5 Isolation and preparation of compounds from *Enicostemma littorale*

3.2.5.1 Isolation of swertiamarin

We established a simple and rapid method for isolation of swertiamarin by direct crystallization after trying several processes. The developed method is commercially viable as good quantity and quality of swertiamarin can be produced. Brief process of isolation is mentioned as below.

n - Butanol fraction was concentrated to dryness to yield a semi solid mass which was further dissolved in ethyl acetate (500 ml x 3). The ethyl acetate soluble material was decanted, pooled together and evaporated on water bath not exceeding 60 ^{o}C. The concentrate of solvent yielded fluffy precipitates which were allowed to settle overnight in refrigerator. The next day, solvent was decanted and the cake of precipitate was kept on water bath for complete removal of solvent. The precipitates were again dissolved in methanol and excess of ethyl acetate was added. This process is repeated three times until pure compound swertiamarin is obtained as a white powder. As the compound was found hygroscopic, the entire process was carried out in less humid conditions and ethyl acetate used for precipitation was dried over anhydrous sodium sulphate before incorporated in butanolic fraction. The compound was placed in air tight container and kept in a cool place.

3.2.5.2 Conversion of swertiamarin to gentianine

Gentianine is a phytochemical present in plants of Gentianaceae and mainly *in Enicostemma littorale* (Valecha, N. *et al.*, 2001, Rai, J. and Thakar, K. A., 1966, Govindachari, T. R., *et al.*, 1956, 1957). Irridoid glucosides, when treated with ammonia, dialdehyde and ammonia react to form dihydropyridines, which are further converted to pyridines, a more stable structure. Swertiamarin and gentiopiocroside are more susceptible to this treatment since they provide good yield of stable pyridine alkaloid, gentianine (Govindachari, T. R. et.al., 1966). There are also some reports mentioning conversion of swertiamarin to gentianine by human intestinal microflora (el-Sedawy, A. I., 1989). This paid our attention to screen gentianine for antimalarial activity as it could be a probable metabolite when swertiamarin is ingested orally. Initially, we had isolated the compound from herb which was of a very low yield as for serial assays, more quantity is required. Later on, we tried to convert swertiamarin to gentianine as the earlier was available in major amount. The detailed methodologies are mentioned as below.

The conversion reaction was carried out as mentioned by Poppov (Popov, S. S., 1988) with little modifications. In brief, 100 mg of swertiamarin was dissolved in 10 ml of methanol. The resulting solution was saturated with ammonia to obtain pH up to 9. Then the solution was transferred to a flask attached with reflux condenser and was allowed to be refluxed for 24 hrs on water bath at temperature not exceeding 60 ^{o}C. After this, reaction mixture was concentrated up to volume of 5 ml and diluted with 10 ml of H_2O. After extraction with EtOAc, the residue were dissolved in methanol and again precipitated with EtOAc to yield gentianine. Gentianine was further confirmed by thin layer chromatography, Modified Dragendorff test for alkaloids and mass fragmentation pattern (Ghosal, S. and Jaiswal, D. K., 1974).

3.2.5.3 Hydrolysis of swertiamarin

It was previously reported that the enzymatic hydrolysis of swertiamarin (a native glucoside in *Swertia japonica*) yielded erythrocentaurin (Kariyone, T. and Matsushima, Y., 1927). So, in context with this, we hydrolyzed swertiamarin to have anlogus of erythrocentaurin. The hydrolytic reaction was carried out by usual method with 10 percent sulphuric acid. In brief, swertiamarin 100 mg was dissolved in 10 ml of methanol and slowly sulphuric acid was added. The hydrolysed sugar was separated as crystals at the bottom of the reaction flask. The solution after 24 hrs was filtered, excess of ethyl acetate was added and the precipitates so obtained were of hydrolysed swertiamarin.

3.2.5.4 Isolation of flavonoid from ethyl acetate fraction

Ethyl acetate fraction was evaporated to yield a dry mass and then washed repetitively with methanol. Methanol soluble portion was allowed to settle at cool temperature. The compound so obtained in form of white precipitates. Initially it was a mixture of two compounds. Then it was purified repetitively by washing with methanol.

3.2.6 Characterization of extracts/compounds

The extracts/compounds were characterized by various techniques as mentioned below.

3.2.6.1 Thin layer chromatography

One mg of compound was dissolved in 25 ml of methanol and applied on a plate pre coated with Silica gel GF_{254} (E. Merck). The test solution was loaded on the plate by fine glass capillary. The plate was then developed in the solvent system of Ethyl acetate: Methanol (8:2) up to 8 cm. After development, the solvent on the plate was evaporated and visualized at UV $_{254}$ nm.

3.2.6.2 Evaluation by Ultraviolet spectrometry:

One milligram of compound was dissolved in 1ml of methanol and was drawn to a quartz cuvette of 1 ml and scanned for the wavelength ranging between 200 – 800 nm on Helios UV spectrophotometer. The wavelength at maxima peak was recorded.

3.2.6.3 Evaluation by melting point

One mg of compound was taken in a capillary tube and placed in silicon oil. The temperature was continuously recorded and the point at which the compound melts was recorded.

3.2.6.4 Evaluation of the compound by mass fragmentation pattern

For mass spectral identification by ESI-MS, 1 mg of compound was dissolved in methanol and was introduced for direct fragmentation in ionization chamber in positive and negative ionization mode. The base peak was recorded and compared with the molecular weight.

3.2.6.5 HPTLC Analysis of selected extracts and compounds from Enicostemma littorale

Twenty five mg of each methanolic and butanolic extract of drug was dissolved in methanol. Methanol soluble portions were pooled together and concentrated to make up the volume of 50 ml in a volumetric flask. In another flask, 8 mg of swertiamarin standard was dissolved in 10 ml of methanol and standard solutions of 32 to 112 µg/ml concentrations were prepared by transferring aliquots (0.4 to 1.4 ml) of stock solution to 10 ml volumetric flask and adjusting the volume to 10 ml with methanol.

Ten µl of each of the standard solutions (320 to 1120 ng per spot) were applied on a precoated silica gel 60 F_{254} TLC plates (E. Merck) of uniform thickness (0.2 mm). The plates were developed in the solvent system ethyl acetate: methanol (8:2) in twin trough chamber up to a height of 8 cm and scanned densitometrically at 243 nm, the

peak area were recorded and the calibration curve was prepared by plotting peak area against concentration of the swertiamarin applied. Ten µl of the test and standard solutions were applied in triplicate on a precoated silica gel 60 F_{254} TLC plate of uniform thickness by using Linomat 5 spotter. The plate was developed in the solvent system and the peak area of swertiamarin was recorded under UV light at 243 nm using CAMAG TLC SCANNER 3. The amount of swertiamarin present in the sample was calculated from calibration curve of swertiamarin.

Part B: Antimalarial activity

3.2.7 Antimalarial activity of extracts / compounds

Detailed methodologies of parasite culture maintenance and schizont maturation inhibition assay (SMI) are mentioned in chapter 2; 2.3. Chloroquine sensitive strain was used for evaluation of antimalarial activity.

3.2.7.1 Preparation of test solutions for antimalarial assay

Five milligrams each of methanolic extract, ethyl acetate and butanol fraction were dissolved in 100 µl of DMSO. The resulting solutions were diluted with medium (without serum) up to 1ml and immediately loaded on plates. While, 1 mg each of swertiamarin, gentianine, hydrolysed form of swertiamarin and swertisin were dissolved in 100 µl of DMSO, volume was adjusted up to 1ml and after then immediately loaded on culture plates.

3.3 Results

3.3.1 Preparation of various extracts/fractions from *Enicostemma littorale*

Table 3.1 represents yields of extracts and fractions calculated gravimetrically on basis of dry weight of plant. Crude extract of the plant yielded around 14 %, while from fractions, it could be stated that plant *Enicostemma littorale* contained more amount of polar compounds as extractive values of *n* - butanol and diethyl ether fractions were measured as high.

Table 3.1: Results of the extraction of *Enicostemma littorale* with different solvents

Plant	Extraction solvent	Weight (g)	% w/w Yield
Enicostemma littorale	Methanol	136.7	13.67
	Chloroform	35	3.5
	Ethyl acetate	15.6	1.56
	n - Butanol	70	7.0
	Diethyl ether precipitates	80	8.0

3.3.2 Characterization of extract/fractions by thin layer chromatography

Figure 3.3 represents comparative thin layer chromatographic pattern of extracts and fractions. From results, it could be seen that spot around R_f 0.5 corresponded to a major compound as swertiamarin present in both methanolic extract and *n* - butanol fraction. Hence, these results give idea about presence of the compound in *n* - butanol fraction.

3.3.3 HPTLC analysis of extracts/ fractions from *Enicostemma littorale*

Amount of swertiamarin was estimated from methanolic extract and butanolic fraction by High Performance Thin Layer Chromatography and presented in table 3.2. The results are shown as percent yield of swertiamarin in both the samples.

From results, it could be seen that *n* - butanol fraction contained swertiamarin as a half of its total weight. Figure 3.4 represents recorded densitograms of methanolic extract, butanolic fraction, swertiamarin isolated and swertiamarin standard at 254 nm. The peak of swertiamarin observed at R_f 0.55 was completely overlapped with peaks of test compounds. Further, peak of swertiamarin in all test compounds was selected and scanned by CAMAG TLC scanner 3 using winCATs software (figure 3.5 a, b & c) and λmax was recorded as 244 nm corresponded to swertiamarin confirmed in each sample.

Table 3.2: Percent yield of swertiamarin in methanolic extract and *n* - butanol fraction

Extract	% Swertiamarin (w/w)
Methanolic extract	10.76
Butanol fraction	54.64

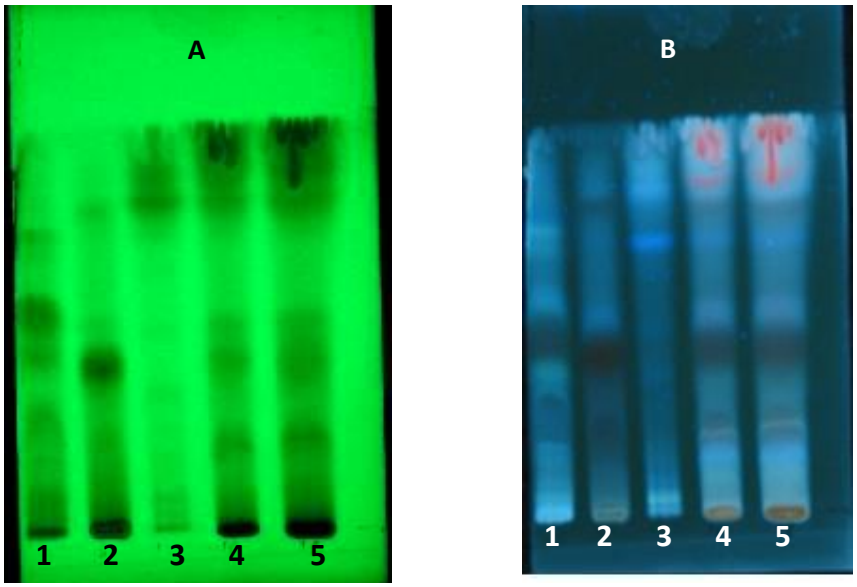


Figure 3.3: TLC pattern representing tracks: 1. Ethyl acetate; 2. *n* – butanol; 3. chloroform; 4 & 5. methanolic extract of *Enicostemma littorale*

Plates observed at: A. UV254 nm; B. UV366 nm

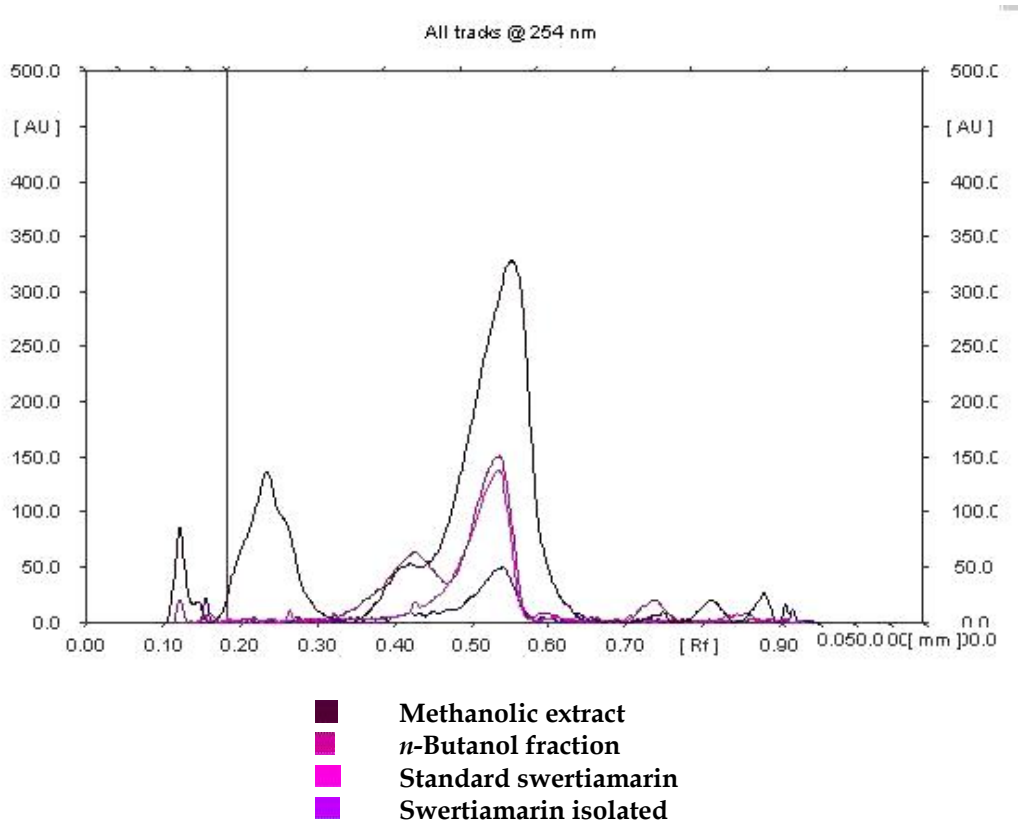
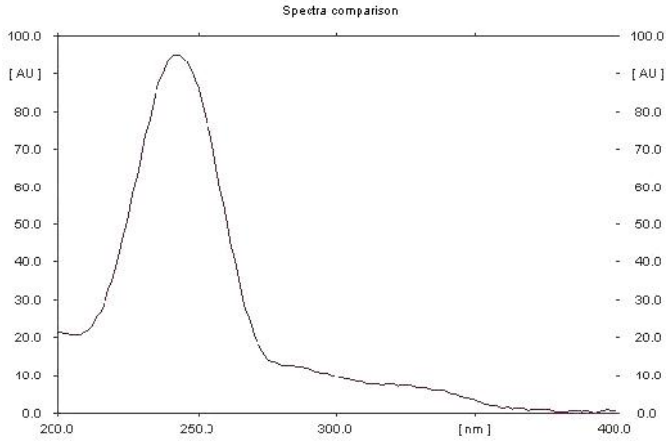
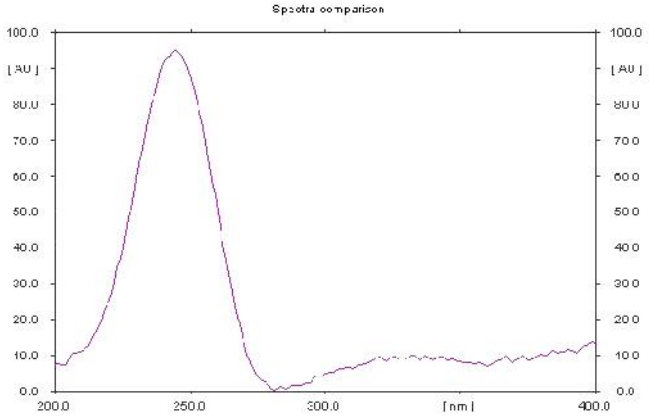


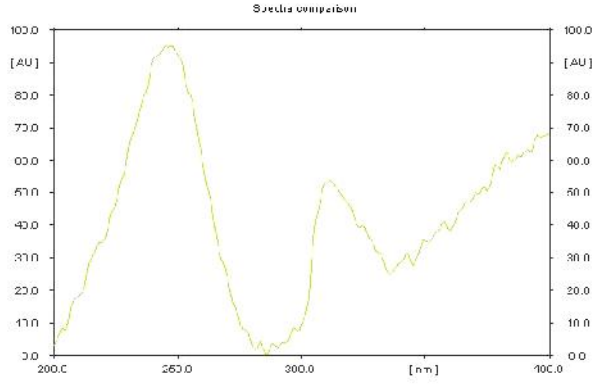
Figure 3.4 HPTLC densitograms of tracks corresponding to swertiamarin present in methanol extract and *n* - butanol fraction of *Enicostemma littorale*



(a)



(b)



(c)

All tracks @ 244 nm

Figure 3.5 Absorption spectra of swertiamarin in corresponding tracks

(a) Methanolic extract (b) *n* - Butanol fraction (c) Swertiamarin standard

3.3.4 Characterization of swertiamarin

n – Butanol fraction when treated with ethyl acetate yielded 7.31 % w/w of swertiamarin as calculated corresponding to methanolic extract. Figure 3.6 represents swertiamarin in methanolic extract, while figure 3.7 represents spots of swertiamarin from non purified to purified stage when compound was washed with ethyl acetate repetitively. HPTLC densitogram represented purity of the compound (figure 3.4) as single peak was obtained and the same was having λmax of 244 nm (figure 3.5 c). We also confirmed reference spectrum of isolated and standard compound and observed overlapping pattern (figure 3.8).

Ultraviolet absorption spectrum has shown the λmax range of 240-245 nm. Melting point of the compound was 190-192 ^{o}C. All these data were checked with the merck index (Maryadele, J. and Neil, O., 2006). Mass fragmentation pattern of the isolated compound had shown peaks at positive ion mode were of m/z 374 and after removal of hexose sugar, 212 (M -162).

3.3.5 Characterization of gentianine

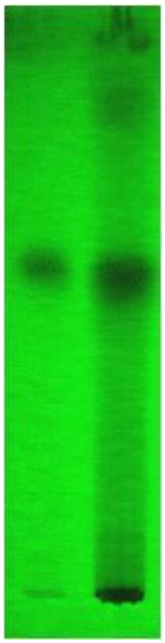
Swertiamarin was converted to gentianine (figure 3.9a) when treated with ammonia solution of pH 9. From 100 mg of compound, 27 mg of gentianine was obtained (27 %). The whole reaction is represented on TLC plate (figure (3.9b, plate A & B.)). Figure (3.9b, plate C) represents the positive dragendorff test for presence of alkaloids. The compound was further confirmed by HPTLC densitogram (figure 3.10a) as a major peak at R_f of 0.86 and having λmax of around 220 nm (figure 3.10b). The compound was also confirmed by mass fragmentation pattern (ESI-MS) showing two main peaks at m/z 158 (base peak, M-H_2O) and 176 (M+H) at positive ionization mode, while 175 (M-H) at negative ionization mode, which corresponded to molecular weight of gentianine.

3.3.6 Hydrolysed form of swertiamarin

The hydrolysed form of swertiamarin had produced a white stable compound, giving a spot at R_f 0.65 a higher than swertiamarin (R_f 0.55) when the plate was developed in the solvent system ethyl acetate:methanol (8:2) (figure 3.11). The compound was completely soluble in methanol.

3.3.7 Compound isolated from ethyl acetate fraction

The ethyl acetate fraction was evaporated at room temperature. Precipitates were obtained when the volume of the solvent was reduced to half. The solvent was decanted and the precipitates were washed with methanol. This process yielded mixture of compounds which were purified with repetitive washes of methanol. Yield of the compound was approximately 500 mg. The compound was confirmed with HPTLC (Figure 3.12a & b) with recorded λmax of 344 nm and Mass fragmentation pattern represents base peak of m/z 447 (M+H) and 326 (M+H-120) as another major peak at positive ionization mode, while, base peak of 445 (M-H) at negative ionization mode.



Swertiamarin

Figure 3.6: TLC pattern representing tracks 1. Swertiamarin; 2. Methanolic extract of *Enicostemma littorale*

Figure 3.7: Spots of swertiamarin from non purified to purified stage (left to right)

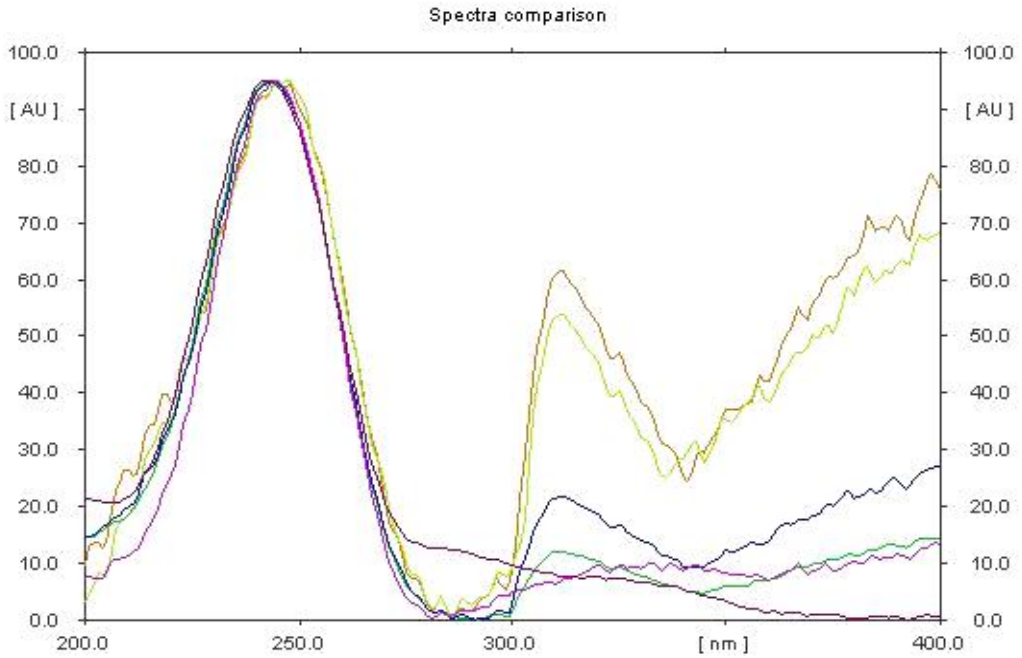
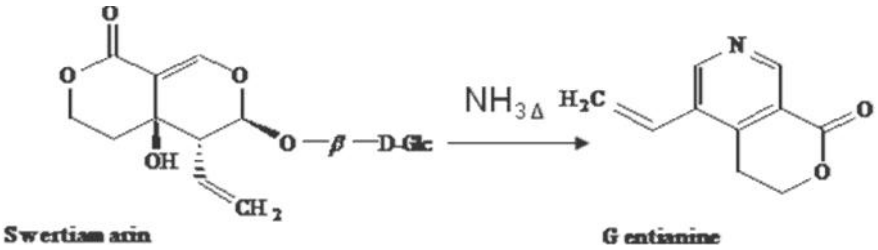


Figure 3.8 Overlay of absorption spectra of swertiamarin isolated in lab and reference standard



3.9 a

3.9b

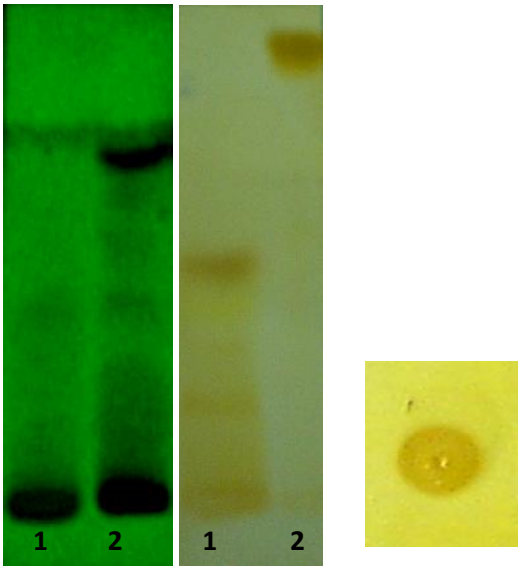


Figure 3.9 a & b: Conversion of swertiamarin to gentianine

Tracks: 1. Reaction mixture containing swertiamarin and ammonia at 0 time; **2**. Final product formed after 24 hrs

Plates: A. Visualized at UV_{254} nm; B. with iodine in natural light; C. Derivatized with dragendorff reagent

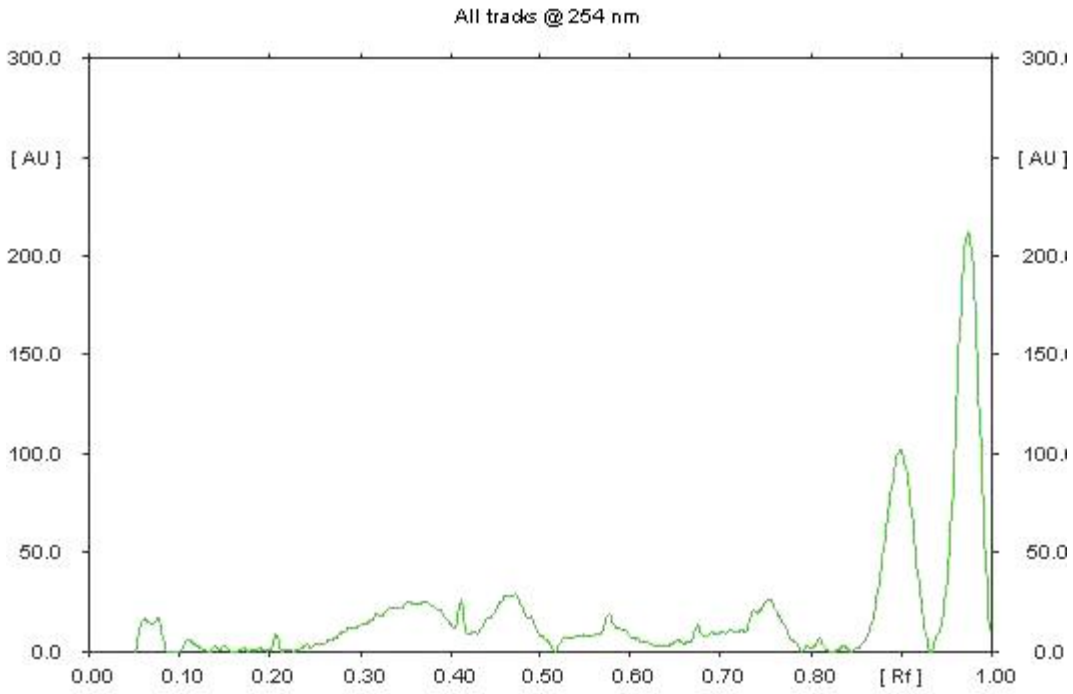


Figure 3.10 a: HPTLC densitogram of gentianine

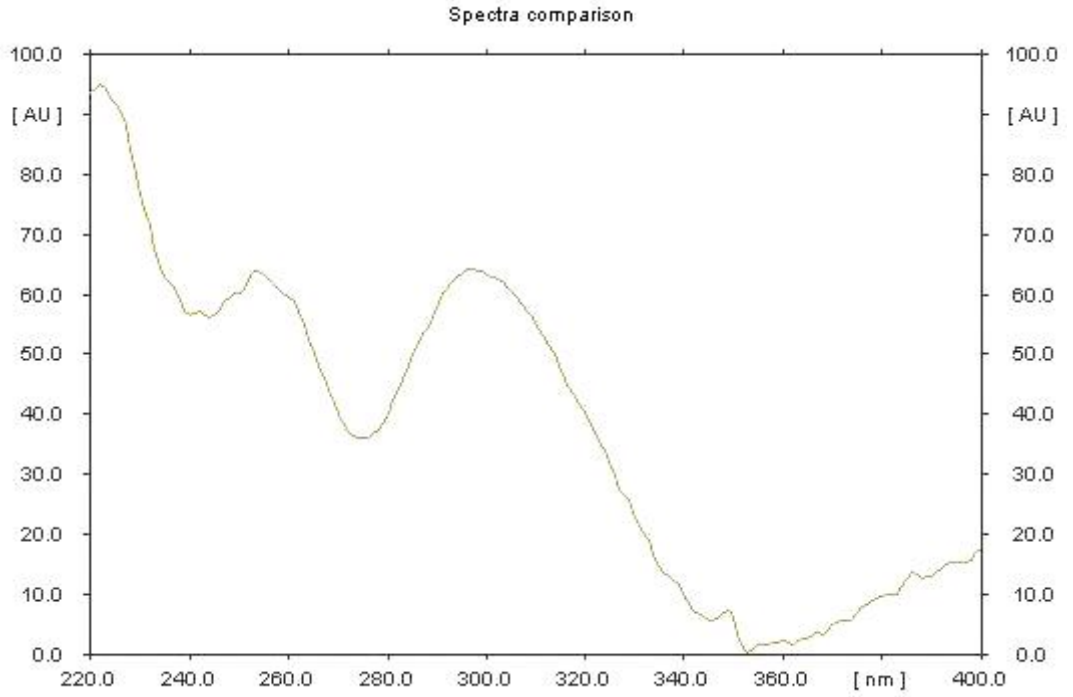


Figure 3.10b: HPTLC spectrum of gentianine

Figure 3.11 TLC of hydrolysed form of swertiamarin; derivatized by anisaldehyde sulphuric acid reagent and observed at natural light

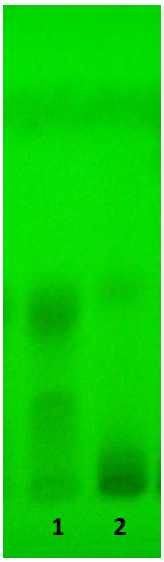


Figure 3.12a Tracks: 1. Precipitates from ethyl acetate fraction; **2,** purified swertisin, visualization UV_{254} nm

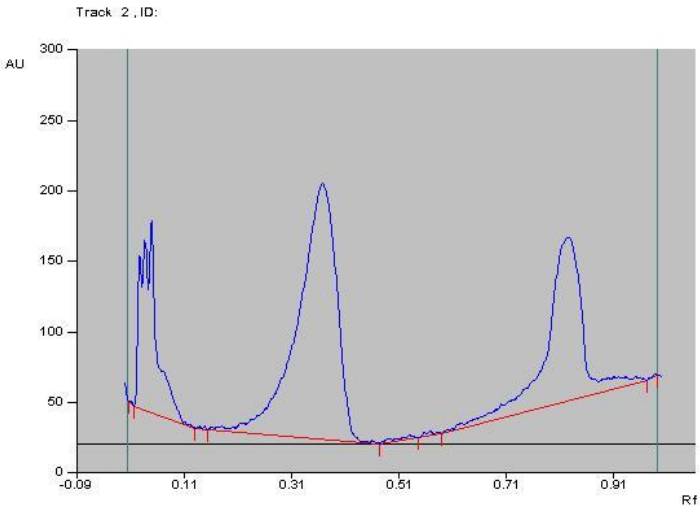


Figure 3.12b: Densitogram of swertisin by HPTLC

3.3.8 *In vitro* antimalarial activity of *Enicostemma littorale* extracts/fractions

Dose dependent antimalarial activities of methanolic extract, irridoid precipitates and *n* - butanol fraction are presented in figure 3.13 when they were evaluated for inhibition of formation of schizonts from trophozoits. Inhibitory concentrations of the same are represented in table 3.3. Methanolic extract of *Enicostemma littorale* was found to produce 100 percent inhibition at concentration of 15.875 mg/ml (figure 3.13a). Irridoids precipitated from the methanolic extracts produced 64 percent inhibition, when the last dose was tested as 7.74 mg/ml (figure 3.13b). *n* – Butanol fraction was at concentration of 0.74 mg/ml could inhibit 100 percent growth of schizonts (figure 3.13c). We also found dead parasites in *n* - butanol treated fraction as unstained parasite in cells when treated with giemsa solution and observed microscopically (figure 3.14). From results, it could be seen that *n* - butanol fraction exhibited highest antimalarial activity amongst all. The order of activities are as *n* – butanol > irridoid mixture > methanolic extract. If we refer results as discussed in part A of this chapter, it was clear that *n* – butanol fraction contained swertiamarin at higher concentration; around half of its extract weight. So, it could be predicted that increase order of activity from extract to fractions was directly proportional to amount of major compound present in them.

Table 3.3 Antiplasmodial activity of extract/fractions

Name of extract/fraction	Inhibitory concentrations (IC) in mg/ml from Schizont maturation inhibition (SMI) assay		
	IC_{50}	IC_{75}	IC_{90}
Methanolic extract	0.529	1.98	7.93
Irridoid mixture	2.13	NA	NA
n – Butanol fraction	NA	0.046	0.185

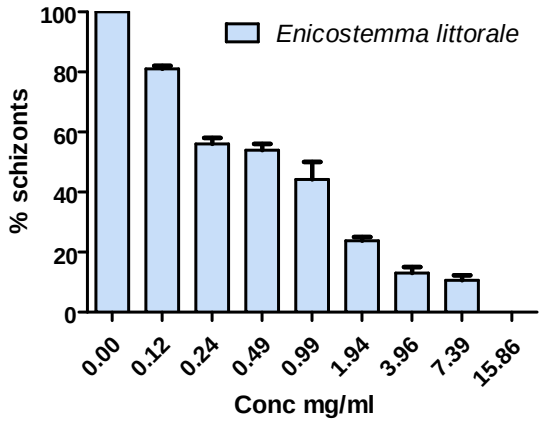


Figure 3.13a: Dose dependent inhibition of schizont formation by *Enicostemma littorale* extract

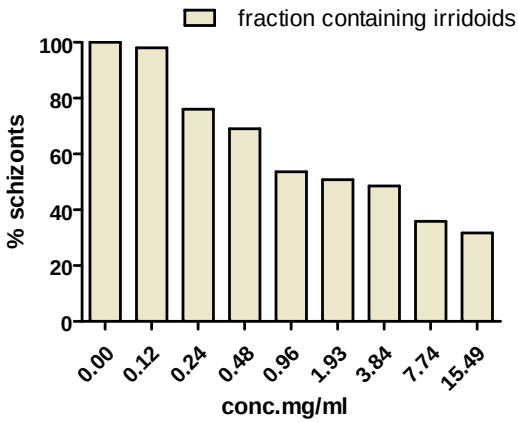


Figure 3.13b: Dose dependent inhibition of schizont formation by fraction containing irridoids

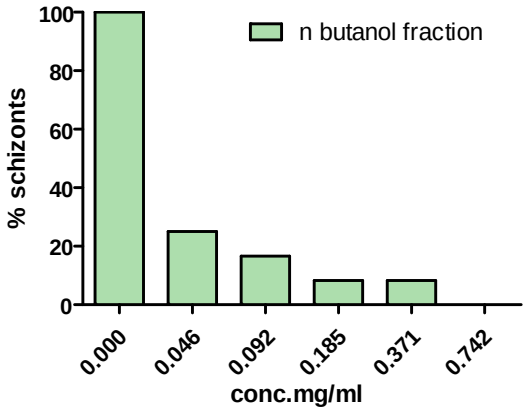


Figure 3.13c: Dose dependent inhibition of schizont formation by *n*- butanol fraction

Figure 3.13: Evaluation of antimalarial activity of extracts/fractions derived from *Enicostemma littorale*

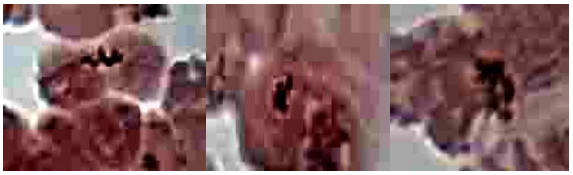


Figure 3.14: Parasites found dead when treated with *n* - butanol fraction

3.3.9 *In vitro* antimalarial activity of compounds from *Enicostemma littorale*

Evaluation of swertiamarin, gentianine and hydrolysed form of swertiamarin was carried out against culture of chloroquine sensitive strain of *Plasmodium falciparum* at 48 hrs of post infection containing 1 - 1.5 % parasitemia. Dose response curves were plotted. Figure 3.15 mentions antimalarial activity of these three compounds in dose dependent manner, while their inhibitory concentrations are presented in table 3.4. From results, it was found that swertiamarin could produce inhibition of schizont formation at dose of 0.82 µg/ml while, gentianine exhibited 50 % inhibition at dose around 11 µg/ml and hydrolysed form of swertiamarin was effective with dose around 3 µg/ml to produce 50 % inhibition in schizont maturation inhibition assay. Hence, swertiamarin was the most effective compound amongst all the tested ones. From results, it could be seen that gentianine was found moderately effective as compared to another compounds. From this study, it can be hypothyzed that swertiamarin, when ingested orally, can be hydrolysed at acidic medium and converted to gentianine at basic medium. Our present investigation postulates that efficacy of the compound may decrease if it is given orally. *In vitro* data suggested around four fold decreases in efficacy of acidified form and 10 fold decreases in basified form. Detailed microscopic observations of parasite developmental stages represent that in swertiamarin treated groups immature ring stages were seen as it inhibited the development of young trophozoits to the mature ones. While, these results were not obtained with gentianine and hydrolyzed form of swertiamarin treated groups.

Swertiamarin was further investigated by incubating with post infection of 48 hrs of plasmodium culture with higher parasitemia of 3 to 4 percent in a stage specific manner. All the parasites developmental stages were observed and counted as relative percentage of trophozoits and schizonts per field (fig.3.16). Comparative time and dose dependent studies were also carried out on parasite cultures with lower parasitemia (1-1.5%) for 24 hrs incubation and with higher parasitemia (3-4%) for the incubation period for 48 hrs (table 3.5). The microscopical representation of

activity of compound on parasite stages in control group *vs* drug treated group is mentioned in figure 3.17.

Table 3.4 Antiplasmodial activity of compounds

Name of Compound	Inhibitory concentrations (IC) in µg/ml from Schizont maturation inhibition (SMI) assay Mean ± S.E.M.		
	IC_{50}	IC_{75}	IC_{90}
Swertiamarin	0.82 ± 0.29	1.86 ± 0.27	2.49 ± 0.6
Gentianine	11.53 ± 4.26*	19.52 ± 7.17	15.42 ± 8.91
Hydrolysed form of swertiamarin	2.93 ± 1.09	14.01 ± 10.7	36.63 ± 13.06
Quinine	0.03 ± 0.002	0.045 ± 0.0	0.066 ± 0.03

***ns** Not significant, $p < 0.05$; p values derived from comparison with reference standard quinine (n=3)**

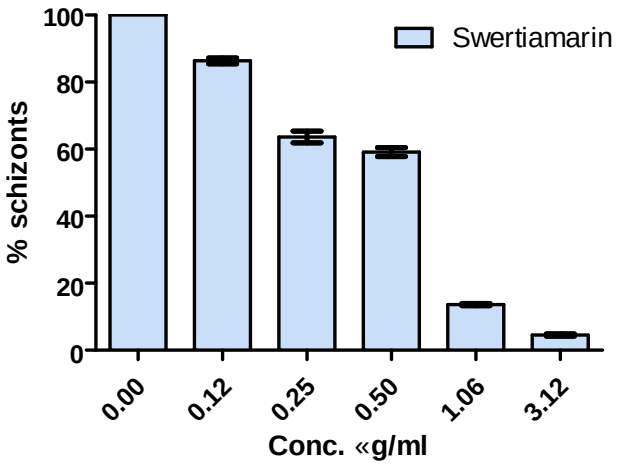


Figure 3.15a: Dose dependent inhibition of schizont formation by swertiamarin

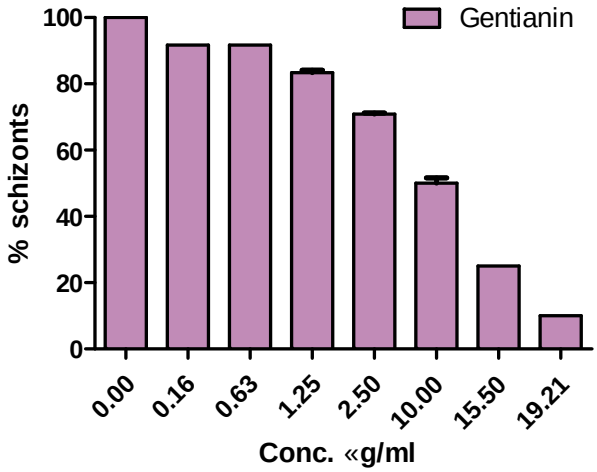


Figure 3.15b: Dose dependent inhibition of schizont formation by gentianin

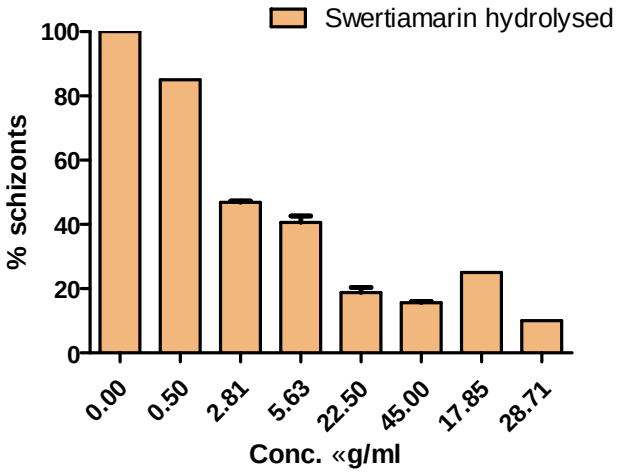


Figure 3.15c: Dose dependent inhibition of schizont formation by swertiamarin hydrolysed

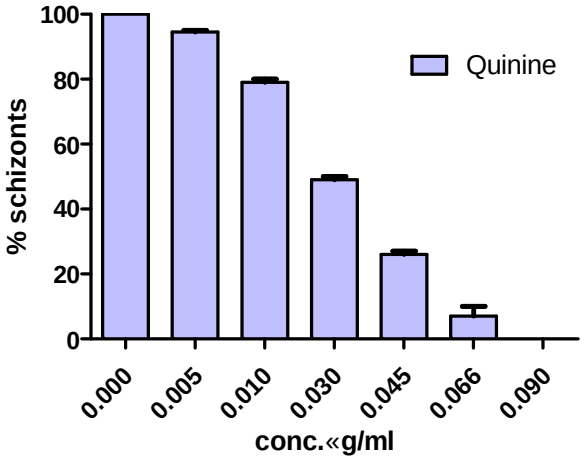


Figure 3.15d: Dose dependent inhibition of schizont formation by quinine

Figure 3.15: Evaluation of antimalarial activities of compounds derived from *Enicostemma littorale*

Table 3.5: Time dependent antimalarial activity of swertiamarin on higher and lower parasitemia of chloroquine sensitive strain *Plasmodium falciparum* MRC 20.

Swertiamarin	Inhibitory concentrations (IC) in µg/ml from Schizont maturation inhibition (SMI) assay Mean ± S.E.M.		
	IC_{50}	IC_{75}	IC_{90}
After 24 hrs in 1 -1.5 % parasitemia	12	19.79	25.29
After 48 hrs in 3 -4 % parasitemia	0.82	1.86	2.49

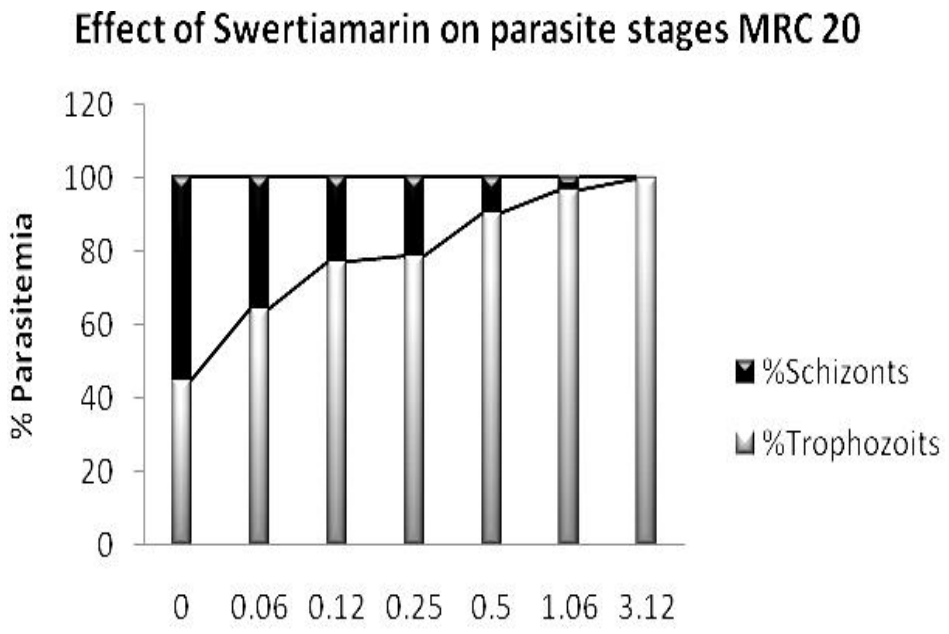
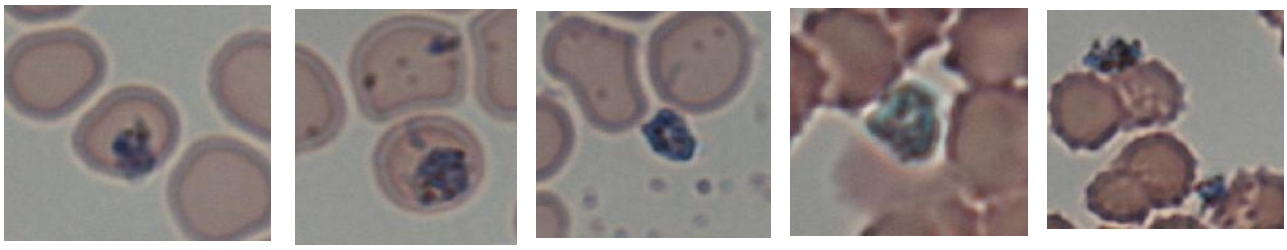


Figure 3.16: Dose dependent activity of swertiamarin on 48 hrs of post infection

Schizonts in untreated groups



Trophozoit stages in swertiamarin treated groups

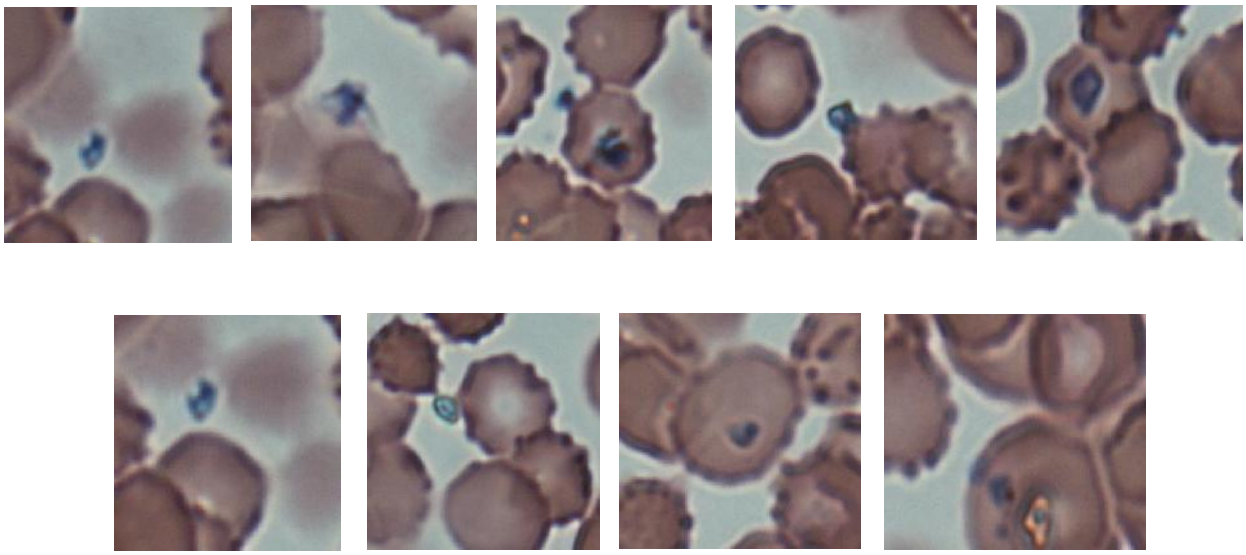


Figure 3.17: Inhibition of schizont formation from trophozoits by swertiamarin when tested on chloroquine sensitive strain of *Plasmodium falciparum* (MRC 20)

3.4 Discussion

Traditional literatures mention use of bitter herbs for treatment of chronic and intermittent fevers like *vishamjvara* (malaria). *Swertia chirata* is one of the potential remedy of malarial fever which contains swertiamarin as a major phyto constituent. Until now this compound remained untouched for its antimalarial investigation. *Enicostemma littorale*, belonging to gentianaceae family, is also a richest source of swertiamarin (Anwar, M. *et al.*, 1996) and it has been studied as a safe and effective compound with very low toxicity. Our present investigation represents *n* - butanol fraction of the plant having good schizonticidal activity, so this encouraged us to further investigate this fraction. We also evaluated ethyl acetate fraction and a flavonoid; swertisin isolated from the same but could not achieve antimalarial activity. Swertiamarin was later on isolated from *n* - butanol fraction possessed potential antimalarial activity by inhibiting formation of schizonts from trophozoits (ring stages). The effective concentration for 50 % inhibition of schizont formation was found as 0.82 µg/ml. This is the first scientific report of the plant having antimalarial activity from our laboratory (Soni, S. and Gupta, S., 2009). Previous methods for isolation of swertiamarin were time consuming with certain conventional techniques like column chromatography. We developed a more commercially viable method to isolate the compound which can be used at industrial scale to achieve the compound with good purity and quantity. To search more leads from this plant, we focused on gentianine as another important phytoconstituent of majority of gentianaceous plants and was previously reported for antimalarial activity (Natarajan, P. N., 1974). Initially, we isolated the compound from plant but could not get good yield. So, we further reviewed possibilities to get the compound in good quantity. Later on, swertiamarin was further converted to gentianine by treating with ammonia and purification with suitable solvents like ethyl acetate yielded around 27 % of the compound. The compound was found moderately active as IC_{50} was 10 µg/ml when tested on *Plasmodium falciparum* culture. There was no change in parasite morphology in drug loaded culture wells. Hence, our present investigation represents gentianine as a less active antimalarial when compared with

swertiamarin. Literature reviews also represents that enzymatic hydrolysis of swertiamarin yields a low molecular weight compound, erythrocentaurin. The sugar component of known irridoid compounds isolated from plants of genus *Gentiana,* family gentianaceae, was investigated as D-glucose; however number and positions of its residue are varying (Jensen, S.R. and Schripsema, J., 2002). In context with this, we hydrolysed swertiamarin with help of strong acid and also investigated antiplasmodial activity of hydrolysed form of swertiamarin against chloroquine sensitive culture of *Plasmodium falciparum* (MRC 20). Form present investigation, we found that the compound is effective at EC_{50} of 2.81 µg/ml. This concentration was approximately 3-4 times higher than that of swertiamarin. So, it can be considered that the sugar moiety attached with compound is essential for activity as parasite identifies sugar as a source of energy. Hence, the present study postulates that swertiamarin can only the effective antimalarial as it is in intact form found in nature.

3.5 Conclusion

From detailed investigation of many fractions of *Enicostemma littorale*, we found that *n* - butanol fraction was showing good activity, swertiamarin was found maximum in the same. We developed a rapid method of isolation of swertiamarin. Later on, amongst all the compounds isolated from *Enicostemma littorale*, swertiamarin was proven a potential anti malarial candidate. Although, gentianine was considered as a main constituent of Gentianaceae family, could not give good antimalarial activity. Hence we further investigated swertiamarin against chloroquine resistant strain of *Plasmodium falciparum* (RKL 19) to check its efficacy against chloroquine resistance. This is mentioned in next chapter.

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4.1 Introduction

4.1.1 *Drug resistance in malaria*

Antimalarial drug resistance has been defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject. In other way it is a gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action". Resistance to antimalarial drugs can cause treatment failure with respect to many responsible factors like incorrect dosing, non compliance to the dosing regimen, poor quality drug or drug interactions, poor diagnosis etc.

Resistance to antimalarial drugs has been demonstrated as a worsening problem throughout the world. *Plasmodium falciparum* has developed resistance to nearly all antimalarials in current use. The appearance of drug-resistance *P. falciparum* strains since 1960, in particular to chloroquine, has made the treatment of malaria increasingly problematic in virtually all malarious regions of the world (Winter, R. W. *et al*., 2006). Massive use of chloroquine, a golden and cost effective treatment for malaria resulted in resistance to *P. falciparum* parasites everywhere in the world and this was transmitted to various malarious areas like Central America (north-west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia. Mefloquine resistance is also frequent in some areas of South-East Asia and has been reported in the Amazon region of South America and sporadically in Africa (Mockenhaupt, F.P., 1995). Significant resistance has also been reported to chloroquine in Indian subcontinents (Bloland, P. B., 2001). In 2007, the Indian National Vector-borne Disease Control Programme (NVBDCP) reported 1,502,742 malaria cases and 1,274 deaths. However, cases and especially deaths may be under-reported. Reasons for under-reporting include management of two thirds of the patients by the private sector, absence of routine death certification in rural areas and poor infrastructure for malaria diagnosis in public health care facilities.

It is believed that resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization (Foley, M. and Tilley, L., 1997). This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones (Krogstad, D. J. *et al*., 1987). Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system (Martin, S. K. *et al*., 1987). It is unclear whether parasite resistance to other quinoline antimalarials (amodiaquine, mefloquine, halofantrine, and quinine) occurs via similar mechanisms (Mockenhaupt, F. P., 1995). Another important mechanism of resistance is as the malaria parasite digests haemoglobin; large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin (malaria pigment).

Several researchers have dedicated efforts to the development of new active compounds, especially from artemisinin, as an alternative to chloroquine. Currently no single drug is effective for treating multi-drug resistant malaria, and effective combination therapy includes artemisinin derivatives such as artesunate, or mixtures with older drugs such as the atovaquone – proguanil combination Malarone® (Winter, R. W. *et al*., 2006, Taylor, W. R. and White, N. J, 2004). Unfortunately first reports on drug resistance to artemisinin-derivatives (Jambou, R. *et al*., 2005) and to drug combination therapies (Wichmann, O. *et al*., 2004) have already appeared. So, in the absence of a functional, safe and widely available malaria vaccine, efforts to develop new antimalarial drugs urgently needed now.

4.1.2 *Role of irridoids and flavonoids in malaria*

There is a consensus among the scientific community that natural products have been playing a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases (Newman, D. J., 2002). Indeed, the vast majority of the existing antimalarial chemotherapeutic agents are based on natural products, and this fact anticipates that new leads may certainly emerge from the

tropical plant sources, since biological chemo diversity continues to be an important source of molecular templates in the search for antimalarial drugs (Ziegler, H. L. *et al*., 2002**,** Kalauni, S. K. *et al.,* 2006**,** Portet, B. *et al.,* 2007**)**.

(A) ***Irridoids***

Previous studies suggest that irridoids and terpenoids are important plant agents with promising antimalarial activity. Phenylpropanoid conjugated iridoids were isolated from *Morinda morindoides* (Rubiaceae) and found to inhibit parasite proliferation with little cytotoxicity (Tamura, S. *et al*., 2010). The iridoid swertiamarin had been obtained from *Enicostemma littorale* (Gentianaceae) and showed promising results *in vitro* in a schizont maturation inhibition assay, with an IC_{50} value of 2.19 µM as discussed in chapter 2 (Soni, S. and Gupta, S., 2009). Thus, by considering its potentiality, we decided to move further with this molecule against chloroquine resistant strains of *Plasmodium falciparum*.

(B) ***Flavonoids***

We focused flavonoids, secondary plant metabolites having vast structural diversity. Flavonoids comprise a large group of naturally occurring, low molecular weight and polyphenolic compounds widely distributed in the plant kingdom as secondary metabolites. A number of reports have demonstrated growth inhibitory effects of flavonoids, particularly of the flavonol quercetin and of the flavone luteolin, on the protozoan parasite genera *Toxoplasma* (Weiss, L. M. *et al*., 1998), *Trypanosoma* (Mamani-Matsuda, M. *et al*., 2004) and *Leish- mania* (Kirmizibekmez, H. *et al*., 2004, Mittra, B. *et al*., 2000, Sen, G. *et al*., 2005). The majority of studies involving flavonoids and malaria describe the antiplasmodial activity-guided fractionation of plants.

Flavonoids (usually along with other compounds) have been identified exhibiting antiplasmodial activity in fractions of many plants, and in some cases have been shown to possess antiplasmodial activity when isolated (Kraft, C. *et al*., 2001; Liu, K. C. S. C. *et al*., 1992; Yenesew, A. *et al*., 2004).

A brief literature review of flavonoids quercetin, hesperidin and curcumin mentioning their therapeutic importance in terms of efficacy and safety are mentioned as below.

Quercetin

The most common flavonol in the diet is quercetin, belonging to a group of polyphenolic substances known as flavonoids and bioflavonoids. Quercetin is present in various fruits and vegetables like onions, tea, apple and berries (Hertog, M. G. L. *et al*., 1992; Häkkinen, S. H. *et al*., 1999). In the United States, the estimated intake of flavonols and flavones was 20 to 22 mg/d, of which 73% to 76% was quercetin (values are for women and men) (Sampson, L. *et al*., 2002).

Quercetin has been reported to exhibit antioxidative (Hayek, T. *et al*., 1997, Chopra, M. *et al*., 2000), anticarcinogenic (Verma, A. K. *et al*., 2000, Deschner, E. E. *et al*., 1991, Pereira, M. A. *et al*., 1996), anti-inflammatory (Ferry, D. R. *et al*., 1996), anti-aggregatory (Pignatelli, F. M. et., al., 2000) and vasodilating (Perez-Vizcaino, F. *et al*., 2002) effects. Antioxidant property of quercetin could be a result of metal chelation (Ferrali, M. *et al*., 1997, Sestili, P. *et al*., 1998), scavenging of radicals (Huk, I. *et al*., 1998, Aherne, A. and O′ Brein, N. M., 2000), enzyme inhibition (Da Silva, E. L. *et al*., 1998, Nagao, A. *et al*., 1999), and/or induction of the expression of protective enzymes (Nagao, A. *et al*., 1999). Anticarcinogenesis, on the other hand, could result from enzyme inhibition, (Agullo, G. *et al*., 1997, Huang, Z. *et al*., 1997), ant oxidation, or effects on gene expression (Hansen, R. K. *et al*., 1997, Piantelli, M. *et al*., 2000, Xiang, N. *et al*., 2001). Altered gene expression could lie behind the anti-inflammatory effect as well (Kobuchi, H. *et al*., 1999). Regarding anti carcinogenesis, it should be noted that in the 1970s, quercetin was actually considered a carcinogen because the compound showed mutagenicity in the Ames test (Bjeldanes, L. F. and Chang, G. W., 1977). However, a number of long-term animal studies subsequently performed with different species have indicated that this is not the case. On the contrary, quercetin has been shown to inhibit carcinogenesis in laboratory animals (Stavric, B., 1994). During the past few years, some reports on the biological activities

of potential quercetin metabolites have been published. In these studies, it has been shown that the site and/or type of conjugation are important determinants of its antioxidant activity (Manach, C. *et al*., 1998, Moon, K. *et al*., 2001, Day, A. J., 2000).

Absorption of quercetin in ileostomists was quantified by Hollman (Hollman, P. C. H. *et al*., 1997) and it was observed that it was 52 ± 5% for quercetin glucosides from onions, 17 ± 15% for quercetin rutinoside, and 24 ± 9% for quercetin aglycone. The plasma quercetin concentration in subjects with an intact colon, after ingestion of fried onions, apples and pure quercetin rutinoside, decreased slowly with elimination half-lives of about 25 h. Thus, repeated dietary intake of quercetin will lead to accumulation in plasma. It is also reported as bioenhancer for some of the important calcium channel blocker agents like verapamil, with post administration, the Cmax and AUC of verapamil was increased two folds (Choi, J. S. and Han, H. K., 2004).

Hesperidin

Hesperidin, a flavonol, is an abundant and inexpensive by-product of Citrus cultivation and is a major flavonoid in sweet orange and lemon. In young immature oranges it can account for up to 14 % of fresh weight of fruit (Barthe, G. A. *et al*., 1988), Hesperidin is a important compound with diversified pharmacological activities *viz.* Decreasing in capillary permeability, chronic venous insufficiency, anti hypercholesterolemic, anti-inflammatory and analgesic activities (Bisset, N. G. *et al*., 1991; Struckmann, J. R. *et al*., 1994; Son, H. S. *et al*., 1991; Galati, E. M. *et al*., 1996). It is also reported to possess anti viral, antifertility, anticarcinogenic, immunomodulatory, anti oxidant, antiallergic, wound healing, anti ulcer and antihaemmorhoidal activities (Middleton, E. J., 1984; Joyce, C. L. and Zaneveld, L. J. D., 1985; Yang, M. *et al*., 1997; Kawaguchi, K. *et al*., 1999; Bouskela, E. *et al*., 1995; Kubo, M. *et al*., 1992a; Suarez, J. *et al*., 1996; Hasanoglu, A. *et al*, 2001; Daftary, S. N. *et al*., 1995).

Citrus bioflavonoids including hesperidin appear to be extremely safe and without side effects even during pregnancy (Pizzorno Jr, J. E. and Murray, M. T., 1999). Based on an experiment in male and female mice (Sieve, B. J., 1952), it was established that phosphorylated hesperidin (PH) was nontoxic both to the organism and to tissues, easily assimilated, non accumulative and caused no allergic reactions.

Hesperidin, when administrated along with diltiazem and verapamil, increased the half life of the drugs (Cho, Y. A. *et al*., 2009, Pio, Y. J. and Choi, J. S., 2008).

Curcumin

Turmeric is used in Indian tradition from a long period in food as well as in medicine. In old Hindu medicine, it had been used in swelling caused by injury (Ammon, H. P. T. and Wahl, M. A., 1991). Curcumin is a main active constituent of turmeric (3-4 %) and responsible for its yellow colour. The antioxidant activity of curcumin was reported (Sharma, O. P., 1976) as early as 1974. It acts as a scavenger of oxygen free radicals (Ruby, A. J. *et al*., 1995, Subramanian, M. *et al*., 1994). It can protect haemoglobin from oxidation. *In vitro*, curcumin can significantly inhibit the generation of reactive oxygen species (ROS) like superoxide anions, H_2O_2 and nitrite radical generation by activated macrophages, which play an important role in inflammation (Joe, B. and Lokesh, B., 1994). Curcumin also lowers the production of ROS *in vivo*. Curcumin exerts powerful inhibitory effect against H_2O_2-induced damage in human keratinocytes and fibroblasts (Phan, T. T. *et al*., 2001) and in NG cells (Mahakunakorn, P. *et al*., 2003). Curcumin reduces oxidized proteins in amyloid pathology in Alzheimer transgenic mice (Lim, G. P. *et al*., 2001). It also decreases lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy, Ach. and Lokesh, B. R., 1994). This is brought about by maintaining the activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidise (Pulla Reddy, Ach. and Lokesh, B. R., 1992). It was observed that curcumin prevents oxidative damage during indomethacin-induced gastric lesion not only by blocking inactivation of gastric peroxidase, but

also by direct scavenging of H_2O_2 and ·OH (unpublished observation). Since ROS have been implicated in the development of various pathological conditions (Bandopadhyay, U. *et al*., 1999, Halliwell, B., 1998, Halliwell, B. and Gutteridge, J. M. C., 1990), curcumin has the potential to control these diseases through its potent antioxidant activity.

Apart from having a potential anti oxidant properties, Curcumin has been shown to protect the stomach from ulcerogenic effects of phenylbutazone in guinea pigs at 50 mg/kg dose (Dasgupta, S. R. *et al*., 1969, Sinha, M. *et al*., 1974). Antispasmodic activity of sodium curcuminate was observed in isolated guinea pig ileum (Srihari Rao, T. *et al*., 1982). Anti flatulent activity was observed in both *in vivo* and *in vitro* experiments in rats (Bhavani Shakar, T. N. and Sreenivasa Murthy, V., 1979). Curcumin also enhances intestinal lipase, sucrase and maltase activity (Patel, K. and Shrinivasan, K., 1996). It decreases the severity of pathological changes and thus protects from damage caused by myocardial infarction (Nirmala, C. and Puvanakrishnan, R., 1996). Curcumin improves Ca^{2+}-transport and its spillage from the cardiac muscle sarcoplasmic reticulum, thereby raising the possibility of pharmacological interventions to correct the defective Ca^{2+} homeostasis in the cardiac muscle (Sumbilla, C. *et al*., 2002). Curcumin has significant hypo cholesteremic effect in hyper cholesteremic rats (Patil, T. N. and Shrinivasan, M., 1971).

Curcumin is anti coagulant (Srivastava, R. *et al*., 1985), inhibits sperm motility (Rithaporn, T. *et al*., 2003), anti diabetic (Arun, N. and Nalini, N., 2002, Sajithlal, G. B. *et al*., 1998), prevent galactose induced cataract formation at very low doses (Suryanarayana, P. *et al*., 2003), anti bacterial (Mahady, G. B. *et al*., 2002), anti fungal, antiprotozoal (Koide, T. *et al*., 2002, Gomes Dde, C. *et al*., 2002, Rasmussen, H. B. *et al*., 2000), anti viral (Hergenhahn, M. *et al*., 2002, De Clercq, E., 2000).

Human clinical trials suggest that curcumin is safe with daily intake of 1-8 gm/day (Chainani-Wu, N., 2003; Aggarwal, B. B., 2003).

Hence, it could be seen that ingestion of Quercetin, Hesperidin and Curcumin as common dietary flavonoids are safe and effective to improve various pathological conditions.

Quercetin, Hesperidin and curcumin are well known compounds from Indian medicinal plants having a diverse therapeutic potential. Flavonoids are reported having anti malarial potential among which quercetin is reported to be effective at IC_{50} of 15 µg/ml against chloroquine resistant 7G8 strain (Adele, M. *et al*., 2008). Curcumin is also a poly phenolic organic molecule derived from turmeric also had been shown to inhibit chloroquine resistant *Plasmodium falciparum* cultures at IC_{50} of 5 µM (Reddy, R. C. *et al*., 2005).

We selected swertiamarin as a previously isolated and identified potential antimalarial agent from our lab for further evaluation of efficacy against chloroquine resistant strain of *Plasmodium falciparum* (RKL 19). By considering the activity of flavonoids against malaria, we selected common dietary flavonoids like curcumin, quercetin and hersperdin which are completely safe and are found as major constituents in nature to generate comparative antimalarial values and to generate a supportive data for evaluating combinatorial effects of upcoming hits from this study.

4.2 Materials and Methods

4.2.1 *Preparation of samples*

Isolation and identification of swertiamrin is discussed in chapter 3 and of hesperidin is mentioned in chapter 2. Stock solutions of swertiamarin and hesperidin (isolated in our laboratory), quercetin (SISCO Research Laboratories, Mumbai), curcumin (Sigma Aldrich) were prepared by dissolving in DMSO to yield a final concentration of 1 mg/ml of each.

4.2.2 *Antimalarial assay*

The detailed description on antimalarial assay is mentioned in chapter 2. We used chloroquine resistant strain of *Plasmodium falciparum* RKL 19 in this study. Chloroquine was used as a positive control in this study.

4.3 Results

Investigation of swertiamarin against chloroquine resistant strain of *Plasmodium falciparum*, has been found most potential as it has IC_{50} of 0.15 µg/ml. While IC_{50} of chloroquine tested on the culture was 0.31 µg/ml. Hence, the results represent more efficacy of swertiamarin when compared with chloroquine. Figure 4.1 represents the images of parasites in the control and drug treated groups respectively. Figure 4.2 represents the percent trophozoits and schizonts in control as well as drug treated group. The compound was effective by inhibition of schizont formation from young trophozoit stages. It was also found that there were undeveloped food vacuoles in the drug treated group when compared to control (wells with no drug). Hesperidin was found effective against resistant strain of *Plasmodium falciparum* (RKL 19) with IC_{50} values in a range of 37.5 µg/ml (91.06 µM). The images of parasite development stages arrested by hesperidin are presented in figure 4.3. Quercetin was found effective at IC_{50} of 10.62 µg/ml (37.11 µM). While, curcumin was found effective at IC_{50} around 1.36 µg/ml (4.55 µM). Dose dependent antimalarial activities of all compounds are presented in figure 4.4. We used chloroquine as a positive standard and respective $IC_{50/75/90}$ values calculated for

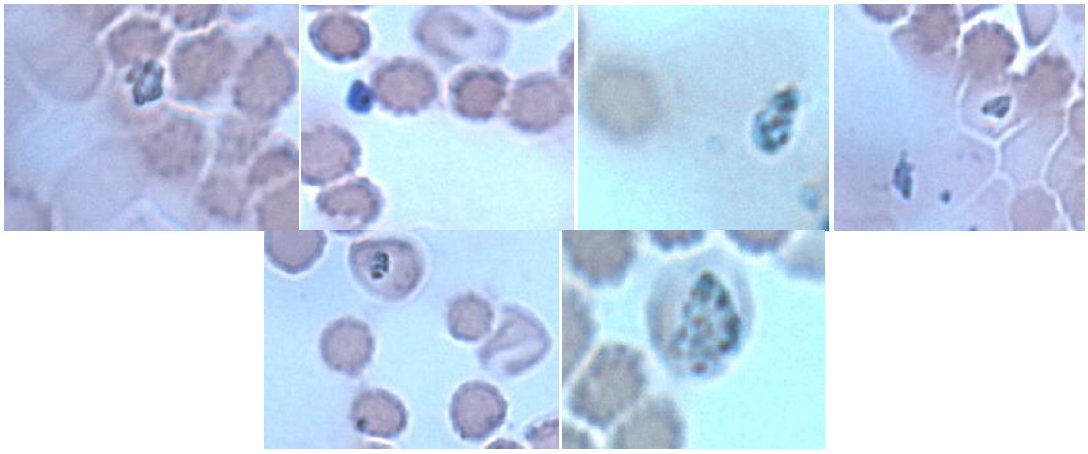
each compound along with chloroquine are presented in table 4.1. From results, it was clear that swertiamarin exhibited highest potential amongst all tested compounds against chloroquine resistant strain of *Plasmodium falciparum*. As mentioned in chapter 2; by following parameters of Batista (Batista, R. *et al*., 2009), the concentration of swertiamarin was 0.4 µM (<1 µM) representing the compound as most active. Curcumin was also found to have good activity (IC_{50} of 1-20 µM). While, hesperidin and quercetin were termed as moderately or low active (IC_{50} of 100 - 200 µM).

Table 4.1 Comparative Inhibitory concentrations of compounds against chloroquine resistant strain of *Plasmodium falciparum* (RKL 19).

Effective Doses	µM Concentration (Mean ± S.E.M.) of compounds against chloroquine resistant strain of *Plasmodium falciparum* (RKL 19) (dose range in µg/ml)				
	Swertiamarin*	**Hesperidin****	**Quercetin****	**Curcumin****	**Chloroquine**
IC_{50}	0.40 ± 0.4 (0.12-0.18)	91.06 ± 16.91 (37.5-78.88)	37.11 ± 0.8 (10.62-11.89)	4.55 ± 0.56 (1.36-2.73)	0.65 ± 0.02 (0.31-0.36)
IC_{75}	0.80 ± 0.81 (0.28-0.32)	164.69 ± 28.92 (77.56-148.33)	54.67 ± 0.72 (16-17.6)	12.51 ± 0.97 (3.41-4.60)	1.09 ± 0.004 (0.56-0.57)
IC_{90}	14.53 ± 0.4 (4.81)	208.86 ± 36.13 (101.6-190)	66.81 ± 0.86 (19.11-21.23)	16.71 ± 1.22 (4.65-6.15)	1.35 ± 0.02 (0.67-0.73)

***p* value not significant; ****p*<0.05 when compared with reference standard chloroquine (n=3)**

Schizonts in untreated groups



Trophozoit stages in swertiamarin treated groups

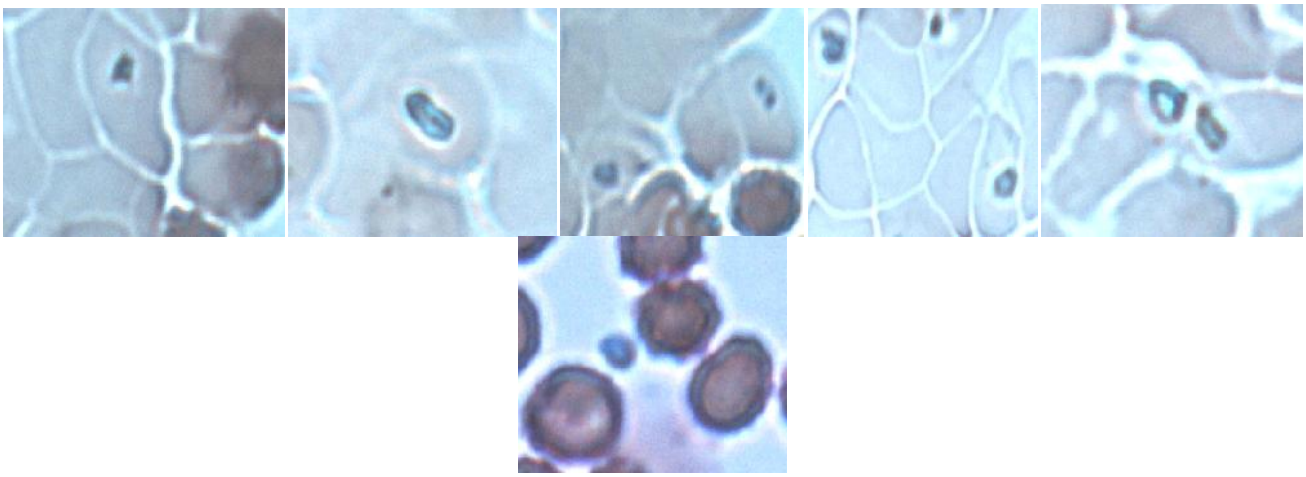


Figure 4.1: Inhibition of schizont formation from trophozoits by swertiamarin when tested on chloroquine resistant strain of *Plasmodium falciparum* (RKL 19)

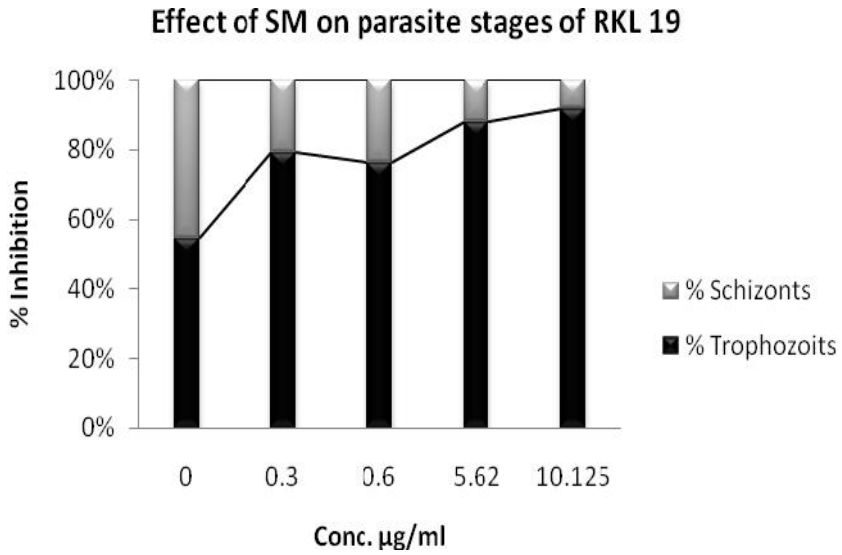


Figure 4.2: Percent trophozoits and schizonts in swertiamarin treated groups

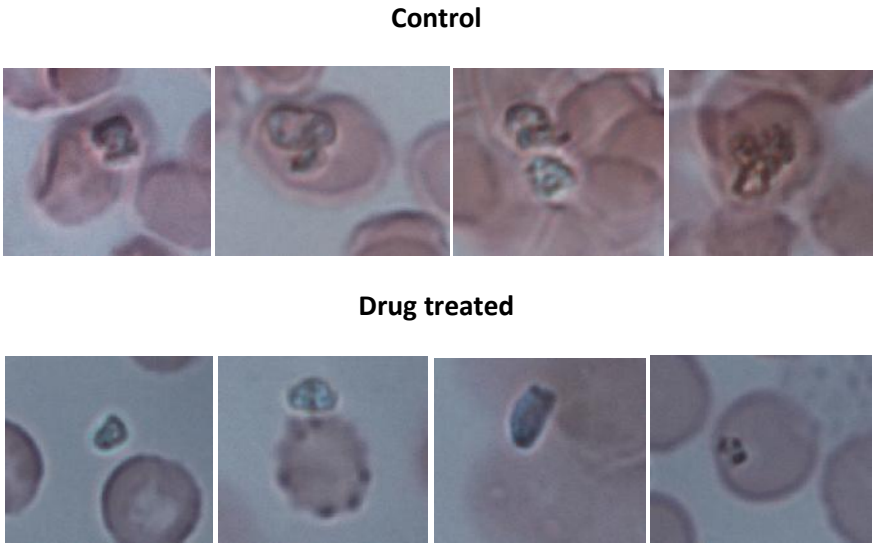


Figure 4.3: Effect of hesperidin on maturation of schizonts in chloroquine resistant strain (RKL 19)

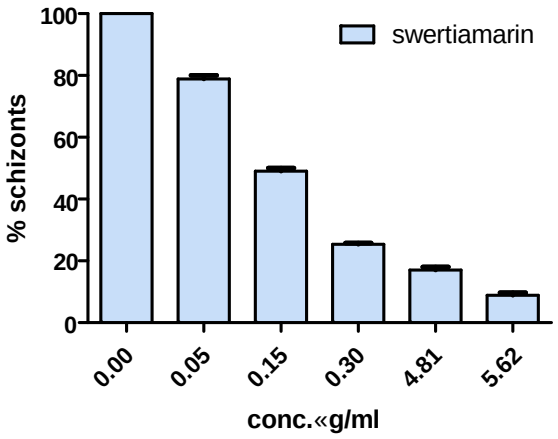


Figure 4.4a: Dose dependent inhibition of schizont formation by swertiamarin

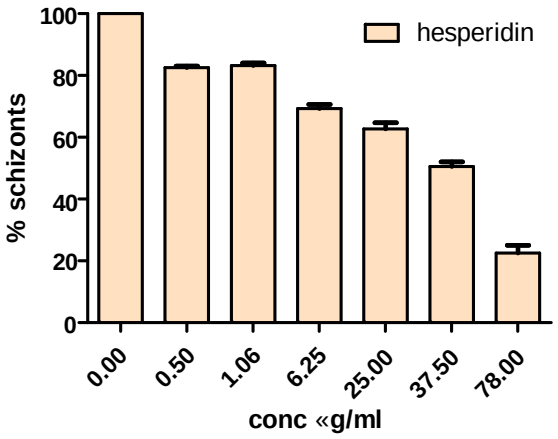


Figure 4.4b: Dose dependent inhibition of schizont formation hesperidin

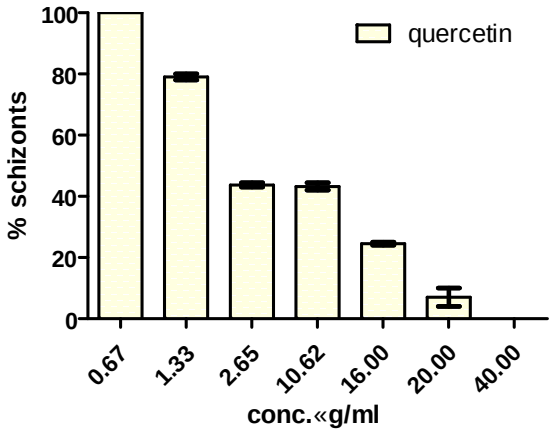


Figure 4.4c: Dose dependent inhibition of schizont formation by quercetin

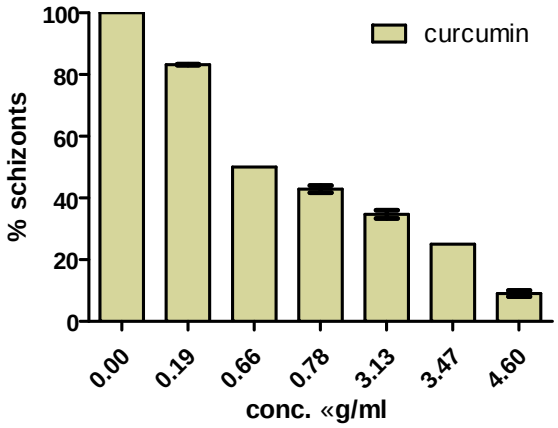


Figure 4.4d: Dose dependent inhibition of schizont formation by curcumin

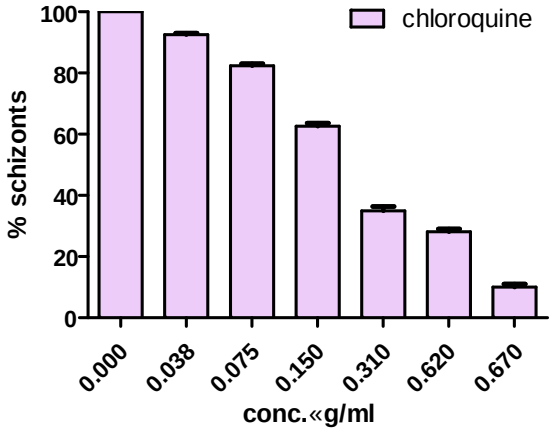


Figure 4.4e: Dose dependent inhibition of schizont formation by chloroquine

Figure 4.4: Antimalarial activities of compounds against chloroquine resistant strain of *Plasmodium falciparum* (RKL 19)

4.4 Discussion

Majority of antimalarials in the clinical use are facing problem of developing resistance throughout the world. Although there are many approaches mentioned in antimalarial drug discovery, plant derived approach is very important which gives a completely diversified molecular leads active against malaria. The success of quinine and artemisinin are better examples for the same. Derivatives of the existing series of compounds having same mode of action, often seems to develop resistance, for example therapeutic failure of quinine derivatives choroquine and mefloquine.

Our present investigation shows activity of a newly diversified compound swertiamarin against chloroquine sensitive and resistant strains of *Plasmodium falciparum* with concentration range of <1µg/ml.

The plant *Enicostemma littorale* and swertiamarin present in it are well documented for their effect on diabetes. But we report first time the potential effect of swertiamarin against chloroquine resistant parasites. Microscopical observations have been found that when compared to control, drug treated groups showed a very early stages of trophozoits with undeveloped or abnormal food vacuoles. The total number of parasites in each field were also very much less when compared to control, almost 50 % reduction of total parasitemia. Thus, the compound not only inhibited formation of schizonts but also lowered the total parasite growth.

Flavonoids have been frequently identified in phytochemical analysis of plants used in traditional system of medicine in various malarious areas (Nundkumar, N. and Ojewole, J. A., 2002). However, there are several flavonoids occurring in plants with a huge structural diversity and there is controversy in their antimalarial reports. Several flavonoids were previously investigated from a popular Chinese antimalrial remedy – *Artemisia annua* for their potential against multidrug resistant parasites (Kraft, C. *et al*., 2003). As flavonoids are commonly found in diet, we used quercetin as flavonone and hesperidin as flavonol representative. In our present investigation of flavonoids and polyphenols on chloroquine resistant culture of *Plasmodium falciparum*, Curcumin was most potential compound having

antiplasmodial activity among all the dietary chemicals tested while, hesperidin was found less potential on both the strains. However, quercetin and curcumin are well known anti malarials. However, this is the first report of effectiveness of hesperidin. We also developed a simple and rapid method through which the compound can be obtained in large quantity with less consumption of equipments and time. If we compare order of activity of these three phytonutrients, it will be curcumin>quercetin>hesperidin.

It has been demonstrated that shikimic pathway has been found in *Plasmodium falciparum* and is required for the parasite growth. Hence, it could be considered that flavonoids and polyphenols as the end product of schikimate pathway occurring in plants act as a potential analogs of pABA (Mc. Robert, L. and Mc Conkey, 2002). Previous studies also suggest role of flavone binding to Pgp-like transporter of *Leishmania tropica* is responsible for reversal of daunomycin resistance of parasite (Perez- Victoria, J. M. *et al*., 2001; 2002). Malaria parasites also generate reactive oxygen species (ROS) during their erythrocytic life stages and are ascribed to both the malaria parasite metabolism and host immune response (Becker, K. *et al*., 2001; Griffiths, M. J. *et al*., 2001; Mishra, N. C. *et al*., 1994; Pabon, A. *et al*., 2003; Muller, S., 2004; Kawazu, S. *et al*., 2004; Rahlfs, S. and Becker, K., 2001) and are highly susceptible to oxidative stress (Becker, K. *et al*., 2003, Turrens, J. F. *et al*., 2004, Ittarat, W. *et al*., 2003, Savvides, S. N. *et al*., 2002). Since, flavonoids are potential antioxidants and free radical scavangers, they are considered to chelate free heme in parasite food vacuoles and stop sequestration to form non toxic hematin.

The present postulate is that high plasma concentrations of antioxidant flavonoids obtained by regular intake of foods like green tea, apple and onions for quercetin, hesperidin from orange peel and curcumin from *Curcuma longa* would permit scavenging of ROS and other free radicals produced by the malaria parasites and host response. The high plasma antioxidant concentration would also mitigate the pro-inflammatory response of the body's immune system to the parasites (Langhorne, J. *et al*., 2002) thereby ablating the cascade of molecular events that exacerbates the pathophysiology of malaria (Miller, L. H. *et al*., 1994, Perimann, P.

and Troye-Blomberg, M., 2000). To evaluate this hypothesis, we also checked the role of these flavonoids and polyphenols for inhibition in formation of beta hematin from heme, this is mentioned elaborately in chapter 5.

4.5 Conclusion

Swertiamarin was the best active compound effective on chloroquine resistant strain of *Plasmodium falciparum* (RKL 19). The activity of swertiamarin was comparable with that of chloroquine. Hence, it can be a potential lead for drug resistance malarial due to massive use of chloroquine. The compound had also been proven its activity against chloroquine sensitive strains as mentioned in previous chapter. Hence, it can be concluded that the compound is a potential antimalarial candidate. Although flavonoids quercetin and hesperidin were moderately active, curcumin exhibited its potential. As discussed above, flavonoids are well known bio enhancers; we further studied synergism of swertiamarin with curcumin, quercetin and hesperidin respectively. Chapter 6 mentions detailed investigation of the same.

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5.1 Introduction

During intra erythrocytic stages, parasite takes haemoglobin from erythrocyte as food by pinocytosis into the cytostol and fuse to the acidic food vacuole (Yayon, A. *et al.*, 1984). The hemoglobin inside the food vacuole is oxidized to methemoglobin in the acidic pH and subsequently hydrolyzed by aspartic proteases (plasmepsin I, II, IV and histo-aspartic protease) into free heme (Fe^{+3}) and denatured globin. Denatured globin is further hydrolyzed by cysteine proteases (falcipain) and zinc containing metallopeptidase (falcilysin) into small peptides (Eggleson, D. *et al.*, 1999). These peptides are then assumed to be transported to the parasite cytoplasm by peptide transporter located in the membrane of food vacuole (Rubio, J. P. and Cowman, A. F., 1996, Kolakovich, K. A. *et al.*, 1997).

Peptides are further hydrolyzed to amino acids by cytosolic exopeptidase and the released amino acids are used by parasite for their protein synthesis (Sherman, I. W., 1998). If the released heme is allowed to accumulate within the food vacuole, free heme (Fe^{+3}) level may reach 300–500 mM (Wright, A. D. *et al.*, 2001a). Free heme (Fe^{+3}) is very toxic because it can generate reactive oxygen species (Kumar, S. and Bandyopadhyay, U., 2005) and may induce oxidative stress leading to parasite death (Vincent, S. H., 1989, Schmitt, T. H. *et al.*, 1993) (Fig. 1). Since free heme (Fe^{+3}) is a lipophilic molecule, it can easily intercalate in the membrane and may cause changes in membrane permeability, lipid organization and induces lipid peroxidation of parasite membrane (Ryter, S. W. and Tyrrell, R. M., 2000, Stojiljkovic, I. *et al.*, 2001). Oxidation of membrane components induced by free heme (Fe^{+3}) promotes cell lysis and ultimately death of the parasite (Schmitt, T. H. *et al.*, 1993). Free heme (Fe^{+3}) may also interfere with the haemoglobin degradation pathway. The cysteine protease, falcipain has been shown to be very sensitive to free heme (Francis, S. E. *et al.*, 1997, Pandey, A. V. *et al.*, 1999). To overcome free heme (Fe^{+3}) toxicity, malaria parasite is equipped with unique heme detoxification protein disulfide isomerase (Harwaldt, P. *et al.*, 2002, Campanale, N. *et al.*, 2003). *P. falciparum* possesses 1-Cys peroxiredoxin (Prx) (Pf1-Cys-Prx), a cytosolic protein expressed at high level during the heme-digestion stage and acts as an antioxidant to cope up with the oxidative burden of free heme. Pf1-Cys-Prx protects parasite against oxidative stress by binding to free

heme, slowing the rate of GSH-mediated heme degradation and consequent iron generation. Besides, it also protects proteins from iron-derived reactive oxygen species, and interferes with the formation of membrane-associated heme (Kawazu, S. *et al.*, 2005).

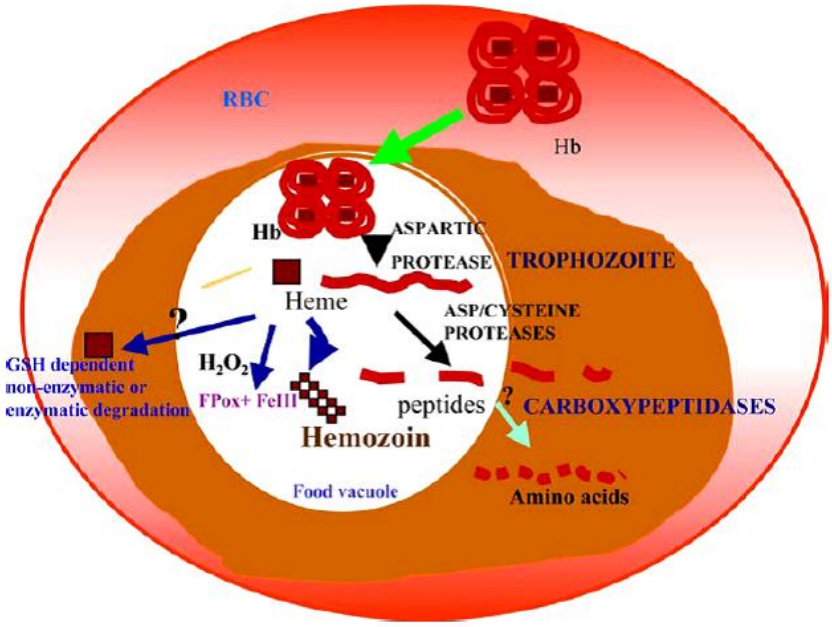

Figure: 5.1 Possible mechanisms of free heme (Fe^{+3}) toxicity in malaria parasite. Hb, hemoglobin; FV, food vacuole; IRBC, infected red blood cell; PVM, Parasitophorous vacuolar membrane; ROS, reactive oxygen species.

5.1.1 Free heme detoxification systems of Plasmodium falciparum

Malaria parasite possesses efficient heme detoxification mechanisms (Figure 5.2) to protect itself from heme-induced oxidative stress. The heme detoxification system can be classified into two; (a) primarily via hemozoin formation (Slater, A. F. and Cerami, A., 1992, Ziegler, J. *et al.*, 2001) in the food vacuole of parasite and (b) secondarily, other systems situated mainly in the cytosol, such as detoxification by GSH, heme binding proteins (Campanale, N. *et al.*, 2003) and degradation of free heme by H_2O_2. The mechanistic details of free heme (Fe^{+3}) detoxification are discussed below.

Detoxification through hemozoin (β-hematin) formation (primary); parasite converts toxic heme into a non-toxic hemozoin by a mechanism known as hemozoin formation and this process is considered to be the most important mechanism for heme detoxification in Plasmodium species (Sherman, I. W., 1998). Although hemozoin formation is generally accepted as a biomineralization process by which free heme is being converted to hemozoin crystal (Pagola, S. *et al.*, 2000, Egan, T. J., 2001, 2002) but many theories have been suggested to explain the mechanism of hemozoin formation (Slater, A. F. and Cerami, A., 1992, Sullivan Jr, D. J., *et al.*, 1996, Egan, T. J., 2004, Tekwani, B. L. and Walker, L. A., 2005) which are as follows:

Spontaneous hemozoin (β-hematin) formation; β-hematin can be spontaneously synthesized, if monomeric hematin is allowed to incubate at 37 °C at pH-4.8, without any parasitic material (Dorn, A. *et al.*, 1998). Incubation of heme in presence of glacial acetic acid at 70–80 °C for about 18 h yields formation of β-hematin (Bohle, D. S. and Helms, J. B., 1993). The process occurs via rapid precipitation of amorphous heme, followed by slow conversion to crystalline β-hematin. Acetate may act as a phase transfer catalyst by solubilizing heme and facilitating its re-deposition as β-hematin (Egan, T. J., 2001). However, yield of β-hematin formed depends on the experimental conditions, particularly on pH and presence of salts. Variation in these conditions in individual assays for β- hematin formation may lead to inconsistent results specifically in the case of evaluation of inhibitor's effect on β-hematin formation. A method of β- hematin formation was standardized to alleviate the problem posed by pH and salts variations, which has

been found to produce consistent results (Parapini, S. *et al.*, 2004). Nevertheless, the above studies could not explain that how synthesis of a material identical to β-hematin occurs so efficiently in the malaria parasite under mild physiological conditions. The parasite digestive vacuole, the physiological site for hemozoin formation, has acidic pH, along with presence of organic acids, lipids and proteins. Similarly, *in vitro* formation of β-hematin under aqueous acidic conditions and physiological temperature may be achieved only in the presence of some biological factors *viz.*, the malaria parasite lysate, organic solvent extracts of the parasite lysate, histidine rich protein II or III, preformed hemozoin or β-hematin and some unsaturated lipids (Dorn, A. *et al.*, 1995, 1998, Sullivan Jr, D. J. *et al.*, 1996, Fitch, C. D. *et al.*, 1999). Similarly, non biological assays have been developed and validated by reacting hematin with organic acids and by maintaining the conditions resembling to that of occurring in parasites (Parapini *et al.*, 2004).

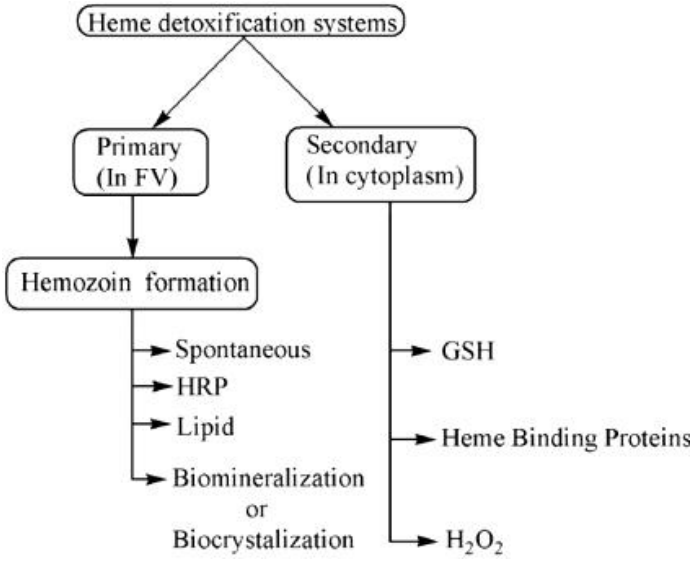

Figure 5.2: Free heme detoxification systems by malarial parasites

We previously mentioned antimalarial activity of compounds from medicinal plants. We also studied role of various dietary flavonoids against resistant culture of *Plasmodium falciparum* (RKL 19). In previous chapter, through reported references we hypothesized role of antioxidants as potential antimalarials. Majority of the flavonoids and polyphenols from plants had been reported for antimalarial activity, particularly, in case of multidrug resistance.

In context with this we evaluated the role of flavonoids *viz.* quercetin, curcumin and hesperidin in the formation of beta hematin. We also tested swertiamarin for the same as it was found as a potential antimalarial from our study. Beta hematin formation inhibition assay using non biological system (BHIA) was performed as previously reported and validated by Parapini (Parapini *et al.*, 2004).

5.2 Material and methods

5.2.1 Preparation of solutions for test compounds

Stock solutions of hesperidin (isolated in our laboratory), quercetin (SISCO Research Laboratories, Mumbai), curcumin (Sigma Aldrich), swertiamarin (Isolated in our laboratory) and chloroquine phosphate (40 mg/ml injection, Ipca Labotaries, India) were used.

Hesperidin, quercetin and curcumin were dissolved in DMSO and volume was made up with distilled water to yield final concentrations of 1 mg/ml. The concentration of DMSO in final stock was maintained not exceeding up to 25 %. Chloroquine and swertiamarin were dissolved in distilled water and used for the assy. Control well were kept for both containing each of respective DMSO and water to compare the respective compounds dissolved in DMSO and water.

5.2.2 Preparation hemin solution

Hemin solution used in the assay was of 8mM. In brief, 0.520 gm of hemin (bovine heme) was first dissolved in 25 ml of DMSO. The mixture was kept on cyclomixer for complete dissolution of hemin in DMSO. The final volume of solution was kept at 100 ml by adjusting volume with DMSO. The hemin solution containing vial was covered with aluminium foil and was immediately transferred in refrigerator at 4 ^{o}C away from light.

5.2.3 Preparation of acetate buffer (pH 5)

In this assay, 8 M acetate buffer was used. The buffer was prepared by dissolving 108.8 gm of sodium acetate trihydrate in 48 ml of glacial acetic acid. The mixture was kept on water bath to dissolve the solid. Temperature was adjusted not exceeding 60 ^{o}C. After complete dissolution, the volume was adjusted up to 100 ml. pH of the reaction mixture was adjusted up to 5 with help of acetic acid glacial.

5.2.4 Assay of beta hematin formation inhibition in ependorff tubes

The assay was performed as reported by Parapini (Parapini *et al.*, 2004). The eppendorf tubes were loaded with 100 µl of distilled water. Solutions of drugs were loaded by withdrawing a 100 µl of stock and loaded in last well of the row and

mixed well with pipette. From this, 100 µl was withdrawn and mixed in next well. Hence, 2 fold dilutions occurred in each drug loaded wells. The first row was used as control in which no drug was loaded while chloroquine phosphate solution was used as positive control. After proper loading, carefully hematin solution was withdrawn from refrigerator and kept in dark to allow the solution to reach at room temperature. A 20 µl of this hemin solution was loaded in each well and mixed gently using cyclomixer to allow mixing to two separated phases of drug solutions and hemin solution. After this, reaction was started by addition of 100 µl of acetate buffer of pH 6. Again the solutions were gently mixed. The eppendorf tubes were kept in dark and incubated at 37 ^{o}C for a period of 24 hrs. After this time period, the tubes were moved to centrifuge at 3000 rpm. The clear DMSO and water phase were decanted. To the obtained pellet, again 500 µl of DMSO was added and tubes were again centrifuged at 3000 rpm. This process was repeated at least 3 times to remove unreacted free heme from the solution as it is soluble in DMSO. Then, the pellet obtained was dissolved in 0.1 M solution of sodium hydroxide to make the volume of 1 ml. Readings were taken on UV spectrophotometer at wavelength of 405 nm. Sigmoidal dose response curve of each drug was prepared by plotting concentration of each drug *versus* percent inhibition of beta hematin formation and $IC_{50/75/90}$ values of each drug were calculated.

5.3 Results

From each dose response curve of compounds, $IC_{50/75/90}$ values were calculated (table 5.1). Quercetin produced 50% inhibition of β hematin formation at concentration of 1.57 µg/ml. Figure 5.3a represents dose dependent inhibition of β hematin by quercetin. Curcumin also produced the same effect at 5.7 µg/ml (figure 5.3b). Hesperidin could produce the effect at 46.80 µg/ml (figure 5.3c). Chloroquine was used as a reference standard in this study, which was found to inhibit 50 % of hematin formation at 41.2 µg/ml (figure 5.3d). From present investigation, it could be stated that among all the compounds tested, quercetin and curcumin were found as they effectively inhibited formation of beta hematin. However, hesperidin was found effective at higher concentration. The activity of quercetin and curcumin were even more than that of chloroquine standard. With respect to IC_{50}, quercetin was more effective than curcumin but as compared to its IC_{75} and IC_{90} values with curcumin, they were found higher. Hence, we considered curcumin as more effective agent than quercetin. Swertiamarin was not found to inhibit the formation of beta hematin. Hence, there can be another mechanism through which swertiamarin possesses antimalarial activity. This study represents that flavonoids are potential compounds to interfere with β hematin formation and can stop heme sequestration process in parasite food vacuole.

Table 5.1: Comparative evaluation of compounds against β hematin formation

Drugs	Inhibitory concentrations (in µg/ml) of β hematin formation Mean ± S.E.M.		
	IC_{50}	IC_{75}	IC_{90}
Quercetin	1.57 ± 0.47	33.24 ± 1.23	53.68 ± 0.96
Curcumin	5.7 ± 2.89	10.90 ± 1.75	14.03 ± 0.53
Hesperidin	46.80 ± 0.98	75.44 ± 4.66*	92.62 ± 4.49
Chloroquine	41.2 ± 0.73	60.19 ± 1.30	71.44 ± 0.22

***p* value not significant; $p<0.05$ when compared with reference standard chloroquine (n=3)**

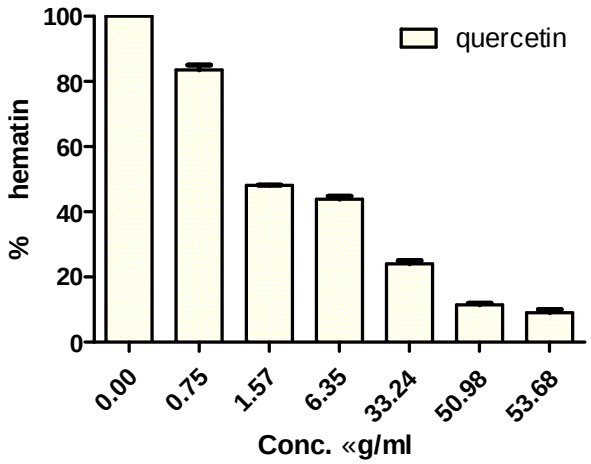

Figure 5.3a: Dose dependent inhibition of β-hematin formation by quercetin

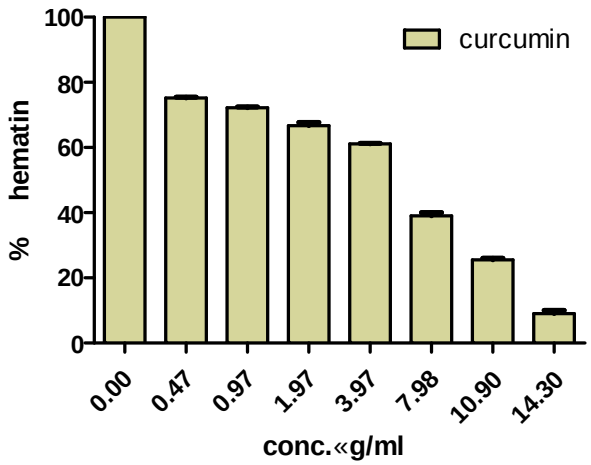

Figure 5.3b: Dose dependent inhibition of β-hematin formation by curcumin

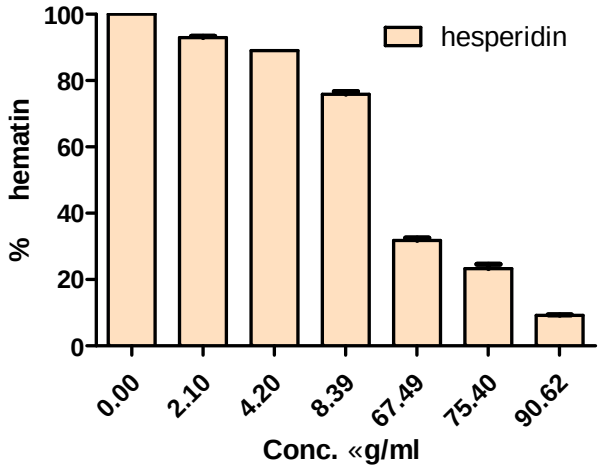

Figure 5.3c: Dose dependent inhibition of β-hematin formation by hesperidin

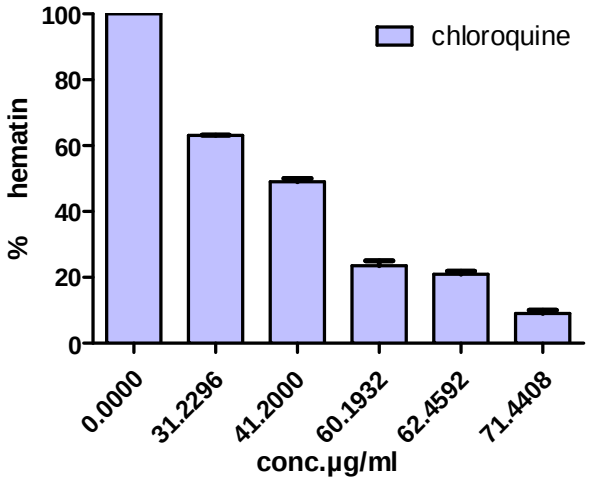

Figure 5.3d: Dose dependent inhibition of β-hematin formation by chloroquine

Figure 5.3: Dose dependent inhibition of β – hematin formation by compounds

5.4 Discussion

In previous chapter we had seen antimalarial profile of flavonoids and polyphenols as three structurally diversified molecules *viz.* quercetin; a flavonone, hesperidin; a flavonol and curcumin; a non flavonoid polyphenolic molecule, on parasite strain resistant to chloroquine. We also mentioned various hypotheses through which these compounds are acting as antimalarials. One of important among them is that parasites during intra erythrocytic phases, digest haemoglobin from host red blood cell (RBCs) and free heme is generated as a product, toxic to parasite. Detoxification of free heme is a spontaneous process in which heme is sequestered in to beta hematin, a non toxic product. Antioxidant compounds are well known to scavenge free radical generated in biological system. Hence, it would be interesting to search role of antioxidants particularly, these three common dietary flavonoids; quercetin, hesperidin and curcumin in formation of beta hematin from heme. As swertiamarin is found most potent antimalarial in our study, we also investigated it for β hematin inhibition assay but did not demonstrate this action.

In present investigation, we screened quercetin, hesperidin and curcumin for betahematin formation inhibition, a non biological target specific assay through which majority of blood schizonticidals act. Hematin was allowed to sequester with acetate buffer to form beta hematin as end product and the compounds were screened along with the same system to check interference of compounds in sequestration of free hematin and found that all these compounds inhibited formation of beta hematin from hematin. If we grade the results of beta hematin formation inhibition, the order should be Curcumin > Quercetin > Chloroquine Phosphate > Hesperidin. Although chloroquine is most potential drug acting on this target, quercetin and curcumin were proven as more effective than chloroquine, may be because of free heme binding profile of antioxidants. Thus, it can be considered from preset study, antioxidants play a vital role in malaria infection.

Previous literature reports also suggest antimalarial profile of flavonoids. Some of them reported as potential antimalarials having IC_{50} values less than 5 μM are catechin -3- gallate and catechin -5-gallate (Ramanandralbe, V. *et al.*, 2008),

artonin F (Namdaung, U. *et al.*, 2006), lonchocarpol A (Khaomek, P. *et al.*, 2008), Ginkgetin, isoginkgetin, sciadopitysin (Weniger, B. *et al.*, 2006). As flavonoids are end products of schikimate pathway, some of the designed analogs were having antimalarial potential (McRobert, L. *et al.*, 2005). Currently, phenols like gallic acid and ellagic acid from *Punica granatum* have shown antimamarial potential (Reddy, M. K. *et al.*, 2007). Epigallocatechin 3 gallate and epigallocatechin gallate from green tea were also found potential antimalarial and also potentiated activity of artemisinin (Sannela, A. R. *et al.*, 2007). Many of polyphenols from *Argania spinosa* like gallic acid, tannins catechin, flavonoids like quercetin, anthocyanins and cyanindin were quantified and good antioxidant and antimalarial activity were obtained for DPPH (2,2–diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3ethylbenzothiazoline-6-sulfonic acid) assays in ethyl acetate extract and decoction of the plant (El, B. F. *et al.*, 2010). Some of previous studies also mentioned role of antimalarials as antioxidants and suggested to use antimalarials like chloroquine and quinacrine as anti inflammatory agents (Miyachi, Y. *et al.*, 1986).

Ayurveda - Indian system of medicine, mentions use of antioxidants like triphala ghrita in treatment of *vishammjvara* symptoms relates to malarial fever. This triphala is rich in phenols and polyphenols like gallic acid and ellagic acid. These are present in all the drugs used viz., *Emblica officinalis* (Amala), *Terminalia chebula* (Harde) and *Terminalia belerica* (Baheda). These compounds now a day have been proven as antimalarials. The present investigation supports such phenomenons as discussed above and represents role of anti oxidant flavonoids working as antimalarials by inhibiting β hematin formation.

5.5 Conclusion

Till date, although there are various reports for antimalarial activity of flavonoids and polyphenols, the mechanism of majority of them remains unclear. So, present investigation proposes one of the most important mechanism through which these compounds are exhibiting antimalarial activity. Although quercetin, curcumin and hesperidin are studied by us for antimalarial activity and except hesperidin, both the compounds are reported in literature as antimalarials. Thus it seems to be the first report of their antimalarial mechanism of flavonoids. It can be considered that due to acidic nature of flavonoids (as they are polyphenols), they can cross parasite food vacuole easily and remain in cytosol for long time because of their poor solubility they interfere with heme sequestration process by chelating free heme in parasite food vacuoles and function as effective antimalarials.

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6.1 Introduction

Combination of drug regimen is preferable for infectious diseases like cancer, AIDS, tuberculosis to achieve better therapeutic efficacy, lowering toxicities and resistance. The growing problem of drug resistance has greatly complicated the treatment for falciparum malaria with available potential therapies *viz.* chloroquine and sulfadoxine/pyrimethamine, antifolates (i.e. pyrimethamine), atovaquone/proguanil and mefloquine but proven effective when the same were used with artesunate and its derivatives (Shanks, G. D., 2006). The combinations of drugs in the treatment of malaria aim at three distinct objectives like action on different stages of parasitic life-cycle, enhancement of antiparasitic activity and prevention of drug resistance (Ogutu, B. R. and Kokwaro, G. O. 2004). Effective antimalarial therapy represented many successful combinations. Some of the examples are combination of atovaquone and proguanil (Malarone), chlorproguanil and dapsone (Lap – Dap), artemisin and lumifentrine (Coretam) (Fivelman, Q. L. et al., 2004).

Definition of synergy from a pharmacological perspective

When two drugs that produce similar effects are present together, certain questions arise: How does a particular effect of the combination compare with the effects of the individual constituents when given at the same doses? What is the expected effect of the combination, and how is that expected effect calculated? Is the observed effect of the combination significantly greater (or less) than the expected effect? Even if one is not administering two drugs together, it is clear that giving even a single drug places it in potential contact with a myriad of other chemicals already present in the system. Hence, a quantitative knowledge of drug combination pharmacology is important—in clinical settings and in all experiments aimed at studying mechanism.

One of the most experimentally convenient methods for defining synergy is "isobole method" of Berenbaum (Berenbaum, M. C., 1989) and so far proposed for proof of synergy effects (figure 6.1); the x and y axes reflect the dose rates of the single individual components. Different dose combinations are investigated for the same effect. An isobole is understood to be a line or curve between the points of the same effect.

According to Berenbaum, the zero (0) – or additive interaction means that the effect of two substances a and b is a pure summation effect (equation 1). Correspondingly, the overall effect with antagonistic interaction is less than expected from the summation of the separate effects (equation 2). A convex curve will be obtained. With the existence of a real synergism with potentiated or over additive effects, the overall effect of two drugs a and b that are applied together a mixture must be larger than it would be expected by the summation of the separate effects. The result is then a concave curve (equation 3).

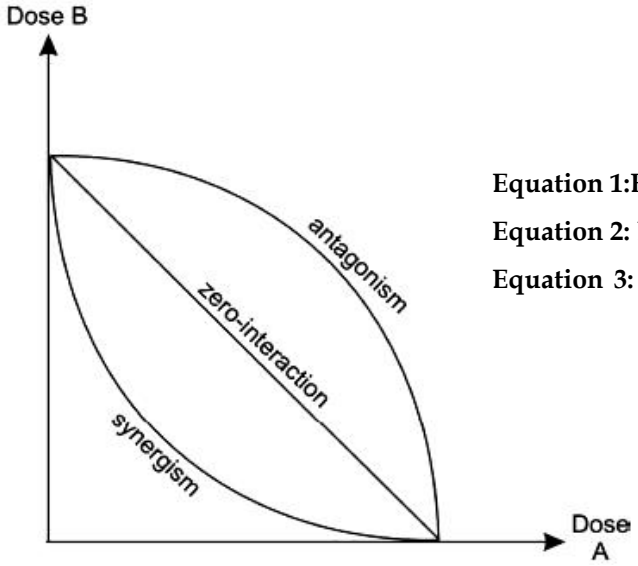


Figure 6.1 Isoboles of zero interactions, synergism and antagonism

(Curves shapes represents: Concave as synergy; convex as antagonism; linear as additivity)

Swertiamarin isolated from *Enicostemma littorale* has been demonstrated as most potential antimalarial compound on *in vitro* cultures of chloroquine sensitive (MRC 20) and resistant (RKL 19) of human malaria parasite, *Plasmodium falciparum*. Hence, interactions between swertiamarin and common dietary phytochemicals quercetin, curcumin and hesperidin against chloroquine resistant strain of *Plasmodium falciparum* RKL 19 have been investigated in this chapter.

6.2 Materials and methods

6.2.1 Parasite cultivation

Detailed methodologies for parasite culture are mentioned in chapter 2. In present study, we used chloroquine resistant strain of *Plasmodium falciparum* (RKL 19).

6.2.2 Preparation of drug solutions used for study

Swertiamarin, hesperidin, quercetin, curcumin, chloroquine and verapamil were used for study. Stock solutions were prepared at 1 mg/ml in dimethyl sulfoxide (0.05%). On the day of the experiment dilutions were prepared from stock solutions with serum-free medium.

6.2.3 Preparation plates for assay

Dose response assays were first carried out to obtain the 50% inhibitory concentration (IC_{50s}) of the individual drugs. For combination study, solutions in µg/ml of quercetin and hesperidin along with swertiamarin were prepared in assay medium at ratios of 6:0, 5:1, 4:2, 3:3, 2:4, 1:5, 0:6; while for curcumin – swertiamarin combinations in µg/ml with the ratios were 10:5, 20:4, 30:3, 40:2, 50:1 were prepared (table 6.1). Hundred µl of each test solution, followed by 2-fold serial dilutions in assay medium of each ratio, allowing IC_{50} to fall approximately at the mid-point of the serial dilution of each drug alone. A final 1% parasitemia at 1% hematocrit was used. The controls, drug-free unparasitized erythrocytes (RBC) and parasitized erythrocytes (pRBC), were similarly prepared. The plates were stacked in sterile desiccators and incubated at 37 ^{o}C for 20 – 24 hr. After incubation, plates were removed in sterile conditions, thin blood smears were prepared and slides were stained with giemsa stain, parasitemia was calculated by percent inhibition as counting number of parasites per 10,000 erythrocytes and compared with non drug loaded wells as controls.

Table 6.1 Preparation of combination solutions of swertiamarin along with flavonoids

Combination of solution	Ratio of swertiamarin with flavonoids		
	Swertiamarin	Hesperidin/ quercetin	Curcumin
1	0	6	60
2	1	5	50
3	2	4	40
4	3	3	30
5	4	2	20
6	5	1	10
7	6	0	0

6.2.4 Data analysis

The concentration of one sample was plotted against that of another sample in combination. The points representing the IC_{50s} of the two samples in combination and the points on the concentration axes representing their individual IC_{50s} were joined through a line, which is known as 'isobole'. As discussed above, concave isobole indicates synergy, a straight isobole denotes indifference and a convex isobole, antagonism (Williamson, 2001). Comparative dose response curves of each drug in combinations were prepared to correspond efficacy of drugs in combination. IC_{50} values were also used to calculate fractional inhibitory concentrations (FIC50/90s), for each drug ratio fractional inhibitory concentration (ΣFIC) were calculated by the following equation and represented as isobolograms:

ΣFIC50/90 = ($IC_{50/90}$ of Drug A in combination/ $IC_{50/90}$ of Drug A alone)
+ ($IC_{50/90}$ of Drug B in combination/ $IC_{50/90}$ of Drug B alone)

An overall mean **Σ**FIC50 or **Σ**FIC90 value for each combination was determined and synergy or antagonism defined as a mean **Σ**FIC < or > 1,

respectively, as reviewed by Bell (Bell, A., 2005). Lack of interaction or 'additivity' was defined as **Σ**FICs = 1.

6.2.5 Sorbitol induced haemolysis assay to find out calcium channel blocking potential of hesperidin and verapamil

The assay was performed by following the principle as; a high parasitized culture (20% parasitemia) when incubated with iso osmotic solution of sugars, parasite infected RBCs will be lysed as the parasites uptake sugars as a source of energy. In the same conditions, when the culture is incubated with calcium channel blockers, sugar entry in parasite infected cells will be inhibited by blockage of cellular channels and the culture will not be lysed by hypertonic sugar solutions.

The effects of the compounds of interest on the parasite-induced permeation pathways were investigated by a sorbitol hemolysis method similar to that described previously (Kirk, K. and Horner, H. A., 1995). Chloroquine-resistant *P. falciparum* RKL 19 was cultured; and trophozoite-stage infected erythrocytes (approximately 36 to 44 h postinvasion, 20% parasitemia) were harvested by centrifugation (600 g, 5 min, 25°C), washed twice with a solution of NaCl (150 mM)–HEPES (20 mM) (pH 7.4; 304 mosM), and suspended in the same solution to give a hematocrit of about 50%. In initial experiments, a single concentration (10 μM) of hesperidin was tested for its ability to inhibit sorbitol-induced hemolysis. Test compound was dissolved in dimethyl sulfoxide (DMSO) to give 10 mM solutions, which were diluted further with a solution comprising 300 mM sorbitol–20 mM HEPES (pH 7.4; 345 mosM) to give stock solutions of 0.1 mM. A total of 100 μl of this solution was combined with an additional 800 μl of the same sorbitol-HEPES buffer, together with 100 μl of the infected cell suspension in NaCl-HEPES. The mixture was incubated at 37°C for 15 min and centrifuged, and aliquots of the supernatant (100 μl) were dispensed into 96-well plates. The absorbances at 540 nm were read to estimate the amount of hemoglobin released and, hence, the degree of sorbitol-induced hemolysis. The absorbances of the control wells containing only test compounds in sorbitol-HEPES solution were determined concurrently, and corrections to the absorbances measured in the test wells were made when necessary. Verapamil was also screened for the same and the results of hesperidin and verapamil were compared.

6.3 Results

The IC_{50} values of each compound tested for antimalarial activity on Chloroquine resistant strain of *Plasmodium falciaprum* RKL 19 are mentioned in table 6.2. Results for combination of various flavonoids with swertiamarin are discussed as below.

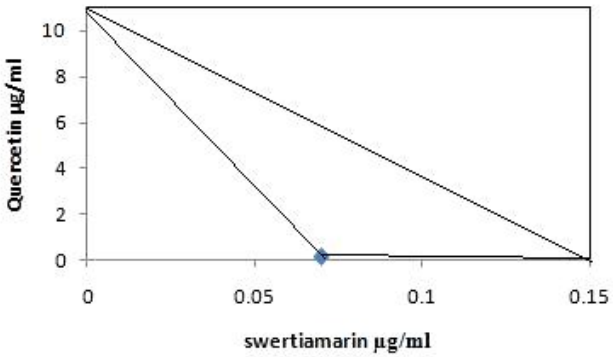
6.3.1. Swertiamarin and quercetin

Swertiamarin in combination with quercetin in ratio of 1:2 could produce 50 % of inhibition on growth of trophozoits when tested for schizont maturation inhibition (SMI) assay. Concentration of swertiamarin in combination was 0.07 µg/ml; almost reduced to half when compared to swertiamarin alone to produce 50 % inhibition. The inhibitory concentrations of swertiamarin in combination are mentioned in table 6.3. Isobologram of the combination is also mentioned in Figure 6.2, represents the combination as synergistic and the fractional inhibitory concentration was less than 1 (ΣFIC = 0.099). Figure 6.3 represents dose response curves of each drug in combination and it is clearly seen that curve of swertiamarin was lifted to left side showing more effectiveness of the compound in combination. We also observed that there was a decrease in total parasitemia in drug treated groups when compared to control (Figure 6.4). Hence, the combination not only inhibited the parasite stages but also found to inhibit their total population.

Table 6.2 Inhibitory concentrations of compounds on Chloroquine resistant strain of *Plasmodium falciaprum* RKL 19

Name of drug	Inhibitory concentrations (µg/ml)		
	IC_{50}	IC_{75}	IC_{90}
Swertiamarin	0.15 ± 0.02	0.3 ± 0.016	5.81 ± 0.04
Quercetin	10.62 ± 0.52	15.13 ± 0.65	18.16 ± 0.86
Curcumin	0.66 ± 0.55	3.47 ± 0.48	4.6 ± 0.61
Hesperidin	46.66 ± 16.41	93.74 ± 28.95	117.93 ± 36.16
Chloroquine	0.23 ± 0.02	0.36 ± 0.004	0.44 ± 0.02

n=3; Mean ± S.E.M.



Isobole index of swertiamarin 0.07/0.15 + 0.15/10.62 = 0.099 (<1)

Figure 6.2: Isobologram of swertiamarin and quercetin

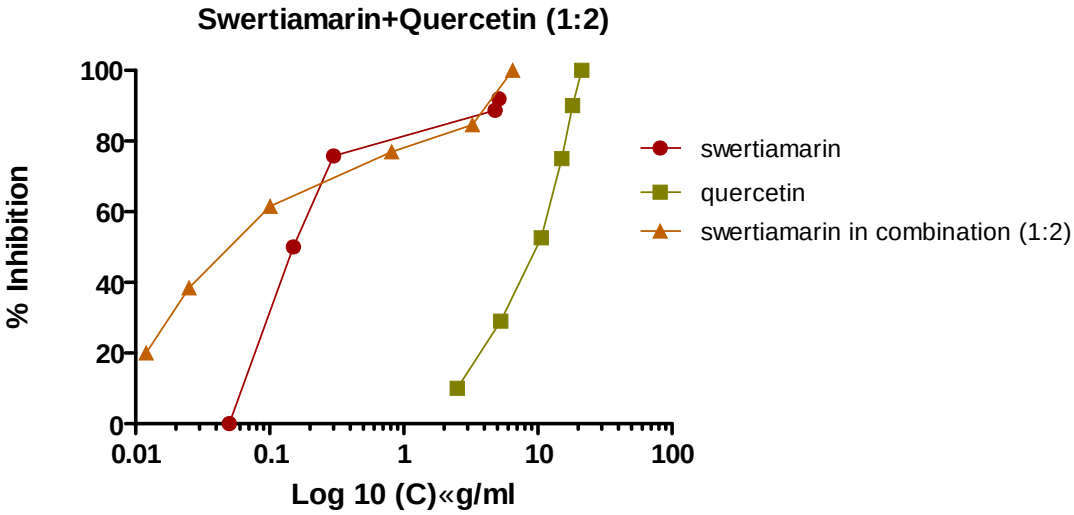


Figure 6.3 Comparative dose response curves of swertiamarin and quercetin in combination

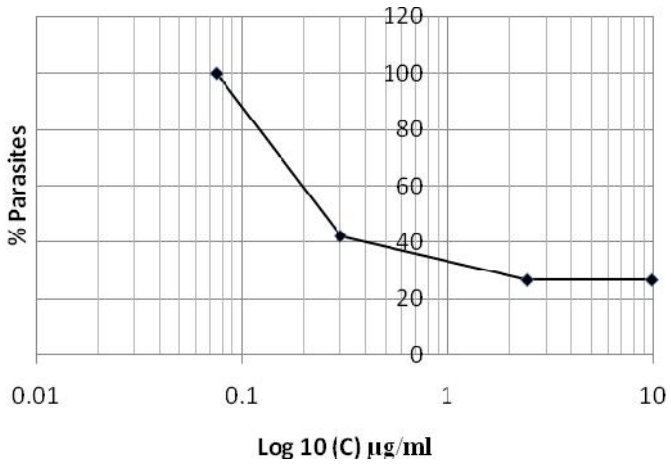


Figure 6.4 Reduction in total parasitemia by swertiamarin and quercetin combination (1:2)

Table 6.3 Effective doses of swertiamarin and quercetin in combination (1:2)

Drug Name	Inhibitory Concentration (µg/ml)		Fractional Inhibitory Concentrations (ΣFIC)	
	IC_{50}	IC_{90}	FIC_{50}	FIC_{90}
Swertiamarin	0.07 ± 0.005	1.13 ± 0.008	0.099	0.31
Quercetin	0.15 ± 0.02	2.25 ± 0.006		

n=3; Mean ± S.E.M.

6.3.2 Swertiamarin and hesperidin

This combination could produce 50 % inhibition on formation of schizonts from trophozoits in ratio of 1:2. There was a reduction of around 7.5 fold in IC_{50} of swertiamarin, when combined with hesperidin. The inhibitory concentrations of this combination are mentioned in table 6.4. Isobologram of the combination is presented in figure 6.5, shows synergism as isobole index or fractional inhibitory concentration (ΣFIC) of swertiamarin was 0.02 (<1). Figure 6.6 also shows comparative dose response curves of the combination along with the individual drug. The curve represents that dose of swertiamarin in combination shifts to left side of the curves of individual swertiamarin and hesperidin mentions synergism. Figure 6.7 also represents reduction in total parasitemia with this combination.

Table 6.4 Effective doses of swertiamarin and hesperidin in combination (1:2)

Drug Name	Inhibitory Concentration (µg/ml)		Fractional Inhibitory Concentrations (ΣFIC)	
	IC_{50}	IC_{90}	FIC_{50}	FIC_{90}
Swertiamarin	0.02 ± 0.06	2.16 ± 0.0	0.02	0.42
Hesperidin	0.038 ± 0.06	4.15 ± 0.0		

n=3; Mean ± S.E.M.; **NA: Not Available**

6.3.3 Swertiamarin and curcumin

Swertiamarin and curcumin in ratio of 1:12 could produce 50 % inhibition of schizont formation from young trophozoits. The IC_{50} of swertiamarin was as 0.025 µg/ml when combined with curcumin. Hence, there was around 6 fold decrease in the concentration of swertiamarin. Inhibitory concentrations of this combination are mentioned in table 6.5. Isobologram of the combination is mentioned in figure 6.8;

represents synergism as isobole index or fractional inhibitory concentration (ΣFIC) of swertiamarin was 0.16 (<1). Comparative dose response curves of each drug as well as their concentration in combinations are presented in figure 6.9 which represents that curve of swertiamarin in combination stays left side of the curve of swertiamarin alone shows the combination is synergistic. We also found reduction in total parasitemia with this combination as mentioned in figure 6.10.

Table 6.5 Effective doses of swertiamarin and curcumin in combination

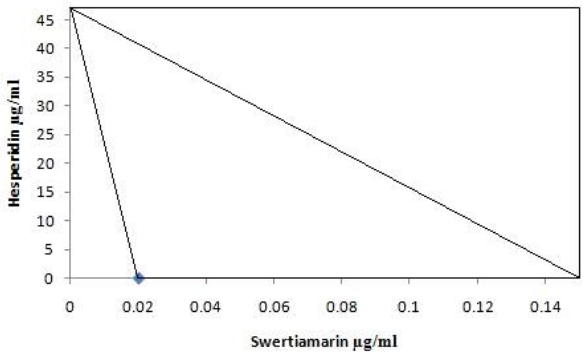
Drug Name	Inhibitory Concentration (µg/ml)		Fractional Inhibitory Concentrations (ΣFIC)	
	IC_{50}	IC_{90}	FIC_{50}	FIC_{90}
Swertiamarin	0.025 ± 0.004	0.36 ± 0.0	0.16	0.066
Curcumin	0.002 ± 0.002	0.03± 0.0		

n=3; Mean ± S.E.M

Hence, all the compounds *viz.* hesperidin, quercetin and curcumin were found to be synergistic with swertiamarin. If we compare the reduction in IC_{50} of swertiamarin with all three combinations, it seemed that combination of compound with hesperidin reduced around 6 fold of the concentration of swertiamarin. Fold decrease in the concentration of swertiamarin with these three flavonoids are mentioned in table 6.6. Figure 6.11 also represents the comparative analysis of all the compounds along with their combinations.

Table 6.6 Fold decrease of inhibitory concentration (IC_{50}) of swertiamarin when combined with quercetin, hesperidin and curcumin

Name of combination	Fold decrease in IC_{50} of swertiamarin
Swertiamarin + Quercetin	2.14
Swertiamarin + Hesperidin	7.5
Swertiamarin + Curcumin	6



Isobole index of swertiamarin 0.02/0.15 + 0.038/46.66 = 0.02(<1)

Figure 6.5: Isobologram of swertiamarin and hesperidin

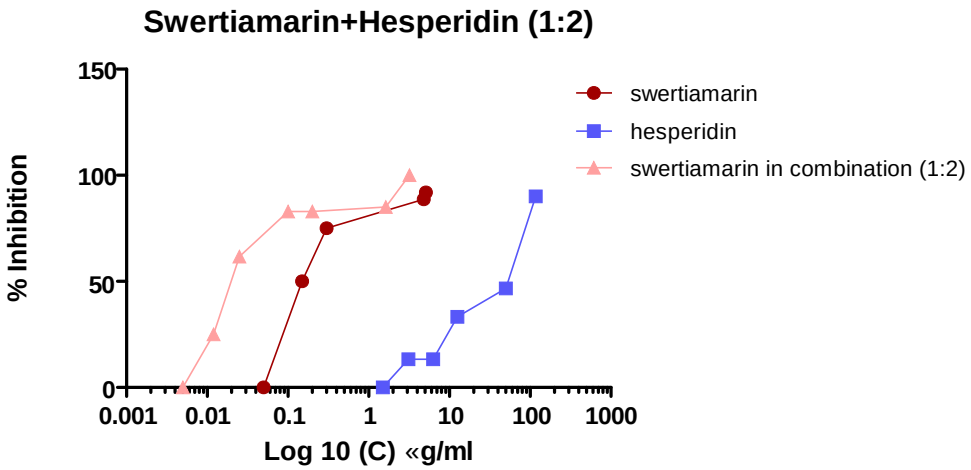


Figure 6.6 Comparative dose response curves of swertiamarin and hesperidin in combination

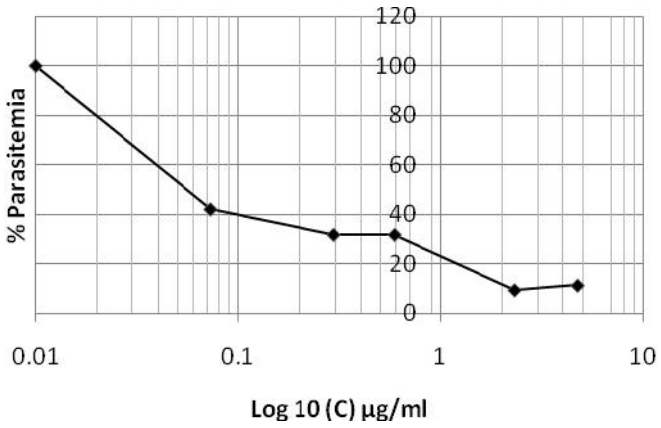
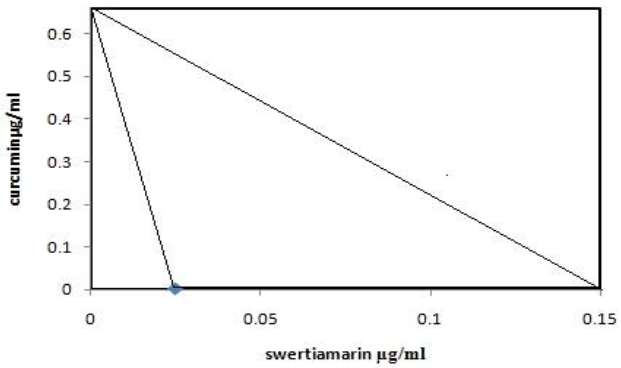


Figure 6.7 Reduction in total parasitemia by swertiamarin and hesperidin in combination (1:2)



Isobole index of swertiamarin 0.025/0.15 + 0.02/0.6 = 0.16 (<1)

Figure 6.8: Isobologram of swertiamarin and curcumin

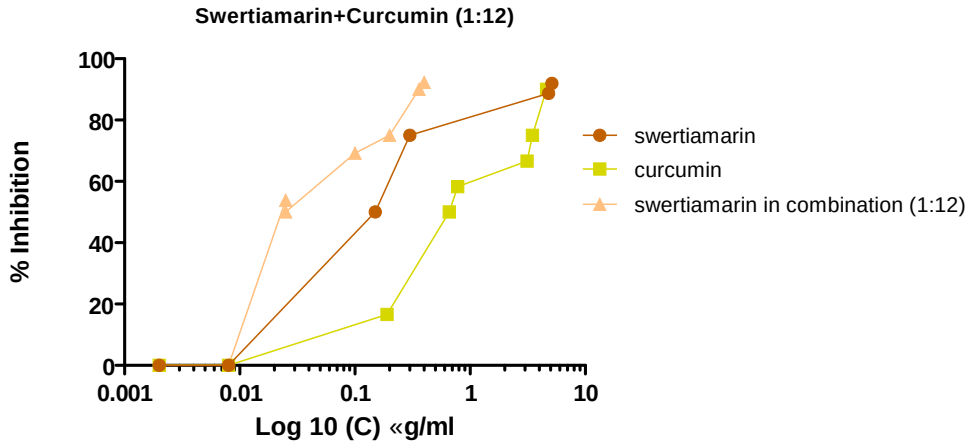


Figure 6.9 Comparative dose response curves of swertiamarin and curcumin in combination

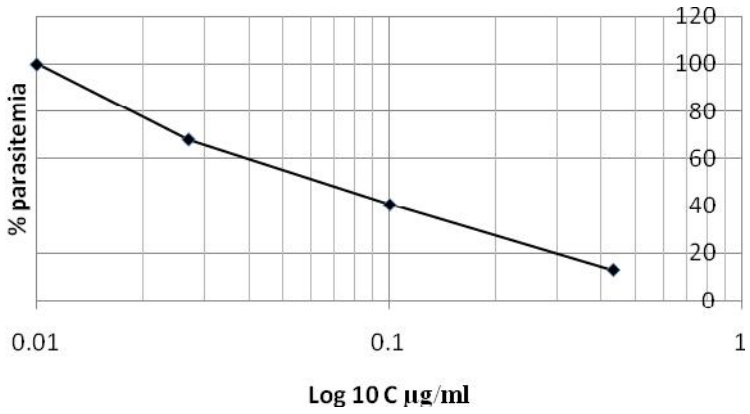


Figure 6.10 Reduction in total parasitemia by swertiamarin and curcumin in combination (1:12)

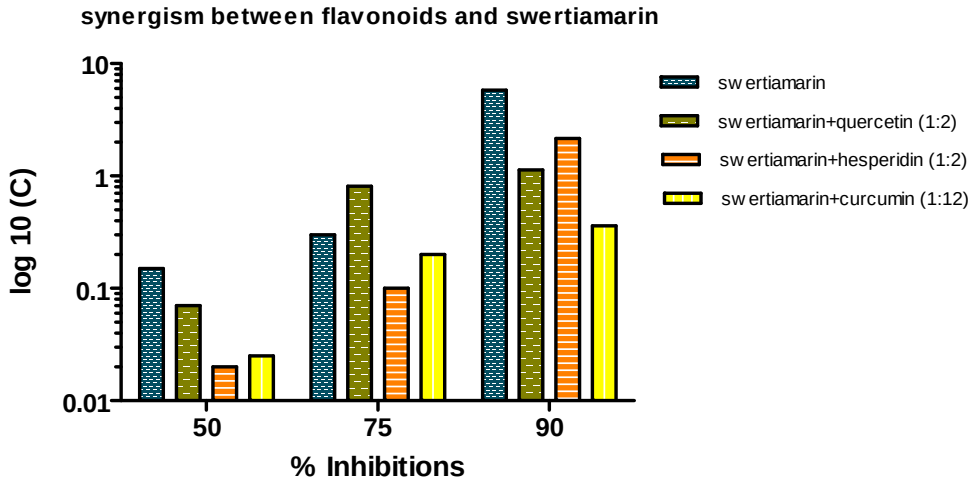


Figure 6.11 Comparative dose response curves of all compounds alone and swertiamarin in combinations

Drug resistant parasites expel the drug from food vacuole faster than the sensitive ones. This results less accumulation of the drug in parasite food vacuole leading to drug failure. Even though chloroquine is facing the same problem, it is previously reported that if chloroquine is given with calcium channel blocker agents like verapamil, the efficiency of the drug is increased against resistant strain (Martiney, J. A. *et al*., 1995). Our present investigation represented that all the flavonoids synergised antimalarial activity of swertiamarin and swertiamarin – hesperidin combination was most potential amongst all. It can be hypothesised that hesperidin can play a role as calcium channel blocker as previous reports also mention calcium channel blocking property of the compound (Morita, Y. *et al*., 1992). So, by considering this, the compound had also been screened with chloroquine to study the synergistic interactions. To validate the results, we compared combination along with chloroquine and verapamil combination. The detailed study is as mentioned below.

6.3.4 Chloroquine with hesperidin

Chloroquine along with hesperidin could produce 50 % inhibition of schizonts at ratio of 1:1. The combination could reduce IC_{50} of chloroquine up to 12 folds than chloroquine alone. Table 6.7 represents effective doses of chloroquine and hesperidin in combination, while figure 6.12 represents isobologram of this combination, representing synergism. Comparative dose response curves of this combination are presented in figure 6.13.

Table 6.7 Effective doses of chloroquine and hesperidin in combination

Drug Name	Inhibitory Concentration (µg/ml)		Fractional Inhibitory Concentrations (ΣFIC)	
	IC_{50}	IC_{90}	FIC_{50}	FIC_{90}
Chloroquine	0.019	0.103	0.017	0.23
Hesperidin	0.022	0.078		

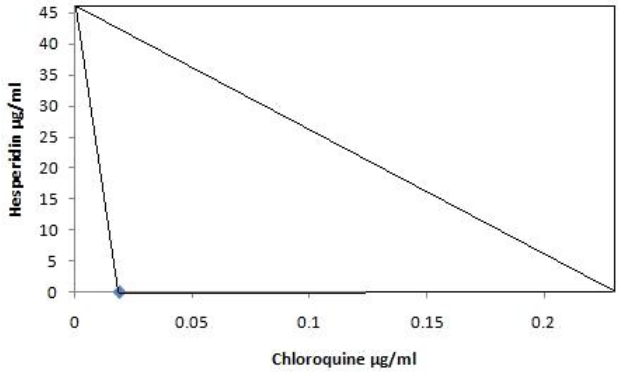
6.3.5 Chloroquine with verapamil

Chloroquine along with verapamil could produce 50 % inhibition of schizonts at ratio of 1:1. The combination could reduce IC_{50} of chloroquine up to 12 folds than chloroquine alone; this also produced similar effect as combination of chloroquine with hesperidin. Table 6.8 represents effective doses of chloroquine and verapamil combination, while figure 6.14 represents isobologram of this combination, representing synergism. Comparative dose response curves of this combination are presented in figure 6.15. Hence, both the combinations of chloroquine produced almost similar effects and found synergistic. The comparative dose response curves of these both combinations along with the individual ingredients are also mentioned in figure 6.16. Verapamil is a calcium channel blocker, produces activity by preventing chloroquine efflux from the parasite food vacuoles. Hesperidin might produce similar actions as some of the evidences mention it as a natural calcium channel blocker.

Table 6.8 Effective doses of chloroquine and verapamil in combination

Drug Name	Inhibitory Concentration (µg/ml)		Fractional Inhibitory Concentrations (ΣFIC)	
	IC_{50}	IC_{90}	FIC_{50}	FIC_{90}
Chloroquine	0.018	0.067	0.117	0.18

Verapamil	0.018	0.067		



Isobole index of chloroquine: 0.019/0.23+0.022/46.66=0.017 (<1)

Figure 6.12 Isobologram of chloroquine and hesperidin

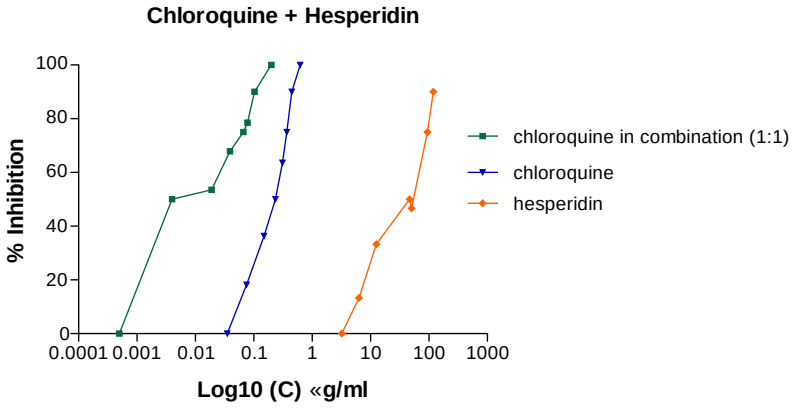
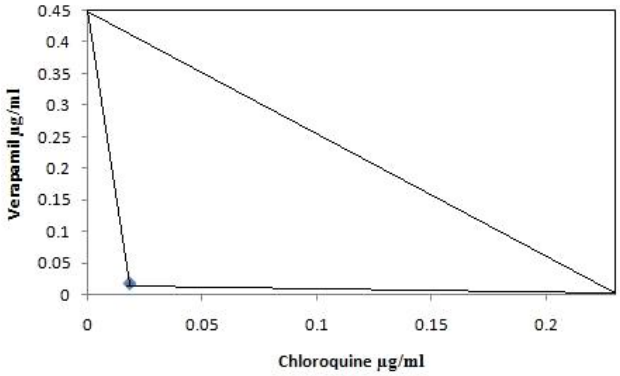


Figure 6.13 Comparative dose response curves of chloroquine and hesperidin in combination



Isobole index of chloroquine 0.018/0.23 + 0.018/0.45 = 0.117 (<1)

Figure 6.14 Isobologram of chloroquine and verapamil

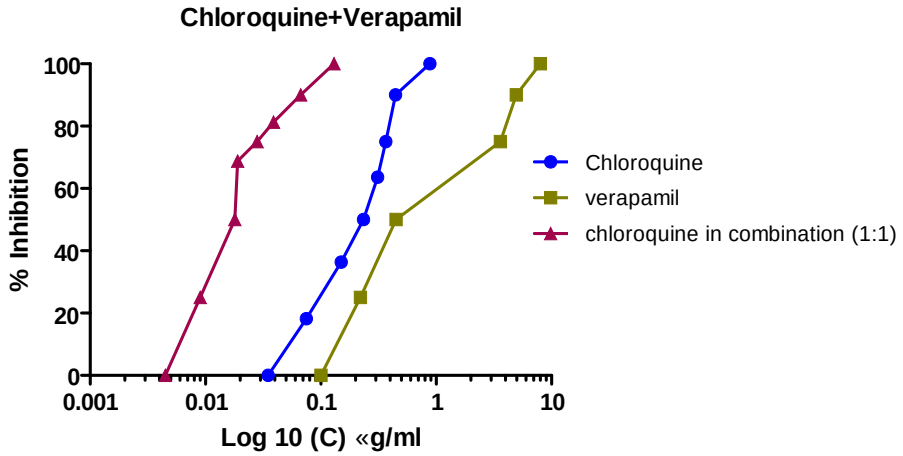


Figure 6.15 Comparative dose response curves of chloroquine and verapamil in combination

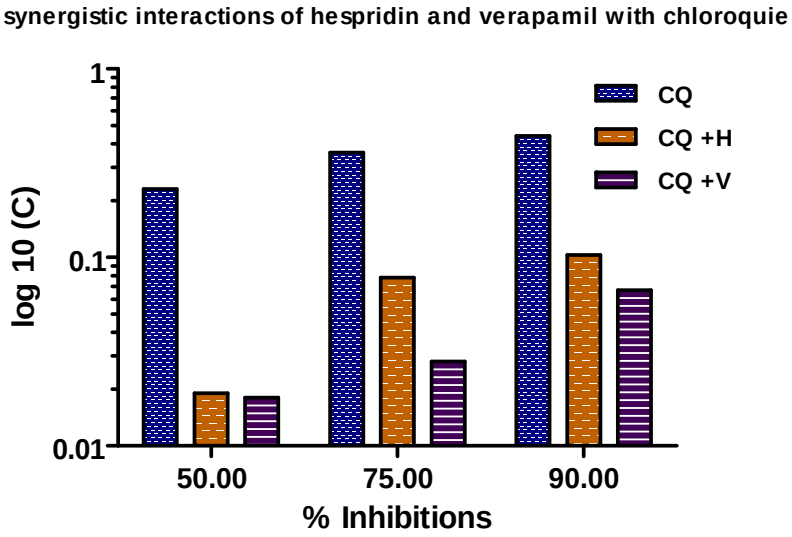


Figure 6.16 Comparative dose response curves of chloroquine in combination with hesperidin and verapamil

6.3.6 Calcium channel blocking potential of hesperidin by sorbitol induced haemolysis assay

We had taken a wide range of concentration and found that hesperidin and verapamil inhibited sorbitol induced lysis of plasmodial parasites with IC_{50} of 6.25 µg/ml. This represents calcium channel blocking efficacy of hesperidin on malaria parasite when compared with verapamil. We also found that the 200 µg/ml concentration of verapamil could lyse the culture but hesperidin could not do the same. The results of effective concentrations of both hesperidin and verapamil are represented in table 6.9.

Table 6.9 Inhibitory concentrations of hesperidin and verapamil against sorbitol induced haemolysis

Drug	**Concentrations of drugs in µM (µg/ml) to inhibit haemolysis**	
	IC_{50}	**IC_{90}**
Hesperidin	10.24 (6.25)	81.96 (50)
Verapamil	13.76 (6.25)	110.132 (50)

6.4 Discussion

Flavonoids (usually along with other compounds) have been identified in the antiplasmodial fractions of many plants, and in some cases they have been shown to possess antiplasmodial activity when isolated (Kraft, C. *et al*., 2000, Liu, K. C. S. C. *et al*., 1992, Yenesew, A. *et al*., 2004).

In many cases there is evidence of synergy, for example, use of herbal teas prepared from the dried leaves of locally grown *A. annua* is being promoted as an alternative treatment for malaria in areas where people do not have access to, or cannot afford effective anti-malarials such as artemisinin combination therapy (Hirt, H. and M'Pia, B., 2008). In a recent study, extracts were obtained from fresh *A. annua* herb either by soaking the herb in water followed by wringing out the juice by hand or by pounding the fresh herb to a pulp followed by squeezing out the juice (Wright, C. W. *et al.*, 2010) and the extracts were then analysed for artemisinin concentration and tested against malaria parasites. It was found that dose of artemisinin was lower in extracts than artemisinin alone. Although it is recognized that the dose of artemisinin contained in herbal preparations is small compared to clinically recommended doses (Wright, C. W *et al*., 2010; Jansen, F. H., 2006, De Ridder, S. *et al*., 2008; Willcox, M. L. *et al*., 2007), it is widely believed that other compounds present in *A. annua*, especially flavonoids, may act synergistically to enhance the action of artemisinin (Liu, K-S. *et al*., 1992; Elford, B. C. *et al*., 1987; Liu, KC-S. et al., 1989).

In this study, we screened three common dietary flavonoids *viz.* quercetin, curcumin and hesperidin in combination with swertiamarin. From results, it could be seen that all these combinations were found as synergistic. Quercetin could reduce the IC_{50} of swertiamarin up to 2 fold. Literature surveys also represent quercetin as bioenhancer for some of the important calcium channel blocker agents like verapamil, as the Cmax and AUC of verapamil was increased two folds (Choi, J. S. and Han, H. K., 2004). Sameway, hesperidin could reduce the IC_{50} of swertiamarin up to 7.5 folds and represented as a potential combination. Literatures also represent that hesperidin, when administrated along with diltiazem and verapamil, increased the half life of the drugs (Cho, Y. A. *et al*., 2009, Pio, Y. J. and Choi, J. S., 2008).

Curcumin had found to lower IC_{50} of swertiamarin up to 6 fold. Some of the recent studies represent its potentiality with artemisinin, as it was found to increase half life of artemisinin which is an important criterion for effectiveness of any antiparasitic drug. Due to poor bioavailability curcumin has been found enhance half life of short acting drugs (Shoba, G. *et al*., 1998). Hence, if we compare the combinations, the order of activity of these three flavonoids along with swertiamarin will be as hesperidin > curcumin > quercetin.

In second phase of the study, we selected hesperidin and combined it with chloroquine. The results were compared with chloroquine-verapamil combinations. From results, it was clear that hesperidin-chloroquine combination was as effective as chloroquine-verapamil combinations. Hesperidin when combined with chloroquine could reduce IC_{50} of chloroquine up to 12 fold. Chloroquine resistance (CQR) in *Plasmodium falciparum* is one of the major causes of the malaria recrudescence in the world. CQR phenotypes in *P. falciparum* were first compared to the multidrug resistance (MDR) status in some mammalian tumour cells (Ginsburg, H., 1991; Karcz, S. and Cowman, A. F., 1991). Altered pharmacology of drugs in MDR cells is related to the P-glycoprotein (P-gp) (Hayes, J. D. and Wolf, C.R. 1989; Endicott, J. A. and Ling, V.1989) and many drugs including calcium channel blockers were shown to interact with this protein (Safa, A. R., 1988) and to restore drug concentrations in resistant strains to levels found in their drug-sensitive parentage (Fojo, A. *et al*., 1985; Rogan, A. M.*et al*. 1989).

The erythrocyte membrane is normally impermeable to sorbitol, but the channels induced by the malaria parasite in the infected cell membrane allow the passage of sorbitol and when mature parasitized erythrocytes are suspended in an isosmotic sorbitol solution, there is a net uptake of sorbitol and water into the erythrocyte, resulting in cell swelling and hemolysis (Kirk, K. *et al*., 1994). Hemolysis was monitored spectrophotometrically by measuring the absorbance of hemoglobin at 540 nm. The rate of hemolysis gives a semiquantitative estimate of the net rate of influx of sorbitol, albeit under nonphysiological conditions. Hemolysis is reduced in the presence of channel inhibitors (lower absorbance), and monitoring of the rates of

hemolysis in the presence of various inhibitors is a convenient means of evaluating the relative potencies of these substances. The present study represents calcium channel blocking potential of hesperidin. The results are compared with verapamil standard and it was found that hesperidin is equivalently effective as verapamil. This was the probable mechanism through which hesperidin could increase potentiality of both swertiamarin and chloroquine and this suggest its use as potential bioenhancer for antiplasmodial activity.

6.5 Conclusion

The present investigation represents the flavoniods *viz.* Hesperidin, curcumin and quercetin produced synergistic interaction with swertiamarin as they lowered the dose of swertiamarin up to 7, 6 and 2 folds respectively. The isobolograms of each combination was also representing synergy. Furthermore, hesperidin was also tested in combination with chloroquine and the results were comparable with chloroquine – verapamil combination.

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7. 1 Summary of the thesis

According to an estimate by World Health Organization, about 350-500 million people are infected from malaria parasites and approximately one to three million deaths reported annually which represents at least one death every 30 sec. The vast majority of cases occur in children under the age of 5 years.

The first gold standard drug for treatment of malaria was quinine, a compound isolated from the bark of a Rubiaceae tree called Cinchona and the drug is still in use. Later on, its derivative was synthesized having more improvised action and lesser side effects like nausea, vomiting and gastric refluxes. The drug is in therapeutic use from around 40 years and is still the cheapest and most effective therapy for malaria. However, due to massive use, resistance with gold standard chloroquine has been widely spread and mortality is on increase in Africa. Treatment of chloroquine-resistant malaria is with alternative drugs or drug combinations, which are rather expensive and sometimes toxic. Furthermore, these combinations are not always based on pharmacokinetic principles due to inadequate knowledge of metabolism and mechanism of action of most antimalarial drugs. Resistance and viability are still problems with other choice of treatment like sulfadoxine pyrimethamine and artemisinins.

The success of the natural products quinine and artemisinin, most potent anti malarial drugs provided thrust to study plants as anti malarial agents. Ethnopharmacological and then phytopharmacological approach for the search of new anti malarial agents from plant sources has proved to be more predictive. In this context, we reviewed many plants mentioned for treatment of *visham jvara* (malarial fever) in Ayurvedic literature. We also reviewed that phytoconstitutes of plants present in major amount, should be logically related to the activity. We aimed to identify the lead which should comply with traditional and chemo rational criteria as well as should be more promising and safe and unique. From review of literature, three plants had been selected *viz. Enicostemma littorale, Adhatoda vasica* and *Embelia ribes* which were not reported for antimalarial activity but having strong traditional backbone to be used for treatment of *visham jvara* (malarial fever). These plants are

used therapeutically as main active components of various formulations and well studied phytochemically as well as pharmacologically.

Enicostemma littorale is well reported for treatment of diabetes. Various studies from our laboratory represent its potentiality in diabetes and related complications to it. Swertiamarin is a major phytochemical present in it. *Adhatoda vasica* is a main active herb used in various formulations for treatment of cough and bronchitis. The plant possesses bronchodilatory property and various studies represent vasicine as main active constituent of the plant. Similarly, *Embelia ribes* is a popular anthelmintic drug having embelin as a main constituent. Many studies represent antimalarial activity of flavonoids, however, there are no reports of antimalarial activity of hesperidin, a major flavonoid found of orange peel. Hence, swertiamarin (a secoirridoid glycoside), vasicine (a quinazoline alkaloid), embelin (a benzo quinone) and hesperidin (a flavonoid) were selected for study and they are structurally diversified compounds when compared with the marketed antimalarials, known to develop resistance.

The primary screening of extracts of selected plants and main active compounds was carried out using chloroquine sensitive strain of *Plasmodium falciparum* (MRC 20). The cultures were maintained in our laboratory by validating various methods to achieve regular parasite life cycle stages occurring in RBCs. For testing of extracts and compounds, we targeted developmental stage of parasite trophozoits to schizonts; an important target through which majority of antimalarial drugs like quinine, chloroquine act and referred as blood schizonticidals. Although many rapid assay techniques are in use to check parasite inhibition, but their validations are always performed microscopically as it is a most reliable technique for studying morphological changes and interference of drugs on parasite life cycle stages.

Order of antimalarial activity of extracts was *Adhatoda vasica* >*Embelia ribes* >*Enicostemma littorale*. But, for compounds it was swertiamarin > embelin > vasicine > hesperidin. From primary screening, we referred swertiamarin as a potential compound as it affected potentially on development of schizonts from trophozoits. Further morphological evaluations also represents that the compound could not

allow parasite food vacuoles development, when drug treated slides were observed after 24 hr. interval. Hence, we selected *Enicostemma littorale* for detailed investigation and to find out most potential leads from the plant.

In Indian traditional literature, extract of *Swertia chirata* and formulations containing the plant like Ayush-64, Sudarshan Ghanvati has been reported for anti malarial activity but active constituent responsible for antimalarial activity has not been reported yet. *Enicostemma littorale* (Gentianaceae) contains higher amounts of swertiamarin (7.7%), a bitter principle than that in *Swertia chirata* (0.94%) which favours more commercial viability of the plant when swertiamarin is to be isolated.

In this context, activity guided fractionation was carried out from the plant *Enicostemma littorale* and it was found that *n* – butanol fraction was found as most potential. First time we observed that this fraction was not only inhibited schizont maturations from trophozoits but also killed the parasites as number was increased with rise in concentration. HPTLC analysis confirmed that swertiamarin in butanol fraction was present at around half of the total weight. Hence, the activity of *n* - butanol fraction could be considered corresponding to its major compound. Later on, swertiamarin had been isolated from this fraction. Although many methods for isolation of swertiamarin are reported, we developed a rapid method of isolation of the compound to achieve pure compound in good quantity. We found a potential antimalarial activity of this compound with IC_{50} of 2.15 µM (0.82 µg/ml) which is considered very good active, as mentioned by Batista (Chapter 2, 2.5).

Furthermore, we tried to investigate gentianine, another important phytochemical present in plants of Gentianaceae and also found in *Enicostemma littorale*. Previous studies suggest that swertiamarin is metabolized to gentianine by human intestinal bacteria. The compound is of alkaloidal nature and had been reported as a responsible antimalarial candidate of gentianaceous plants. To obtain the compound in good quantity for assays, we converted swertiamarin to gentianine chemically. From antimalarial assays, we found that gentianine was less potential than swertiamarin as the IC_{50} of compound was 57.14 µM (10 µg/ml) and categorized as moderately active. We also hydrolysed swertiamarin as a probable derivative in gastric pH. Both derivatives of swertiamarin as probable metabolites

were screened for antimalarial activity. Our findings demonstrate that both gentianine and hydrolysed form of swertiamarin were found less active than swertiamarin.

The next phase of study was to investigate most potential antimalarial compounds on chloroquine resistant strain of *Plasmodium falciparum*. Resistance of parasite to antimalarial drugs has been demonstrated as a worsening problem throughout the world. *Plasmodium falciparum* has developed resistance to nearly all antimalarials in current use. The appearance of drug-resistance *P. falciparum* strains since 1960, in particular to chloroquine, has made the treatment of malaria increasingly problematic in virtually all malarious regions of the world. Massive use of chloroquine, a golden and cost effective treatment for malaria, resulted in resistance to *P. falciparum* parasites everywhere in the world and this was transmitted to various malarious areas like Central America (north-west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia. Mefloquine resistance is also frequent in some areas of South-East Asia and has been reported in the Amazon region of South America and sporadically in Africa.

Three common dietary flavonoids *viz.* querctin, curcumin and hesperidin were also selected for study. Flavonoids (usually along with other compounds) have been identified exhibiting antiplasmodial activity in fractions of many plants and in some cases have been shown to possess antiplasmodial activity. We also isolated a flavonoid, swertisin from *Enicostemma littorale* and tested for antimalarial activity but could not found any changes in parasite developmental stages. While, curcumin was found to demonstrate good activity, whereas quercetin and hesperidin had moderate activity. The compound swertiamarin was found most potential as it is more effective than chloroquine standard. IC_{50} of the compound was 0.15 µg/ml; less than 1 µM.

Furthermore, we evaluated synergistic interactions of swertiamarin along with these three dietary flavonoids *viz.* quercetin, curcumin and hesperidin. In case of multiple drug resistance, it is well known that rather than a single drug, combinations are often more effective by lowering dose of active compounds and

acting on multiple targets. Many of traditional therapies for malarial fever are in decoction known to contain flavonoids as bioenhancers by improving solubility of more lipophilic compounds in water. Several flavonoids were previously investigated from a popular Chinese antimalrial remedy – *Artemisia annua* for their potential against multidrug resistant parasites.

In this context, various combinations each flavonoid along with swertiamarin were studied and isobolograms were prepared to investigate interactions of the compounds. From results, it could be seen that all these combinations were found as synergistic. Quercetin could reduce the IC_{50} of swertiamarin up to 2 fold with the combination of one part of swertiamarin and two parts of quercetin. Literature surveys also represent quercetin as bio enhancer for some of the important calcium channel blocker agents like verapamil, as the Cmax and AUC of verapamil was increased two folds. Same way, hesperidin could reduce the IC_{50} of swertiamarin up to 7.5 folds and represented as a potential combination with the ratio of swertiamarin: hesperidin (1:2). Literatures also represent that hesperidin, when administrated along with diltiazem and verapamil, increased the half life of the drugs. Curcumin had found to lower IC_{50} of swertiamarin up to 6 fold when 12 parts of compound were combined with 1 part of swertiamarin. Some of the recent studies represent its potentiality with artemisinin, as it was found to increase half life of artemisinin which is an important criterion for effectiveness of any antiparasitic drug. Curcumin alone has poor oral bioavailability and has been found to enhance half life of short acting drugs. The order of activity of these three flavonoids along with swertiamarin will be as hesperidin > curcumin > quercetin.

In second phase of the study, we selected hesperidin and combined it with chloroquine. The results were compared with chloroquine-verapamil combinations. From results, it was clear that hesperidin-chloroquine combination was as effective as chloroquine-verapamil combinations. Hesperidin when combined with chloroquine could reduce IC_{50} of chloroquine up to 12 fold. Further, we were interested to investigate a probable pathway through which hesperidin possesses activity. Many of clinical studies represent usage of orange juice in hypertension. Hesperidin had also been reported to enhance of calcium channel bloking agents like

verapamil. A simple assay was performed to investigate calcium channel blocking potential of hesperidin. Parasite cultures (with high parasitemia; 10 %) were incubated in a hypertonic solution of sorbitol. As a source of energy, parasite uptake sugar and due to hypertonicity of the solution, parasite infected RBCs are lysed. If we incubate the same with calcium channel blockers, invasion of sugars in parasite infected cells are prohibited. Hesperidin was found to possess activity by the same mechanism. Hence, it could be stated that hesperidin enhanced antimalarial property of both swertiamarin and chloroquine by blocking parasite calcium channels.

Parasites during intra erythrocytic phases digest haemoglobin from host red blood cell (RBCs) and free heme is generated as a product, toxic to parasite. Detoxification of free heme is a spontaneous process in which heme is sequestered in to beta hematin, a non toxic product. Antioxidant compounds are well known to scavenge free radical generated in biological system. Hence, it would be interesting to search role of antioxidants particularly, common dietary flavonoids; quercetin, hesperidin and curcumin in formation of beta hematin from heme. We screened quercetin, hesperidin and curcumin for β - hematin formation inhibition, a non biological target specific assay through which majority of blood schizonticidals act. Hematin was allowed to sequester with acetate buffer to form beta hematin as end product and the compounds were screened along with the same system to check interference of compounds in sequestration of free hematin and found that all these compounds were inhibiting beta hematin formation from hematin. If we grade the results of beta hematin formation inhibition, the order is Curcumin > Quercetin > Chloroquine Phosphate > Hesperidin. Although chloroquine is most potential drug acting on this target, quercetin and curcumin were proven as more effective than chloroquine, these may because of free heme binding profile of antioxidants. Thus, it can be considered from preset study, antioxidants play a vital role in malaria infection.

7.2 Conclusions

- Swertiamarin, a major phytoconstituent of *Enicostemma littorale* possessed very good antimalarial activity against chloroquine sensitive strain of *Plasmodium falciparum* by inhibiting parasite development from trophozoit to schizont stage.
- Swertiamarin also possessed potential antimalarial activity against chloroquine resistant strain of *Plasmodium falciparum*.
- Combinations of swertiamarin with common dietary flavonoids *viz.* quercetin, curcumin and hesperidin were found synergistic, among which, swertiamarin – hesperidin combination was most potential.
- Common dietary flavonoids possesses antimalarial action by inhibiting β hematin formation; the detoxification process in parasite food vacuoles.
- Combination of chloroquine – hesperidin was as potential as chloroquine – verapamil combination.
- Hesperidin enhanced antimalarial potential of both swertiamarin and chloroquine by calcium channel blocking mechanism.

Future studies and recommendations

- *In vivo* antimalarial activity of swertiamarin and swertiamain - hesperidin combination
- To study acute and chronic toxicity of swertiamarin with regard to establish its therapeutic dose
- Pharmacokinetic studies of swertiamarin to ensure the half life
- *In vivo* metabolism of swertiamarin and characterization of its metabolites