Molecular Epidemiology of Malarial Parasite from Punjab, Pakistan and its Control through Plants Based Extracts and Nanoparticles

By

Huma Fatima

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

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Molecular Epidemiology of Malarial Parasite from Punjab, Pakistan and its Control through Plants Based Extracts and Nanoparticles

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بسم الله الرحمن الرحيم


بسم الله الرحمن الرحيم

Dedicated to my

Parents

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Student Name: **Ms. Huma Fatima** Signature: _____

Examination Committee:

a) External Examiner 1: Signature: _____

Dr. Akram Shah
Professor, Department of Zoology,
University of Peshawar, Peshawar

b) External Examiner 2: Signature: _____

Prof. Dr. Afsar Main
Department of Biology
Arid Agriculture University Rswalpind

Supervisor Name: **Dr. Naveeda Akhtar** Signature: _____

Name of HOD: **Dr. Sajid Malik** Signature: _____

Date: 26-12-2018

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Huma Fatima

Contents

List of Abbreviation

ABER	Annual Blood Examination Rate
API	Annual Parasite Incidence
ALP	Alkaline Phosphatase
AST	Amino Transferase
ALT	Alanine Amino Transferase
CDC	Center for Disease Control and Prevention
ED_{50}	Effective Dose for 50% survival of tested population
EDTA	Ehtlene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FATA	Federally Administered Tribal Areas (Part of KPK)
FTIR	Fourier transformed infrared spectroscopy
Hb	Hemoglobin
IC_{50}	Inhibitory concentration at 50 % cells death
PCR	Polymerase Chain Reaction
KPK	Khyber Pakhtunkhwa
LD_{50}	Lethal Dose at 50% lethality of tested population
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean Cell Volume
NCBI	National Center for Biotechnology and Information
NPs	Nano Particles
PVC	Packed Cell Volume

PRBCs	Parasitized Red Blood Cells
RDT	Rapid Diagnostic Test
SEM	Scanning Electron Microscopy
SPR	Slide Positivity Rate
TBE	Tris/Borate/EDTA
TEM	Transmission Electron Microscopy
UV- vis	Ultra Violet Visible
USA	United State of America
WHO	World Health Organization
XRD	X-Ray Diffraction Analysis

List of Tables

List of Figures

General Abstract

Malaria is one of the serious diseases distributed worldwide, especially in the underdeveloped countries of tropical and subtropical regions. It is caused by protozoan parasites of genus *Plasmodium*, which is transmitted in humans a mosquito vector of the genus *Anopheles*. In human, the five species of *Plasmodium* are yet diagnosed causing malaria i.e. *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum* and *P. knowlesi* of which *P. falciparum* and *P. vivax* are widely distributed. The available epidemiological data on malaria from Pakistan is insufficient. Moreover the malarial patients are showing resistance against the prime antimalarial drugs like chloroquine, pyrimethamine, mefloquine and artemisinin. Therefore the present work is planned to identify different species of *Plasmodium*, their incidence in the ten selected cities of Punjab, Pakistan situated in the Northern and Southern Punjab. The seed extract of five medicinal plants and nanoparticles of silver and iron of three effective plants that showed antiplasmodial activity were evaluated in an *in vivo* experiment using BALB/c mice infected with *Plasmodium berghei* strain.

The data of (n=16075) malaria suspected cases and blood samples of microscopically positive samples (n=925) were collected from different hospitals of ten cities of Punjab. i.e. Rawalpindi, Jhelum, Gujrat, Chakwal, Gujranwala, Dera Ghazi Khan, Rajanpur, Rahim Yar Khan, Multan, and Bahawalpur in whole year of 2015 from January to December under all biosafety and bioethical rules. The blood samples were evaluated for distribution of malaria cases among the study area, seasonal variation, age group, gender, and for species diagnosis on the basis of microscopy and molecular analysis. After that prevalence was estimated on the basis of molecular results. Some of the positive PCR products were analyzed by DNA sequence and evolutionary history was inferred using the Neighbor-Joining method.

The aqueous seed extracts of five medicinal plants, i.e. ***Trachyspermum ammi***, ***Punica granatum***, ***Cydonia oblonga***, ***Benincasa hispida*** and ***Saussurea lappa*** were obtained via soxhelet apparatus and identified for major constituents by phytochemical analysis. The experiment was designed using BALB/c mice as a model animal infected

with *Plasmodium berghei* pathogen to find antiplasmodial activity of selected plants. The three plants *T. ammi, P. granatum* and *S. lappa* were used to synthesize silver and iron nanoparticles. The presence of nanoparticles was confirmed by UV-visible spectroscopy; Fourier transformed infrared spectroscopy, X-ray diffraction analysis and Transmission electron microscopy.

In order to calculate LD_{50} the mice were given four different oral doses of each plant extract, observed for their physical behavior and other toxicity symptoms like convulsion, coma and death. The LD_{50} was calculated by Probit analysis. The mice were grouped and infected with *Plasmodium berghei*. After seventy two hours of post infection mice were treated with different concentrations of experimental plants and their silver and iron nanoparticles. The effect of seed extracts and nanoparticles were studied by general symptoms, parasite count, chemosupression, survival time measurement, histopathology, biochemical and hematological analysis along with the positive and negative control on randomly selected five mice from each group.

The slide positivity rate, annual parasite incidence and annual blood examination rate were 5.75%, 0.12 per 1000 population and 0.22% respectively in all recruited cities Punjab. The seasonal variation showed that highest cases were recruited in summer season, i.e. from June to September then its incidence decreases. The age wise distribution of malarial patients was maximum among 1-20 years and lowest among 41-60 years. The gender wise distribution indicated that *Plasmodium* infection was dominant in males than females in all study areas. Out of the 925 microscopically positive recruited malarial infection 66.70% (n=617) were diagnosed as *P. vivax,* 23.67% (n=219) as *P. falciparum* and 9.62% (n=89) as mixed containing both *P. vivax* and *P. falciparum* whereas no case of other *Plasmodium* species was observed. The PCR results showed that 53.40% (n=494) as *P. vivax*, 16.70% (n=173) as *P. falciparum* and 12.86% (n=119) as mixed species and 15.02% (n=139) were not amplified through PCR. The overall prevalence of treatment seeking malarial patients in all recruited cities of Punjab was 4.88%. The DNA sequenced sample of *P. vivax* and *P. falciparum* found in this study were more related to India, Brazilian western Amazon and Korea species of phylogenetic trees.

The qualitative phytochemical analysis of experimented plants revealed the presence of alkaloids, flavonoids, saponins and steroids, etc. The maximum antiplasmodial activity (80.06±4.89) was observed for the *S. lappa* that was treated at the dose of 750 mg/kg. The *P. granatum, B. hispida, T. ammi, and C. obloga* showed antiplasmodial activity as (77.58±2.09) > (71±6.12) > (69.76±2.01) > (68.76±2.01) at the dose of 500 mg/kg. The maximum antiplasmodial activity was observed for the Silver nanoparticles of *P. granatum* (85.92 ±0.5) > *S. lappa* (85.32 ±0.82) > *T. ammi* (83.50 ±0.65) that was treated at the dose of 150 mg/kg.

The iron nano particles results showed that antiplasmodial activity was observed in an order of *S. lappa* (82.51±4.68), >*P. granatum* (81.46±3.10) > *T. ammi* (77.14±4.18) at the dose of 300 mg/kg. However the nonsignificant (P>0.5) results was shown when the chemosupression was compared with chloroquine treated group. The mean survival time, weight change, biochemical, the hematological and histopathological analysis also showed a marked difference in infected untreated group as compared to groups treated with nanoparticles, plant extracts and chloroquine.

The slide positivity rate, annual parasite incidence, and blood examination rate was high in Northern Punjab as compared to Southern Punjab. Season wise distribution of malaria indicated the high incidence in summer and postmoon season. The incidence of malaria was high in low and middle age group as compared to the old age groups. The gender wise distribution indicated that *Plasmodium* infection was dominant in males. The present studies confirmed the presence of two *Plasmodium* species *i.e. P. vivax and P. falciparum* from the clinical isolates of the ten cities of Punjab, Pakistan. The overall prevalence of treatment seeking malarial patients in all recruited cities of Punjab was 4.88%.

The seeds of *S. lappa, P. granatum* and *B. hispida* exhibited significantly more antiplasmodial activity as compared to *T. ammi* and *C. oblonga.* Both silver and iron nanoparticles of all the three experimented plants exhibited antiplasmodial activity and also no adverse effects on the hematology, histology and biochemical parameters were observed. It is therefore suggested that the emerging nanotechnology can be used in drug

formulations. The synthesized nanoparticles can be used to develop new agents for parasite control.

In this study non-specific targeting of nanoparticles were studied, but special targeted nanoparticles can be designed that can help the immune system to attack the species of malaria parasite after it enters the body and before it has a chance to hide and aggressively spread. In this way nanoparticles can also help in the vaccine formation by stimulating the immune system.

General Introduction

General Introduction

The pathogen of malaria is an apicomlexan protozoan of class Haemosporidia (Irshad *et al.*, 2013) belonging to the genus *Plasmodium.* It is spread by female *Anopheles* mosquitoes. Nwazue *et al* (2013) estimated that about 165 *Plasmodium* species infect vertebrates through different ways. However, five species of *Plasmodium* infect humans that are *P. ovale, P. vivax, P. knowlesi,* P. *malariae* and *P. falciparum.* In European countries most cases are due to *P. malariae and P. vivax* (Voittier *et al.*, 2008).

This disease has become a public health issue in developing countries, and approximately 50% of total world population is at danger of contracting this disease (WHO, 2008), with 250 million cases and one million deaths being reported globally per year (WHO, 2009). The majority of the malaria cases have been reported in the Ethiopian region (78%) then in South East Asia (15%) next is the Eastern Mediterranean (5%) area (WHO, 2010).

Inspite being a avoidable and treatable disease, malaria is one of the public health worldwide challenge (Irungu et al., 2012). Among the global prevalence in 2017, about 219 million cases of malaria were estimated (WHO, 2018). According to the WHO, report of 2015, there were 660,000 deaths (WHO, 2016) but the death no decreases to 435,000 in 2017 (WHO, 2018). WHO (2011) reported that high risk areas of malaria consists of 3.3 billion population of the world. In disease-prevalent areas of Africa, 54% of hospitalized children who were aged less than one died due to malaria (Schellenberg, 1999).

Pakistan is present in Eastern Mediterranean region of WHO with an estimated 4.5 million suspected cases of malaria (WHO, 2012). Malaria is the most prevalent disease in Pakistan, with 177 million out of 180 million inhabitants of Pakistan being at risk of malaria and 3.5 million cases reported annually. Despite having a well established malaria control program, there is no remarkable decrease in malaria prevalence in Pakistan (Mukhtar, 2006). Furthermore, 97% of Pakistan's inhabitants are at hazard of being attacked by malaria pathogens (WHO, 2010).

All the provinces of Pakistan have a high rate of malaria endemicity (Raza and Beg, 2013). The highest incidence of malaria was observed in Baluchistan and Sindh

as compared with that of Punjab. The Punjab and Khyber Pakhtunkhwa (KPK) were reported to have moderate incidence whereas FATA, Sindh and Baluchistan are among the highly malaria prevalent regions (Kondrachine, 2008).

In Punjab due to variation in seasons, malarial incidences changes with temperature and unbalanced in this province. The malaria prevalence along with seasons in a year also varies years to years. The considerable variations in its prevalence are the cause of floods and heaviy rainfall in Southern Punjab and Northern Punjab (Bouma and Van Der Kaay, 1996).

The pathogen of malaria is transmitted through mosquito that belongs to the genus *Anopheles* (Tyagi and Chaudhary, 1997; Tyagi *et al.*, 1995). There are 460 species of genus *Anopheles* reported around the world (Wells *et al.*, 2009). Out of these 23 species are the vectors of malaria parasite. However *A. culicifacies* and *A. stephens* are important malarial vector and *A. culicifacies* is more common in rural areas (Singh, 2011; Dash *et al.* 2007).

However in KPK province of Pakistan many species of *A. culicifacies* and *A. stephensi* are reported for malarial transmission, but *A. stephens*is is more prominent than *A. culicifacies* which is a vector of *Plasmodium* species in Afghanistan, Pakistan and India (WHO, 2009). Ekpuka *et al.*, (2013) reported that no any human malarial pathogen reservoir other than human is yet reported (Ekpuka *et al.*, 2013).

The malaria vector population is influenced by ecological factor. The specific habitat is necessary for the reproduction of mosquitoes i.e. water condition for reproduction and moisture for their survival. The development rate of both the vector and parasite population is influenced by temperature (Ceccato *et al.*, 2005).

Pakistan is an agricultural country and due to extensive agricultural practices, monsoon rain, extensive irrigation network and snow melting all these factors promote the development of malaria vector and increase in the transmission of malaria (Tasawer *et al.*, 2003). The most favorable months for malaria transmission are from June to November in Pakistan (Safi *et al.*, 2010).

The occurrence of *P. falciparum,* and *P. vivax* is comparatively higher in the tropical and subtropical regions. *P. ovale* is widespread in the West Africa and *P. knowlesi* in South East Asia (Krettii *et al*, 2001).

Pakistan is in habitant of P. *falciparum* and *P. vivax* no other species are reported yet now may be due to there less prevalance (Asif, 2008). Among all these species *P. falciparum* is the most virulent infecting brain (Clarkson *et al.*, 2004, Sahar *et al.*, 2010). Endemic areas have *P. vivax* as dominant species except in Africa. About 100 million cases of *P. vivax* infection are recorded every year in the endemic areas (Bozdech *et al.*, 2008).

Infants and young children are more vulnerable of *P. vivax* infection in its prevalent areas (Poespoprodjo *et al.*, 2009). Khattak *et al.*, (2013) showed that approximately 64% malarial infection is caused by *P. vivax* and 34% is due to the *P. falciparum*. Malaria infection is different in different age groups. The age of infants less than six months are immune to malaria due to the existence of maternal antibodies (Larru *et al.*, 2009).

The rate of *P. falciparum* incidence is infrequent in highly endemic areas after 5 years of age. More adults are not infected in high transmission area while young children are more affected in moderate transmission areas, and all age groups are at risk to be infected in low transmission area (Dondorp *et al.*, 2008).

The bite of infected female mosquito results in the transmission of infectious sporozoite to the mammalian host (Aly et al., 2009). Single bites of an infected mosquito, inject hundreds of sporozoite that ultimately makes their way to the liver attacking tissues (Lindner *et al.*, 2012). Sporozoite play important role in host cell invasion (Ishino *et al.*, 2000). With the successful invasion of liver cell the sporozoites develops into trophozoites (Jayabalasingham et al., 2010).

Upon development the trophozoites enters schizogony and produce large number of liver schizonts (Stanway et al., 2011). The shizont differentiates into the merozoites that infect blood. The red blood cells are ruptured and merozoites are released into the blood and infect other erythrocytes (Bannister and Sherman, 2009). Approximately 6 to 32 daughter merozoites are produced by each merozoite in 24 to 72 hours but the number varies from species to species (Anstey *et al.*, 2009).

Some merozoites differentiate into micro and macro gametocytes, which enter into the gut of mosquitoes with their blood meal where they undergo the sexual reproduction and complete their life cycles (Bannister and Sherman, 2009).

The general symptoms of malaria include high temperature, trembling, joint pain and nausea and more often weight loss (Beare *et al.*, 2006; Nadjm and Behrens, 2012).

The malaria can be diagnosed commonly by three methods i.e Microscopy, Rapid diagnostic test (RDT) and PCR. The Giemsa staining is used to stain thick and thin smears for oberservation of microscopic diagnosis of malaria. The percentage parasitemia can be found by counting infecting RBC's on the thin film (Bailey *et al.*, 2013). The microscopic examination requires long time and constant attention and need skilled experts (Khairnar *et al.*, 2009).

The available medical facilities are limited in the rural areas. Due to the lack of equipments, paramedical staff and electricity, the quick and commonly used technique for the detection of *Plasmodium* is RDT or immunodiagnostic tests. The principle of this method is the identification of antigens. These antigens are released from infected erythrocytes. The specific RDT test that is commercially available for *P. falciparum* is para HITf test. It identifies histidine rich protein11 (Taviad *et al.*, 2011). The antigen lactate dehydrogenase is produced by *P.falciparum, P. malariae, P.vivax,* and *P. ovale* (Panchal and Desai, 2012).

However the performance of RDT varies with parasite species, environmental occupation and geographical location. The low parasitic densities decrease the sensitivity of RDT. The RDTs give different results in the laboratory and field conditions (Kim *et al.*, 2008). It gives results within 15 to 20 minutes and don't require skilled technicians. However, it is less specific and sensitivite than microscopy (Okiro *et al.*, 2009).

The more accurate species diagnosis and phylogeny is made through DNA extraction and PCR. The nested PCR approach is used for diagnosis of *Plasmodium* even in the low level of parasitemia. The 18s ribosomal RNA region of the parasite is used for the identification of *Plasmidium* species (Kebede *et al.*, 2014). The other type of PCR used for the detection of *Plasmodium* is Real-time PCR. Through Real time PCR can detect 0.01 to 0.02% parasitemia in the patient specimens with 0.2 genomes

analytical sensitivity was estimated per reaction (Mangold *et al.*, 2005 and Chaudry *et al.*, 2012).

The origin of *P. vivax* is very complicated. The *P. vivax* shows a close relation with *P. simium* (monkey parasite). Many studies are in agreement that the host switch event occurs and one of *P. vivax* or *P. simium* species emerged in South America (Lim *et al.*, 2005). The source of *Plasmodium*, related to *P. vivax* which not only links the monkeys and humans, but also link the chimpanzees and gorillas (Liu *et al.*, 2010).

Along with these general symptoms malaria infection also results in the hematological and neurological disorders (Dzeing-Ella *et al.*, 2005). Hematological infection outcomes in the anemia (Wickramasinghe and Abdalla, 2000) by damage of uninfected and infected erythrocytes, deficient erythropoiesis and low proportion of RBCs in circulation (Kumar, 2009).

The clinical indications of malaria are mainly associated with destruction of erythrocytes by merozoites (Trampuz *et al.*, 2003). In early stages patients experience fever, anxieties and headaches with other common symptoms like dizziness, sickness, abdominal pain, vomiting and dry cough (Genton and Acremont, 2001). During pregnancy malaria can cause early delivery, low birth weight and fetal death (Carpenter *et al.*, 1999).

After proliferation of the parasites the patients develop the periodic fever or malarial paroxysm consisting of three consecutive phases i.e. cold, hot and sweating stage. In *P. ovale* and *P. vivax* the schizonts grow after 48 hours and the periodicity is tertian while in *P. malariae* (Quartan malaria) schizonts develop after every 72 hours (Baron, 1996). Cerebral malaria is observed in the majority of serious case of malaria caused by *P. falciparum* (Baron, 1996). Malarial hepatitis is common in case of P. falciparum infection (Anand *et al.*, 1992) and is rare in P. vivax malaria (Chakravorty and Craig, 2005).

Together the host and parasite associated elements contribute to the pathogenicity of the disease (Autino *et al.*, 2012). General signs of malaria fever happen after the schizont ruptures. The toxins and other products of parasites are released from the ruptured RBCs. Fever is caused by triggering of the immune system

and release of proinflammatory cytokines (TNF-α) in response to the parasite material (Rowe *et al.*, 1997).

Inside the RBCs the schizonts metabolize the globin part of hemoglobin while the heme part, that is toxic to the parasite is converted into an insoluble pigment called hemozin (Ihekwereme *et al.*, 2014). The hemozin present inside the parasitized RBCs or act as a free crystal. This pigment disturbs the general cellular function and physiology of host while the parasite is not affected (Schwarzer *et al.*, 2008). The presence of monocytes containing hemozin is linked with severe malarial anemia (Novelli *et al.*, 2010), immunosuppression (Arese and Schwarzer, 2008) and cytokines deregulation (Ochiel *et al.*, 2005).

Cytoadherence or the adherences of the parasitized RBCs in the endothelial lining of circulatory passages cause severe malaria and local anoxia (Baron, 1996). Sequestration of *P. falciparum* causes of cerebral malaria in brain (Grau and Craig, 2012). The pathogenesis of anemia is associated with destruction of infected and non-infected RBCs and reduces erythrocytes production (Autino *et al.*, 2012). The disfunctioning of liver is associated with reduced blood flow in portal passage due to the adherence of pRBCs and high parasitemia that suppress bilirubin excretion (Bhalla *et al.*, 2006).

Currently, many drugs are in use for curing malarial infection e.g. quinine, resochin (chloroquine), sontochin proguanil, sulfones, sulfonamides proguanil or pyrimethamine) mefloquine and artemisinin (CDC, 2005). *P. falciparum* and *P. vivax* are two main agents that produce infection in human but unfortuantly they has developed resistance against antimalarial drugs. *P. falciparum* become resistant to all drugs although the extent of resistance fluctuates significantly (Gelband *et al.*, 2004: Looareesuwan *et al.*, 1997).

The first antimalarial drug Quinine was derived from *Cinchona officinalis bark. C. officinalis* has origin in South America in 1820 (Phillipson, 1991). Since that quinine is used as a potent candidate for malaria treatment, irrespective of the fact qunine resistance is being developed randomly. However, a case of resistance against quinine was reported in 1910 (WHO, 2003).

Hans Andersag worked for Bayer IG Farbenindustrie in Elberfeld, Germany discovered sontochin and resochin as an alternate of quinine. *P. falciparum* has developed resistance against chloroquine in four different localities. In pakistan the case of chloroquin resistance was reported in 1984 for the first time and latter on confirmed in Punjab and Landi kotal near Pak-Afghan border (Mockenhaupt *et al.*, 1995: Robinson *et al.*, 1984).

The chloroquine and primaquine are synthetic drugs prepared to overcome the resistance to quinine (Baird and Hoffman, 2004). All of these drugs interfere with parasites in the production of toxin i.e. hemozin, although qunine is also in use for malaria cure (WHO, 2009).

The proguanil, is a derivative of pyrimidine. It was introduced into the pipeline of antimalarial drugs in 1948. Further study on its chemical class led to the discovery of pyrimethamine. The antifolate drugs, next to the first line drugs; restrict the folic acid synthesis by hindering the parasite enzyme dihydro folate reductase DHFR (Wells *et al.*, 2009). With the mutation in the DHFR gene, *P. falciparum* resistant strain against proguanil (Peterson *et al.*, 1990).

The combined therapy strategy was used in which sulfones and sulfonamides combined with proguanil or pyrimethamine in order to increase efficacy (Arrow *et al.*, 2004). But resistance against antifolate emerged more quickly than chloroquine (Wells *et al.*, 2009). It was noted first time in Tanzania than in Thailand in 1967 and spread rapidly throughout South-East Asia and Africa (Mockenhaupt *et al.*, 1995).

Next the mefloquine came in the pipeline of antimalarial drug in 1974. mefloquine's prevented the *p. falciparum* infection effectively (Carpenter *et al.*, 1991). mefloquine was very effective against *P. falciparum* (WHO, 1990) but the side effects were associated with nervous and psychiatric problems (Lobel *et al.*, 1991). Mefloquine resistance developed 1985 for the first time Asia. Mefloquine resistance is frequent in some areas of South East Asia and has been prevalent in Africa and amazon region of South (Mockenhaupt *et al.*, 1995).

With emerging resistance of parasite, a new drug artemisinin was extracted from Artemisia annua (shrub, wormwood plant) in 1972 by the Chinese researcher (Tu, 2016). Artemisinin is very effective against the asexual stages of *Plasmodium*.

Commonly used derivatives of artemisinin are artemether and artesunate. The *Plasmodium* species become successful in getting resistant against artemisinin in many part of the world and all other drugs, so the Artemisinin combine therapy is recommended by WHO (Carpenter *et al.*, 1991). Resistance to artemisinin combine therapy was first recorded in 2009 (WHO, 2010).

The possible reasons for the antimalarial drugs resistance is the unusual genetic structure of malarial parasites the parasite changes its cell surface protein e.g , merozoite surface proteins of *P. vivax* (Pvmsp-3α and Pvmsp-3β) genes and *P. falciparum,* (Pfmsp-1 and two of Pfmsp-2) in order to escape from antimalarial drug used (Khatoon *et al.*, 2010). The fake or poor quality treatments and unfettered or inadequately managed treatment with drugs is another cause of antimalarial drug resistance.

Plants are used for the treatment of various ailments in last few decades. The quinine and artemisinin both are plant derivatives and have been obtained from *Cinchona* species (Phillipson, 1991) and *Artemisia annua* (Greenwood, 1992) respectively.

The modern age of science have focus on developing new drugs and ways to treat malaria. The malaria pigment, hemozin is considered to be effective in vaccine development. The hemozin was reported to induce the humoral and cellular immunity in the body, but commercially no malarial vaccine is available till date (WHO, 2015).

Medicinal plants have been used traditionally from the very beginning, but in the recent years, many plants have proved to be a good source of malaria control (Adzu *et al.*, 2007). In Africa about 80% people use traditional and plant based medicine due to their low cost and less access to the modern health facilities (Kassaye *et al.*, 2006).

A plant having good antiplasmodial activity is *Croton macrostachyus* (family: Euphorbiaceae). It is widely used in Ethiopia for malaria cure In Nigeria many plant species having antimalarial activity, e.g. *Emilia chlorantha* (African yellow). *Eupartorium odorantum* (Ogbogbo), *Cajanus cajan* (Pigeon pea in English), *Pisidium guajava* (guava), *Mangifera indica* (mango) are reported (Gidey *et al.*, 2006).

There are many plants with antiprotozoan activity i.e. *Aspergillus racemosus, Acacia tortilis* and *Albizia coriaria* (Kigondu *et al.*, 2009). Ethyl acetate extract of

Murraya koenigii leaves has been reported to have *in vivo* antimalarial effect against *P. berghei* (Kamaraj *et al.*, 2014). These all facts triggers to screen and search for plant based natural remedies against malaria.

Some of the medicinal plants and their parts have great pharmaceutical importance. So the medicinal plants that were selected in order to check their antiplasmodial activity in this study were ***Trachyspermum Ammi, Punica granatum, Cydonia oblonga, Benincasa hispida*** **and** ***Saussurea lappa.***

The ***Trachyspermum ammi*** commonly known as Carum or Ajwain belong to family Apiaceae. **The *T.ammi*** is a medicinal plant its seeds are best remedy for curing many infections cough undigestion, abdominal pain and also used as spices in cooking food (Kumar *et al.*, 2009). The major phytochemical constituents of *T.ammi* are phenolic compounds one of that is thymol which exhibit antimicrobial properties (Nagalakshmi *et al.*, 2000).

The ***Punica granatum*** generally recognized as Pomegranate or Anaar dana is an inclusive fruit cultivated all over the Mediterranean regions. Antioxidant properties of pomegranate are elaborated through various studies (Jurenka, 2008).

The ***Cydonia oblonga*** commonly known as Quince seed or Bahee Dana belongs to the family *Rosaceae*. The quince seeds are used for the treatment of G.I.T disorders, inflammation of joints, and hemorrhage of the bowel and for soothing of eyes, It is used for the treatment of lung diseases and pneumonia in Iran (Hamideh *et al.*, 2013). Its seeds are also used as medicine in the cosmetic industry (Jones *et al.*, 1979)

The ***Benincasa hispida*** commonly known as white gourd or Pethakaddu It belongs to the family *Cucurbitaceae*. It is grown for its fruits. Its fruit is used as a vegetable, especially in Asian countries. Its fruit has nutritional and medicinal importance. It has been used as antidiarrheal, antiulcer, anti-obesity and antioxidant (Vongtau *et al.*, 2004). It is also used as antipyretic (Al-Ghamdi, 2001).

The fruit of *B. hispida* is used as a local medicine for the cure of, ulcer, epilepsy and other nervous disorders like antidepressant (Qadrie *et al.*, 2009).

The ***Saussurea lappa*** commonly known as Costus or Kust-e shirin fit in to the group *Asteraceae.* It is an inhabitant plant of India, Pakistan and China and grows in the Himalaya region at 2 500-3 500 m altitude (Rao *et al.,* 2013).

It has been observed that when the dimensions of a material become very small, its physical and chemical properties changed from those of the same material in bulk amount. Nanoparticles size range of 1-100 nanometers. This technology recently has various applications due to the small size of nanoparticles and it shows the dissimilar behavior like the bulk materials.

Because of their small size they have a large surface to volume ratio, they specifically interact with human cells and tissues (Navaladian *et al.*, 2007). These properties also increase the efficiency of nanoparticles to interact with microorganism (Lamsal *et al.*, 2011). Nano particles have distinict physical properties and chemical properties (Panigrahi *et al.*, 2004).

Nanotechnology is applied as nanomedicine on a large scale in health side for diagnotic and therapeutic purposes. In the field of nanoscience it is of great interest to prepare and explore various nanoparticles (Kalaiarasi *et al.* 2013). Generally three methods are used to prepare the nanoparticles e.g. physical (Sreeram *et al.*, 2008), chemical (Starowicz *et al.*, 2006) and biological (Begum *et al.*, 2007) methods.

The chemical method consists of the reduction of metal ions using the water as solutions and in the presence or absence of stabilizing agents (Starowicz *et al.*, 2006). The physical methods of nanoparticles synthesis include photo reduction and chemical reduction in reverse micelles and thermal decomposition in organic solvents. These methods are not only expensive, but also involve toxic and piosnious agents that may be proved dangerous for ecosyystem (Sreeram *et al.*, 2008).

The green syntheses of nanoparticles have many benefits because biological materials possess no chemical toxicity on applications as well as on the environment. They are environmental and best suited for drug delivery and biomedical purposes (Begum *et al.*, 2007). The active biological components found in the biological materials used for nanoparticles synthesis itself acts as a reducing and steady mediator, in this manner, dropping on the whole cost of the production procedure (Klaus *et al.*, 1999).

Nanoparticles synthesized by chemical methods are extremely reactive in nature and tend to form aggregates, which eventually results in loss of reactivity (Song and Kim, 2009). The conventional method applied for the preparation of nanoparticles contains some toxic substances also so not good to use for preparing drugs (Kim *et al.*, 2008). The use of nonpolar organic solvents for the formation of nanoparticles is also not preferable for their medicinal use (Hyeon *et al.*, 2003).

Many microorganisms, such as yeasts, bacteria and other fungi, also used to synthesize Au and Ag nanoparticles (Huang *et al.*, 2007). In recent years, the plant based biological syntheses of nanoparticles are cost effective and environment friendly. Although biosynthesis of silver nanoparticles by plants from leaves of *Andrographis paniculata* (Panneerselvam *et al.*, 2011), aqueous and ethanolic extracts of leaves of *Solanum tricobatum, Syzygium cumini* and peel powder of *Citrus sinensis* (Logeswari, Silambarasan, and Abraham, 2013), aqueous extracts of leaves of *Catharanthus roseus* (Ponarulselvam *et al.*, 2012), aqueous extract of leaves of *Pargonium graveolens* (Shankar, Ahmad, and Sastry, 2003) have been reported.

The silver nanoparticles and spherical gold nanoparticles have been synthesized using sun-dried mass of the leaves of *Cinnamomum canphora* (Huang *et al.*, 2007). The gold and silver nanoparticles have been prepared from Aloe Vera leaves extracts (Chandran *et al* 2006). More research is needed to explore the potential of the plants as biological materials for the synthesis of nanoparticles (Panneerselvam *et al.*, 2011).

There is a requirement to build up harmless, low cost and eco-friendly nanoparticles. The biological method of nano-synthesis utilize natural agents i.e. microbes, enzymes and plants (Song and Kim 2009). The biosynthetic method is clean, simple and safer than other conventional methods (Sharma & Yangard, 2009). In bio-synthetic process the active phytochemical and enzyme present in plants and micro-organism reduces the metals to synthesize nanoparticles (Panigrahi *et al.*, 2004).

Green synthesis is more useful and valuable than using microorganism (Huang *et al.*, 2007). Green synthesis requires low concentration of plant extracts and the method is safe, simple and performs at low cost. In green synthesized nanoparticle the

plant phytochemical acts to reduce and stabilize the metal ion into nanoparticle (Klaus *et al.*, 1999).

It is of great interest to synthesize silver nanoparticles because of their protective role against various microbes (Sharma *et al.*, 2009). From the very past silver has been recognized as antibacterial and inhibiting various microbes (Lok *et al.*, 2007). Silver nanoparticles has been recognized as anti-fungal (Kim *et al.* 2009), anti-inflammatory (Nadworny *et al.* 2008), and anti-viral (Rogers *et al.* 2008). The Ag nanoparticles are less toxic and some textiles also contain silver however the consumer products release only very small amount of this element not hazardous to human health (Ray *et al.*, 2009).

Magnetic nanoparticles are the new class of important nanoparticles. They possess many exceptional properties, e.g. superparamagnetic and high coercively. These nanoparticles are now emerging as medicine. However, green synthesis of magnetic nanoparticles has remained relatively unexplored research area with the majority of papers published only in the last two years (Herlekar *et al.*, 2014).

There are many applications of nanoparticles due to the distinctive physicochemical properties, e.g. drug delivery and targeting (Hu *et al.*, 2009) cancer therapy (Peer et al., 2007) biosensors (Lord and Kelley, 2009) food additives and cosmetics (Nohynek et al., 2007).

Keeping in view the medicinal importance of *T. ammi*, *P. granatum*, *C. oblonga*, *B. hispida* and *S. lappa* the extract of seeds of these plants have been used for the evaluation of their protozocidal activity. The Ag and Fe nanoparticles are synthesized, using the aqueous extracts of seeds of T. ammi, *P. granatum* and *S. lappa* and their antiplasmodial activity are also evaluated against *Plasmodium berghei* in the BALB/c mice.

Aims and Objectives

1. To study the distribution of *Plasmodium* species from different areas of Punjab, Pakistan.
2. To study the phylogenetic relationship of the variant forms of *Plasmodium* species from study areas.
3. To determine the *in vivo* antiplasmodial activity of five medicinal plants..
4. To evaluate the antiplasmodial activity of green synthesized silver and iron nanoparticles of the plants that show the high antiplasmodial activity.

Prevalence and Distribution of Human *Plasmodium* Infection from Northern and Southern Punjab, Pakistan

Abstract

Pakistan, with 35% of the country's landmass is classified as endemic for malaria and sixty percent population lives in such regions. Epidemiologically, Pakistan is considered as a moderate malaria endemic country where outbreak of malaria in different areas is varied in accordance to its population and temperature. The present study was based upon the survey of various government and private hospitals in ten cities of Northern and Southern Punjab and to find out the spatiotemporal pattern of microscopically positive cases of malarial infection in 2015.

The 925 microscopically malaria positive blood samples were evaluated for distribution among the study area, seasonal variations, age groups and gender. However the suspected cases during the research period were 16075. The parasite species were identified both microscopically and on molecular basis. The Phylogenetic relationship of *Plasmodium* species was determined by DNA sequence analysis using software MEGA vs 7.

The area wise distribution of malarial infection revealed that out of 925 microscopically positive cases, 364 cases were recruited from Northern Punjab with the highest from Rawalpindi (n=93, 25.5%) and the lowest from Chakwal ((n=58, 15.9%). However 561 cases were observed from Southern Punjab with highest values from Rajanpur (n=120, 21.4%) and lowest one from Multan and Rahim Yar Khan i.e. (n=104, 18%). The slide positivity rate, Annual Parasite Prevalence and Annual Blood Examination Rate (in all recruited cities of both zones of Punjab were found to be 5.75%, 0.12 per 1000 population and 0.22% respectively. Seasonal variation of malarial prevalence revealed that it began to increase in the spring, peak in summer and Post monsoon, while showed minimum cases in winter. The age wise prevalence of malaria in 2015 revealed that it was highest among the young ones (1-20 years) while lowest cases were observed among people at the age of (41-60 years). However the gender wise

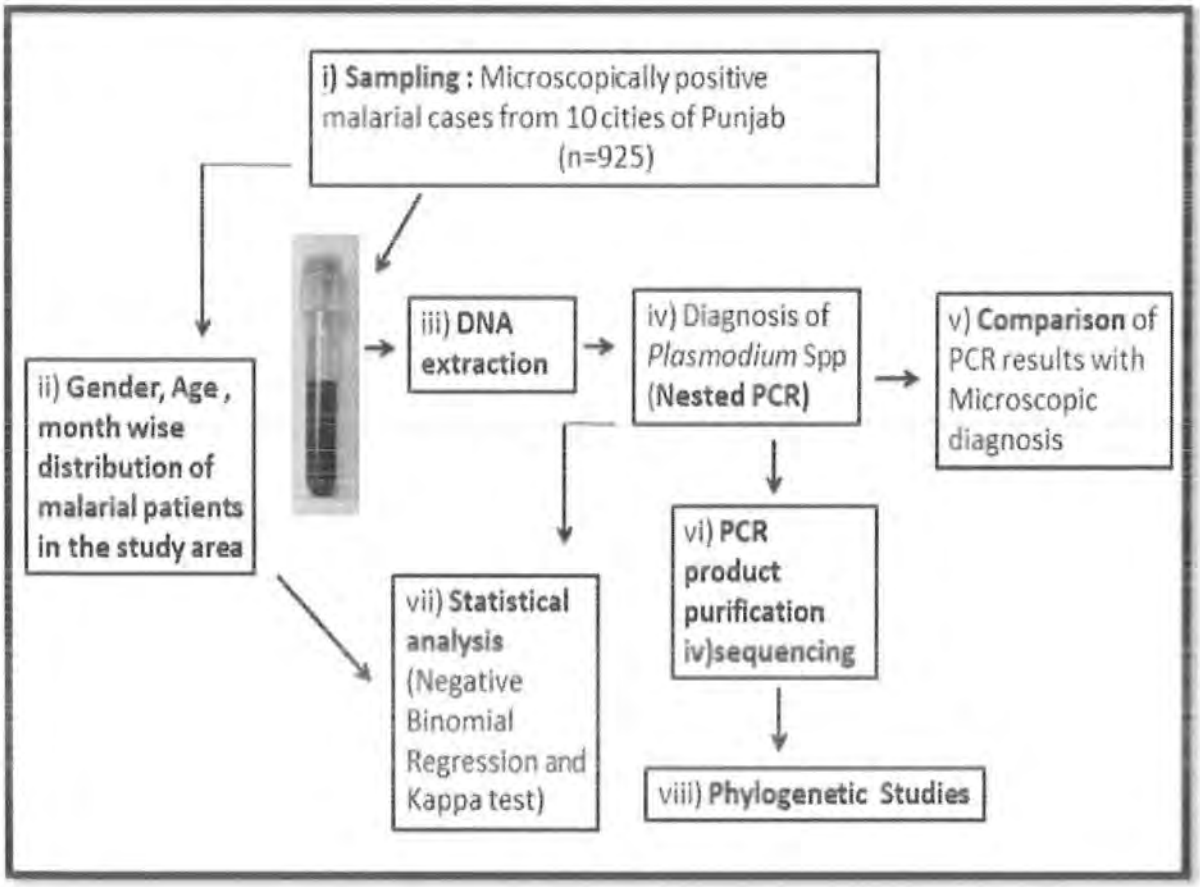
prevalence indicated that the higher cases in males as compared to females and no age to gender wise relationship was observed. Microscopic and molecular diagnosis of malarial parasites revealed the occurrence of only two species of *Plasmodium* i.e. *P. vivax* and *P. falciparum*.

The microscopic studies of 925 cases indicated the high abundance of *P. vivax* 16.7% (n=617) followed by *P. falciparum* 219 (23.7%) in the blood of recruited cases however 89 (9.62%) cases showed the mixed infection. Molecular analysis also revealed the same trend i.e. *P vivax* showed the higher occurrence followed by *P. falciparum* and the mixed infection. The overall prevalence of treatment seeking patients in all recruited cities of Punjab was 4.88%. However the comparison of Northern and Southern Punjab indicated that the prevalence was higher in Southern Punjab (5.54%) as compared to the Northern Punjab (4.01%).

The overall results indicated that malarial infection is most prevalent in the Southern Punjab as compared to Northern Punjab and it is most prevalent during summer and moonsoon. Moreover people under the age of 20 were most susceptible and over all *P. vivax* showed the higher occurrence in the patients as compared to other *Plasmodium* species.

Chapter # 1

Graphical Abstract



Graphical representation of molecular epidemiological study of malarial species from Punjab, Pakistan

Introduction

Infectious diseases are considered as the major cause of worldwide morbidity and mortality. The history of malaria encapsulates our failure to combat global epidemics as yet it is the leading threat to public health, economic growth and development in many countries (Khatoon *et al.*, 2010). Human malaria has been known to be caused by five species of *Plasmodium* that are *P. vivax, P. falciparum, P. ovale, P. malariae* and *P. knowlesi*. Regardless of massive and costly control efforts over many decades, malaria has re-emerged as a serious health problem in Asia.

World is currently not on track to achieve the milestones of 2020 of WHO Global Technical Strategy (GTS) of malaria 2016-2030. The mile stone of 2020 is to reduce malaria death and disease by 40% but there is a continuous increase in the prevalence of malaria cases from 2015 to 2018 (WHO, 2018). The number of malaria cases globally estimated were 214 million in 2015 that increased to 217 million in 2016 then to 219 million in 2017 (WHO, 2016: WHO, 2017: WHO, 2018)

Sixty percent population of Pakistan lives in malaria endemic regions. Two prevalent species found in these areas are *P. vivax* and *P. falciparum* account for 64% and 36%, respectively (WHO, 2013). As reported by WHO (2012) Pakistan is one of the 6 countries within WHO Eastern Mediterranean Region with areas of high malaria transmission with 100% of the population living at risk. The endemicity of malaria varies in different Provinces and even in different cities of a province having variable climates. The Balochistan and Sindh have higher prevalence of malaria as compared to Punjab and Azad Jamu Kashmir (AJK) whereas Baluchistan and Federally Administered Tribal Areas (FATA) while Sindh and Khyber Pakhtunkhwa was reported with a moderate prevalence (Kakar *et al.*, 2010).

The endemicity of malaria varies in Punjab being the land of five rivers Sutlej, Chenab, Beas, Ravi and Jhelum generate a major breeding ground for malaria vector. It is the most populous province of Pakistan with an estimated population of 101,391,000 according to 2015 Censes. Punjab experiences all types of four seasons, spring, autumn, summer and winter. The onset of the South West monsoon is predictable to reach in the

Punjab in the month of May. However, weather pattern has been irregular since early 1970s. The spring monsoon caused the rain to be occurred so hard and it results in floods. The month of June and July are very warm and temperature up to 46 °C was noted. This overbearing heat is interrupted by the rainy season in August that lead to the end of hard part of the summer. But cooler weather does not come until late October (Ali *et al.*, 2010).

Malaria in Punjab Province is recurring and uneven and its epidemics repeated with thw interval of almost eight years difference. Variations in malaria transmission from year to year are due to the floods in the Southern Punjab and heavy rainfall in the monsoon season in the Northern Punjab. Recently the cyclical climate patterns are linked to variation in the transmission pattern of Malaria (Herrel *et al.*, 2004).

Malaria is more prevalent in the rural areas due to the low socioeconomic conditions (Kaker *et al.*, 2010). The prevalence of *P. falciparum* is high among the Afghan refugees than the local population. Several epidemiological studies have revealed that there was 24 to 36% increase in malaria prevalence of Pakistan due to influx of Afghan refugees (Basseri *et al.*, 2010).

The transmission of malaria is seasonal in Pakistan, and a gradual increase in cases can be noted after the July–August monsoon (Khan and Khattak 2006). The *P. vivax* infection is restrained to two peaks per year, the main peak was in late spring as a result relapses of earlier infections and additional peaks occurred in summer and autumn by recent transmissions (Khatoon *et al.*, 2010). In contrast to *P. vivax*, *P. falciparum* malaria shows an increasing correlation with August-September temperatures (Rowland *et al.*, 2002). The wide canal irrigation network throughout the country, agricultural practices and monsoon rains provide a favorable environment for the development and growth of malarial vectors (Binello *et al.* 2014).

The national malaria control program has reported a six-fold increase in *P. falciparum* infection in the last decade. The increase of *P. falciparum* infection across the country can be associated to chloroquine drug resistance (Shah *et al.*, 1997) also in this part of the world temperature in autumn is warmer that increase the transmission rate and

due to inappropriate measures vector control activities are weakened (Yasinzai and Kakar, 2008).

The malaria epidemiology is influenced by environmental factors that supports the vector development and in turn enhances the parasite and host relationship. The socioeconomic conditions also influence it (Zakeri *et al.*, 2010). Beside these, various other factors like urbanization, exponential population growth, migration of Afghan refugees, environmental changes due to excessive monsoon rains, floods, and extensive irrigation projects also favor malarial parasite transmission (Khatoon *et al.*, 2010: Erdman and Kain., 2008).

The correct diagnosis of malaria is necessary; to treat the different types of *Plasmodium* infection, particularly for *P. falciparum* that is the most lethal infection it is carried out by microscopy and PCR. The microscopy is not possible in the rural areas where there is no diagnostic lab and electricity as well, so there was the need for new, simple, fast, cost-effective and accurate tests for detection of parasites of malaria, to over look the deficiencies of light microscopy test. These issues led to the development of rapid diagnostic test for malaria, which are fast and easy to perform, and do not require electricity or specific equipment (Bell *et al.*, 2006). There are approximately 86 malaria RDTs by 28 different manufacturers (WHO, 2006). The principle of RDTs is to detect antigen of malaria present in the blood that contain specific antimalaria antibodies. Many of these products, target the specific protein of *P. falciparum* (most virulent *Plasmodium* infection in humans). The proteins of *P. falciparum* like histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH). Some tests detect *P. falciparum* specific aldolase or pan-malaria pLDH and distinguish non *P. falciparum* infections from mixed malaria infections. Nevertheless most of RDT products are suitable for *P. falciparum* malaria diagnosis, but there are the RDTs that can effectively diagnose *P. vivax* (Park *et al.*, 2006: Kim *et al.*, 2008). The new RDT method has been developed, for the detection of *P. knowlesi* (McCutchan and Piper, 2008). The RDTs supports the detection of parasite-based diagnosis of malaria that is not possible through light microscopy. Another advantage of RDTs is the management of febrile illnesses in distant malaria endemic areas. The RDT performance for diagnosis of malaria is reported as admirable (Chilton *et*

al., 2006: Noedl *et al.*, 2006) however from remote malaria endemic areas different variations in sensitivity has been reported (Doderer *et al.*, 2007).

The other diagnostic test used for malaria detection is the serological tests method. It is based on the detection of antibodies against asexual blood stage malaria parasites. Immunofluorescence antibody testing (IFA) has been a reliable serologic test for malaria diagnosis (She *et al.*, 2007). It is highly sensitive and specific, but time-consuming (Doderer *et al.*, 2007). The specific antibodies within 2 weeks of initial infection the *Plasmodium* species produces and persist for 3-6 months after parasite clearance IFA detect these antibodies. The IFA uses specific or crude antigen and quantifies both IgG and IgM antibodies in patient serum samples. However, it requires and skilled technicians and fluorescence microscopy. However readings can be inclined depending on the training of the technician or may be due to the low antibody titers of serum samples. This IFA tests are useful for correct prevalence and epidemiological estimation but not appropriate for the detection of sensitive malaria (Chotivanich *et al.*, 2006).

The most advanced, proficient, sensitive and precise method for the detection of *Plasmodium* species is polymerase chain reaction (PCR) based analysis. It has proved to be a method for diagnosis of mixed infections, low parasitemia and species identification (Berry *et al.*, 2008). The PCR based diagnosis has overcome all the restrictions and limitations of the conventional diagnostic methods (Hawkes *et al.*, 2009). It can also be used to find the drug resistance and follow-up therapeutic response (Chotivanich *et al.*, 2006). The present study is based on distribution of malaria with respect to age, gender and seasonal comparison in the two opposite zones of Punjab in order to compare the difference in prevalence of malaria in the highly developed Northern Punjab to least developed remote areas of southern Punjab. As the geographical localities of both Northern and Sothern Punjab varies greatly which has great impact on temperature, precipitation humidity and other climate changes. Temperatures and humidity varies in both regions so these regions were selected for study.

Materials and Methods

Study area

The study areas comprised of ten cities of the Punjab from approximately two opposite zones i.e. Northern (Rawalpindi, Gujar Khan, Gujrat, Chakwal and Gujranwala) and Southern (Dera Ghazi khan, Rajanpur, Rahim Yar Khan, Multan, and Bahawalpur) (Figure 1.1).

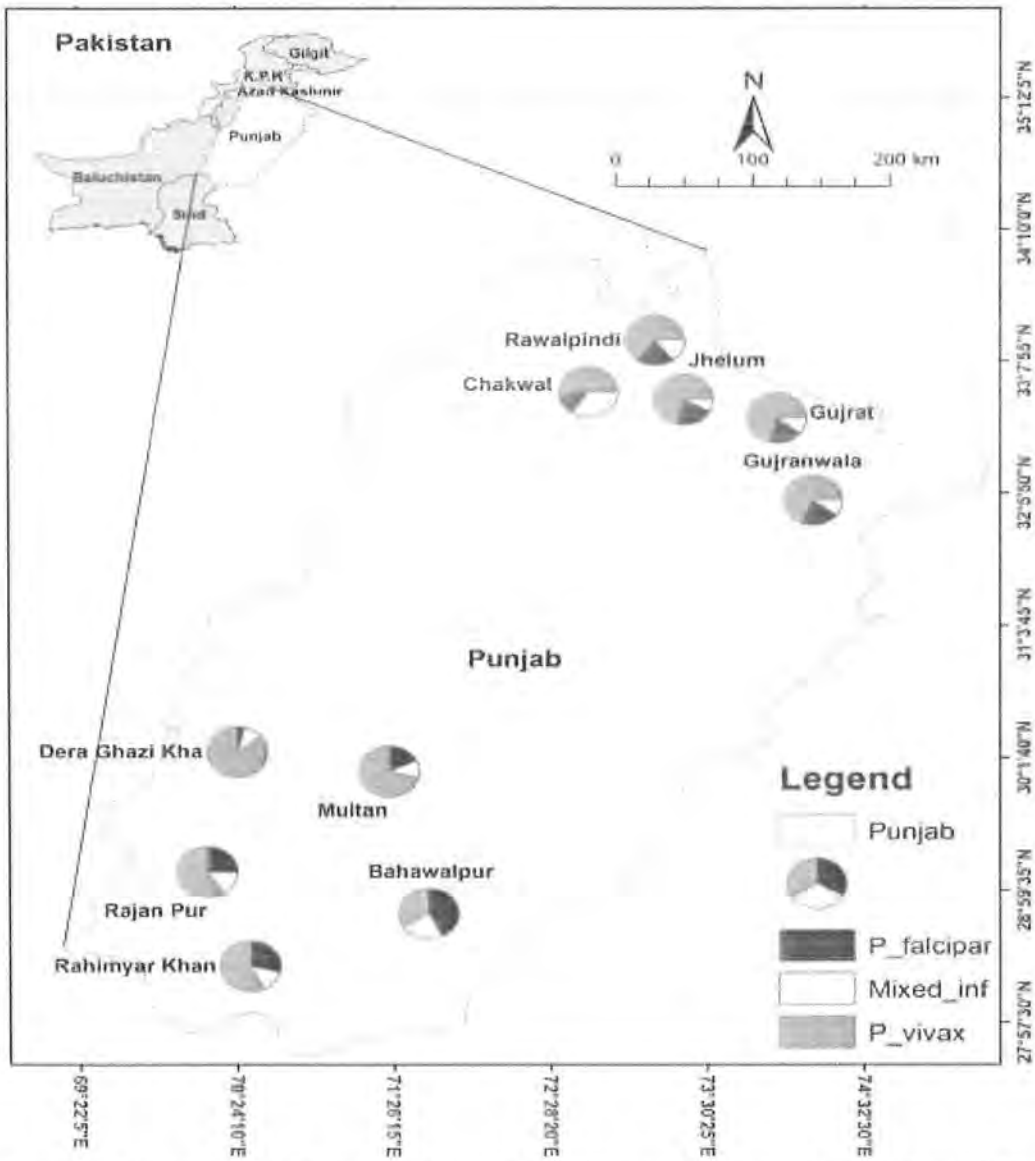


Figure 1.1: Sample sites and distribution of *Plasmodium* species across Punjab, Pakistan

Sample Collection

A survey was conducted from January to December 2015 based upon 16075 subjects of study area. The blood samples of nine hundred and twenty five microscopically malaria positive patients were collected from different hospitals and clinics of study areas in the presence of medical superintendents after taking oral and written consent from patients or their guardians. The venous blood (3 ml) was taken through disposable syringe and collected in EDTA vacutainers for further analysis. The venipuncture technique was used for blood collection as follows; Patient was asked to sit on chair and the tourniquet was placed in his upper arm, a suitable site was selected for venipuncture and blood was drawn (Kim *et al.*, 2006). The samples from distant areas were brought to the Parasitology lab of Department of Animal Science Quaid-i-Azam University Islamabad in the containers containing dry ice with in one day.

Demographic investigation of Malarial Patients

The information about the patients like age, sex, locality etc. was recorded. Slide positivity rate (SPR), Annual parasite prevalence (API) and Annual blood examination rate (ABER) were calculated by following formulas (Laghari *et al.*, 2014)

$$\mathbf{SPR} = \frac{\text{Total positive cases}}{\text{Total observed cases}} \times 100$$

$$\mathbf{API} = \frac{\text{Total positive samples in a year}}{\text{Total Population}} \times 1000$$

$$\mathbf{ABER} = \frac{\text{Total slides examined}}{\text{Total population}} \times 100$$

The prevalence on the basis of molecular diagnosis was calculated according to Khattak *et al.*, (2013).

$$\textbf{Prevalence} = \frac{\text{PCR positive cases}}{\text{Total suspected cases}} \times 100$$

DNA Extraction

The DNA was extracted by using the Gene jet genomic DNA Purification kit (Thermo Scientific, (EU (Lithuania), according to the manufacturer's instructions. The DNA was stored at 4 °C for further use.

Molecular Examination

The high sensitive detection method was adapted for human malarial parasites detection, i.e. by the use of nested polymerase chain reaction (PCR). Following a set of primers of the small subunit (SSU) ribosomal RNA (rRNA) gene were used for the detection of genus *Plasmodium* in the first round of PCR (Snounou *et al.*, 1993) (Table 1.1).

Table 1.1 Primer sets used for the detection of *Plasmodium* genus

Genus	**Primer**	**Primer sequence**	**Amplification product size (bp)**
Plasmodium	Forward PLU5	5'- CCT GTT GTT GCC TTA AAC TTC -3'	1100
	Reverse PLU6	5'- TTA AAA TTG TTG CAG TTA AAA CG -3'	

For all samples that were positive, a species- specific nested PCR was performed using primers of *Plasmodium* Species i.e. *P. vivax P. falciparum, P. malariae* and *P. ovale*

in the second, third, fourth and fifth round of PCR, respectively (Snounou *et al.*, 1993) (Table 1.2). All primers were taken from Invitrogen Company.

Table 1.2 Primer sets used for the detection of *Plasmodium* Species

***Plasmodium* Species**	**Primer**	**Primer sequence**	**Amplification product size (bp)**
P. vivax	Forward fV	5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC -3'	120
	Reverse rV	5'- AAG GAA AGA AAG TCC TTA -3'	
P. falciparum	Forward fF	5'-TTAAACTGGTTTGGGAAAACCAAA TATATT-3'	205
	Reverse rF	5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3'	
P. malariae	Forward fM	5'- TAA CAT AGT TGT ACG TTA AGA ATA ACC GC -3'	144
	Reverse rR	5'- AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA -3'	
P. ovale	Forward fO	5'-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA -3'	780
	Reverse rO	5'- GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG -3'	

For PCR amplifications total 50 µL reaction mixtures were used for every reaction. A master mix was prepared containing all reagents except DNA and Taq polymerase enzyme. It was added to the master mix when the DNA was shifted in the PCR tubes and master mix was added to it immediately. All the reagents were vortexed before use. To avoid contamination only one tube was opened at one time.

The ingredient used to make fifty microliter recipe include $MgCl_2$ 5 µL, Taq buffer 4 µL, dNTPs 1 µL, Forward Primer 2 µL, Reverse primer 2 µL, distilled water 31.5 µL, *Taq.* DNA Polymerase0.5 µL and extracted DNA 4.0 µL.

For nested reactions the recipe was same as above except 3 µL of an aliquot of the PCR product of first reaction was used as a template for the amplification of *P. vivax*, *P. falciparum, P. malarial* and *P. ovale*. The cyclic conditions of PCR used for genus detection were as follows:-

Table 1.3 Cyclic conditions for PCR

Stage	Step	Temperature	Time	Cycles
Initial denaturation	1	95 °C	5:00 min	1
Denaturation	1	94 °C	45 Sec	35
Annealing	2	56 °C	45 Sec	
Extension	3	72 °C	45 Sec	
Final extension	1	72 °C	10:00 min	1

The cyclic conditions for nested PCR used for species identification were same as above except the annealing temperatures. Annealing temperature for *P. vivax* was 65 °C. The PCR machine was set on the gradient temperature for the amplification of *P. falciparum* forward annealing temperature was 59.24 °C and reverse annealing temperature was 66 °C. Annealing temperatures for *P. malariae* was 63 °C and *P. ovale* was 59 °C.

Gel electrophoresis

The visualization of amplified PCR products was conducted by using 1.5-2.0% agarose gel stained with ethidium bromide and visualized under UV-trans-illuminator (Extragene, USA).

Purification of amplified product and sequencing

The smear free amplified PCR products were purified using kit for gene jet PCR purification (Thermo Scientific, EU Lithuania) (cat# K0701) and sent to Korea (Macrogen) for sequencing. The evolutionary tree was constructed using Mega 7 software from Google.

Statistical analysis

The groups of age, gender and seasons were analyzed using bionomial regression model. To measure the level of agreement between two diagnostic tests i.e. PCR and microscopy Cohen 'Kappa was calculated. The kappa values were categorized as poor (<0.2), fair (0.2 - 0.4), moderate (0.40 - 0.6), good (0.61 - 0.80), and very good (0.8) in order to check the strength of agreement between PCR and microscopic examination. The Cohen 'Kappa tests were conducted on the SPSS vs 19.0 while negative binomial regression analysis was performed on R language.

Results

Area wise distribution and prevalence

The overall slide positivity rate (SPR) was 5.75% in all recruited cities of Punjab however the SPR was higher in Southern Punjab (6.11%) as compared to the Northern Punjab (5.27%). The SPR within the recruited cities of Northern Punjab was highest in Jhelum 6.80% lowest in Chakwal i.e. 3.15% percent respectively. Among all recruited cities of Southern Punjab the SPR was maximum in Rajanpur (7.10%) and minimum in Dera Ghazi Khan (5.40%) (Table 1.4).

The API was 0.12 per 1000 population in all recruited cities. Similarly like SPR, the API was also high in Southern Punjab 0.16 per 1000 population as compared to the Northern Punjab 0.09 per 1000 population. The API within the Northern Punjab was high in Jhelum and it is equal to API in Chakwal (0.4 per 1000 population) lowest in Gujranwala (0.04 per 1000 population). Among the analyzed cities of Southern Punjab the API was maximum in Rajanpur and minimum in Multan i.e. 1.21 and 0.06 per 1000 population respectively. The ABER was 0.22 in Punjab and it was greater in Southern Punjab as compared to the Northern Punjab 0.26 > 0.17. The ABER within the Northern Punjab was high in Chakwal and minimum in Gujranwala i.e. 1.33 and 0.07 percent respectively. The ABER in Southern Punjab was highest in Rajanpur (1.70%) lowest in Multan (0.25%) (Table 1.4).

Seasonal variations in malaria infection

The month and season wise distribution of malaria in Northern Punjab indicated that the percentage of malaria began to increase from March and April i.e. in spring season 3.02% (n=11) and 6.31% (n=23) respectively. The highest percentage was observed in summer from July to Sep 16.2%, (n=59) > 20.3% (n=74) > 15.9% (n=58) respectively. There was a low percentage of malaria in winter season, i.e. in the months of November 4.67% (n=17), December 2.19% (n=8) followed by January (0.54%) (n=2) and February 0.54% (n=2) (Table 1.5)

The month and season wise distribution of malaria in southern Punjab showed an almost similar pattern as it was observed in the Northern Punjab. The incident of malaria began to increase in the spring seasons from March and April, 3.02% (n=18) and 6.41% (n=36) respectively. The highest prevalence was observed from June to September, 11.6%, (n=65), 14.2% (n=80), 21.9% (n=123) and 15.1% (n=85) respectively. There was a low prevalence of malaria in winter and autom seasons i.e. the months of November 3.92% (n=22), December 2.13% (n=12) followed by January (0.71%) (n=4) and February 1.60% (n=9) (Table 1.5).

The negative binomial regression analysis indicated strong effect of season on malarial prevalence in Punjab. Autumn (used as control in the analysis) had higher rate of prevalence as compared to spring and winter (with expected prevalence on log scale) of -0.5671 and -2.1374, respectively. The summer was the peak season with expected log (prevalence) of 0.3737 higher than that of autumn holding other variables constant (Figure 1.2 and Table 1.7)

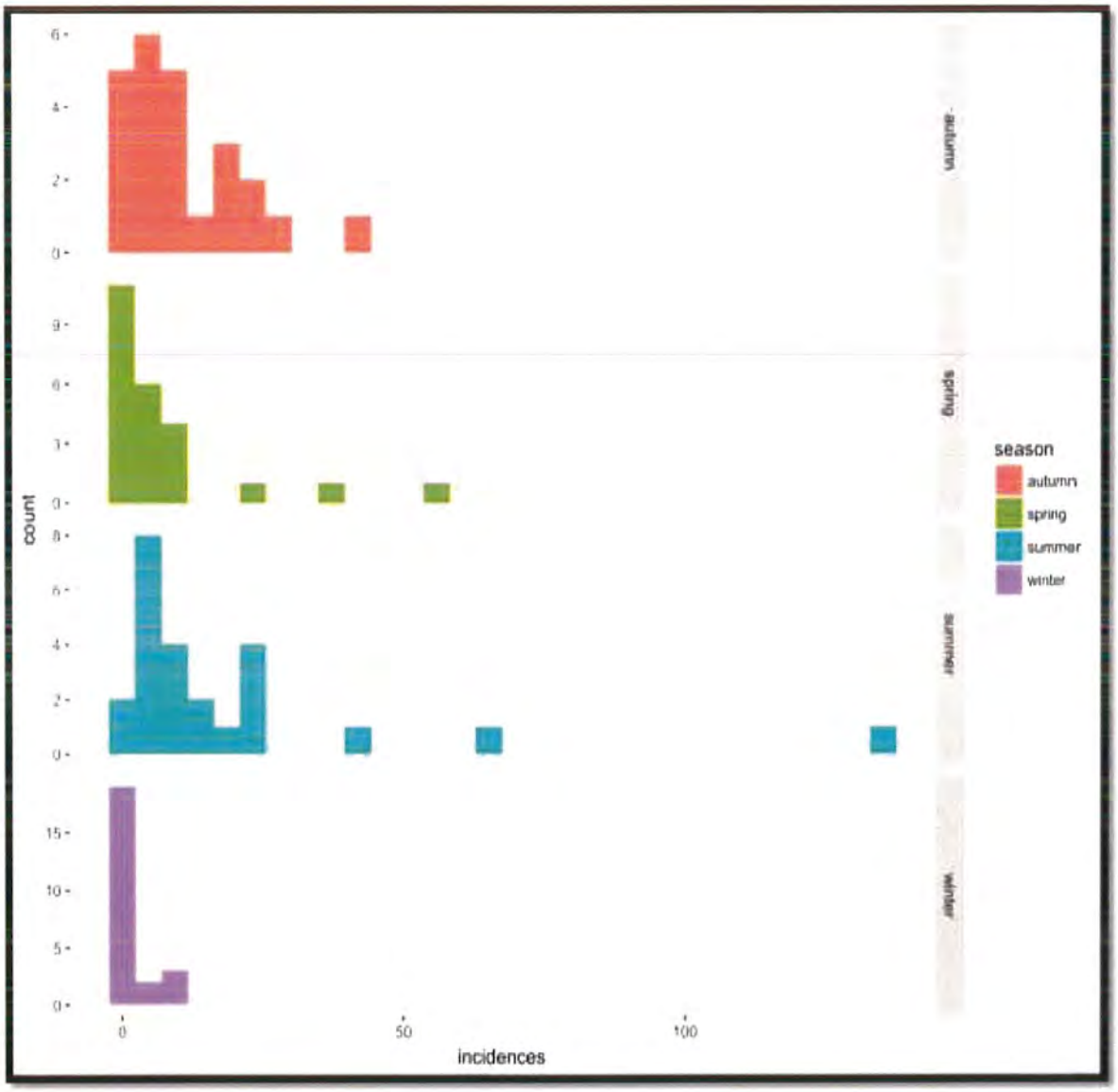


Figure 1.2 Season wise distribution of malaria in all recruited cities of Punjab

Table 1.5: Area and month wise distribution of malaria in Northern and Southern Punjab

Region	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Northern Punjab	**2 (0.54)**	**2 (0.54)**	**11 (3.02)**	**23 (6.31)**	**36 (9.89)**	**40 (10.98)**	**59 (16.20)**	**74 (20.32)**	**58 (15.93)**	**34 (9.34)**	**17 (4.67)**	**8 (2.19)**	**364**
Rawalpindi	1	1	5	7	11	15	16	20	10	3	2	2	93 (25.5)
Chakwal	0	0	1	3	4	6	10	14	9	6	3	2	58 (15.9)
Gujranwala	1	0	2	6	7	6	13	14	11	10	7	3	80 (22.0)
Gujrat	0	0	1	4	6	5	11	12	13	9	2	0	63 (17.3)
Jhelum	0	1	2	3	8	8	9	14	15	6	3	1	70 (19.2)
Southern Punjab	**4 (0.71)**	**9 (1.60)**	**18 (3.20)**	**36 (6.41)**	**60 (10.69)**	**65 (11.58)**	**80 (14.26)**	**123 (21.92)**	**85 (15.15)**	**47 (8.37)**	**22 (3.92)**	**12 (2.13)**	**561**
Bahawalpur	1	0	6	9	12	15	18	23	15	10	7	3	119 (20.3)
D G Khan	0	0	3	11	15	16	18	24	10	7	6	4	114 (20.3)
Rahimyar Khan	1	4	2	8	9	8	9	20	26	16	3	0	104 (18.5)
Multan	0	2	4	6	8	8	10	28	24	9	3	0	104 (18.5)
Rajanpur	2	3	3	2	16	18	25	28	10	5	3	5	120 (21.39)
Whole Punjab	**6 (0.64)**	**11 (1.18)**	**29 (3.13)**	**59 (6.37)**	**96 (10.37)**	**105 (11.35)**	**139 (15.02)**	**197 (21.29)**	**143 (15.45)**	**81 (8.75)**	**39 (4.21)**	**20 (2.16)**	**925**

Age and gender wise distribution of malarial patients

The age of all recruited cases in the present study of both genders was ranged between 5 months to 78 years with median age 22 and 25 years for the Northern and southern Punjab respectively. All subjects were local inhabitants of their native settlements. The gender wise distribution indicated that *Plasmodium* infection was higher in males than female among in both zones of Punjab. In the Northern Punjab male were 28.54% (n=264) and 10.8% (n=100) female were infected. The male ratio was higher in southern Punjab like 42.16% (n=390) male and 18.4% (n=171) female. out of total 925 samples (Table 1.6).

Table 1.6 : The gender and age wise distribution of *Plasmodium* infection among subjects from ten different cities of Northern and Southern Punjab.

Area	Male N (%)	Female N (%)	Total N (%)	Age in years median (Y) (range)
Northern Punjab	**264 (28.5)**	**100 (10.8)**	**364 (39.4)**	**22 (1-78)**
Gujranwala	60 (6.48)	20 (2.16)	80 (8.64)	24 (2-78)
Gujrat	40 (4.32)	23 (2.48)	63 (6.81)	23 (1-64)
Jhelum	55 (5.94)	15 (1.62)	70 (7.56)	19 (1-56)
Chakwal	39 (4.21)	19 (2.05)	58 (6.26)	26 (3-67)
Rawalpindi	70 (7.56)	23 (2.48)	93 (10.04)	25 (3-65)
Southern Punjab	**390 (42.2)**	**171 (18.4)**	**561 (60.6)**	**25 (0.5-76)**
Bahawalpur	85 (9.18)	34 (3.67)	119 (12.86)	30 (2-76)
Rahimyar Khan	71 (7.26)	33 (3.56)	104 (11.24)	25 (1-75)
Multan	77 (7.40)	27 (2.91)	104 (11.24)	17 (1-70)
Rajanpur	78 (8.43)	42 (4.54)	120 (12.97)	23 (1-73)
Dera Ghazi khan	79 (8.54)	35 (3.78)	114 (12.32)	7 (0.5-56)

Age is strong indicator of malaria prevalence, the young individuals (<20 years) were susceptible to the disease, and risk declined gradually with older age classes (Table 1.5). For example prevalence in age group 21-40 was -0.1271 lower (on log scale) than that of age group of 1-20 years holding other variables constant.

Similarly prevalence in age groups 41- 60 and 61_above were -0.9392 and -2.4390 lower, respectively, than that of age group 1-20. It means most prevalence was observed among age group of 1-20 > followed by 21-41 than 41-60 year age groups. The distribution of malarial parasites was least common in older age groups (Figure 1.3 and Table 1.7).

Males had an expected log (prevalence) of 0.8466 higher than that of females holding other variables constant. The *P. vivax* had an expected log (prevalence) of 1.0308 higher than that of *P. falciparum*, however mix infections are -0.8562 lower than *P. falciparum* (Table 1.5 and Figure 1.3). In conclusion, age, gender, species and season are significant predictors of malarial prevalence in Punjab.

Table 1.7 Negative binomial regression analysis showing effects of age, gender, species and season on prevalence of malaria

Coefficients					
	Categories sub type	**Estimate**	**Std. Error**	**Z value**	**Pr(>|z|)**
Intercept	**1_20 Years**	2.1917	0.1800	12.174	< 2e-16 ***
Age groups	**21_40 Years**	-0.1271	0.1496	-0.849	0.395796 ***
	41_60 Years	-0.9392	0.1653	-5.680	1.34e-08***
	61_100 Year	-2.4390	0.2298	- 10.614	< 2e- 16***
Gender	**Male**	0.8466	0.1256	6.741	1.57e-11 ***
Species Types	***P. vivax & P. falciparum***	-0.8562	0.1751	-4.889	1.01e-06 ***
	P. vivax	1.0308	0.1410	7.311	2.65e-13 ***
Seasons	**Spring**	-0.5671	0.1669	-3.397	0.000681 ***
	Summer	0.3737	0.1512	2.472	0.013436 *
	Winter	-2.1374 - 9.249	0.2311	-9.249	< 2e-16 ***

Significant codes: P= 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

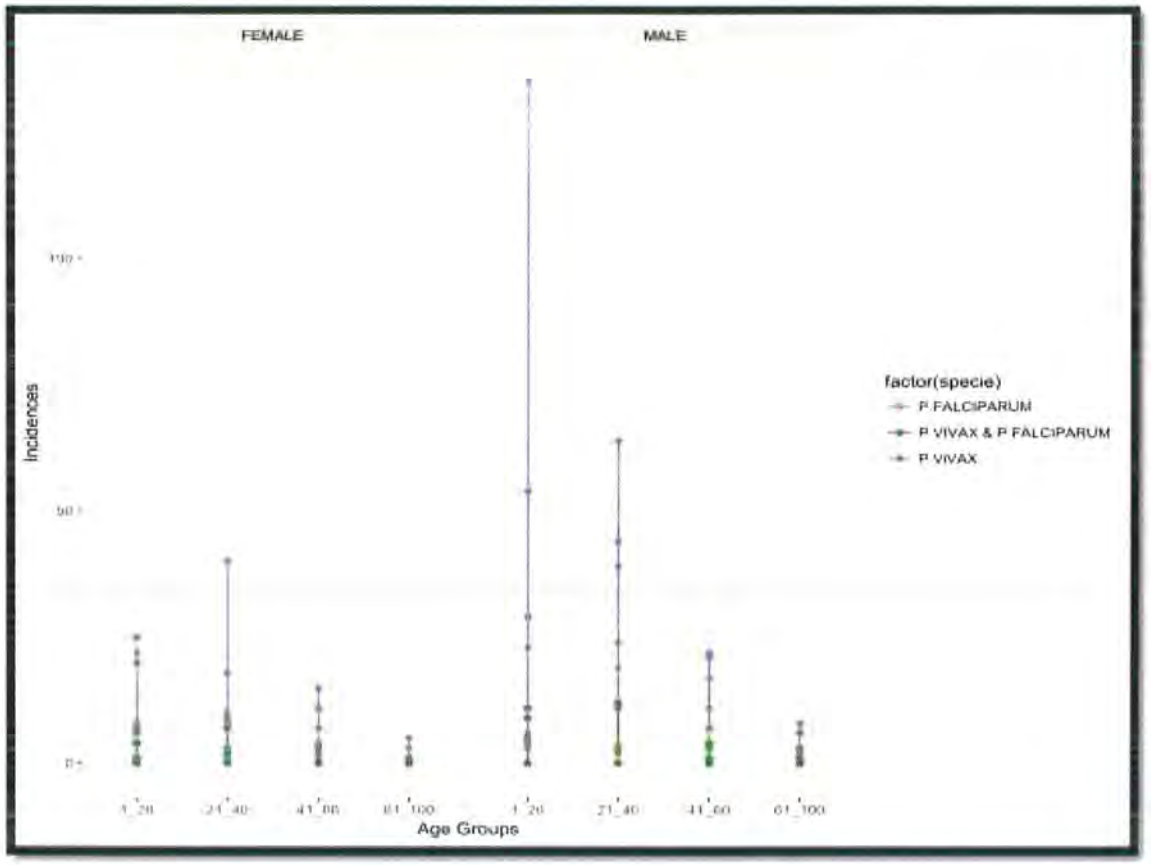


Figure 1.3 Gender and age wise distribution of *Plasmodium* species in all recruited cities of Punjab**.**

Distribution of *Plasmodium* species in Northern and Southern Punjab

The species wise distribution of malaria, according to the microscopy showed 66.70% (n=617) were *P. vivax* 23.67% (n=219) were *P. falciparum* and 9.62% (n=89) were mixed infections out of 925 recruited cases (Table 1.8).

However, molecular analysis showed that 53.40% (n=494) as *P. vivax*, 16.70% (n=173), *P. falciparum* and 12.86% (n=119) as mixed species and 15.03% (n=139) were PCR negative cases. However, no case of *P. ovale* and *P. malariae* was found both through microscopy and PCR (Figure 1.4).

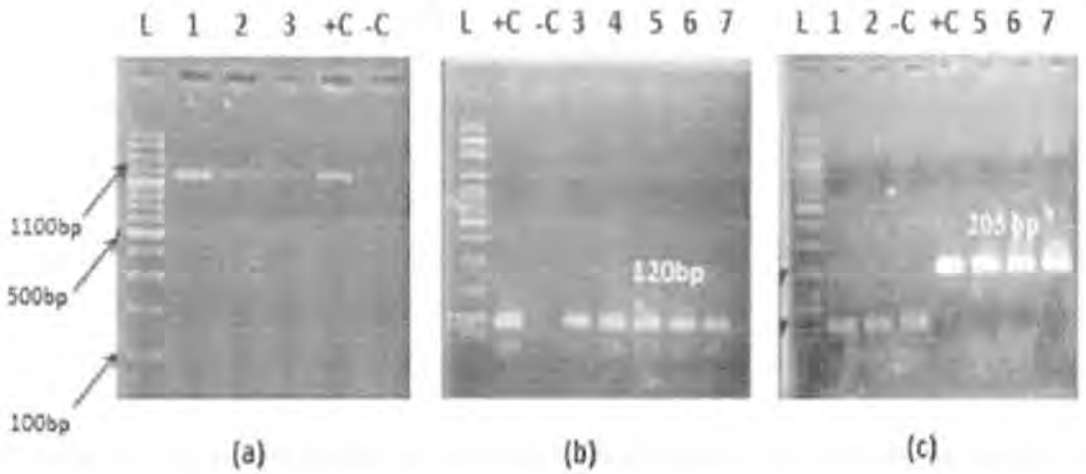


Figure 1.4: Nested PCR results (a) genus *Plasmodium* (b) *P. vivax* and (c) *P. falciparum* Lane L: 100 bp ladder, Lane +C: Positive control, Lane –C: Negative control.

In Northern Punjab 74.2% (n=270) were microscopically positive for *P. vivax*, 19% (n=69) were *P. falciparum* and 6.5% (n=25) had mixed species out of 364 cases. The PCR results showed that 50.3% (n=183) were of *P. vivax*, 14.8% (n=54) *P. falciparum*, 11% (n=40) mixed species and 24% (n=87) did not amplify for any species (Table 1.8).

Similarly, in Southern Punjab 561 cases were recruited. Out of them 61.9% (n=347) were microscopically positive for *P. vivax,* 26.7% (n=150) *P. falciparum* and 11.4% (n=64) of mixed species. The PCR results showed that 55.4% (n=311) were of *P. vivax*, 21.2% (n=119) *P. falciparum,* (14.1%) (n=79), mixed species and 9.3% (n=52) PCR negative. However, in comparison the prevalence of malaria between the Northern and Southern Punjab the overall prevalence was high in the Southern Punjab as compared to Northern Punjab (Table 1.8).

Table 1.8 Microscopy and PCR based diagnosis of clinical isolates collected from both zones of Punjab.

Species	Punjab		Northern Punjab		Southern Punjab	
	Microscopy N (%)	PCR N (%)	Microscopy N (%)	PCR N (%)	Microscopy N (%)	PCR N (%)
P. vivax	617 (66.7)	494 (53.40)	270 (74.2)	183 (50.3)	347 (61.9)	311 (55.4)
P. falciparum	219 (23.7)	173 (16.70)	69 (19.0)	54 (14.8)	150 (26.7)	119 (21.2
Mixed (*P. vivax, P. falciparum*)	89 (9.62)	119 (12.86)	25 (6.5)	40 (11)	64 (11.4)	79 (14.1)
Negative	0	139 (15.02)	0	87 (23.9)	0	52 (9.3)
Total	925	925	364	364	561	561

The coincidental adjustment of kappa statistics specified that overall agreement in the presence or absence of *Plasmodium* species was good (Kappa= 0.79). However, for the detection of *P. vivax* the agreement between microscopy and PCR was fair (Kappa= 0.38). But for *P. falciparum* and mixed infection, it was moderate (Kappa= 0.53, 0.59) respectively.

Molecular epidemiology

The overall prevalence of treatment seeking patients in total sample was 4.88%. However the comparison of Northern and Southern Punjab indicated that the prevalence was comparatively higher in Southern Punjab 5.54% as compared to the Northern Punjab 4.01%. Within Northern Punjab prevalence was higher in Rawalpindi 5.0% than in Gujrat 4.94%. The prevalence was lower in Chakwal city 2.33%. In Southern Punjab the prevalence was higher in Rajanpur 6.74 > in Bahawalpur 5.34 and other cities like Rahim Yar Khan and Multan. The prevalence was lower in Dera Ghazi Khan (Table 1.9).

Table 1.9: Prevalence of *Plasmodium* species on the basis of PCR based results

Localities	Suspected cases	* Prevalence			
		P. vivax N (%)	*P. falciparum* N (%)	Mixed infection N (%)	All species N (%)
Northern Punjab	**6900**	**183 (2.65)**	**54 (0.78)**	**40 (0.58)**	**277 (4.01)**
Gujranwala	1580	48 (3.03)	12 (0.75)	8 (0.51)	68 (4.30)
Gujrat	930	30 (3.22)	9 (0.10)	7 (0.75)	46 (4.94)
Jhelum	1030	25 (2.42)	10 (0.97)	9 (0.87)	44 (4.27)
Chakwal	1840	30 (1.63)	8 (0.43)	5 (0.27)	43 (2.33)
Rawalpindi	1520	50 (3.28)	15 (0.13)	11 (0.72)	76 (5.0)
Southern Punjab	**9175**	**311 (3.39)**	**119 (1.30)**	**79 (0.86)**	**509 (5.54)**
Bahawalpur	1710	65 (3.80)	21 (1.21)	14 (0.81)	100 (5.84)
RahimYar Khan	1895	59 (3.11)	25 (1.31)	18 (0.95)	102 (5.38)
Multan	1770	54 (3.05)	22 (1.24)	13 (0.73)	89 (5.02)
Rajanpur	1690	70 (4.14)	26 (1.54)	18 (1.06)	114 (6.74)
Dera Ghazi khan	2110	63 (2.98)	25 (1.29)	16 (0.76)	104 (4.92)
All	**16075**	**494 (3.07)**	**173 (1.07)**	**119 (0.74)**	**786 (4.88)**

* Prevalence was calculated by dividing the PCR positive cases with suspected cases and multiplied with 100.

Phylogenetic analysis

The DNA sequences with highly alignment scores were used to construct the phylogenetic tree. The four DNA sequences of *P. vivax* (two from Northern Punjab and two from Southern Punjab) and two DNA sequences of *P. falciparum* one from Southern Punjab and one from Northern Punjab was included in order to construct the phylogenetic tree.

The phylogenetic tree indicates two dissimilar clades of *P. vivax i*,e. from Northern and southern Punjab matched in one group, whereas all the *P. falciparum* isolates from the Northern and Southern Punjab in another clade. *Plasmodium vivax* clustered into four and *P. falciparum* clustered into two sub clusters within the main clade. Neighbor-Joining method was applied to infer evolutionary history. The branch length = 6.06796875 was shown and percentage of replicating trees was shown next to the branches.

The p-distance method was used to evaluate evolutionary distance and it was in the units of the number of base differences per site. The analysis involved 21 nucleotides DNA sequences. The 1st+2nd+3rd+Noncoding includes the codon position. Missing data were eliminated because all positions contained gaps. There were total of 85 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 (Figure 1.5).

Two DNA sequence of *P. vivax* one from Northern Punjab and one from southern Punjab showed closest relationship with the *P. falciparum* partial sequence of 18 S ribosomal RNA gene from Brazilian Western Amazone. Another sample of *P. vivax* from Northern Punjab showed the closest association with the *P. vivax* partial sequence of 18 S ribosomal RNA gene from Korea. However one sample of *P. vivax* and *P. falciparum* from southern Punjab and one sample of *P. vivax* from Northern Punjab showed association with *P. vivax* partial sequence of 18 S ribosomal RNA gene from border area of Yunnan Province China (Figure 1.5).

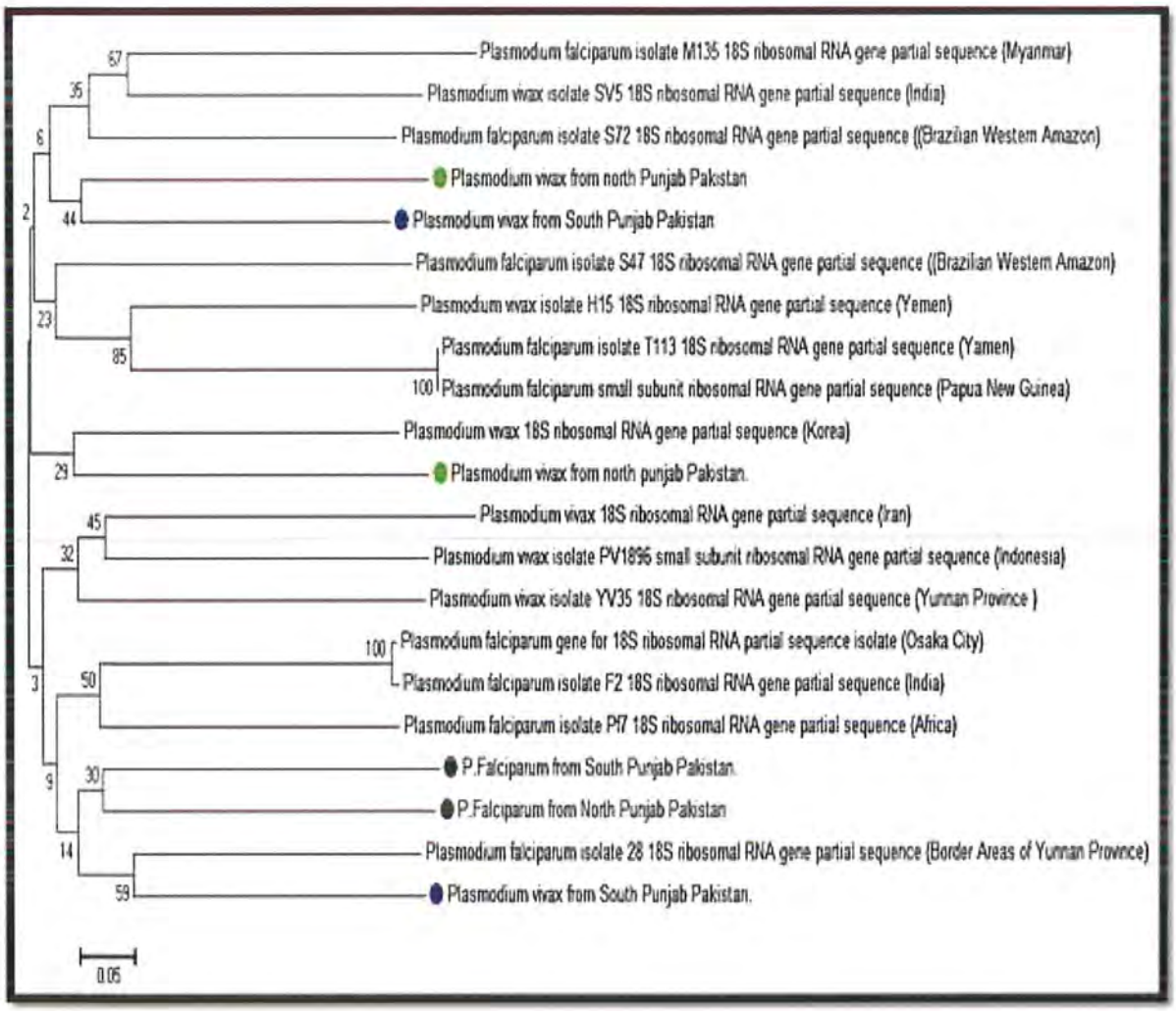


Figure 1.5: Evolutionary relationships of *P. vivax* and *P. falciparum* isolates from Northern and Southern Punjab

Discussion

Malaria is a major health problem in Pakistan Its cases vary significantly in different areas and cannot be assessed accurately due to lacking complete information (Khatoon *et al.*, 2009). The parasite species of malaria also have unequal distribution throughout the Pakistan and their prevalence changes in seasons. Therefore it is challenging to assess the accurate prevalence of malaria infection in Pakistan. The current study implies on the survey of malaria from ten endemic cities of Punjab, Pakistan is experiencing all four seasons with extreme weather conditions.

As reported by the WHO (2014) 3.3 billion people in 97 countries are at risk of malaria, and 1.2 billion are at risk. Greater than 1 case of malaria is per 1000 population each year was observed. WHO, 2011 reported about one million microscopically positive malarial cases in 2010 from the Eastern Mediterranean region out of them 22% were from Pakistan. The definite and accurate estimate of *Plasmodium* infections can also be useful in scaling up malaria surveillance and control measures in Pakistan (Yasinzai *et al.*, 2009).

In the present study 16075 suspected cases of malaria were ascertained of which 925 were microscopically positive. Concerning spatiotemporal distribution, greater number of cases was recruited from Southern Punjab as compared to Northern Punjab. The Rawalpindi from Northern and Rajanpur from Southern zone has the highest cases of malaria among all recruited malaria cases.

The overall SPR in two zones of Punjab was 5.75%. However, the SPR was higher in Southern Punjab 6.11% > 5.27% as compared to the Northern Punjab. Mahmood *et al* (2005) studied 348 patients with fever in Karachi and reported 35% SPR. Yasinzai *et al.,* in (2008) found the SPR between 19.8 to 58.13% among different age groups. Leghari *et al* in 2014 studied the five year surveillance of Malaria in Bahawalpur City only from 2007 to 2011. They observed the SPR from 0.03 to 0.22%. However, in the current study the SPR was observed as 5.75%.

The SPR the API was also higher in Southern Punjab 0.16 > 0.09 per 1000 population as compared to the Northern Punjab. Leghari *et al* in 2014 found the API in

five years from 0.007 to 0.09 per 1000 population. The ABER was 0.22% in Punjab and it was high in Southern Punjab as compared to the Northern Punjab 0.26 > 0.17%.

The incident of malaria began to increase in the spring season i.e. from March and April. The seasonal variation showed that highest cases were recruited in summer season i.e. from June to September then its prevalence become to decrease. However November to February is the coldest season months showed lowest cases.

The age wise distribution of malaria was also evaluated in the present study. The age of all recruited cases in the present study of both genders was ranged between 1 to 78 years. The high prevalence was observed among age group of 1-20 > 21-41 then among 41-60 year age groups. However the distribution of malarial parasites was low in old age groups. The gender wise distribution indicated that *Plasmodium* infection was dominant in males than females among in both zones of Punjab. In the present study 71% males and 39% females were infected.

The females in the Southern Punjab (30.5%) were comparatively high as compared to the Northern Punjab (27.47%). The possible reason may be their role in the agricultural activities. Khattak *et al.*, (2013) reported that males were 64% and females were 36% infected in Punjab. Different reports from different areas of Pakistan showed the males being more affected by Malaria than females (Daud*et al.*, 2014; Khan *et al.*, 2006; Tareen *et al.*, 2012) which are comparable to the present findings. This gender wise difference in malaria transmission is probably due to the socioeconomic norms of Pakistan like the participation of females in agricultural activities and low access of females to the health care centres and hospitals.

The species wise distribution of malaria, according to the microscopy showed 66.70% had *P. vivax,* 23.67% had *P. falciparum* and 9.62% had mixed infections out of 925 recruited cases. However, molecular analysis showed that 53.40% as *P. vivax*, 16.70%, *P. falciparum* and 12.86% as mixed species and 15.03% were PCR negative cases. According to the microscopy 90 samples were diagnosed as mixed infection, but when PCR results showed 119 samples found to having mixed infection. Many species that were diagnosed as *P. vivax* or *P. falciparum* has been diagnosed as a mixed infection

through PCR. The present findings revealed that mixed infection was high in Southern Punjab as compared to Northern Punjab.

A study conducted by Sheikh *et al* (2005) investigated the endemicity of malaria in Quetta. Their study reported 66.8% *P. vivax* and 30.7% *P. falciparum* infection. The research study of Khattak *et al* (2013) showed the distribution of human infection in Pakistan, *P. vivax* prevalence was 76% and *P. falciparum*18% and mixed infection 6%. However, in Punjab Khattak *et al* (2013) reported 73% *P. vivax*, 22% *P. falciparum* infection and mixed were 5%, but his studies cover only five major cities of Punjab. In the present studies it is reported that out of ten cities of Punjab the highest prevalence was observed in Rawalpindi 25.5% (n=93) and from Southern zone and highest prevalence was observed in Rajanpur 21.4% (n=120).

A report of malarial infection in the general population of Lal Qilla Lower Dir by Ahmed *et al* (2013) showed the 99.4% were *P. vivax* infection and 0.53% was *P. falciparum*. However, in the present study *P. vivax* infection was 66.70% and *P. falciparum* 23.67% and the ratio of mixed infection (*P. vivax* and *P. falciparum*) was found to be 9.62% by microscopy. According to the PCR analysis the percentage infection of *P. vivax* was 53.40%, and that of *P. falciparum* was16.70%. The percentage of mixed infection was observed to be 12.86%. Total 139 (15.02%) microscopically positive samples did not amplify with the PCR analysis that may be due to the false positive results of microscopy or may be due to the DNA damaged by anticoagulant used in EDTA tubes.

The high prevalence of *P. falciparum* in contrast to *P. vivax* (65% vs 35%) in 100 children who were positive for malaria infection in a university hospital (Akbar, 2002). Mahmood *et al* (2005) studied 348 patients with fever in Karachi and reported 35% SPR with high percentage infection of *P. falciparum* 88.5% as compared to *P. vivax* 9%. The high rate of *P. vivax* 60.5% and a low prevalence of *P. falciparum* 37.20% were observed in Multan by Yar *et al* (1998). The highest percentage infection of *P. vivax* 90.4% was detected in Kashmiri refugees shifted in Muzaffarabad (Jan and Kiani, 2001).

The prevalence of malaria was higher in the rural areas of Pakistan (WHO, 2010). Similarly, in this study the prevalence of malaria was high in the Southern Punjab as compared to the Northern Punjab due to the floods after unprecedented monsoon rains.

The present survey reported that Southern Punjab cities indicated a high prevalence as compared to Northern Punjab; this discrepancy may be more due to low literacy rate of Southern Punjab than Northern, lack of access to health care facilities, use of presumptive treatment and more flooding in that area during monsoon season.

All cities of Southern Punjab indicated that *P. vivax* had a high prevalence except Bahawalpur where P. *falciparum* was predominant species. Similarly, in Northern Punjab *P. vivax* had highest prevalence and Chakwal was accounted for highest mixed infection. The recent flee of IDPs (Internal displacement people) from high malaria prevalence areas such as FATA (Karim *et al.*, 2016) and Swat (Yasinzai *et al.*, 2008) to Northern Punjab due to war operations against terrorism has contributed to the high prevalence of *P. vivax* in Northern Punjab.

The phylogenetic kinship of *P. vivax* and *P. falciparum* isolates is rather not restricted to one region, but seems to be widespread across different geographical regions of the world. The isolates of *P. vivax* from Northern and Southern Punjab showed homology to the *P. vivax* isolates of Yamen and India. All isolates of *P. falciparum* were clustered together and showed homology of *P. falciparum* isolates from Yunnan, Africa and India. Similar origin of *P. falciparum* was observed by Joy *et al.*, (2003).

However, *P. vivax* showed a more diverse population than *P. falciparum*. The significant rise in temperature and humidity, resulting in an increase in reproduction rate of vector and the vectorial capacity of *Anopheles* that may enhance the *Plasmodium* infection and its reemergence (Hussain *et al.*, 2016: Mitsui *et al.*, 2010)

The microscopy is a conventional method for the detection of *Plasmodium* species. It is cheaper and less time consuming. Its accuracy of the result mostly depends upon the experience of technicians along with other factors like slide staining pattern, parasite level in the blood and microscope condition also influence the results. PCR is used for the most accurate diagnosis of all the five species of *Plasmodium* but its expense level is more than microscopy.

The epidemiological study of malaria conducted in Orakzai Agency showed higher positive results of microscopy as compared to PCR (Asad *et al.*, 2016). Shahzadi *et al* (2013) conducted a molecular study for the diagnosis of malaria and found that the PCR based diagnosis is more sensitive, specific and accurate as compared to microscopy that is standard for routine laboratory diagnosis of malaria. In case of low parasite density and mixed infections, microscopy is not sensitive test. According to Steenkeste *et al* (2009), PCR based diagnosis is the best option for the large scale epidemiological studies especially for the diagnosis of mixed infections.

The overall prevalence rate of malaria in the Southern Punjab found to be high as compared to the Northern Punjab which may be due to the flood in Southern Punjab. *P. vivax* was found to be more prevalent than *P. falciparum*. The mixed infection is also very high in the Punjab. Pakistan is facing many problems and tasks in the control and prevention of malaria, including false diagnosis, scarcity of diagnostic facilities, and use of ostensible treatment. Facts and figures about the burden and species distribution of malaria are acute for guiding national and provincial efforts in effective control measures.

In Punjab the use of presumptive treatments of the infected individuals, improper waste management, no access to health centers, low socioeconomic conditions are some of the hurdles faced and the government need to pay attention especially in the rural areas of Punjab. Therefore, it is essential to improve the accuracy of malaria diagnostic techniques especially to determine species-specific and low parasitemia and to assist with diagnostic method procurement decisions in a more skilled way. The correct estimation of mixed infection of *Plasmodium* species and the record of patient's treatment with antimalarial drugs is possible through this technique. The growing malarial prevalence annexed new ecotypes twisted by, industrial growth, and urban development resulting and the green revolution in model shifts towards man-made malaria. The special attention should be given by the government in the flood infected areas of Southern Punjab and many areas of Sindh in order to eradicate malaria from these regions.

In vivo Antiplasmodial Activity of Different Medicinal Plants against *Plasmodium berghei*

Abstract

Malarial parasites are producing resistance against existing drugs e.g. chloroquine, mefloquine and artimisnin. It is therefore required to explore new plants for their antiplasmodial activity in order to synthesize new drugs. Five plants were selected by keeping in mind about their medicinal importance and their background as Tibetan medicine. The biological activities were already reported however the antiplasmodial potential was not studied.

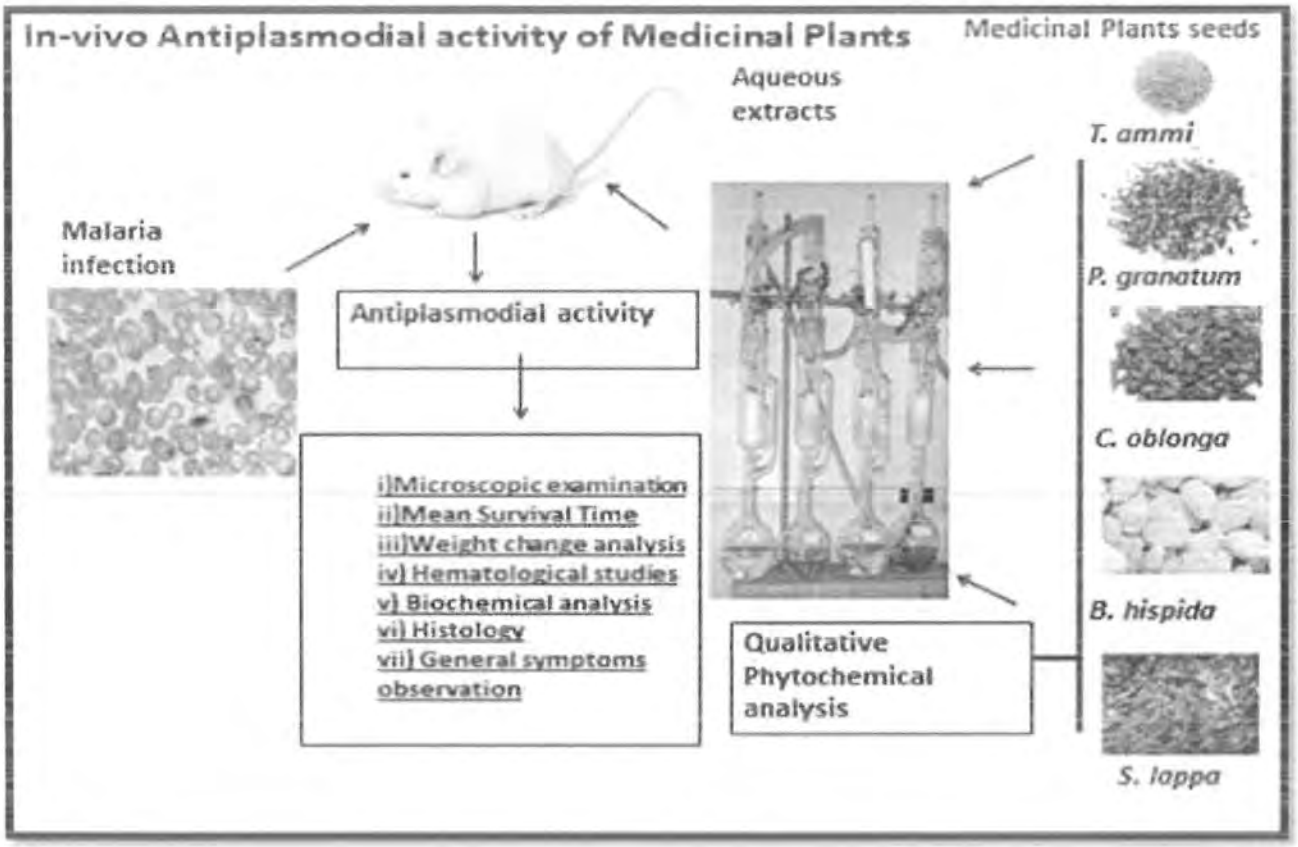
The aqueous seed extracts of five selected plants i.e. *Trachyspermum ammi*, *Punica granatum*, *Cydonia oblonga*, *Benincasa hispida* and *Saussurea lappa* were obtained via soxhelt apparatus. The male BALB/c mice were used as model animals and for the antiplasmodial activity the strain of *Plasmodium berghei* as malarial parasites was selected due to its similarity in mode of action with *P. falciparum*. The toxicity of each plant was observed by feeding mice with four different concentrations of each plant seed extract and calculated LD_{50} by probit analysis. Mice were injected with 50 µL of *P. bherghei strain* via intraperitoneal (IP) injection. The growth of parasites was monitored by tail vein blood sampling and Giemsa-stained thin blood smear microscopy after each 24hours, starting from day 3 of post-inoculation. When the parasitemia reached at 10-30% level, blood was collected from the infected mice. The infected blood with 1×10^6 parasitized red blood cells (RBCs) was injected to mice of each group except normal control. After seventy two hours of post infection; the mice were placed in separate cages with 5 mice in each group and treated with extracts of different concentrations i.e., 250, 500 and 750 mg/kg of each experimented plant. The control experiments were run parallel as (i) infected but treated with water (ii) infected and treated with chloroquine (iii) infected but none treated (iv) non infected and non-treated. The effect of seed extracts was studied by various parameters like general symptoms, parasite count, chemosupression, survival time measurement, histopathology, biochemical and hematological analysis.

Among all the five experimented plants, the LD_{50} of *T. ammi*, *P. granatum*, *C. oblonga*, *B. hispida and S. lappa* was observed as 831, 1989, 1335, 1800, and 1194

mg/kg respectively. The maximum antiplasmodial activity (80.06±4.89) was observed for the *S. lappa* that was treated at the dose of 750 mg/kg. The *P. granatum, B. hispida, T. ammi, and C. oblonga* showed antiplasmodial activity as 77.58±2.09 > 71±6.12 > 69.76±2.01 > 68.76±2.01 at the dose of 500 mg/kg. The results revealed signifiant drop ($P<0.001$) in parasitemia when compared with water treated group. The mean survival time was high in the *S. lappa* than in *P. granatum* treated groups. The biochemical, hematological and histopathological analysis also showed a marked difference in infected untreated group as compared to groups treated with extracts and chloroquine. A progressive decrease in the weight was also shown in non-treated group in compared to the treated ones.

It is concluded from the present study that the seeds of *S. lappa*, *P. granatum* and *B. hispida* exhibit more antiplasmodial activity as compared to other experimented plants thus these plants are more favorable for further use in the experiments of nanotechnology for the present study.

Graphical Abstract



Graphical representation of *in vivo* antiplasmodial activity of five medicinal plants

Introduction

The malaria infection rate is increasing day by day in the developing countries. Although a number of medicines are available for its cure e.g. quinine, resochin (chloroquine), sontochin, proguanil, sulfones and sulfonamides proguanil or (pyrimethamine), mefloquine or artemisinin (CDC, 2005) yet the malarial pathogens are gradually becoming resistant against these drugs.

Among other species of *Plasmodium* causing malaria the increased resistances of *P. falciparum* to antimalarial drugs weaken the line of defense against malaria with the therapeutic collection. The rate of resistance against the existing antimalarial drug is high as compared to the development of novel effective drugs.

So the increased resistance of the parasite to many accessible antimalarial backs the need for new antimalarial drugs that may be cheap and accessible remedies with novel and effective mechanisms of pharmacological action. The plant based products remained the source of biologically active compounds and may have great potential as antiplasmodial compounds and may be used for drug formulation for malaria (Oseni and Akwetey, 2012).

Many medicinal plants have been identified for their antimicrobial activities like *Cassia fistula*, Psidium guajava, Phyllanthus niruri, *Ocimun basilucum* (basil) (thyme) *Rosmarinus officinalis* (rosemary), Ehretia microphylla (Piper betle), *Thymus vulgaris* (Shahid *et al.*, 2011: Parivuguna, 2008 and Hamilton, 2004:). Many have protozocidal potential e.g. *Suregada zanzibariensis, Albizia coriaria, Aspergillus racemosus, Acacia tortilis* (Kigondu *et al.*, 2009) and many others for controlling parasites.

Some of the medically important Plants were selected like ***Trachyspermum ammi*** (Carum or Ajwain) belongs to family *Apiaceae*. It has medicinal importance and traditionally it is used as spice. It is widely used for the treatment of digestive disorders like toxicity produced by hepatic free radical stress, (Anilakumar *et al.*, 2009). The major compound in *T.* ***ammi*** is the phenolic content Thymol. It has been reported to have antibacterial, anti-protozoal, and antifungal agent as well as broncho-dilating actions, Nematicidal, Anthelmintic and Antifilarial activity (Jeet *et al.*, 2012).

The ***Punica granatum*** (Pomegranate or Anaardana) is an inclusive fruit which is cultivated all over the mediterranean regions. Antioxidant properties of pomegranate are elaborated through various studies (Jurenka, 2008). Pomegranate comprises on thocyanidins and flavonoids that showed potent antioxidant activity (Mori-Okamoto *et al.*, 2004). It is used against diarrhoea, microbial infections, haemorrhage, acidosis, dysentery respiratory pathologies and protozociadal properties (Sánchez-Lamar *et al.*, 2008).

The ***Cydonia oblonga*** (Quince seed or bahee dana) belongs to family *Rosaceae*. It is native to Persia, but also cultivated in Turkey. Due to its medicinal importance now a day, it is cultivated throughout the world. It is used for the treatment of digestive disorders like gastrointestinal inflammation, and treatment of diarrhea, inflammation of the joints and for soothing of eye. The quince seeds are used as a remedy for pneumonia and lung disease in Iran (Sajid *et al.*, 2015).

The ***Benincasa hispida*** (White gourd /Pethakaddu) belongs to the family *Cucurbitaceae*. It is one of the well-known crops grown basically for its fruits, especially in Asian countries. It is used as vegetable and it is a richest source of natural sugars, organic acids, amino acid, vitamins and mineral elements. Its medicinal importance can be reviewed through literature.

It has been used as antidiarrheal, antiulcer, anti-obesity antioxidant and antinociceptive (Vongtau *et al.,* 2004). It is also used as antipyretic (Al-Ghamdi, 2001). Its phytochemical analysis revealed that it is a rich source of significant bio actives and therapeutics such as phenolics, sterols, triterpenes, and glycosides. Its fruit is used as a local medicine for the cure of, ulcer, epilepsy and other nervous disorders (Qadrie *et al.*, 2009). Its fruit is used in antidepressant-like activity (Dhingra and Joshi, 2012).

The Saussurea ***lappa*** (Costus or Kust-e shirin) belongs **to** family, *Asteraceae* is a native plant of China, India and Pakistan. It is a perennial herb and grows in the Himalaya region at 2500-3500 m altitude. Medically it is an important plant. Its active ingredients are mainly terpenes, but it also contains anthraquinones, alkaloids and flavonoids. Traditionally it has been used as medicines without any apparent adverse effects (Rao *et al.*, 2013).

The ***S. lappa*** is used as an important medicine for gout, promotes spermatogenesis and erysipelas. Its roots have great medicinal value and used in many Tibetan formulae for the treatment of chronic inflammation of the lungs, chest, and congestion (Tsarong 1994). Its roots possess anthelmintic, carminative, and analgesic properties. It stimulates the brain and used for the medications of blood, liver and kidney disorders (Cho *et al.*, 1998).

Different preparations of *S. lappa* are prescribed in helminthic infestations, tuberculosis, rheumatism, cold and intestinal carcinogenesis (Malik *et al.*, 2011) antibacterial activity (Khalid *et al.*, 2011), anti-inflammatory effects (Yashvanth *et al.*, 2010) and for cancer control (Robinson *et al.*, 2008: ko *et al.*, 2005). *S. lappa* also showed the inhibitory effects of sesquiterpenes on the overproduction of nitric oxide and TNF-α release in LPS-activated macrophages (Zhao *et al.*, 2008). On the basis of a number of biological importances and easy availability of *T. ammi*, *P. granatum, C. oblonga, B. hispida* and *S. lappa,* the seed extracts of these plants were decided to explore their antiplasmodial potential.

Materials and Methods

Model animal acclimatization

Male BALB/c mice of 6 to 8 weeks old were taken from National Institute of Health, Islamabad. The animals were housed in small steel cages in the animal house of Quaid-i-Azam University Islamabad. The animals were nourished on standard Pallet diet and water *ad libitum*. The room temperature was maintained from 26 to 29 °C with 12 hours light/dark cycle. They were acclimatized for one month prior to the experiment. Animals were handled properly and provided with a proper hygienic environment by daily removal of feces and spilled feed from cages.

Experimental plants

The seeds of five selected plants ***T. ammi P. granatum, C. oblonga, B. hispida and S. lappa*** were taken and identified from Department of Botany of National Agricultural Research Centre Islamabad in May, 2015.

Plants extraction formation

The seeds of selected plants were washed, dried and ground finely with electrical grinder. The finely ground seed powder (25 g) of each plant was subjected to the soxhelt apparatus (SYD-260) in water and set at five cycles per hour. The solvent was evaporated with Rotary evaporator (R-205 V). The dried extract was weighed and percentage yield was calculated. The extract was stored at -20 °C for further use. The yield of the extract was calculated according to the following formula (Zhang *et al.*, 2007).

$$\text{E} \qquad \text{y} \qquad (\%) = \frac{\text{weight of the final product}}{\text{weight of the original sample}} X\ 100$$

Acute toxicity test

A trial study was performed to find the acute toxicity of the different plant extracts to be used. Four doses were used 250, 500, 1000, and 2000 mg/kg for all selected plants. The 200 and 500 mg/kg were low dose and 1000 and 2000 were very high dose. These low and high doses were given to four different groups of healthy male BALB/c mice. There were fifteen mice in each group. The animals were observed for physical behavior, motor and feeding activities change other toxicity

symptoms like coma, and convulsion and death for ten days. Then the LD_{50} value of each plant seeds extract was calculated by probit analysis.

Experimental design

The experiment was designed using chloroquine sensitive *Plasmodium berghei* NK 65 strains (attained from MR4/ American Type Culture Collection). Male BALB/c mice (30 to 36 g average weight) and age nearly six to eight weeks old were selected and infected with chloroquine sensitive *Plasmodium berghei* NK 65 strains. The one serial passage of *P. berghei* was conducted by injecting with 50 µL of *P. berghei strain* as via intraperitoneal (IP) injection. It was a singal dose that was enough for causing infection. The growth of parasites was monitored by tail vein blood sampling and Giemsa-stained thin blood smear microscopy daily, starting on day 3 post-inoculation. When the parasitemia reached the 10 - 30% blood was collected from the infected mice and injected to the other mice in this way strain was propagated. The parasitized red blood cells (RBCs) 1 x 10^6 were injected to mice of each group except normal control group A. Malaria parasites were collected at the ring or early trophozoite stage. A stock of parasitized erythrocytes was taken in by heparin-coated syringe from infected mice by cardiac puncture. The cell concentration of the stock was determined and diluted with phosphate buffer saline such that 0.2 ml of the final inoculums contained 10^6 parasitized red blood cells (RBCs). The experimental animals were divided into 18 groups containing 5 mice in each group including controls (Table 2.1). Each mice was inoculated intraperitoneally with the 0.2 ml diluted infected blood except one group of mice (group A, Table 2.1). Drug and extracts were administered after 72 hours of the post infection for five days (Figure 2.2)

Microscopic examination

The blood (2 µL) was taken from the tail vein of each experimented animal started after 72 hrs, of inoculation of parasite strains and continued up to 30^{th} day of the experiment. Thin and thick blood smears were prepared. For thin preparation smears were air-dried, and fixed in methanol for 30 seconds on glass slides and stained with Giemsa for 10 minutes. For thick film preparations, the smears were air dried for 15 minutes, exposed to acetone for 10 minutes and stained with Giemsa. The

prepared slides were washed with tap water; air dried and examined at 10 X 100 X magnification with the light microscope (B350 Optika, Italy).

Table 2.1: Different experimental groups of mice for antiplasmodial activity

Groups	Dose
A	Non infected non treated (Normal control)
B	0.5 ml distilled water (Infected control)
C	5 mg/kg Chloroquine (chloroquine control)
D 1	250 mg/kg of *T. ammi*
D 2	500 mg/kg of *T. ammi*
D 3	750 mg/kg of *T. ammi*
E 1	250 mg/kg of *P. granatum*
E 2	500 mg/kg of *P. granatum*
E 3	750 mg/kg of *P. granatum*
F1	250 mg/kg of *C. oblonga*
F2	500 mg/kg of *C. oblonga*
F3	750 mg/kg of *C. oblonga*
G1	250 mg/kg of *B. hispida*
G2	500 mg/kg of *B. hispida*
G3	750 mg/kg of *B. hispida*
H1	250 mg/kg of *S. lappa*
H2	500 mg/kg of *S. lappa*
H3	750 /kg of *S. lappa*

- There were five mice in each group and each group was repeated in triplicate. Three mice were sacrificed on tenth day for hematology and biochemical analysis. Parasitemia was observed up to 30th day of post infection.

Monitoring of parasitemia and antiplasmodial activity:

The parasitemia was estimated by calculating the 500 red blood cells from 10-15 random fields on prepared slides under light microscope (B350 Optika, Italy). The parasitized erythrocytes were counted out of 500 total red blood cells. The percentage

parasitemia, chemosupression and survival time were calculated by using following formulas as suggested by Amelo *et al.*, (2014).

$$P \quad pc \quad = \frac{T \quad n \; o \; p \quad r \; b \quad c_i}{T \quad n \; o \; r \quad b \quad c_i} X\,100$$

The average percentage of Parasitemia suppression was calculated as

$$P_i \quad s \quad = \frac{P \quad ae: \quad i_1 \; N \quad C \quad g \quad - P \quad i_1 \; t_i \quad g}{P \quad i_1 \; N \quad C \quad g} X\,100$$

The mean survival time for each group was calculated by finding the average survival time (days) of the mice (post-inoculation) in each group over a period of 30 days (D0–D29).

$$M \quad s \quad ti \quad = \frac{S_t \quad o \quad s_i \quad ti \quad o \quad a \quad m \quad i_1 \; u \; g}{T \quad n \; o \; m \quad i_1 \; tha \; g}$$

Hematological and Biochemical analysis

The hematological analysis three mice were sacrificed from each groups on the tenth day of the experiment. Approximately 1 cc to 3 cc blood was collected in EDTA Vacutainers® by cardiac puncture. Following parameters were observed to see the hematological disorders like anemia and thrombocytopenia as occur in malaria. Whole blood was analyzed for complete blood picture (CBP), that is, red blood cell (RBC) count, hemoglobin (HB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell (WBC) count, and platelet (PLT), neutrophils and lymphocytes count using the fully automated using BC -3200 Auto Hematology Analyzer. The 53 μL of blood was aspirated for sample analysis.

The biochemical analysis was carried out on serum separated from blood (1 to 1.5 ml) samples by centrifugation at 3000 rpm for 15 mins. The liver function tests were analyzed by determining the concentration of Plasma enzymes like aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase

(ALT). The creatinine and uric acid levels were analyzed for the kidney function tests by using their respective standards of Randox diagnostic kitson chemistry analyzer (Motenu MTN-658C).

Histopathological study

The three experimented animals were dissected randomly after giving anesthesia with chloroform for the histopathological study at the tenth day of the experiment. Three organs including liver, spleen and kidneys were collected from the dissected animals. Tissues were fixed in 10% formalin for 24 to 48 hours, dehydrated with different alcohol grades in an ascending order. After dehydration tissues were placed in xylene I for 60 minutes and then in xylene II for more 60 minutes and embedded in paraffin wax for section cutting.

The Paraffin wax was melted and poured in the boat. Tissue was embedded in the molten wax, boat and wax was allowed to solidify. Tissues embedded paraffin wax was trimmed and mounted on the wooden blocks for section ribbon cutting by microtomy. The thin filmed sections were stained for observations. The morphological changes within the tissues were observed under light microscope (B350 series Optika, Italy) at 10 x 100 X magnifications and compared with controlled.

General symptoms observation

The morphological symptoms of malaria like body weight, ruffled fur, wobbly gait, limb paralysis, convulsion, and coma and survival rate were recorded from first day up to 30^{th} day of the experiment after each 24 hours interval of all the groups.

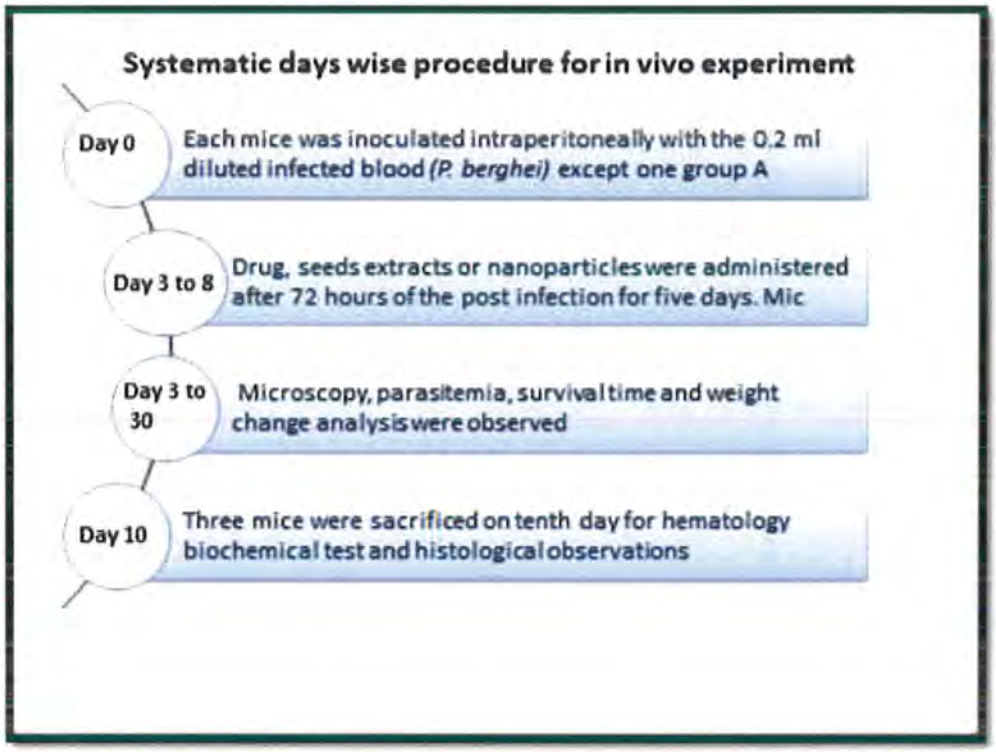


Figure 2.1: Day wise procedural steps for *in vivo* experiment of antiplasmodial activity

Statistical analysis

The results were evaluated statistically using Minitab version 16. The values of the different groups were compared using one-way ANOVA (analysis of variance) and multiple comparison tests (Tukey's test). For the comparison of each sample with control 2 sample t-tests were applied. All results were expressed as S.E.M (standard Error of the mean). $P<0.05$ was taken as statistically significant level. Probit analysis was conducted to measure the ED_{50} and LD_{50}.

Results

Extract yield

The percentage yield of *T. ammi*, *P. granatum*, *C. oblonga*, *B. hispida*, and *S. lappa* was observed as 4.8, 7.2, 8, 4.4, and 5.6 with the same initial quantity used, i.e. two hundred and fifty grams (Table 2.2).

Acute toxicity test

The aqueous seeds extract of *T. ammi*, *P. granatum*, *C. oblonga*, *B. hispida*, *and S. lappa* were administered orally to the different group of mice at the concentration of 500, 1000, 1500, 2000 mg/kg of body weight the LD_{50} value of these extracts was observed as 831 and 1989, 1335, 1800, and 1194 mg/kg respectively (Table 2.2).

Table 2.2: Percentage yield and LD_{50} of plants used

Plants	Family	Percentage yield (%)	LD_{50} (mg/kg)
T. ammi	*Apiaceae*	4.8	831
P. granatum	*Punicaceae*	7.2	1989
C. oblonga	*Rosaceae*	8.0	1335
B. hispida	*Cucurbitaceae.*	4.4	1800
S. lappa	*Asteraceae.*	5.6	1194

- Percentage was calculated according to the above mentioned formula and LD_{50} was calculated by adding information about the concentrations of the dose and mortality observed through probit analysis on Minitab.

Morphological observations

The ruffled fur was observed in all the groups from third to fifth day of infection except in the normal control group A but in Group B it was observed up to 10^{th} day of the infection. Although the limb paralysis was observed only in group of infected and non-treated group (B) and three and two mice from D1 and D3 that was treated with 250 mg/kg and 500 mg/kg of *T. ammi* respectively (Figure 2.2) Convulsion and coma was not observed among all groups.

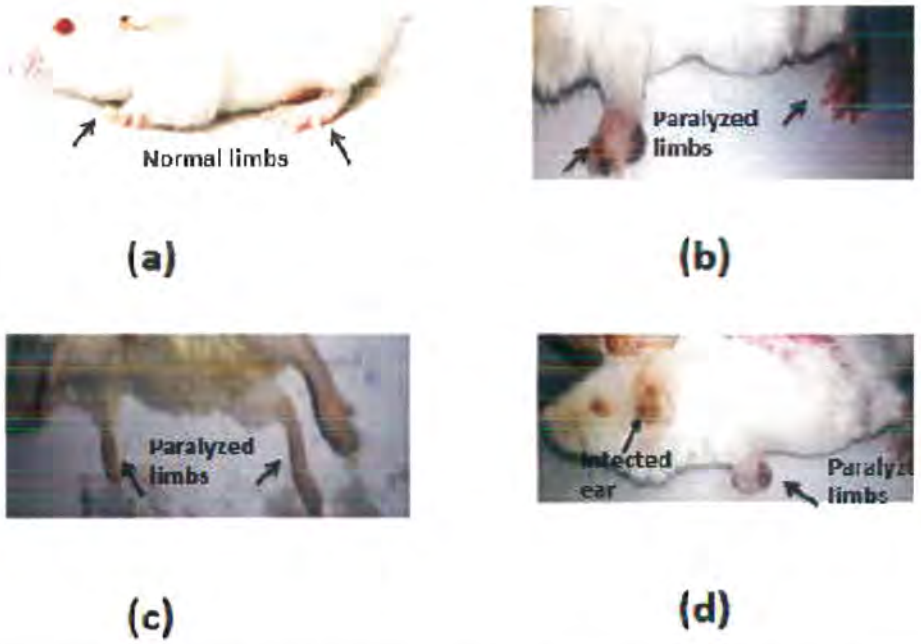


Figure 2.2: Limb paralysis in mice in seed extracts treated groups VS control.

(a) Normal limb of mouse of group A (b) Two limbs of a mouse are paralyzed in infected but treated with water group. (c) Two limbs are paralyzed in a mouse of group F1 (d) Two limbs plus one ear is infected of a mouse in group F1 (All are indicated with arrow).

P. berghei early stage in infected liver and erythrocytes

The early life cycle stages of *Plasmodium berghei* like exoerythrocytic stage were studied during the histological studies of liver (Figure 2.3). The erythrocytic stages were observed during microscopy. Early trophozoite and late schizont stages were detected (Figure 2.4).

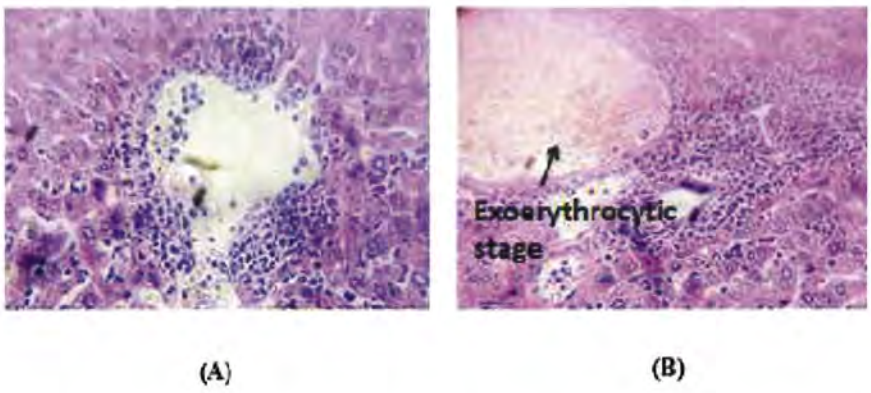


Figure 2.3: Exoerythrocytic form of *Plasmodium berghei* observed in the liver of infected but non treated group B as seen in the figure (B) as compared to the normal group A (A)

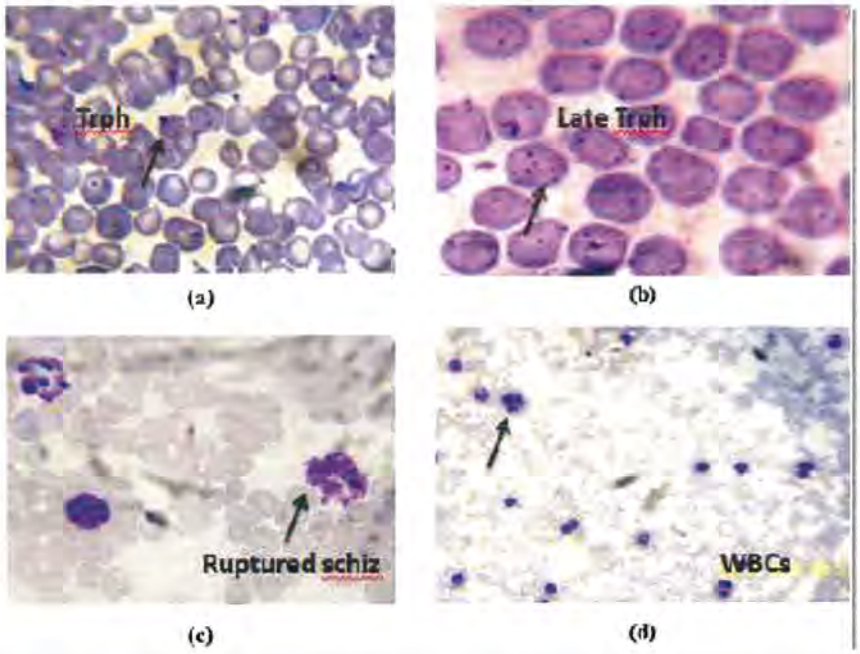


Figure 2.4: Different stages *of* Plasmodium *berghei* observed in erythrocytes.

(a) Early trophozoites (Trph) stage (b) late stage trophozoites (Trph) (multiple trophozoites infect the same cell), (c) late stage schizonts. Ruptured schizonts (Schiz) can be seen indicated by arrow. (d) The number of white blood cells increased in the late stage of infection. Observed under light microscope at 10 X 100 except (d) magnify at 10 X 40.

Percentage parasitemia

Maximum percentage parasitemia was observed in the infected control group B and there was significantly high difference (P=0.001), when it was compared with the drug (chloroquine) treated group C and other seed extracts treated groups like D2 treated with 500 mg/kg *T. ammi* seed extract, E2, E3 treated with different concentrations of *P. granatum* seed extract.

The maximum parasitemia in seed extract treated group was observed in order D3, G1, D1, F1, G3, F3, G2, F2, E1, H1, E2, H2 and then in H3 and the difference was significantly higher for all groups ($P \leq 0.001$) respectively when compared to the non-treated group B on the fifteenth day of the experiment.

The highest level of parasitemia was observed on the day four to six for almost all groups and continuously increased in group B and gradually decreased in the treated group (Figure 2.5 b).

The plant seed extract treated groups like D1, D2, D3, E1, E3, F1, F2, F3, G1, G2, and G3 exhibited the significant difference ($P \leq 0.05$) however the seed extracts

treated groups D1, D3, F1 and G1 showed high significant difference ($P \leq 0.001$) when their percentage parasitemia was compared with chloroquine treated group C.

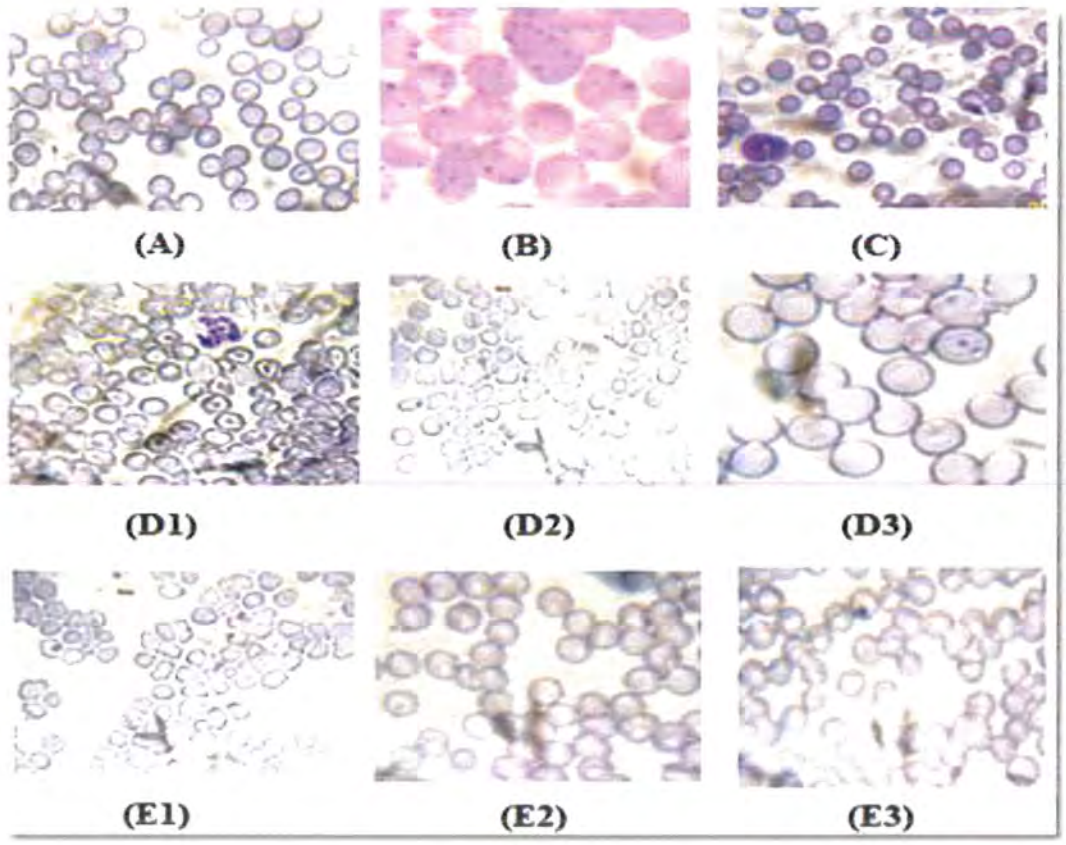


Figure 2.5: Thin smears of control groups and seed extract treated with *T. ammi* and *P. granatum* at 10th day of post infection.

Multiple trophozoites infect the same cell in the infected but treated with water group B can easily be seen as compared to the normal control (A) and treated with plant seed extract (D1-E3) groups.

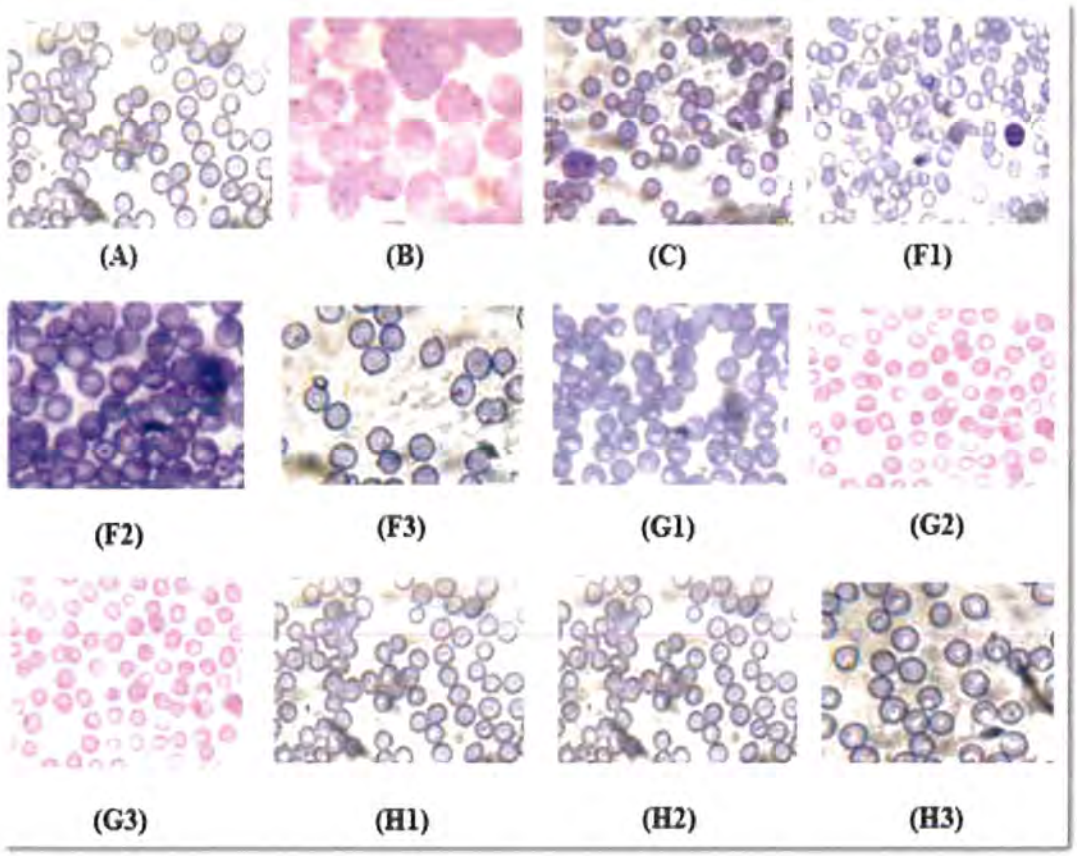


Figure 2.6: Thin smears of control groups and seed extract treated with *C. oblonga* , *B. hispida* and *S. lappa* at 10th day of post infection.

Multiple trophozoites infect the same cell in the infected but treated with water group B can easily be seen as compared to the normal control (A) and treated with plant seed extract groups (F1-H3).

Antiplasmodial activity

Maximum antiplasmodial activity was observed for the group treated with chloroquine, however it differs non-significantly (P=0.057) and (P=0.163) from the groups that were treated with concentrations of 250 and 500 mg/kg of *P. granatum* in E1 and E2 groups, respectively. The groups D2 in D3 treated with 500 and 750 mg/kg of *T. ammi also* showed non-significant difference (P= 0.154) and (P=0.062) in antiplasmodial activity respectively. On the other hand, groups D1 and E3 that was treated with 250 mg/kg *T. ammi* and 750 mg/kg *P. granatum* showed significant difference (P= 0.030) and (P= 0.043) respectively, when compared with drug treated group C (Figure. 2.7 b).

The groups F1, F3 and G1 exhibited the significant difference (P≤0.01) while G2, F2 and G3 groups indicated the low non-significant difference (P≤0.1) and F2, H1, H2, and H3 revealed high non-significant (P≤0.5) when compared with antiplasmodial activity of chloroquine treated group C (Figure 2.8 b). Highest antiplsmodial activity was observed for H3, E2, H2, G2, D2, then for F2 group which means these groups, exhibit the highest when compared with the chloroquine treated group C (Figure 2.8 b, Table. 2.3 and 2.4).

Mean survival time

The **mean survival time** was highest for normal control mice group A days when compared with the infected but non-treated group B.

Table 2.3: Antiplasmodial activity and median effective dose of aqueous seeds extract of *T. ammi and P. granatum* against *Plasmodium berghei*

Group Names	Treatment	Dose mg/kg	ED_{50} mg/kg	ED_{90} mg/kg	Parasitemia (%) Mean ± SE	Chemosupression (%) Mean ± SE	Survival time Mean ± SE (days)
(A)	None	-	-	-	-	-	>25.6 ±1.64 {a}{b}
(B)	Water	-	-	-	19.97±0.74	-	10.4±1.064 {a}[c]
(C)	Chloroquine	5	-	-	3.32±0.67 (a)	83.48±2.15	16.2±0.4 {b}(c)
(D1)	*T. ammi*	250	143.04	586.62	7.29±0.78 (a) [a]	59.95±2.15 [c]	13.60±0.98 {c}(c) [c]
(D2)		500			6.44±1.22 (a) [c]	69.76±2.01 [d]	15.4±0.57 {d}(c) [d]
(D3)		750			9.33±2.19 (b) [a]	46.90±1.15 [c]	13.4±0.57 {c}(d) [c]
(E1)	*P. granatum*	250	124.45	290.17	5.53±0.60 (a) [c]	70.89±2.1 [d]	12.8 ± 0.46 {c}(e) [c]
(E2)		500			4.26±0.26 (a) [e]	77.58±2.09 [c]	17.2 ± 0.385 {d}(c) [c]
(E3)		750			6.44±1.22 (a) [c]	67.10±1.5 [c]	12.14±0.79 {c}(e) [c]

Values are presented as Mean ± SEM (n=5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a]–P ≤0.001, [b]–P≤0.01 [c]=P≤0.05, [d]=P≤0.1 [e]– P≤0.5.

Table 2.4: Antiplasmodial activity and median effective dose of aqueous seeds extract of *C. oblonga, B. hispsida and S. lappa* against *Plasmodium berghei*

Group names	Treatment	Dose mg/kg	ED_{50} mg/kg	ED_{90} mg/kg	Parasitemia (%) Mean ± SE	Chemosuppression (%) Mean ± SE	Survival time Mean± SE (days)
(F1)	*C. oblonga*	250	189.26	567.49	7.106 ± 0.6 [a] [a]	59.89± 4.12 [b]	13.8±0.63 {c}(d) [c]
(F2)		500			5.23 ± 0.7 [a] [c]	68.71±6.12 [d]	15.2±0.98 {d} (c) [d]
(F3)		750			6.062 ± 0.7 [a] [c]	64.42±4.12 [c]	13.00±0.99 {c} (d) [c]
(G1)	*B. hispida*	250	80.86	206.55	8.22 ± 0.6 [a] [a]	58.62 ± 6.17 [b]	11.8±0.53 {b} (d) [c]
(G2)		500			6.016 ± 0.8 [a] [c]	71.7±2.75 [e]	14.60±0.25 {c} (c) [d]
(G3)		750			6.58± 0.87 [a] [c]	65.18±5.99 [c]	11.06±0.2 {b} (d) [c]
(H1)	*S. lappa*	250	66.45	153.56	5.348 ± 0.6 [a] [d]	70.18 ± 5.18 [d]	16.6±1.07 {d} (c) [e]
(H2)		500			4.604± 0.8 [a] [d]	75.7±6.01 [e]	20.01±0.76 {e} (b) [b]
(H3)		750			3.54 ± 0.87 [a] [e]	80.06±4.89 [e]	23.6±0.19 {e} (b) [b]

Values are presented as Mean ± SEM (n=5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a]=P ≤0.001, [b]=P≤0.01 [c]=P≤0.05, [d]=P≤0.1 [e]= P≤0.5.

The survival time of the chloroquine treated group was days. The difference was significantly higher for group A (P=0.006) and only significant (P=0.05) for group C respectively when compared with infected but non-treated group B.

The mean survival time of the groups treated with the different concentration of *T. ammi* and *P. granatum* i.e. D1, D3, E1, E3, F1, F3, G1and G3 showed significant difference (P≤0.05) and the groups D2, E2, F2, and G2 treated with 500 mg/kg of *T. ammi, P. granatum, C. oblonga* and *B. hispida* showed the non-significant difference (P≤0.1) when compared with the normal group of mice A.

The groups H1, H2 and H3 treated with the different concentration of *S. lappa* showed the high non- significant difference ($P \leq 0.5$) in the survival time when compared with normal group A.

The groups D3, E1, and E3 treated with 750 mg/kg of *T. ammi* and 250 mg/kg and 750 mg/kg of *P. granatum* showed the non-significant difference ($P \leq 0.1$) when compared with the non-treated group B. However the mean survival time of the seed extract treated group D1, D2, and E2 showed the significant difference ($P \leq 0.05$) with the mean survival time of mice of non-treated group B (Figure. 2.7 c).

There was significant difference ($P \leq 0.05$) for F2, G2 and H1 seed extract treated groups. The groups H2 and H3 that was treated with 500 and 750 mg/kg of *S. lappa* revealed high significant difference ($P \leq 0.01$) when compared with non-treated group B (Figure. 8). But the groups F1, F3, G1, and G3 showed non- significant difference ($P \leq 0.1$) when compared with non-treated group B.

The maximum survival time was observed for H3, H2, E2 group, H1, D2 and F2 which were treated with different concentrations of medicinal plant's seed extracts. The survival time of H3, H2 E2 and H1 was still greater than chloroquine treated group C while in other groups D2, F2, and G2 only showed the non- significant difference in the mean survival time with drug treated group (Figure 2.7 c and 2.9 a).

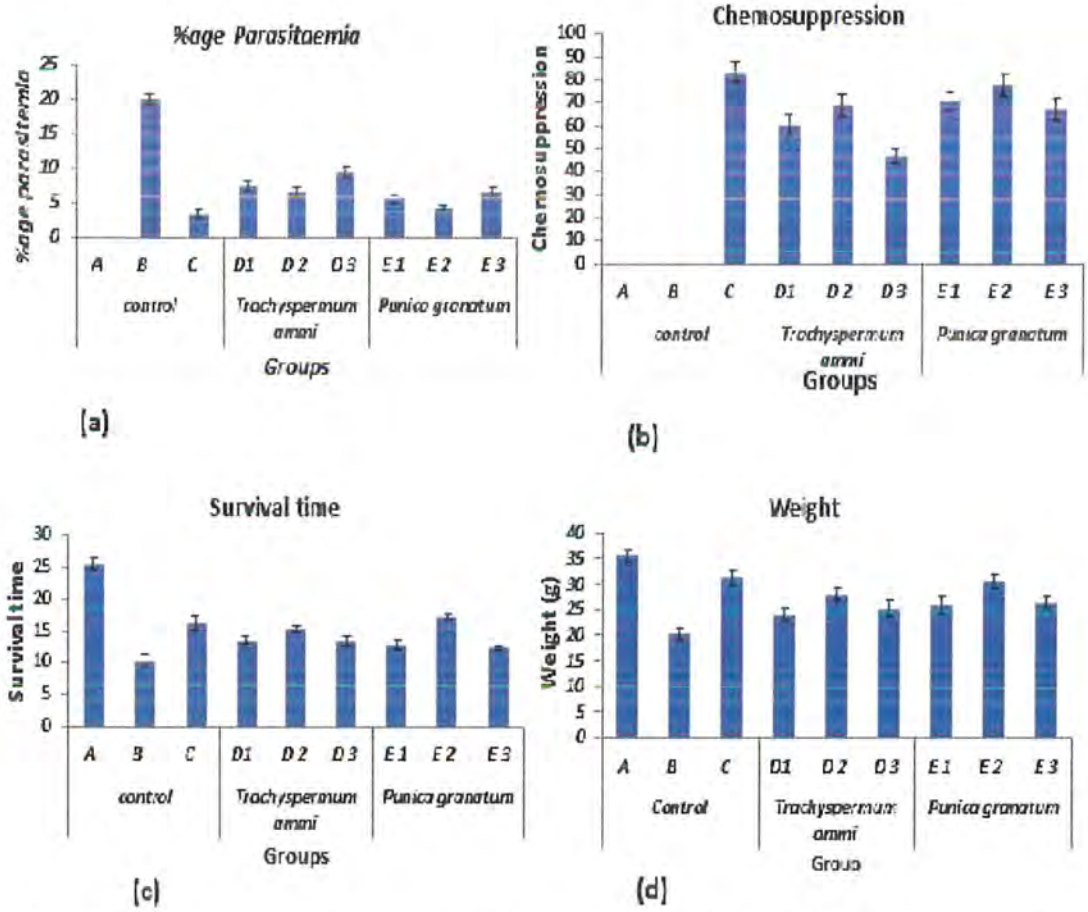


Figure 2.7: The figure shows the (a) Percentage Parasitemia, (b) Chemosuppression, (c) Survival time, (d) weight change of control and the groups treated with different concentrations of *T. ammi* and *P. granatum* seed extracts.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, $P<0.009$ for (a), $P<0.054$ for (b), $P<0.01$ for (c) $P<0.05$ for (d)

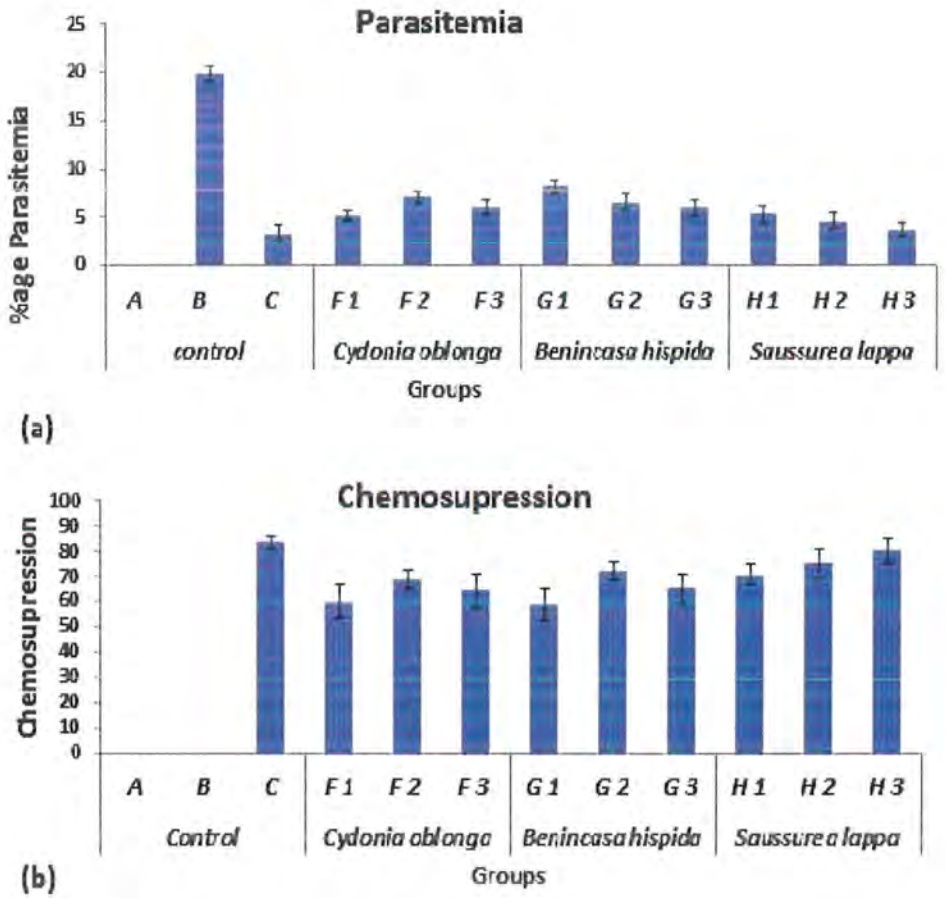


Figure 2.8: The figure shows the (a) Percentage Parasitemia, (b) Chemosuppression of control and the groups treated with different concentrations of *C. oblonga, B. hispida* and *S. lappa* seed extracts.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, $P<0.005$ for (a) $P<0.075$ for (b)

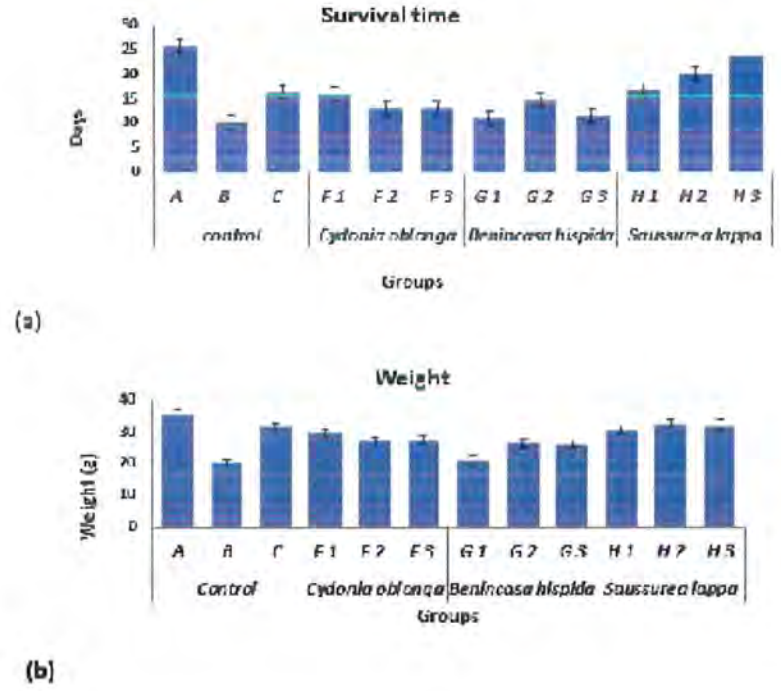


Figure 2.9 The figure shows the (a) Survival time, (b) weight change of control and the groups treated with different concentrations of *C. oblonga, B. hispida* and *S. lappa* seed extracts.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, $P<0.007$ for (a) $P<0.05$ for (b)

Weight analysis

There was a gradual increase in weight of the control group A till the fifteenth day of the experiment, whereas a progressive decrease in body weight was observed in infected but treated with water group B and the difference was significantly high (P =0.001). However, there was a non-significant difference (P = 0.33) for the change in weight of normal control group A and chloroquine treated C was observed.

The change in weight between seed extracts treated groups D2, D3, E1, E2, E3, F2, G2, G3, H2 and H3 were non-significant (P≤0. 5) When compared with the normal group A. However, other seed extracts treated groups D1, F1 and G1 exhibited the high significant difference (P≤0.01) and F3 and H1 exhibited the low significant difference (P≤0.05) in the weight change when compared with the normal mice of group A. There was non-significant difference (P≤0.1 and P≤0.5) for

percentage weight change for all plant seed extract treated groups when compared with the normal control group (A).

The seed extracts treated groups D2, D3, E1, E2, E3 F2, G3, H2 and H3 showed the high significant difference (P≤0.001) and other seed extracts treated groups like D1, F1, F2, F3, G1, G2 and H1 showed the significant difference (P≤0.05) when their weight change was compared to the infected but non-treated group B. However percentage weight change for all plant treated groups were highly significant (P≤0.001) when compared to the infected but non-treated group B.

When the weight change among seed extract treated groups, D2, D3, E1, E2, E3, F2, G2, H2 and H3 compared with the chloroquine treated group (C) a non-significant (P≤0.5) was observed. However, there was a highly significant difference (P≤0. 01) for F1, F3, G1 and significant difference (P≤0.05) for H1 and D1 groups in comparison with the chloroquine treated group (C).However percentage weight change for all plant treated groups were non-significant (P≤0.1 and P≤0.5) except F1group (P ≤0.05) when compared with the chloroquine treated group (C) (Figure 2.7 d and 2.9 b).

The treated groups showed a gradual loss in weight till the day four as compared to the first day of the infection after that a steady gain in weight occurred in the groups treated with the medicinal plants used in this study. The percentage weight change was calculated on the fifteenth day in comparison with the first day of the experiment (Table 2.5).

Hematological analysis

Red Blood Cell (RBC) count

The **Red Blood Cell (RBC) count** among all the experimental groups also varies and showed a noticeable difference when compared with control. The no. of RBC in infected and non-treated group B was differ significantly (P=0.05) when compared with the normal control group A. The infected and treated with chloroquine group (C) showed significant difference (P=0.033) when compared with the normal control group A.

The groups treated with plant *T. ammi* with the dose of 250 mg/kg, 500 mg/kg, 750 mg/kg contained RBC as D1, D2 and D3 that exhibited the significant difference (P≤0. 01) when compared with the normal control group (A).

Table 2.5. Comparison of body weight and percentage weight change among seed extracts treated groups Vs controls

Group	Treatment	Dose mg/kg	Body weight (g) Mean±SE		Percentage weight change (%) Mean±SE
			Day 0	Day 15	
(A)	None	-	35.83±0.764	40.31±0.153 [a][e]	12.51±1.02 [a][e]
(B)	Water	-	33.90±0.265	19.87±3.87 [a][a]	-41.38±1.65 [a][a]
(C)	Chloroquine	5	34.40±0.107	37.60±0.393 [e](a)	9.30±0.85 [e](a)
(D1)	*T. ammi*	250	33.25±0.274	32.40±0.841 [b] (c) [c]	2.40±0.76 [b] (a) [c]
(D2)		500	33.86±0.40	36.99±1.09 [e] (a) [e]	9.24±0.87 [e] (a) [e]
(D3)		750	36.33±0.322	39.03±0.313 [e] (a) [e]	7.45±0.23 [d] (a) [e]
(E1)	*P. granatum*	250	34.11±0.495	36.88±0.168 [d] (a) [e]	8.12±0.45 [d] (a) [e]
(E2)		500	35.27±0.255	39.9±0.470 [e] (a) [e]	11.67±0.34 [e] (a) [e]
(E3)		750	33.82±0.371	36.42±0.540 [d] (a) [e]	7.68±0.34 [d] (a) [e]
(F1)	*C. oblonga*	250	33.20 ± 0.6	34.00 ± 0.42 [b] (c) [b]	2.94±0.38 [b] (a) [c]
(F2)		500	33.01± 0.5	36.03 ± 0.56 [d] (a) [e]	9.14±0.45 [d] (a) [e]
(F3)		750	32.27 ± 0.7	34.30 ± 0.40 [c] (b) [d]	5.42±0.78 [c] (a) [d]
(G1)	*B. hispida*	250	32.13 ± 0.9	33.25 ± 0.72 [b] (c) [c]	3.48±1.45 [b] (a) [d]
(G2)		500	33.01 ± 1.13	35.10 ± 0.63 [d] (b) [d]	6.33±1.67 [d] (a) [d]
(G3)		750	34.07 ± 1.1	36.32 ± 1.74 [d] (a) [e]	6.60±1.45 [d] (a) [e]
(H1)	*S. lappa*	250	33.00 ± 0.5	34.96 ± 0.5 [c] (c) [d]	5.93±1.20 [c] (a) [d]
(H2)		500	33.27 ± 1.27	36.11 ± 1.1 [d] (a) [e]	8.53±1.56 [d] (a) [e]
(H3)		750	33.02 ± 1.17	36.50 ± 0.99 [d] (a) [e]	10.53±1.34 [d] (a) [e]

Values are presented as Mean ± SEM (n=5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a]=P≤0.001, [b]=P≤0.01 [c]=P≤0.05, [d]=P≤0.1 [e]= P≤0.5.

The groups treated with plant *P. granatum*'s seeds at the dose of 250 mg/kg250 mg/kg, 500 mg/kg, 750 mg/kg showed RBC as in group E2 ≥ E1 and E3. The RBC of E2 showed non-significant difference (P=0.162) and E1 and E3 depicted the significant difference (P≤0.05) when compared with control group A. The RBC count of other seed extract treated group F1, F2, F3 , and G1 differ significantly (P≤0. 05) while the group G2, G3, H1, H2, and H3 depicted the non-significant difference (P≤0.5) when compared with the normal group of mice A.

The significant difference (P= 0.003) of RBC number was observed between infected but treated with water group B and chloroquine treated group C. When the RBC the groups D1, D2 and D3 that was treated with different dose of *T. ammi* was compared with the infected, but treated with water group B there was a non-significant difference (P=0.147) for D1 but D2, and D3 groups showed the significant difference (P= 0.049) (P=0.012) respectively. But the highest significant difference, i.e. (P= ≤0. 01) was detected for the *P. granatum* treated groups E1, E2, E3 when compared with the infected, but treated with water group B.

The groups treated with the different concentration of *C. oblonga, B. hispida* and *S. lappa* e.g. F1, F2, F3, G1, G2, G3, H1, H2 and H3 revealed the significant difference (P≤0.01) when compared with the infected but non treated group B. The highest significant difference was observed for the E2, G1, H2 and H3 groups.

There was a non-significant difference (P≤0.5) of RBC when the chloroquine treated group C compared with different seed extracts treated groups D1, D2, D3, E1, E2, and E3. The other seed extracts treated groups F1, F2, F3, G1, G2, G3, H1, H2, and H3 treated groups also showed the non-significant difference (P≤0.1) when compared with the chloroquine treated group C. It means that the RBC numbers in the plant extract treated groups was compatible with chloroquine treated group C.

Hemoglobin

The normal control group A (11.3 ±0.497) showed highly significant difference (P=0.002) of Hemoglobin with infected, but untreated group B and highly non-significant difference (P=0.312) with the group C treated with chloroquine. The comparison seed extract treated group D1, D2, and D3 with the normal control group (A) showed the non-significant difference (P=0.408) for D2, and significant difference (P= 0.007 and 0.043) for D1, and D3 respectively. There was a highly non-

significant difference (P=0.950) and (P=0.087) for hemoglobin of E2 and E3 group significant difference (P=0.023) for E1 (8.3 ±0.328) when compared with the normal control group A.

The groups treated with other medicinal plant like *C. oblonga, B. hispida* and *S. lappa* F1, F2, F3, G1, G2, G3, H1, H2 and H3 respectively also showed the non-significant (P≤0.5) when compared with normal control group A. The highest non-significant difference was observed for E2, H1, F3, and G2. There was highly significant difference (P=0.001) of hemoglobin when infected but non treated group B (3±0.346) and the drug treated group C was compared.

When the hemoglobin of seed extracts treated groups were compared with the infected but treated with water group B then the group D2 and D3, indicated the significant difference (P= 0.002) (P=0.025) respectively whereas D1showed the non-significant difference (P=0.158). There was the highest significant difference (P=0.002, P= 0.020, and 0.012) for the groups treated with different doses of *P. granatum*, E2 than E3 and E1 groups respectively. The groups treated with Plants *C. oblonga, B. hispida,* and *S. lappa* F1, F2, F3, G1, G2, G3, H1, H2 and H3 also indicated significantly higher difference (P≤0.01) when compared with the infected but non treated group B. The highest significant difference was observed for the D2, E2, F2, G1, H1, H2 and H3 group.

Besides those groups showing significantly higher values of Hb compared with infected and non-treated groups the seed extracts treated groups like D2, D3, E2, and E3 exhibited the non-significant difference (P≤0.5), for Hemoglobin when compared with drug treated group C (10.5 ±0.87). However the groups D1 and E1 showed significant difference (P =0.007 and 0.032) when compared with chloroquine treated group C.

The groups F1, F2, F3, G1, G2, G3, H1, H2 and H3 treated with other plants also showed the high non-significant difference (P≤0.5) when compared with chloroquine treated group C.

Hematocrit (packed cell volume)

The results of **Hematocrit /packed cell volume (PCV)** were also comparatively **analyzed.** It was found that the group A showed significant difference (P=0.006) of PCV as compared with infected, but non medicated group (B) and

significant difference (P=0.038) with the group C, treated with chloroquine. When the **Hematocrit** level of the seed extract treated group was compared with the normal mice group A. The seed extracts treated groups D1, D2, D3 and E1 indicated the significant difference (P≤0.05) whereas groups E2 and E3 revealed the non-significant P > 0.05 i.e. 0.089, 0.078 difference as compared with the normal control group (A).

When the Hematocrit value of normal control group (A) was compared with the other seed extracts treated groups F1, F2, F3 which bared the significant difference (P≤0. 01) while G1, G2, G3, H1, H2 and H3 exposed the non- significant difference (P≤0. 5).

There was a significant difference (P= 0.023) when the Hematocrit value of non-treated group B was compared with the chloroquine treated group C. There was non-significant difference (P≤0.1) when the seed extracts treated groups D1, D2 and D3 the E1, E2 and E3 compared with the infected but treated with water group B.

It was observed that the hematocrit level of seed extracts treated groups F1, F2, F3, G1 and G2 showed the non-significant difference (P≤0.5) and seed extracts treated groups G3, H1, H2 and H3 depicted the significant difference (P≤0.01) when their hematocrit level was compared with the infected but treated with water group B.

There was non-significant difference (P≤0.5) for **Hematocrit** level of chloroquine treated group C was compared with the seed extracts treated groups D1, D2, D3, E1, E2, and E3 groups. It was observed that the other seed extracts treated groups F1, F2, F3, G2 and G3 also showed the non-significant difference (P≤0.5) except for H1, H2 and H3 which showed the significant difference (P≤0.01) when compared with the chloroquine treated group C.

Mean cell volume (MCV)

The group (A) showed highly significant difference (P=0.000) of **MCV** with infected, but non medicated group (B) and significant difference (P=0.019) with the group (C) treated with chloroquine. The probability was highly significant because a normal control group was compared with the infected but non-treated group and drug treated group (Table 2.6). The groups treated with different plant extracts D1 and D3 showed significant difference (P≤0.05) while D2, E1, E2 and E3 indicated the non-significant difference (P≤0.1) as compared with the normal control group (A).

The other seed extracts treated groups only F1, F2 and F3 showed the significant difference (P≤0.01) and G1, G2, G3, H1, H2, and H3 revealed the non-significant difference (P≤0.5) compared with normal control group (A).

There was a highly significant difference (P= 0.005) for MCV of non-treated group B and chloroquine treated group C. It was observed that the seed extracts treated groups D1, D2, D3, E1, E2 and E3 indicated a significant difference (P≤0. 01) when compared with the infected, but treated with water group B. Similarly, other plants treated groups F1, F2, F3, G1, G2, G3, H1, H2 and H3 also revealed the significant difference (P≤0.01) when compared with infected but non treated group B.

There was non-significant difference (P≤0. 5) for MCV between C group and different seed extracts treated groups D1, D2, D3, E1, E2, E3, F1 and F2 groups. But the groups F3, G1, G2, G3, H1, H2 and H3 showed significant difference (P≤0.01) when compared with chloroquine treated group C.

Mean cell hemoglobin (MCH)

The group (A) showed a highly significant difference (P=0.000) of MCH with infected, but treated with water group B) and significant difference (P=0.021) with the group C treated with chloroquine. The seed extracts treated groups D3 and E3 showed significant difference (P= 0.024, 0.004). While D1, D2, E1and E2 indicated the non-significant difference (P≤0.5) when compared with normal group of mice (A). However the other plants treated groups like F2, F3, G1, G2, G3, H1 and H2 showed the non-significant difference (P≤0.5). But the groups F1and H3 indicated the significant difference (P≤0.01) when compared with the normal group A.

There was a highly significant difference (P= 0.007) of **MCH** between infected, but non treated group B and chloroquine treated group C. It was observed that the seed extracts treated groups D1, D2, E1, E2 and E3, F2, F3, G2, G3, H1 and H2 and H3 showed the significant difference (P≤0.5). However the groups F1 and G1 showed the non- significant difference (P≤0.5) when compared with the infected but treated with water group B.

There was a non-significant difference (P≤0.5) for MCH level of chloroquine treated group C and all seed extracts treated groups also revealed the non- significant difference (P≤0.5) when compared with the chloroquine treated group.

Mean cell hemoglobin concentration (MCHC)

The group A showed a highly significant difference (P=0.000) of **MCHC** with infected, but treated with water group B and significant difference (P=0.05) with the group C) treated with chloroquine drug. The seed extracts treated groups D2, E1, E2, and E3 groups showed the non- significant difference (P≤0.5). But the seed extract treated group D1 showed the significant difference (P=0.06) when compared with the normal control group A. The seed extracts treated groups *C. oblonga, B. hispida and S. lappa* with all doses showed the non-significant difference (P≤0. 5) when compared with the normal control group A.

There was a highly significant difference (P= 0.00) between infected, but treated with water group B and chloroquine treated group C. All plants treated groups indicated the significant difference (P≤0. 01) except for the G1, which indicated the non-significant difference (P= 0.092) when compared with the infected but non treated group (B).

There was non-significant difference (P≤0.5) for MCHC between chloroquine treated group and different Seed extracts treated groups of *T. ammi, P. granatum, C. oblonga, B. hispida and S. Lappa* also showed the highest non-significant difference (P≤0. 5) when compared with the Chloroquine treated group C.

Platelets

The group (A) (325000 ±27.9) showed a highly significant difference (P=0.000) and (P=0.004) when compared with the **Platelets** of infected but treated with water group (B) (178000 ±18.1) and with the chloroquine treated group C (274000± 23.6) respectively. The Plant seed extracts treated groups D1, D2, D3, E1 , and E3 showed very high significant difference (P≤0.001) and E2 showed the only significant difference (P≤0.05) when compared with the normal control group (A).

It was observed that the other seed extracts treated groups like F1,F2, F3, G1, G2, G3, and H2 groups also showed the significant difference (P≤0.01) except the H1 and H3 which exhibited the non-significant difference (P≤0.1) when compared with normal control group (A).

There was highly significant difference (P= 0.000) between B and C group. The seed extract treated with the different concentration of *T. ammi* and *P. granatum*

seed extracts of also indicated the high significant difference (P≤0.1) for D2, and for E1, E2 and E3 group. The group D1 treated with the 250 mg/kg of *T. ammi*'s seed showed significant difference and D3 group that was treated with the 750 mg/kg *T. ammi*'s seed showed highly non-significant difference (P≤0.5) of when compared with the infected but treated with water group (B) .

The other treated groups that was treated with other medicinal plants i.e. *C. oblong, B. hispida,* and *S. lappa* F1, G1, H1, H2,and H3 showed the high significant difference (P≤0.01) however F2, F3, G2, and G3 showed the only significant difference (P≤0.05) when compared with the infected but treated with water group (B).

There was significant difference (P≤0.001) between chloroquine treated group C and D1, D2, D3, E1, and E2 groups except for E3 showed non-significant difference (P= 0.37) where P > 0.05 that was our level of significance.

It was observed that other seed extract treated groups, i.e. F1, F3, G1, G2, H1, H2 and H3 showed the non-significant difference (P≤0.5) and but F2 and G3 indicated the significant difference (P≤0.05) for Platelet count when compared with the chloroquine treated group C.

Neutrophils

The group (A) (24 ±0.882) showed highly significant difference (P=0.008) of **neutrophils** with infected but treated with water group (B) and non-significant difference (P=0.263) with the group (C) treated with chloroquine. The seed extracts treated groups D1, D2, E2 indicated the non-significant level (P≤0.5). But the groups D3, and E3 did differ significantly (P≤0. 05) when compared with the normal control group (A).

It was observed that other seed extracts treated groups F1 indicated the high non- significant difference (P= 0.343) and G1, G2, G3, H1, H2 and H3 showed the non-significant difference (P≤0.1) except for F2 and F3 which indicated the significant difference (P≤0.05) when compared with normal control group (A).

There was a significant difference (P= 0.034) among neutrophils of infected but treated with water group B and chloroquine treated group C. The groups treated with plant extract D1, D2, E1, and E2 showed the significant difference (P≤0.05)

except for groups D3 and E3 which exhibited the non-significant difference (P≤0.5) when compared with the infected but treated with water group B.

The plants treated with the *C. oblonga, B. hispida,* and *S. lappa* F1, F2, F3, G1, G2, G3exhibited the non- significant difference (P≤0.01), but H1, H2, and H3 revealed significant differences (P≤0.05) when compared with the group infected but treated with water group B. There was non-significant difference (P≤0.5) between neutrophils of drug treated group C group and all the groups treated with *T. ammi, P. granatum, C. oblonga, B. hispida*, and *S. lappa* plants.

Lymphocytes

The group (A) showed a highly significant difference (P=0.002) of **lymphocytes** with infected, but treated with water group (B) and non-significant difference (P=0.687) with the group (C) treated with chloroquine. The groups treated with the 250 and 500 mg/kg of *T. ammi* D1, D2 respectively, showed the non-significant difference (P≤0.1) and the remaining doses of this plants, i.e. 750 mg/kg and all the low and high dose of *P. granatum* indicated the high non-significant difference (P≤0.5) when compared with the normal control group A.

The lymphocytes in the other seed extracts treated groups like F1, F2 and H3 showed the non-significant difference (P≤0.1) however F3, G1, G2, G3, H1, H2 exhibited the high non-significant difference (P≤0.5) when compared with the normal control group A.

There was a significant difference (P≤0.01) among the lymphocytes of non-treated group B, chloroquine treated group C different seed extracts treated groups D1, D2, D3, E1, E2 and E3 groups.

Other plants treated groups like, F3, G1, G2, G3, H1 , H2, and H3 also showed the high significant difference (P≤0.01) and F2 showed the low significant difference(P= 0.05) except for F1 indicated the non-significant difference (P= 0.056) when compared with the non-treated group B.

There was no significant difference (P≤0.5) between drug treated group C (66±1.23) and all plants treated groups also indicate the non- significant difference (P≤0.5) when compared with the lymphocytes of the chloroquine treated group C (Table 2.6 and 2.7).

Table 2.6: Comparison of Hematological parameters of groups treated with different concentrations of the seed extract of *T. ammi* and *P. granatum* along with control groups

Heam-paramerters	Groups								
	A	B	C	D1	D2	D3	E1	E2	E3
RBC (X10^6/µL)	8.51± 0.4 (c)[c]	3.97±0.16 {c}[b]	6.00±0.48 {c}(b)	4.86±0.48 {b}(e)[d]	5.34±0.58 {b}(c)[e]	5.73±0.37 {c}(c)[e]	6.63±0.46 {c}(b)[d]	7.36±0.52 {e}(b)[e]	6.16±0.524 {c}(b)[e]
HB (g/dL)	11.3±0.50 (b)[e]	5.43±0.35 {b}[a]	10.5±0.9 {e}(a)	6.83±0.62 {b}(e)[b]	10.6±0.46 {e}(b)[e]	8.53±0.73 {c}(c)[d]	8.3±0.33 {e}(b)[c]	11.37±0.80 {d}(c)[e]	9.20±0.59 {d}(c)[e]
Hematocrit (%)	38.50 0.55(b)[d]	26.87±1.1 {b}[c]	34.53±0.7 {d}(c)	30.80±0.44 {b}(d)[d]	32.03± 1.10 {c}(d)[d]	33.70±1.50 {c}(d)[e]	32.50± 8.13 {c}(d)[d]	36.37±1.1 {d}(c)[e]	35.3±1.0 {d}(d)[e]
MCV (fL)	53.43±0.62 (a)[c]	43.23± 0.66{a}[a]	48.07±1.10 {c}(a)	47.57.9 ±0.92{a}(c)[e]	50.87±0.82 {d}(b)[d]	48.33±1.05 {c}(b)[e]	51.57± 0.46{d}(b)[d]	53.70±2.11{e }(a)[d]	50.57±1.18 {d}(b)[e]
MCH (Pg)	16.9±0.2 (a)[b]	12.9±0.20{a }[b]	15.23±0.41 {b}(b)	15.13±0.55{d }(b)[e]	15.90±0.71 {e}(b)[e]	15.36±0.38 {d}(b)[e]	17.03±0.8 1{e}(b)[e]	17.43±0.29 {e}(b)[d]	14.5-±0.62 {b}(d)[e]
MCHC (g/dL)	32.13±0.94 (a)[c]	26.16±1.48 {a}[a]	31.86±0.95 {c}(a)	29.97±0.95 {c}(c)[e]	32.76±0.961 {e}(b)[e]	30.4±1.25 {e}(b)[e]	33.3±1.64 {e}(b)[e]	31.4±1.42 {e}(b)[e]	31.6±0.87 {e}(b)[e]
Platelets (/µL)	325000±27.9 (a)[b]	178000±18 .1{a}[b]	274000± 23.6 {b}(b)	183000 ±30.0 {a}(c){a}	225000± 32.4{a}(b)[b]	179000±13. 4{a}(e)[a]	352000± 21.2 {c}(a)[b]	375000± 24.3 {b}(a)[a]	267000± 19.6 {b}(b)[e]
Neutrophils (%)	24±0.882 (b)[e]	33±1.5{b}[b]	26±1.2{e}(b)	27±0.58 {e}(c)[e]	24±0.88 {e}(b)[e]	33±0.57 {b}(e)[d]	28±1.22{c}(c)[e]	25±0.75 {e}(b)[e]	29±1.1 {c}(d)[e]
Lymphocytes (%)	65±1.1 (b)[e]	83±0.71 {b}[b]	66±1.23 {e}(b	69±1.2 {d}(b)[e]	60±0.7 {d}(a)[e]	69±1.2 {d}(b)[e]	67±0.8 {e}(b)[e]	62±0.3 {e}(a)[e]	67±1.5 {e}(b)[e]

Table 2.7: Comparison of Hematological parameters of groups treated with different concentrations of seed extract of *C. oblonga*, *B. hipida* and *S. lappa* VS control

Hematological parameters	Groups								
	F1	F2	F3	G1	G2	G3	H1	H2	H3
RBC ($X10^6/\mu L$)	5.73±0.24 {c}{c}[e]	6.21±0.19 {c}{c}[e]	6.49±0.41 {c}{b}[e]	6.38±0.43 {c}{b}[e]	7.98±0.45 {e}{c}[e]	7.22±0.12 {e}{c}[e]	7.26±0.55 {e}{b}[e]	7.03±0.37 {e}{b}[e]	8.38±0.35 {e}{a}[e]
HB (g/dL)	10.2±0.43 {e}{b}[e]	10.76±0.39 {e}{b}[e]	12.43±0.94 {e}{b}[e]	10.967±0.24 {e}{b}[e]	13.367±0.48 {e}{b}[e]	11.867±0.90 {e}{a}[e]	11.66±0.62 {e}{a}[e]	12.93±0.73 {e}{a}[e]	12.96±0.28 {e}{a}[e]
Hematocrit (%)	30.7±0.85 {b}{c}[d]	31.8±0.85 {b}{c}[d]	31.46±0.55 {b}{c}[d]	34.56±0.86 {e}{b}[e]	34.89±0.60 {e}{c}[e]	40.2±1.60 {e}{b}[e]	37±0.30 {d}{b}[d]	38.23±0.49 {d}{b}[d]	37.33±0.39 {d}{b}[d]
MCV (fL)	48.53±0.29 {b}{b}[e]	47.6±0.75 {b}{b}[e]	42.06±1.12 {b}{c}[e]	51.2±0.49 {e}{a}[e]	53.26±0.86 {e}{b}[b]	53.8±0.87 {e}{a}[b]	53.13±1.23 {e}{a}[b]	55.56±0.93 {e}{a}[b]	55.76±0.53 {e}{a}[b]
MCH (Pg)	13.86±0.45 {d}{e}[e]	16.53± 0.90 {e}{c}[e]	16.43±0.28 {e}{c}[e]	14.5± 0.70 {d}{b}[e]	17.06± 0.33 {e}{c}[d]	15.53±0.68 {d}{c}[e]	15.63± 0.24 {d}{c}[e]	16.9±0.50 {e}{b}[e]	17.00±0.36 {d}{b}[e]
MCHC (g/dL)	32.26±0.66 {e}{b}[e]	32.13±0.55 {e}{b}[e]	32.8±0.68 {e}{b}[e]	30.93±0.85 {d}{b}[e]	30.3±0.95 {d}{b}[e]	29.53±0.44 {d}{d}[e]	30.6±0.66 {d}{b}[e]	33.6±0.23 {e}{b}[e]	32.4±0.68 {e}{b}[e]
Platelets (/μL)	246000±13.4 {b}{b}[	230000±14.48 {b}{c}[e]	226000±21.64 {b}{c}[e]	240000±50.208 {b}{c}[e]	253000±21.73 {b}{c}[e]	221000±17.67 {b}{c}[e]	286000±16.49 {d}{c}[e]	260000±10.21 {b}{b}[e]	296000±16.13 {d}{b}[e]
Neutrophils (%)	26±0.58 {e}{b}[e]	29±0.57 {c}{b}[e]	30.66±1.20 {c}{b}[d]	29.66±0.33 {c}{b}[d]	28.66±0.98 {d}{b}[d]	29.33±0.58 {c}{b}[d]	25.33± 0.8 [e]{b}[e]	25.66±0.78 {c}{b}[e]	21.667±0.63 {c}{b}[e]
Lymphocytes (%)	73±1.52 {c}{b}[c]	73±0.57 {c}{b}[c]	64.3±0.33 {e}{b}[e]	65.33±0.88 {e}{b}[e]	63.33±2.02 {e}{b}[e]	71±1.52 {c}{b}[c]	62±1.15 {d}{a}[d]	62.33±1.45 {d}{a}[d]	59 ±0.57 {c}{b}[c]

Biochemical analysis

To analyze the combined toxic nature of the seeds extracts and infection plasma level of ALT, ALP and AST was analyzed as the liver function tests. Elevated level of **ALT,** was observed in infected but non-treated group B as compared to the normal control group A and the difference was statistically significant (P = 0.005). The plant's seed extracts treated groups, i.e. D2, D3, E1, and E3 showed a significance difference (P≤0. 05) however the drug treated group C and the group D1 treated with the 250 mg/kg of *T. ammi* and E2 500 mg/kg of *P. granatum* depicted the non-significant difference (P≤0.1) when compared with the ALT level of mice of normal control group A.

The seed extracts treated groups F1, F2, G1, G2, G3, H1, and H2 showed the only significant difference (P< 0.05) but F3 groups indicated the high significant difference (P ≤ 0.001) and only H3 group depicted the non-significant difference (P= 0.078) when compared with the normal group.

The ALT range of drug treated group C, and seed extracts treated groups D1, D2, D3, E1, E2, and E3 the group H1 that was treated with the 250 mg/kg of *S. lappa* showed high significant difference (P≤0.001) for all groups when compared with non-treated group B. The ALT range of other seed extracts treated groups F1, F2, F3, G1, G2, G3, H2 and H3 showed the significant difference (P≤0.01) when compared with infected but treated with water group B.

The seed extracts treated groups like D2, D3, E1, E2, E3, H1 and H3 showed the non-significant difference (P≤0.5.) and other seed extracts treated groups D1, F1, G1, G2, G3, H2 revealed the significant difference (P≤0.05) while F2, F3 groups exhibited the high significant difference (P≤0.01), (P ≤ 0.001) respectively when compared with chloroquine treated group C (23.56 ± 2.3) (Figure 2.9 (a) and 2.10 (a).

The difference between the **ALP** level was highly significant (P=0.007, 0.002) when the normal group A and drug treated group C were compared to the infected but treated with water group B respectively. The comparison of seed extracts treated groups D2 and D3 significant difference (P≤0.5) and non- significant difference (P≤0.1) for D1, E1, E2, E3 and when compared to the normal group of mice A (Figure 2.10 (a) and 2.11 (a).

The difference between the **ALP** levels was highly significant (P≤0.01) when the normal group A and drug treated group C were compared to the infected but treated with water group B. The seed extracts treated groups F1, H3 showed non-significant difference (P≤0.1) and G1, G2, H1, H2 also revealed high non-significant difference (P≤0.5). But the groups treated with 500, 750 mg/kg *C. oblonga,* and 750 mg/kg *B. hispida* F2, F3 and G3 showed the significant difference (P≤0.05) when compared with the normal group of mice A. The all plant seed extract treated groups showed significant (P≤0.01) when compared with the infected but non treated group B.

The ALP level of seed extracts treated groups D2, E1. E3, F1, G1, G2, G3, H1, H2 and H3 groups showed non-significant difference (P≤0.5). But the seed extracts treated groups D1, D3, E2, F2 and F3 groups indicated the significant difference (P≤0.05) when compared with the chloroquine treated group C (Figure 2.10 a and 2.11 a).

The elevated level of **AST** was observed in infected but non-medicated B group as compared to the normal control group A and chloroquine treated group C, statistically it was highly significant (P = 0.002 and 0.000) respectively. The AST value of the groups treated with different concentrations of *T. ammi* and *P. Granatum* show significant difference (P≤0. 5) except for the D3, which depicted the non-significant difference (P = 0.076) when compared to the normal control Group A.

The AST level of other seed extracts treated groups like F1, G1, showed the low significant difference (P≤0.05) and for F2, G2, G3 indicated the high significant difference (P≤0.01). While the groups treated with the 750 mg/kg of *C. oblonga* and different concentrations of like 250,500,750 mg/kg of *S. lappa* F3, H1, H2 and H3 indicated the non-significant difference (P≤0.5) when compared with normal group A.

The comparison of AST of all seed extracts treated groups showed the significant difference (P≤0.01) when compared with non-medicated B group.

The seed extracts treated groups D1, E1, G1, G2 and G3 groups showed high non-significant difference (P≤0.5). The seed extracts treated groups D2, E2 and F1 showed the low non- significant difference (P≤0.1) in the AST level. However, other seed extracts treated groups D3, E3, F2, F3, H1, H2 and H3 indicated the significant difference (P≤0.05) when compared with the chloroquine treated group C.

The Kidney function test was determined by Creatinine and uric acid level. Increased levels of **Creatinine** was observed in the non-treated group B as compared normal group A and drug treated group C the difference was statistically significant (P=0.001) and (P= 0.002) respectively. The groups D1, D2, D3 and E3 treated with 250, 500 and 750 mg/kg of *T. ammi* and 500 and 750 *P. granatum* (0.29±0.020) indicated significance difference (P≤0.05) but the and E2 treated with the 500 mg/kg *P. granatum* exhibited the non-significant difference (P≤0.1) when compared with normal group A.

The groups treated with the *C. oblonga, B. hispida and S. lappa* showed the significant difference (P≤0.01) and only H3 showed the non-significant difference (P= 0.057) in the creatinine level when compared with normal group A.

The groups treated with different concentration of *T. ammi* and *P. granatum* D1, D2, D3, E1, E2, and E3 showed the marked difference (P≤0.01) in creatinine level with non-treated group B. The other plant extract treated groups like F1, F3, G1 G2, G3 showed the low significant difference (P≤0.01) but F2, H1, H2 and H 3 revealed high significant difference (P ≤ 0.001) with non-treated group B. However the group F1, G1 and G3 indicated the low significant difference (P≤0.05) in the creatinine level when compared with of infected but non-treated group B.

The comparison of creatinine level of drug treated group C with the seed extracts treated groups D1, E3, F1, F2, F3, G2, G3 and H2 showed the non-significant difference (P≤0.1) E1 H1, H3 depicted the very high significant difference (P≤0.5) however the groups D2, E2 and D3 displayed the significant difference (P≤0.01) (Figure 2.10 b and 2.11 b).

Uric acid level in the non-treated group B was significantly high (P= 0.01) as compared normal group A. The drug treated group C (16.84± 0.593) indicated the non-significant difference (P≤0.5) in the uric acid level when compared normal group A. The drug treated group C indicated the significant difference (P≤0.01) when compared with non-treated group B. The comparisons of normal control group A, with the medicinal plant seeds extract treated groups D1 and E2 showed the low significant difference (P≤0.05). On the other hand the plant seeds extract treated groups like D2, D3 and E3 showed high significant difference (P≤0.01) but non-

significant difference (P=0.066) was observed for the group treated with the 250 mg/kg of *P. granatum* (E1).

Uric acid level in the non-treated group B was significantly higher (P≤0. 01) and non- significant (P≤0.1) for drug treated group C when compared normal group A. The plant extract treated group F1, F3, G1, showed a highly significant difference (P ≤ 0.001) and G2 and G3 low significant difference (P≤0.01) but F2, H1, H2 and H3 groups indicated the non- significant difference (P≤0.5) when compared with the normal group of mice A.

The seed extracts treated groups D1, E1, E2, and E3 exhibited highly significant difference (P≤0.01) except for the D2 and D3 that indicated the non-significant difference (P≤0.5) as compared to the non-treated group B. Similarly the other seed extracts treated groups like F1, F2, H1, H2 and H3 showed the significant difference (P≤0.01) and F3, G1, G2 and G3 groups indicated the non-significant difference (P≤0.5) when compared to the non-treated group B.

The groups treated with the different plant extracts D2, D3, F1, F3, G1, G2 and G3 showed the high significant difference (P≤0.01). The seed extracts treated groups i.e. D1, E1, E3 and F2 showed the low significant difference (P≤0.05), however the other seed extracts treated groups H1, H2 and H3 showed the non-significant difference (P≤0.5) respectively when compared with chloroquine treated group C (Figure 2.10 c and 2.11 c) and (Table 2.8 and 2.9).

Table 2.8: Comparison of liver and kidney function test of groups treated with different concentration of *T. ammi* and *P. granatum* VS con trols

Group names	Treatment	Dose mg/kg	Liver function test (U/L)			Kidney function test (mg/dl)	
			ALT	ALP	AST	Creatinine	Uric acid
(A)	None	-	20.36± 1.01 $^{(b)[e]}$	43.63±2.73$^{(b)[b]}$	57.59 ±1.36$^{(a)[a]}$	0.29±0.02$^{(a)[a]}$	18.49±0.81$^{(b)[e]}$
(B)	Water	-	89.62± 6.61$^{\{b\}[a]}$	108.35±9.3$^{\{b\}[b]}$	250.15± 4.17$^{\{a\}[a]}$	1.04±0.04 $^{\{a\}[b]}$	29.789±0.44$^{\{b\}[b]}$
(C)	Chloroquine	5	23.56 ± 2.3$^{\{e\}(a)}$	62.84±7.03$^{\{b\}(b)}$	91.66 ± 5.9$^{\{a\}(b)}$	0.48±0.03$^{\{a\}(b)}$	16.84± 0.593$^{\{e\}(b)}$
(D1)	*T. ammi*	250	17.16 ± 2.19$^{\{d\}(a)[c]}$	42.84±7.13$^{\{d\}(a)[c]}$	105.03± 5.14$^{\{c\}(b)[e]}$	0.65±0.04$^{\{d\}(a)[d]}$	22.11±0.62$^{\{c\}(b)[c]}$
(D2)		500	27.34 ± 4.12$^{\{c\}(a)[e]}$	81.8±8.43$^{\{c\}(c)[d]}$	100.31± 6.82$^{\{c\}(b)[d]}$	1.02±0.02$^{\{a\}(e)[c]}$	27.72±0.74$^{\{b\}(e)[b]}$
(D3)		750	33.75 ± 4.96$^{\{c\}(a)[d]}$	79.08±8.5$^{\{c\}(c)[c]}$	57.59± 1.36$^{\{e\}(a)[e]}$	0.89±0.045$^{\{b\}(c)[b]}$	28.32±0.63$^{\{b\}(e)[b]}$
(E1)	*P. granatum*	250	24.14± 1.33$^{\{c\}(a)[e]}$	65.44±5.46$^{\{d\}(b)[e]}$	92.93 ± 7.65$^{\{c\}(b)[e]}$	0.92±0.04$^{\{c\}(c)[b]}$	19.85±0.45$^{\{d\}(b)[c]}$
(E2)		500	16.87± 2.66$^{\{e\}(a)[d]}$	38.17±2.73$^{\{e\}(a)[c]}$	80.02± 9.09$^{\{c\}(b)[d]}$	0.72±0.05$^{\{d\}(a)[e]}$	16.36±0.54$^{\{c\}(a)[e]}$
(E3)		750	27.93± 3.49$^{\{c\}(a)[e]}$	65.73±8.18$^{\{d\}(b)[e]}$	115.21±9.28$^{\{b\}(b)[e]}$	0.88±0.01$^{\{c\}(a)[c]}$	22.65±0.85$^{\{c\}(c)[c]}$

Values are presented as Mean ± SEM (n=5). The probability value in bracket {}, () and [] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. ^{a}=P≤0.001, ^{b}=P≤0.01 ^{c}=P≤0.05, ^{d}=P≤0.1 ^{e}= P≤0.5.

Table 2.9 Comparison of liver and kidney function test of the groups treated with different concentration of *C. oblonga, B. hispida, and S. lappa*

Group Names	(Drug /Extract used)	Dose mg/kg	Liver function test (U/L)			Kidney function test (mg/dl)	
			ALT	ALP	AST	Creatinine	Uric acid
(F1)	*C. oblonga*	250	52.96 ±9.58$^{\{c\}(b)[c]}$	58.17±4.0$^{\{b\}(b)[c]}$	81.19±2.40$^{\{c\}(b)[c]}$	0.787±0.06$^{\{b\}(c)[d]}$	21.71±1.68$^{\{a\}(b)[b]}$
(F2)		500	52.96±4.57$^{\{c\}(b)[b]}$	69.9±5.53$^{\{c\}(b)[c]}$	74.49±2.59$^{\{b\}(b)[c]}$	0.576±0.01$^{\{b\}(a)[d]}$	19.99±2.00$^{\{e\}(b)[c]}$
(F3)		750	63.43±2.48$^{\{b\}(b)[a]}$	70.9±3.15$^{\{c\}(b)[c]}$	63.14±2.78$^{\{e\}(b)[d]}$	0.69±0.01$^{\{b\}(b)[d]}$	26.45±3.613$^{\{a\}(e)[b]}$
(G1)	*B. hispida*	250	41.03±5.26$^{\{c\}(b)[c]}$	39.4±0.79 $^{\{e\}(a)[e]}$	90.79±3.24$^{\{c\}(b)[c]}$	0.83±0.04$^{\{b\}(c)[c]}$	28.57±1.929$^{\{a\}(e)[b]}$
(G2)		500	42.77±4.85$^{\{c\}(b)[c]}$	48.2±3.28$^{\{e\}(b)[e]}$	96.01±2.14$^{\{b\}(b)[c]}$	0.9±0.03$^{\{b\}(c)[d]}$	24.71±0.587$^{\{b\}(e)[b]}$
(G3)		750	44.81±0.58$^{\{c\}(b)[c]}$	53.6±2.41$^{\{c\}(b)[e]}$	87.88±0.77$^{\{b\}(b)[c]}$	0.7±0.026$^{\{b\}(b)[d]}$	22.86±0.709$^{\{b\}(e)[b]}$
(H1)	*S. lappa*	250	25.31±0.46$^{\{e\}(a)[e]}$	48.2±5.95$^{\{e\}(b)[e]}$	65.47±2.15$^{\{e\}(b)[d]}$	0.523±0.03$^{\{b\}(a)[e]}$	16.93±2.317$^{\{e\}(b)[e]}$
(H2)		500	42.96±7.27$^{\{c\}(b)[c]}$	44.9±2.92$^{\{e\}(b)[e]}$	57.61±2.02$^{\{e\}(b)[d]}$	0.623±0.013$^{\{b\}(a)[d]}$	18.3±1.679$^{\{e\}(b)[e]}$
(H3)		750	28.37±0.58$^{\{e\}(b)[d]}$	60.9±3.96$^{\{b\}(b)[c]}$	54.7±1.05$^{\{e\}(b)[d]}$	0.4±0.023$^{\{d\}(a)[e]}$	15.13±2.00$^{\{e\}(b)[e]}$

Values are presented as Mean ± SEM (n=5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. ^{a}=P≤0.001, ^{b}=P≤0.01 ^{c}=P≤0.05, ^{d}=P≤0.1 ^{e}= P≤0.5

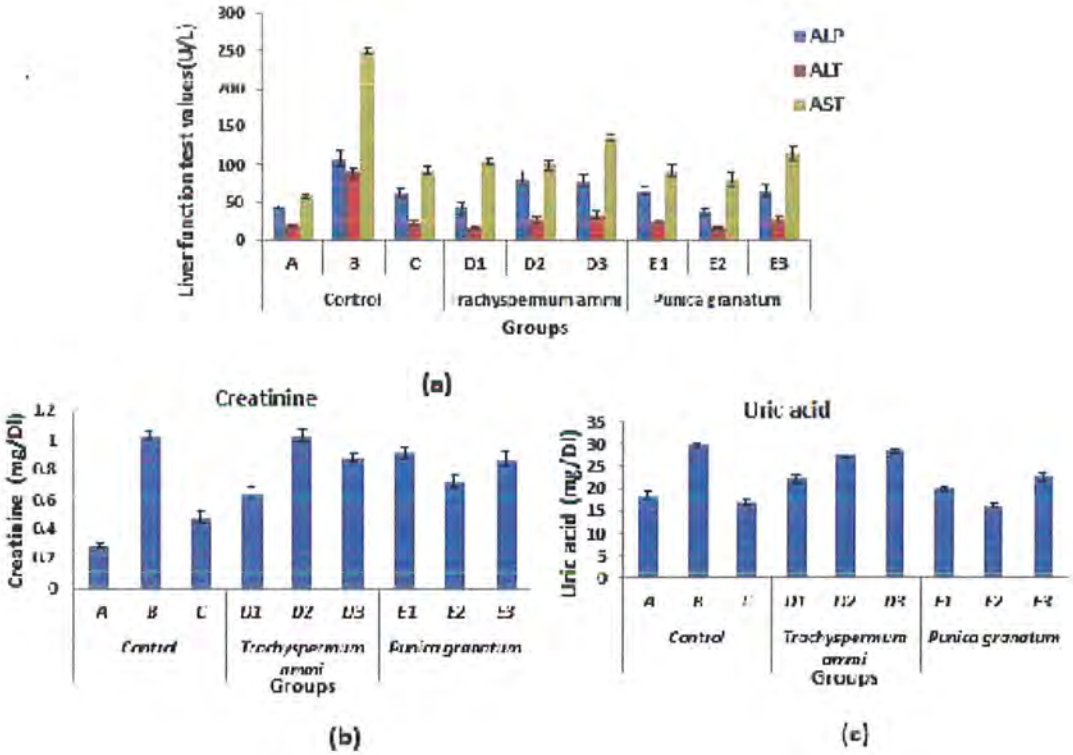


Figure 2.10: Plasma concentrations of ALP, ALT, AST (a), Creatinine (b), and Uric acid (c) of control and groups treated with seed extracts *T. ammi and P. granatum* are shown in this graph.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is shown as Mean ± SE with N= $P<0.06$ for (b) and $P<0.05$ for (c) .

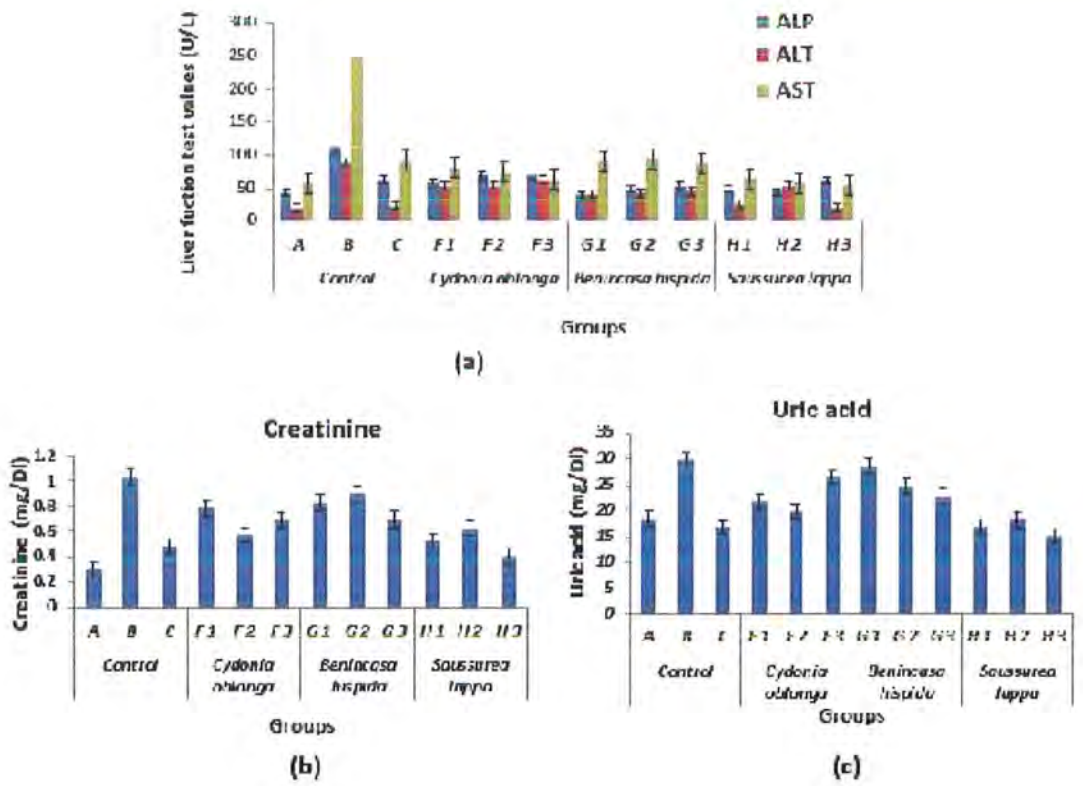


Figure 2.11: Plasma concentrations of (a) ALP, ALT, AST, (b) Creatinine and (c) Uric acid of control and groups treated with the different concentrations of seeds extracts of *C. olonga, B. hispida* and *S. lappa.*

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is shown as Mean ± SE with N=5, $P<0.04$ for (b) and $P<0.05$ for (c)

Post-mortem observations of organs

The Post-mortem examinations of the internal organs revealed the darkening of the liver, spleen, lungs and the kidneys of the malaria-infected mice less adverse effects were observed for seed extract treated groups (Figure 2.12 and 2.13 and Table 2.10)

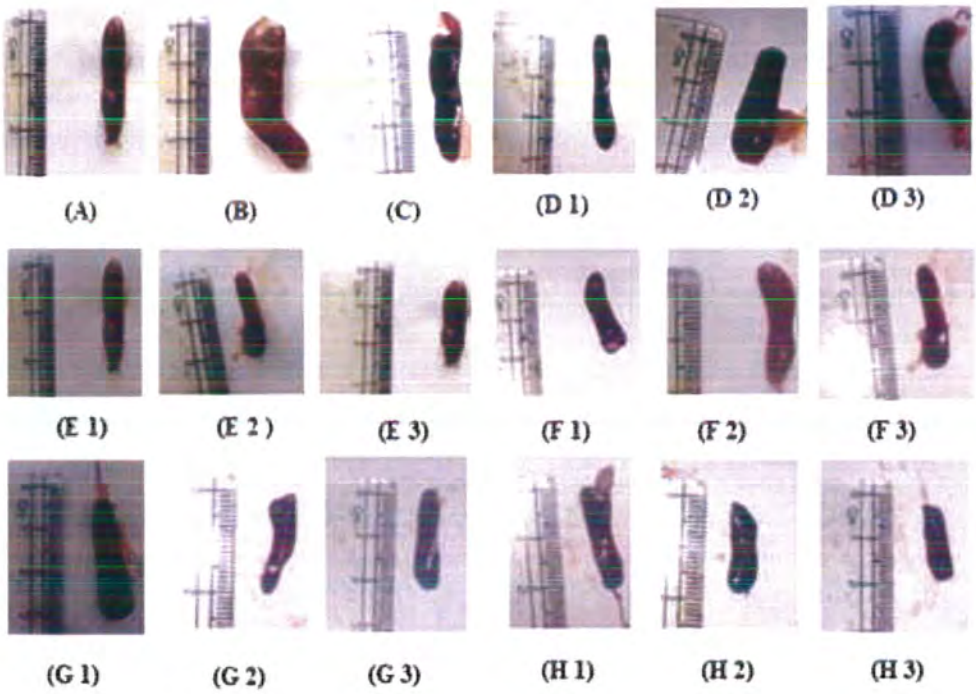


Figure 2.12: Post mortem observations of spleen of control and plant extract treated groups

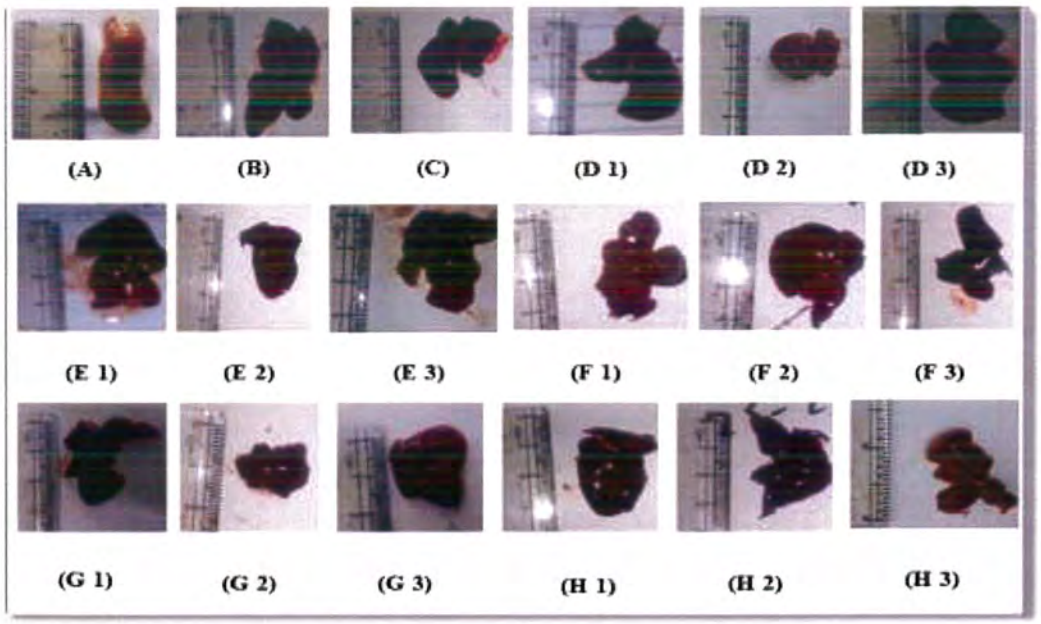


Figure 2.13 Post mortem observations of liver of control and plant extracts treated groups

Table 2.10: Post mortem observations of major organs of control and the groups treated with seed extracts *T. ammi and P. granatum C. oblonga, B. hispida and S. lappa*

Observation	A	B	C	D1	D2	D3	E1	E2	E 3	F 1	F2	F3	G1	G2	G3	H1	H2	H 3
Enlargement of the size of spleen (splenomegaly)	–	+++	++	++	–	++	+	–	–	–	–	+	+	–	–	+	–	–
Discoloration of the spleen	–	+++	+++	+	–	++	+	–	–	++	+	+	+++	–	++	–	++	+
Enlargement of the size (hepatomegaly)	–	++	+	+	–	++	++	–	+++	–	+	+	+	–	+	–	+	–
Discoloration of the liver	–	++	+++	++	++	++	+	–	+	–	–	++	++	+	+	+	++	–
Discoloration of the kidneys	–	+++	++	++	+	++	+	–	+	+	–	+	++	–	+	+	–	+
Discoloration of the lungs	–	+++	+	++	+	+	+	+	+	+	+	–	+	–	+	–	–	+

Indicator: (-) absent; (+): mild; (++): moderate; (+++): sever

Histopathological observation

Histopathological studies revealed the marked differences between the control and treated groups. The sequestrations of Parasitized RBCs in the microvasculature were observed in the liver, spleen and kidney of non-treated group as compared to the treated groups. The liver of infected but treated with water group showed atrophy of hepatocytes, hyperplasia and hypertrophy of the kupffer cells. The exoerythrocytic stage of the *P. berghei* was obvious in the liver the malarial infected but non-treated group of mice (Figure 2.14).

The hyperplasia and hypertrophy of some kupffer cells were also observed in C, D1, D3 and E1. The atrophy of hepatocytes was also observed in C, D1, D3 and E1. Congestion of blood vessels was observed in B, C, D3 and little bit in E3 group (Figure 2.14).

The hyperplasia and hypertrophy of some kupffer cells were also observed in FI, F3, and little bit in G3. The hypertrophy of hepatocytes was also observed in C, F2, G1, G3, and little bit in H1 group. Congestion of blood vessels was observed in C, F1 and little bit in H1 group (Figure 2.15).

The Malaria pigments **haemozoin** was seen in the spleen tissue. The congestion of blood vessels was also observed (Figure 2.16). The kidney tissues were presented with abundant of sequestrations of Parasitized RBC and haemozoin in the interstitium. The congestions and haemorrhages were also seen (Figure 2.18).

The microvascular sequestrations of PRBCs were observed in B and little bit in C, D 1 and D 3. Haemozoin pigment was also observed in D1, D3, and E3. Tiny proportion of Haemozoin pigment was also observed in F1, G1, G2 and H1. The microvascular sequestrations of PRBCs were also present B and a few in F1 and G1 (Figure 2.17).

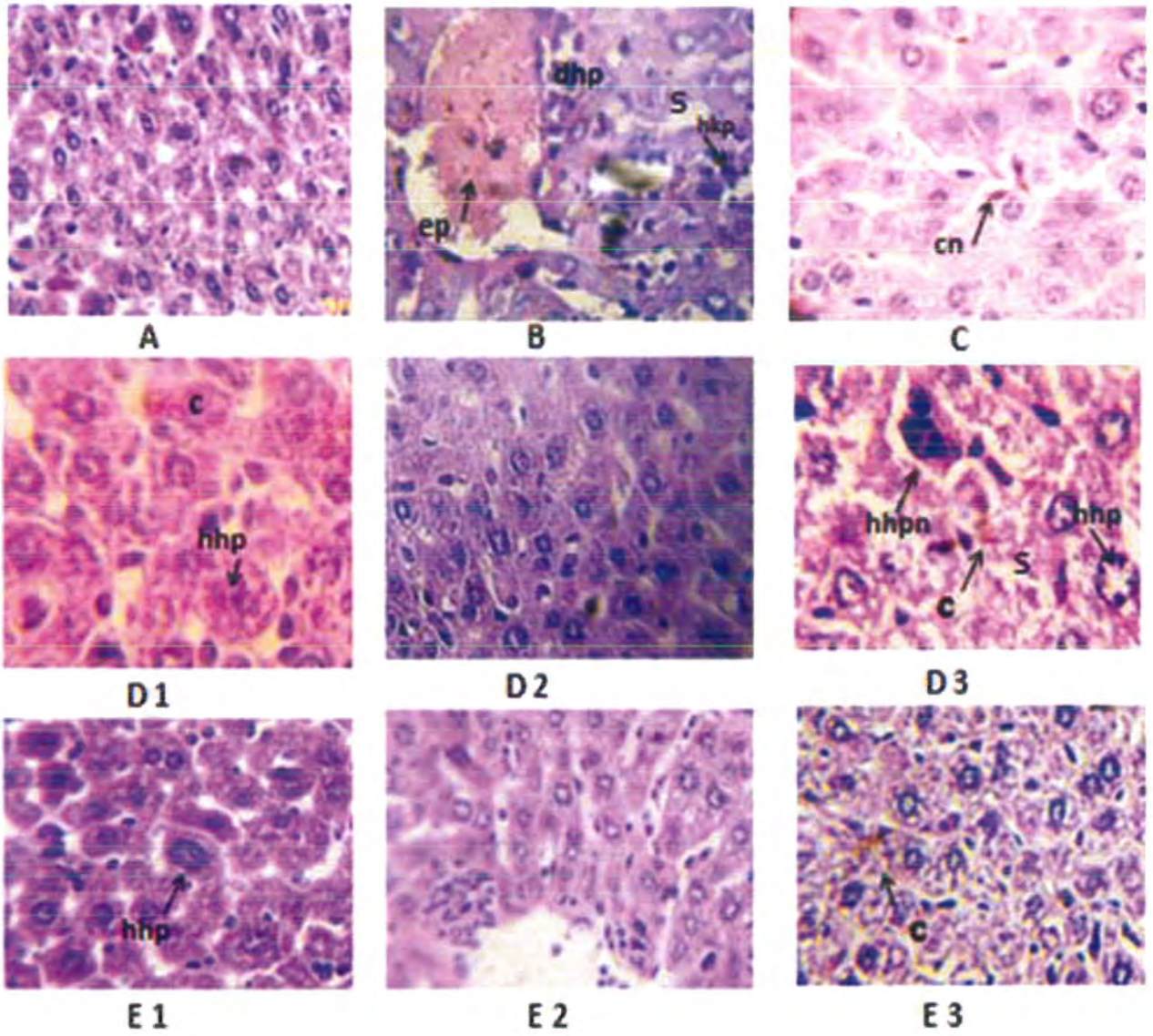


Figure 2.14: The histological studies of liver of *T .ammi and P. granatum* treated groups VS control.

The liver of malaria-infected mice, exoerythrocytic form of *Plasmodium berghei* was observed in B (ep), hyperplasia and hypertrophy of kupffer cells (hkc) and vacuolar degeneration and atrophy of hepatocytes (dhp) and wide sinusoidal space as compared to normal A and other the groups treated with different concentrations of *T. ammi* and *P. granatum* seeds extracts. Hyperplasia and hypertrophy of some kupffer cells were also observed in C, D1, D3 and E 1 (along with atrophy of hepatocytes) groups. Viewed under light microscopy at 10 x100 X magnifications

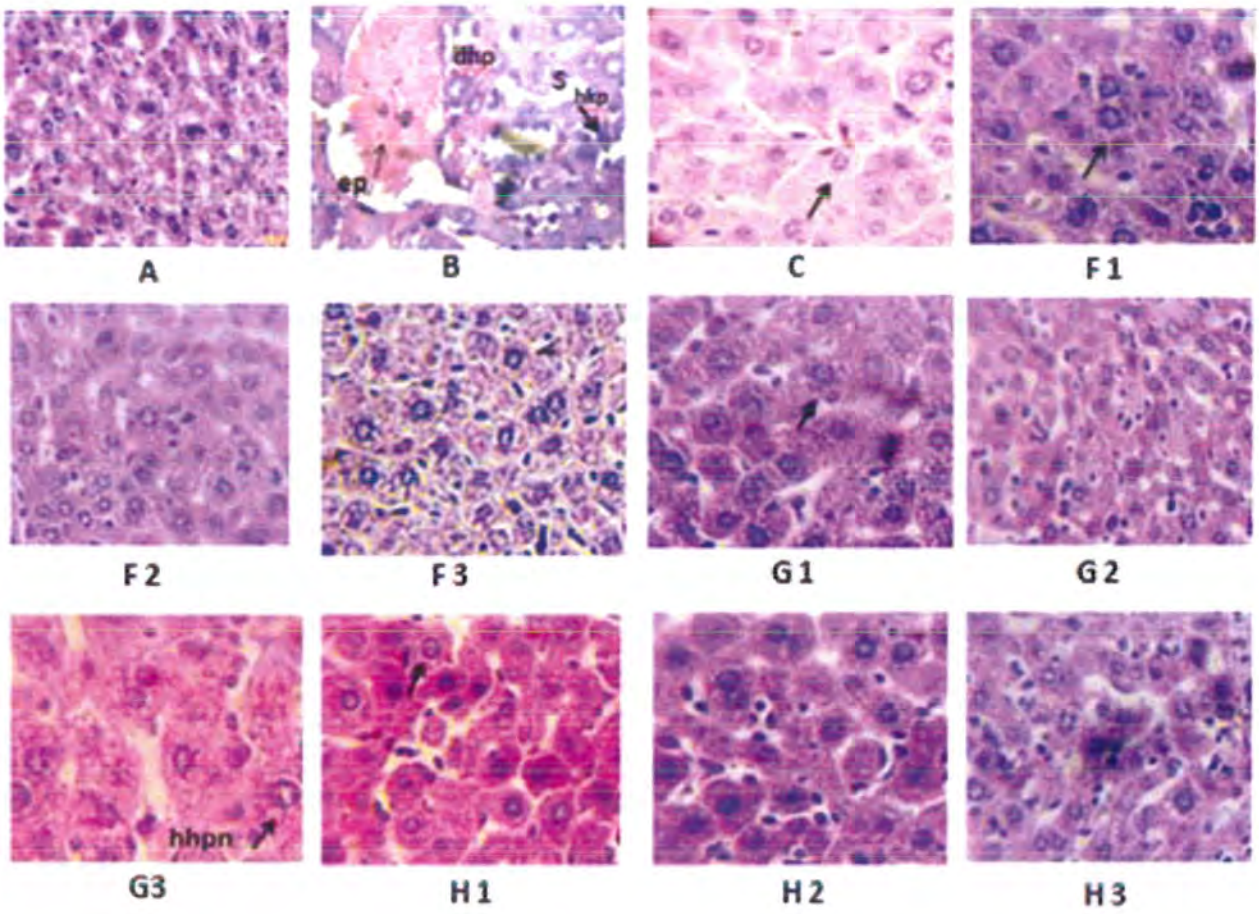


Figure 2.15: The histological studies of liver of experimented animals treated with *C. oblonga, B. hispida, and S. lappa* VS Control.

The liver of malaria-infected mice, exoerythrocytic form of *Plasmodium berghei* was observed in B (ep), hyperplasia and hypertrophy of kupffer cells (hkc) and vacuolar degeneration, atrophy of hepatocytes (dhp) and wide sinusoidal space (s) as compared to normal A and the other groups treated with different concentrations of *C. oblonga , B. hispida* and *S. lappa* seeds extracts. Kupffer cells hyperplasia and hypertrophy were also observed in FI, F3, and a little bit in G3. Hypertrophy of hepatocytes was also observed in C, F1, G1, G3, and H1 group observed under light microscopy at 10 x100 X magnifications.

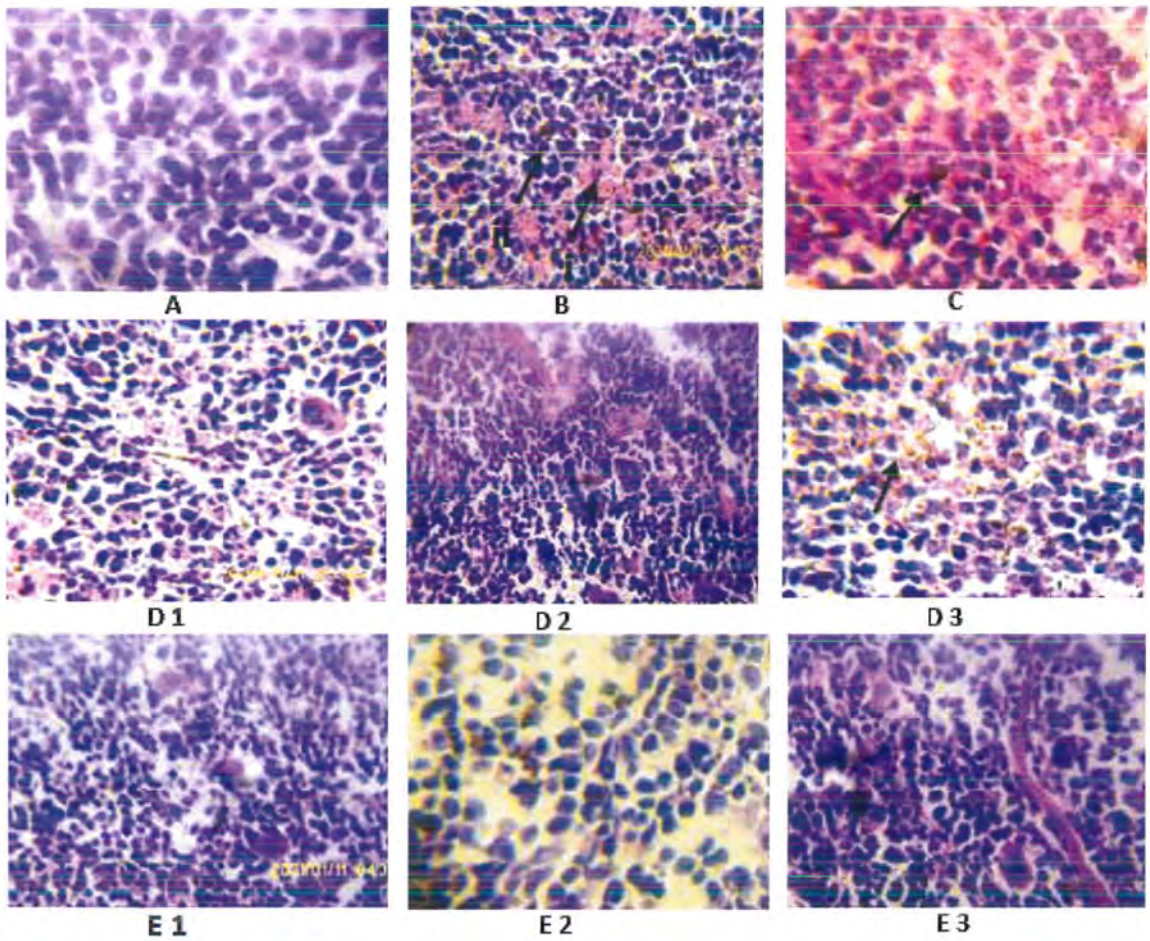


Figure 2.16: The histological studies of spleen of experimented animals treated with *T .ammi and P. granatum* VS control.

The accumulation of more malarial pigments, Amazon, was more in the pulp histiocytes and sinusoidal lining cells of the spleen in the B group (i) as compared to a normal control group A and other groups treated with different concentrations of *T. ammi* and *P. granatum* seeds extracts.. Microvascular sequestrations of PRBCs were also present B (ii) and a little bit in C, D 1 and D 3. Viewed under light microscopy at 10 x100 X magnifications

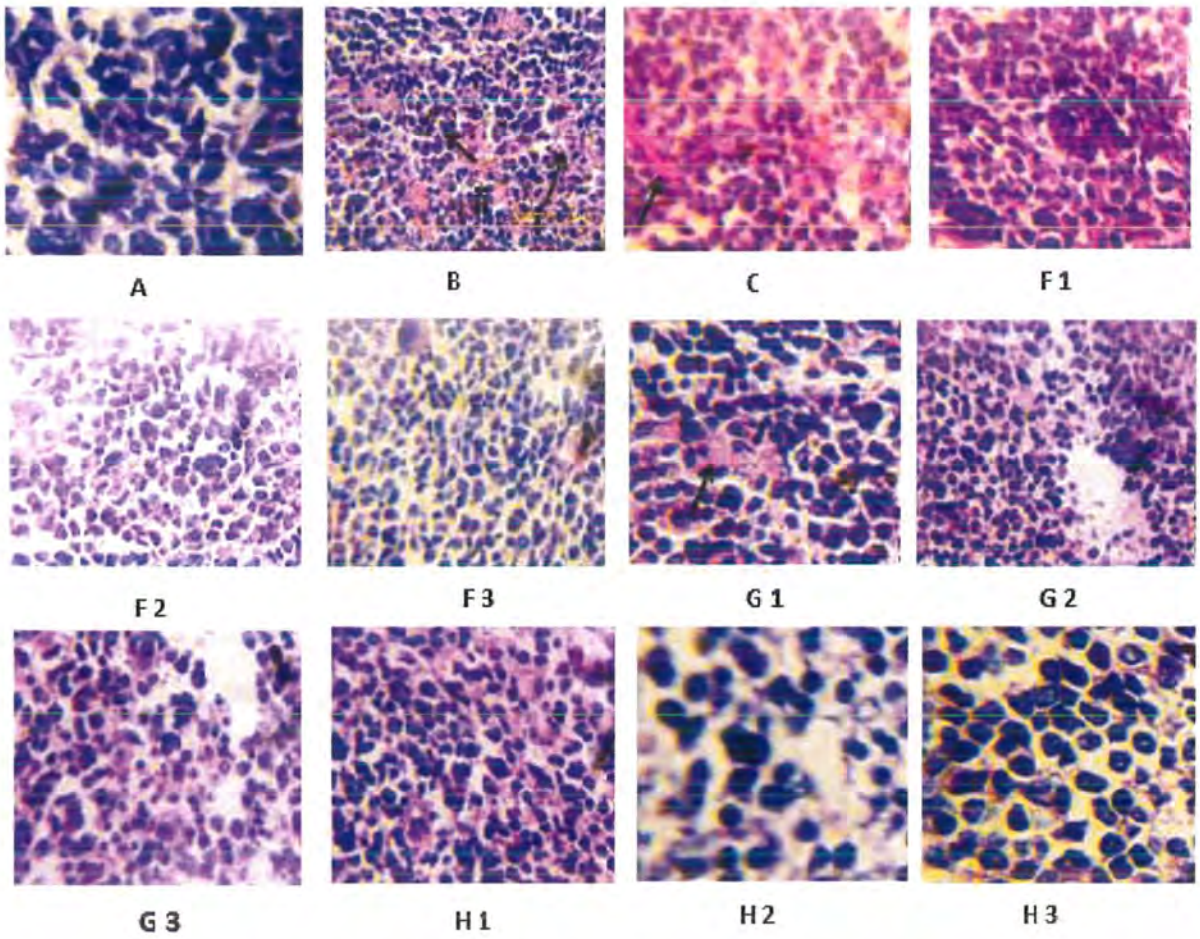


Figure 2.17: The histological studies of Spleen of experimental animals treated with *C. oblonga, B. hispida, and S. lappa* VS Control.

Accumulation of, haemozoin, was more in the spleen in the group B (i) as compared to a normal control group A and the other groups treated with different concentrations of *C. oblonga ,B. hispida*and *S. lappa* seeds extracts. Little bit Haemozoin pigment was also observed in F1, G1, and H1. Sequestrations of PRBCs in microvascular were present B (ii) and a few in F1 and G1. Observe under light microscopy at 10 x100 X magnifications.

The kidney tissues were presented with abundant of sequestrations of Parasitized RBC and haemozoin in the interstitium and also in microvasculature. Congestions and hemorrhage were seen in the infected but non treated group as compared to the other groups treated with plant extracts. Microvascular sequestrations of PRBCs were also observed in D3 (Figure 2.18). Microvascular sequestrations of PRBCs were also observed in G1 (Figure 2.19).

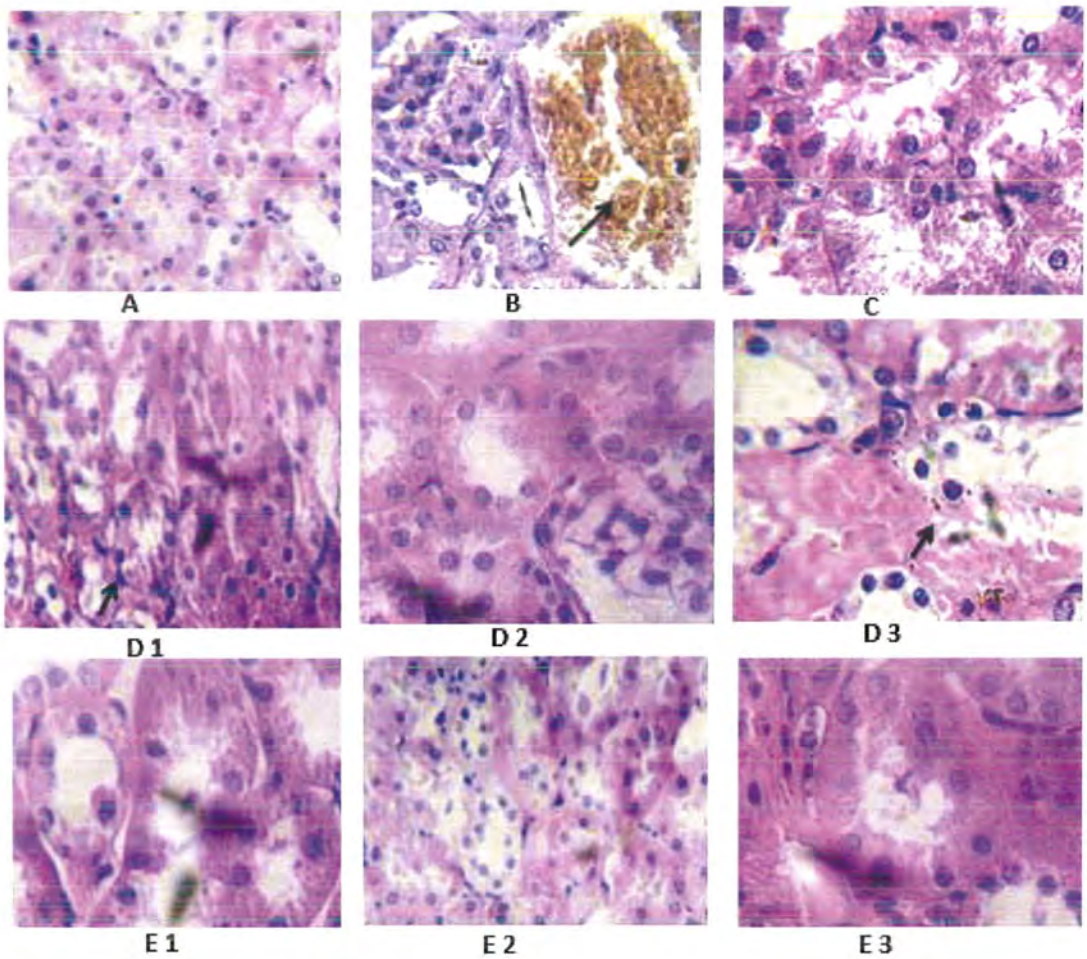


Figure 2.18: The histological studies of liver of experimental animals treated with *T .ammi and P. granatum* VS control.

The kidney from infected mice was presented with widespread sequestration of PRBCs, Hemorrhages and congestion of blood vessels (B), as compared to the to normal control group A and other treated groups. Viewed under light microscopy at 10 x 100 X (A, B, C, D1, E2) and 10 x 200X (D2, D3, E1 and E3) magnification.

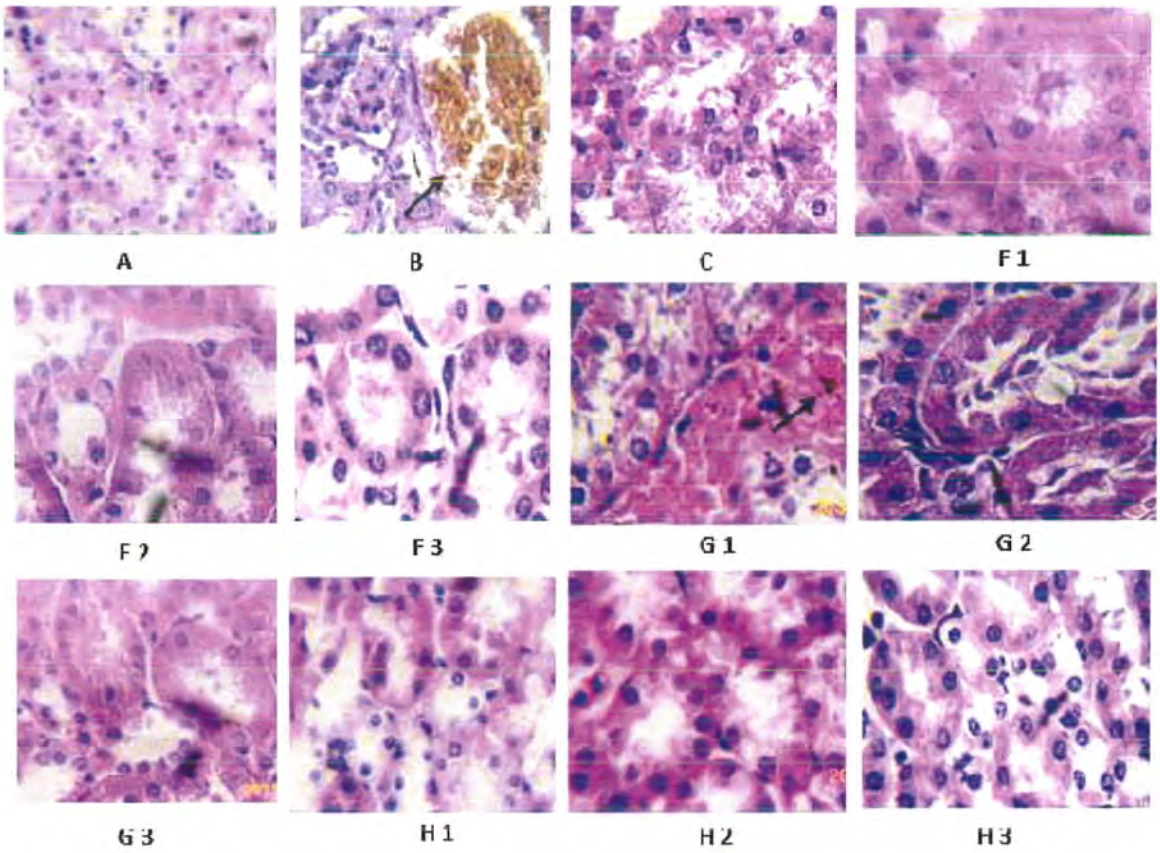


Figure 2.19: The histological studies of kidney of experimental animals treated with *C. oblonga, B. hispida, and S. lappa* VS control.

The kidney of mice infected but treated with water group was presented with widespread sequestration of PRBCs, Haemorrhages and congestion of blood vessels (B), as compared to the to normal mice of group A and the other groups treated with different concentrations of *C. oblonga , B. hispida* and *S. lappa* seeds extracts. Microvascular sequestrations of PRBCs were only observed in B and G1. Observed under light microscopy at 10 x 100 X (A, B, C, H1) and 10 x 200 X (F1, F2, F3, G1, G2, G3, H2 and H3) magnification.

Discussion

Considering the lethality of malarial parasites it is mandatory to search for its cure. The existing drugs are not sufficient and the pathogens got resistant against some of them whereas still others are at risk (CDC, 2005). It is reported that plants are the vital source because they have been used for the treatment of various diseases since ever. Basir *et al* (2012) suggested that disease pathogenesis and its treatment can be comprehended by the in vitro studies, but for proper physiological conditions and full chemistry of the body, *in vivo* model is required. The present study was based upon the *in vivo* use of medicinal plants to cure malaria. The BALB/c mice were used as animal model.

The malaria in BALB/c mice is caused by *P. berghei* and this infection is very similar to a human *P. falciparum* infection. The *P. berghei* infection cause anemia, hypoglycemia, weight loss, changes in body temperature and occasional death and these symptoms are very similar to human malaria infection (Stephens et al., 2012). There is no other animal model except *Aoutus* monkey (available in USA) and C57BL/6 mice, the carrier of human *Plasmodium* species. Thus the BALB/C mice were supposed to be the best to use as a model animal in Pakistan to explore new anti-malarial agents from Plants. Another advantage of using BALB/C mice is that it has 99.9% similarity with humans.

The bioassay was run for each seed extracts of each plant. It was observed from present investigations that *T. ammi, P. granatum, C. oblonga, B. hispida, and S. lappa* exhibited the LD_{50} as 831 and 1989, 1335, 1800, and 1194 mg/kg respectively. The highest LD_{50} value was observed for *P. granatum* as compared to the *T. ammi C. oblonga, B. hispida, and S. lappa.*

The efficiency of Plant seed extracts used in the present study was observed at 500 mg/kg for *T. ammi, P. granatum, C. oblonga, B. hispida* and 500 mg/kg and, 750 mg/kg for *S. lappa*. The median lethal dose of the studied plants seeds extract (*T. ammi P. granatum, C. oblonga, B. hispida, and S. lappa*) was calculated as 143.04, 124.45, 189.26, and 80.26, and 66.45 respectively. The minimum effective median lethal dose ED_{50} was observed in the following order *S. lappa* 66.45, *B. hispida 80.26 P. granatum* 124.45 *T. ammi* 143.02 than for *C. oblonga*189.26.

Antiplasmodial activity of three doses used for *T. ammi* did not differ from each other but for *P. granatum*, *C. oblonga*, *B. hispida* the difference between three doses like 250, 500, 750 mg/kg did not differ so much especially between 500 and 750 mg/kg. But *S. lappa* produced dose dependent Chemosupression causing significant reductions in Parasitemia. The *S. lappa* aqueous seeds extract at 750 mg/kg exhibited the highest antiplasmodial activity within the group as well as among all the groups.

There is still no report regarding *in vivo* effects of *T. ammi* seed extract against malarial parasite however there are number of reports of in vitro studies. Kamaraj *et al* (2012) reported that Seed extract of *T. ammi* prepared in ethyl acetate showed the antiplasmodial activity up to (25±0.7) against *P. falciparum* in in vitro culture. A study by Moein *et al.*, (2014) reported that the fraction of the essential oil of *T. ammi* was evaluated for possible antibacterial and antifungal activities.

Medicinally, it has been recognized that *T. ammi* possesses different pharmacological activities, for example antifungal, cytotoxic activity, antioxidant, antimicrobial, broncho-dilating actions, Nematicidal, Anthelmintic, and Antifilarial activity. Its nutritional and medicinal properties have been verified through lots of papers (Jeet *et al.*, 2012).

The crude methanol extracts of fruit *P. granatum* showed antiplasmodial activity in in-vitro cultures against *P. falciparum* (Al-Musayeib *et al.*, 2012). In India it is used as anti-malarial home remedy. A study conducted by Dell'agli *et al*, (2010) fruit rind of the immature fruit of this plant is used as an herbal Medicine in India, for the treatment and prophylaxis of malaria. They used the Methanolic extract that inhibited the parasitemia in vitro. The tannins fraction enriched within punicalagins (29.1%), ellagic acid and punicalins (13.4%) and its glycoside are more active than others.

The current study supports the medicinal importance of selected plants as described in previous studies of previous studies conducted on the same plant, but presently, seeds are explored to have antiplasmodial activity in the aqueous extracts. Aqueous extracts are economical as compared to the other organic solvent extracts. Sánchez-Lamar *et al.*, (2008) confirmed it's following properties, microbial

infections, acidosis, dysentery, diarrhea, helminthiasis, respiratory pathologies, and hemorrhage. The present study revealed the aqueous extracts of this plant show highest antiplasmodial activity at 500 mg/kg.

There is no study reported on the antiplasmodial activity of *C. oblonga*. Although other biological activities has been reported like antidiabetic and antioxidant etc. An *in vivo* study on the diabetic rats *C. oblonga* leaf (500 mg/kg) extract caused a decrease in blood glucose levels by 34% (Aslan *et al.*, 2010). Hydroalcoholic extract of the fruits of *C. oblonga* Miller (quince) increased the aphrodisiac activity in Wistar rats. It is regarded as a strong libido Invigorator in Unani System and Tib-e-Nabvi Medicine (Aslam and Sial. 2014). The seeds of *C. oblong* were used for the treatment of constipation (Siddiqui, et *al.*, 2002), Conjunctivitis, Cough, and Bronchitis (Ghanadi, 2003).

*The **B. hispida*** methanolic extracts of seeds showed the antioxidant activity in the in vitro by DPPH free radical scavenging assay (Sharma and Verma, 2013). Its antibacterial activity in methanol extract was also elucidated against gram positive and gram negative bacteria (Obayed Ullah *et al.*, 2013). It is a plant with very high medicinal importance like it is used as central nervous effects anxiolytic, muscle relaxant, an antidepressant, in the treatment of Alzheimer's disease, anti-inflammatory, analgesic, nephron protective, antiasthmatic, antidiabetic, diuretic, and antimicrobial effects (Al-Snafi, 2013). The antiplasmodial activity of *B. hispida* was first time reported in the present study.

The ***S. lappa*** is used as an important medicine for gout. Its root has great medicinal value and used in many Tibetan formulae for the treatment of chronic inflammation of the lungs, chest, and congestion (Tsarong 1994). Its roots possess antihelmintic, carminative and analgesic properties. It stimulates the brain and used for the medications of blood, liver and kidney disorders. Different preparations of *S. kappa* is prescribed in helminthic infestations, leprosy, ulcer and skin disease (Malik *et al.*, 2011). The *S. lappa* possess the anti-parasitic activities but its antiplasmodial activity is not up to the date. In this study the antiplasmodial activity has been reported and aqueous extracts of the seeds of this plant exhibit the maximum antiplasmodial activity as compared to the other plants used in the present study.

According to another study (Chung *et al.*, 2009) anti-malarial activity of 6-(8'Z-pentadecenyl)-salicylic acid from *Viola websteri* was determine in mice and LD_{50} was calculated as 500 mg/kg while highest antiplasmodial activity 82.1% was observed at 25 mg/kg. The n-butanol fraction of *Dodonaea angustifolia* was given to the mice infected with *P. berghei*. LD_{50} was greater than 2000 mg/kg but highest antiplasmodial activity 67.51% was observed at 600mg/kg dose (Amelo *et al.*, 2014).

An *in vivo* study conducted on the solvent fraction of methanolic root extract of *Dodonaea angustifolia* against *Plasmodium berghei* infected mice. LD50 was observed above than 2000 mg/kg and antiplasmodial activity as 67.51% at an oral dose of 600 mg/kg of n-butanol fraction. Lower dose 200 mg/kg and 400 mg/kg produce the activity as of the fraction also resulted in Parasitemia suppression of 38.02% and 55.85%, respectively (Amelo *et al.*, 2014). Similarly in the present investigations the *P. granatum, C. oblonga, B. hispida, and S. lappa* antiplasmodial activity of medium and high dose did not differ significantly, although the antiplasmodial activity was higher than n-butanol fraction of root extract of *Dodonaea angustifolia.*

The *T. ammi* seed extract (3–100 mg/kg) caused a dose-dependent reduction in arterial blood pressure in anesthetized rats. Treatment of rats with *T. ammi* seeds extract with the dose of 500 mg/kg orally for 2 days prohibited the paracetamol and CCl4 prompted a rise in serum alkaline phosphatase (ALP) and aminotransferases (AST and ALT). The same dose of this extract was used to inhibit the CCl_4-induced prolongation in pentobarbital-induced sleeping time in mice confirming its hepato protectivity. These results indicate the presence of calcium antagonist (s) in these seeds (Ahsan *et al.*, 1990)

The Qnais *et al.* (2007) conducted an in-vivo study on the aqueous extract of *Punica granatum* peels used against diarrhea in rats. The extract was delivered through intra-peritoneal injections and LD_{50} of the extract was reported as 1321 ± 15 mg/kg. In the present study the seed extract of *P. granatum* was administered orally and LD_{50} was calculated as 1989 mg/kg.

A study conducted by Hafiz *et al*, (2016) in order to evaluate the activity of methanolic peel extract of *P. granatum* against malaria-induced hepatic injury and

oxidative stress in *P. chabaudi* infected mice. The *P. granatum* extract successfully reduced the anemia, hepatic injury and oxidative stress. The Methanolic extract of *P. granatum* reduced Parasitemia oxidative stress and inflammation of spleen in the *P. chabaudi* infected mice (Mubaraki *et al.* 2016) similarly in this study the experimented plants reduced the inflammation of liver and spleen.

The alcoholic extract of fruits of *B. hispida* was administered orally to different groups of mice to evaluate the anxiolytic effects with different dose levels and found that the dose of 2000 mg/kg body weight is safe and did not produce the toxicity (Nimbal *et al.*, 2011). In this study the LD_{50} of *B. hispida* was calculated as 1800 mg/kg body weight, which is approximately accordance with the work of Nimble *et al* 2011.

The effects of plant extracts on kidney and liver function were also studied in the present studies. Among these serum ALT, ALP, and AST are the bio markers for liver health and urea and creatinine concentration are used for the evaluation of renal efficiency. The chloroquine also increased the enzymatic activities as well as the autophagy of cells. Hence, this can probably be explanatory because there is the elevation in enzyme activity of liver and kidney of, chloroquine treated mice in the present study. Similarly the artemisinin is also reported to cause a significant increase in serum aspartate aminotransferase, ALP and alanine amino transferase ALP (Udobre *et al.*, 2009). The elevated level of liver bio mark, i.e., ALT, ALP, and AST in chloroquine treated group was observed as compared to the group treated with *P. granatum* (500 mg/kg) was also observed in the present study. There was significant increase in serum ALT, ALP and AST, urea and creatinine in infected control as compared to normal. Elevate level of ALP and creatinine was observed in the infected group as compared to the treated steroidal alkaloid conessine isolated from the bark of *Holarrhena antidysenterica* treated groups (Dua *et al.*, 2013) and against the use of homeopathic compound Nosode 30 and 200 (Bagai *et al.*, 2012).

The Studies revealed that parasite burden in malaria infection is associated with the peripheral blood. So the hallmark of malaria is hematological abnormalities, especially during *P. falciparum* infection (Perrin *et al.*, 1982). The severe malarial anemia is considered a main cause of mortality in animals. The mechanism of anemia

due to malaria is similar to those of human anemia which is due to the loss or damage of infected RBCs or may be due to loss of non-infected RBCs (Lamikanra *et al.*, 2007). There was a marked reduction in RBC, HB, HCT, MCV, MCH, MCHC of infected but non treated group as compared to the treated groups.

A study conducted by Kuthala *et al* (2013) in order to find out the efficacy of artemisinin derivatives on hematological parameters on C57BL/6J mice infected with *Plasmodium falciparum*. It was observed that there was the decrease of RBC, WBC, hemoglobin, packed cell volume, mean cell volume, and platelet count in infected mice as compared to the treated mice. Hence, the hematological results of this study are in accordance with the hematological results of the present study.

A study conducted by Hafiz *et al*, (2016) on the methanolic peel extract of *P. granatum* significantly reduced the anemia in mice infected by *P. chabaudi*. The present study also revealed the protective effects on the hemoglobin concentration and erythrocyte count in the seed extracts treated groups as compared to the infected but non treated groups.

Hence the malaria infects multi-organs and drug may also affect many organs. So the histopathological studies were performed on the three majorly infected organs during malaria i.e. liver, spleen, and kidneys. The sequestration of PRBCs, in the microvasculature was observed. The macrophages engulfed the PRBCs and results in the lysis of the blood cells and leave the haemozoin behind in the malaria infected liver. The splenomegaly and discoloration of the spleen of infected mice in this model is the same condition as occurred in the *P. falciparum* infection in humans, in which, spleen enlargement is a very common due to the malaria haemozoin pigment accumulation in the pulp element (Basir *et al.*, 2012).

The sequestrations of PRBC were observed in the kidney. These histological changes were clearly observed in the infected, but non treated group as compared to normal and treated with plant groups and these results are comparable with Histopathological study on ICR mice infected with *Plasmodium berghei* ANKA strains, as a model of cerebral malaria (CM). He observed the splenomegaly, hepatomegaly and discolorations of major organs and severe anemia due to the high parasitemia (Basir *et al.*, 2012).

Similarly in the present study all the symptoms were observed for the infected but not treated group. The histological results were also comparable with the findings of (Hafiz *et al.*, 2016) on liver and Mubaraki *et al.*, 2016) on spleen of mice infected with malaria.

The seeds of experimented plants in the present study are frequently used in our daily life. The aqueous extracts of these plants might contain a potential lead molecule for the development of a new drug for treatment of malaria. The extracts of the experimented plants can form the basis for selection of new natural bioactive compounds and be subject for the isolation and characterization of bioactive components via HPLC or GC-MS or for the production of nanoparticles to be used as antimalarial agents for drug delivery.

Chapter # 3

Qualitative Phytochemical Analysis

Abstract

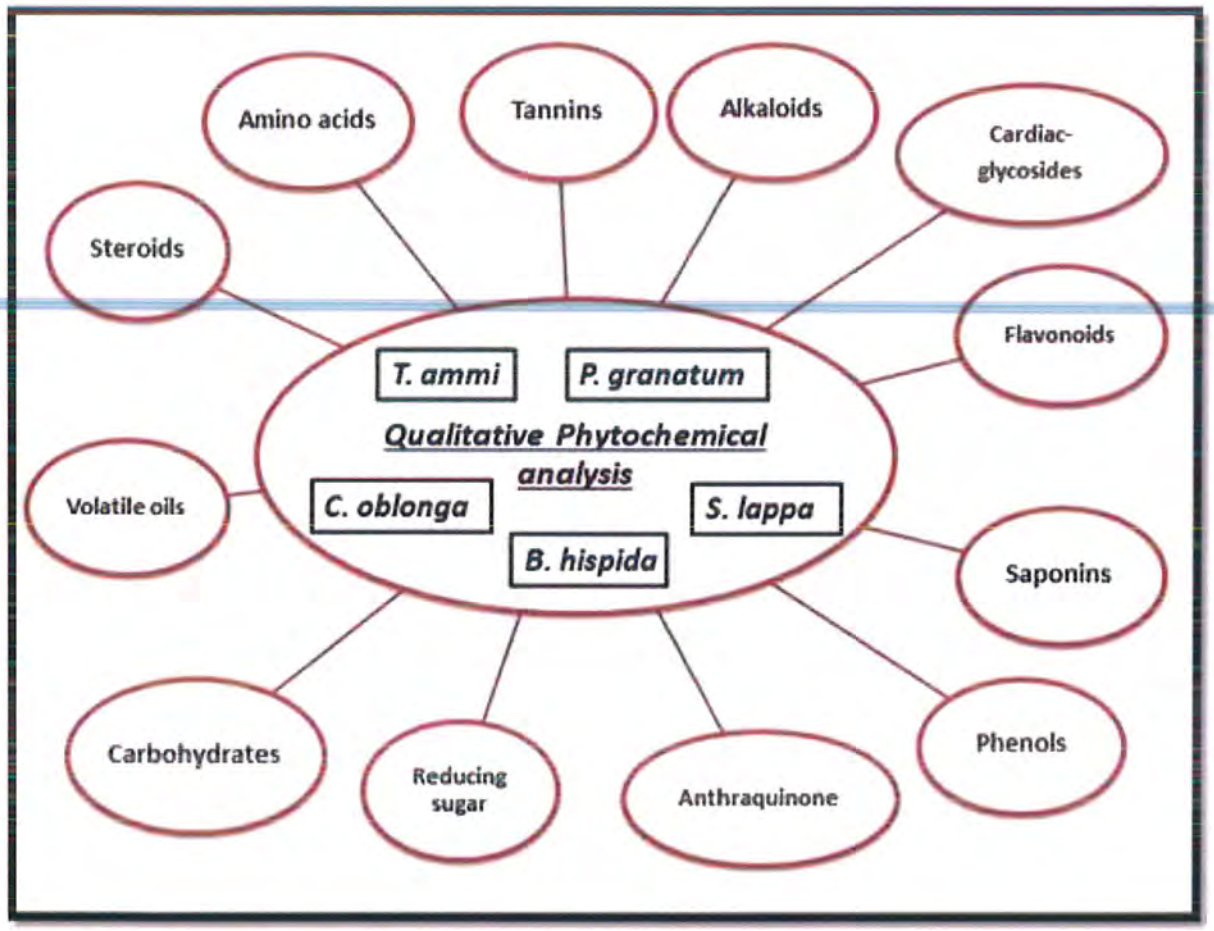
Herbal medicines are commonly used for the treatment of various aliments in traditional systems. The screening of phytochemical constituents is important to step up towards the drug formulation step.

The preliminary qualitative phytochemical analyses were conducted to know about the constituents present in the seeds of *T. ammi. P. granatum, C. oblonga, B. hispida* and *S. lappa* according to the standard procedures.

The alkaloids, tannins, carbohydrate and steroids were present in all five experimented Plants. The Cardiac glycosides and reducing sugars were present in *T. ammi. P. granatum, C. oblonga,* and *S. lappa*. Saponin was absent only in *P. granatum*. Anthraquinone was only present in the *B. hispida,* Amino acid only in *C. oblonga* and volatile oil in *T. ammi* seeds. Tannins were found in *T. ammi. P. granatum* and *C. oblonga.* However the Phenols were found in *T. ammi P. granatum* and *S. lappa.*

The major constituents were observed in the five experimented plants. The generated data from the five medicinal plants like *T. ammi. P. granatum, C. oblonga, B. hispida* and *S. lappa* provided the basis for its wide uses in the traditional and folk medicines, especially for the drug formulation. The active constituents can be isolated from the seed extracts of *P. granatum, B. hispida and S. lappa* that showed the highest antiplasmodial activity in the present study.

Graphical Abstract



Graphical representation of Qualitative phytochemical analysis of five medicinal plants

Introduction

The therapeutic efficacy of many indigenous plants for various diseases has been described by traditional herbal medicinal practitioners. Natural products are the source of synthetic and traditional herbal medicine.

The major compound is the phenolic content Thymol. Pomegranate comprises on thocyanidins and flavonoids that show potent antioxidant activity (Okamoto *et al.*, 2004).

The Phytochemical analysis of *C. oblonga* revealed that it contains oxalic acid, malic acid, quinic acid (72.2%), and citric acid (13.6%) (Oliveira *et al* 2008), shikimic acid, ascorbic acid, 3-caffeoylquinic acid, fumaric acid, polymeric procyanidin and 5-caffeoylquinic acid, 3 procyanidin polymers and many other compounds (Wojdy *et al.*, 2013, and Carla *et al.*, 2007). The aphrodisiac potential of this plant may be due to the metabolites such as flavonoids, tannins, glycosides, and phenolic compounds present in the extract (Kumar, 2000).

The phytochemical analysis of *B. hispida* revealed that it is a rich source of significant bio actives and therapeutics such as phenolics, sterols, triterpenes, and glycosides Qadrie *et al.*, (2009). Many researchers analyzed *S. lappa* collected from different regions chemically and found its constitution as terpenes, anthraquinones, alkaloids and flavonoids. Terpenes that mainly have anti-tumor properties and anti-inflammatory, such as costunolide, dihydrocostunolide, dihydrocostus lactone, (Yang *et al.*, 1998) HD 12-methoxydihydrocostunolide and dehydrocostus lactone, α-hydroxydehydrocostus lactone, β-hydroxydehydrocostus lactone, lappa dilactone, mokko lactone, betulinic acid, betulinic acid methyl ester (Sun *et al.*, 2003), cynaropicrin, reynosin, santamarine (Choi *et al.*, 2009) saussure amines A-C (Yoshikawa *et al.*, 1993), α-cyclocostunolide, alantolactone, isoalantolactone Zhao *et al.*, 2008).

Material and Methods

Qualitative Phytochemical Analysis

The qualitative phytochemical analysis of seeds of all experimental plants like *T. ammi P. granatum, C. oblonga, B. hispida and S. lappa* were analyzed. Different chemical tests were conducted using standard procedures as described by Sofowara (1993) (Table 3.1).

Table 3.1 Qualitative phytochemical analysis of seeds of experimented Plants

Sr.#	Constituents tested	Reagents added	Physical Observation
1	Tannins	0.5 g of the extract, 1 ml of distilled water and 10% $FeCl_3$	Bluish green
2	Alkaloids	0.5 g of the extract, 0.5 ml of 1% HCL Few drops of distilled water, Few drops of Wagners reagent.	Reddish brown Precipitates
3	Cardiac-Glycosides	0.5 g of the extract, 2 ml of glacial acetic acid Few drop of $FeCl_3$ solution, 2 ml of concentrated H_2SO_4	Brown color ring
4	Flavonoids	2 ml of extract, 2 ml of diluted NaOH	Yellow
5	Saponins	0.5 g of the powdered seeds, 12 ml of distilled Water	Foam Produced (Persist for 10 minutes)
6	Phenols	Equal volume of plant extract and FeCl3 (1:1)	Deep bluish green
7	Anthraquinone	0.5 g of the extract, 10 ml of benzene and 10% of Ammonia	Pink, red or violet color
8	Reducing Sugar	3 ml of extract, 5 ml of distilled water and Fehling solution A and B	Red precipitate
9	Carbohydrates	0.5 g of the extract, Few drops of distilled water few drops of Molish reagent and 1 ml of conc. H_2SO_4	Brown ring
10	Volatile oils	0.5 g of the extract, 90% ethanol and few drops of $FeCl_3$	Green color
11	Steroids	0.5 g of the extract, 3 ml of chloroform and few drops of conc. H_2SO_4	Reddish brown color
12	amino acids	1 ml of extract, few drops of Ninhydrin reagents	purple color

Results

The Phytochemical Analysis

The qualitative phytochemical analysis of seeds of *T. ammi, P. granatum, C. oblonga, B. hispida* and *S. lappa* revealed the presences of different constituents such as alkaloids, flavonoids, carbohydrates, and steroids etc.

The alkaloids, flavonoid, carbohydrates and steroids were present in all five experimented Plants. The Cardiac glycosides and reducing sugars were present in *T. ammi. P. granatum, C. oblonga,* and *S. lappa.* Saponin was absent only in *P .granatum.* Anthraquinone was only present in the *B. hispida,* Amino acid only in *C. oblonga* and volatile oil only in *T. ammi* seeds. Tannins were found in *T .ammi. P. granatum* and *C. oblonga.* However the Phenols were found in *T .ammi P. granatum* and *S. lappa.* The major constituents were observed in the five experimented plants (Figure 3.1).

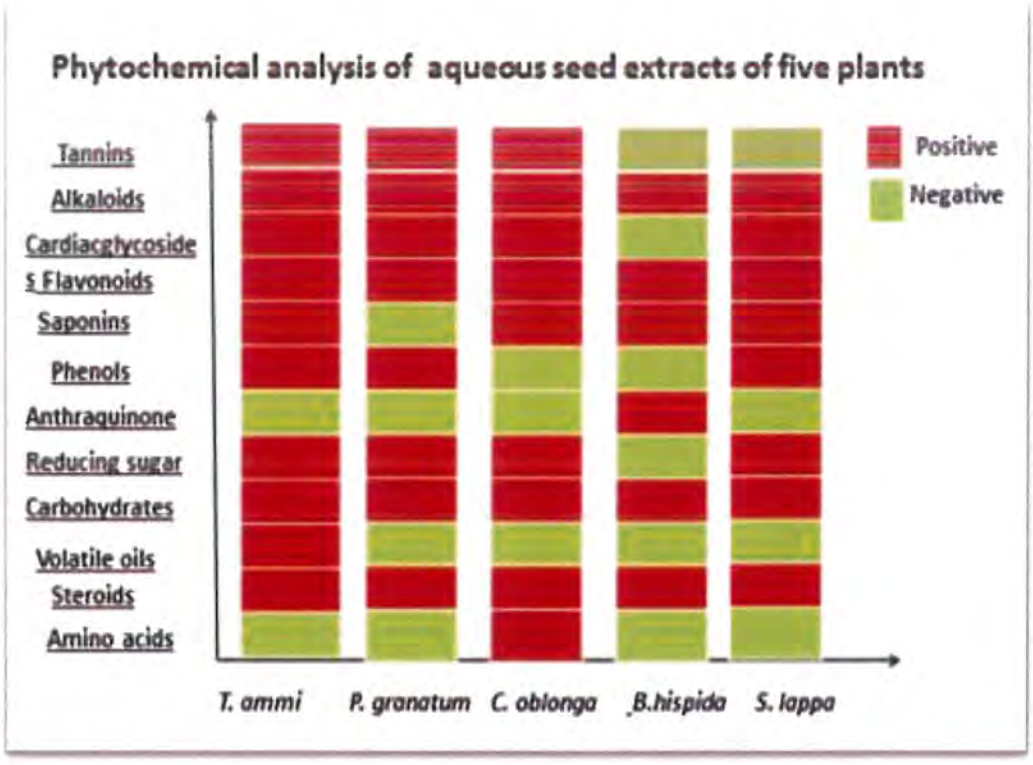


Figure 3.1: The Qualitative Phytochemical analysis of aqueous seed extracts of five experimented plants

Discussion

The preliminary phytochemical screening tests are used for the detection of bioactive compounds that subsequently lead to the drug discovery and development. These tests provide the basic knowledge about the estimation and qualitative separation of pharmacologically active chemical compounds (Varadarajan et al., 2008).

According to the literature the phytoconstituents of *T. ammi* seeds include saponins, phenolic compounds, carbohydrates, glycosides, volatile oil (para-cymene thymol, and γ -terpinene), protein, fat, fiber and mineral matter containing, phosphorous, calcium, iron and nicotinic acid (Jeet *et al.*, 2012). The result of the present study also showed many similar compounds like Saponins, phenolic and carbohydrates, etc. as described by Jeet *et al.*, (2012).

Javed *et al.*, (2012) showed the phytochemical constituents of ajwain as flavonoids, tannins and steroids. The nutrition analysis indicated higher energy value (314.55%), carbohydrates (47.54%), protein (20.23%), fat (4.83%), and moisture (11.6%), fibre (4.3%) and ash (11.5%).

Pomegranate comprises thocyanidins and flavonoids, which show potent antioxidant activity (Okamoto *et al.*, 2004). The most important constituents of pomegranate peel are gallic acid, phenolic compunds; and other fatty acids; flavonols; flavones, flavanones; and anthocyanidins (Jurenka, 2008).

The phytochemical analysis of the seeds of *P. granatum* revealed that it is a rich source of significant bioactive and therapeutic compounds such as Alkaloids, Tannins, cardiac glycosides, Phenols, Flavonoids, reducing sugar, carbohydrate and steroids as confirmed by the above mentioned studies.

However the Phytochemial analysis of *C. oblonga* seeds showed the presence of Alkaloids, Tannins, Cardiac glycosides, Flavonoids, reducing sugar, carbohydrate and steroids and amino acids. The Phytochemical analysis revealed that it contains oxalic, quinic, citric and, malic, ascorbic, fumaric acids, and many other compounds (Wojdy *et al.*, 2013, Oliveira *et al* 2008 and Carla *et al.*, 2007). The aphrodisiac potential of this plant may be due to the metabolites such as glycosides, flavonoids, tannins, and phenolic compounds present in the extract (Kumar, 2000).

The active ingredients observed during qualitative phytochemical analysis of *B. hispida* were anthraquinones, alkaloids flavonoids, saponins, tannins, carbohydrates, steroids and saponins. Its phytochemical analysis conducted by Qadrie *et al.*, (2009) showed that it is a rich source of significant bioactives and therapeutics such as phenolics, sterols, triterpenes, phenolics, and glycosides.

The phytochemical analysis of the seeds of *S. lappa* revealed that it is a rich source of significant bioactive and therapeutic compounds such as alkaloids, cardiac glycosides, phenols, flavonoids, reducing sugar, carbohydrate and steroids. Its active ingredients are mainly terpenes, but it also contains anthraquinones, alkaloids and flavonoids (Rao *et al.*, 2013).

The information about presence of different phytoconstituents provides basic information for the potential use of plants in preparing medicines. The antiplasmodial activity of *S. lappa P. granatum and B. hispida* may be due to their phytochemical constituents. The generated data from the five medicinal plants provided the basis for its wider uses in the traditional and folk medicines especially for the drug formulation. The active constituents can be isolated by GC-MS and HPLC from the seed extracts of *P. granatum, B. hispida and S. lappa.*

Chapter # 4

Toxicology and Clinical Potential of Green Synthesized Silver and Iron Nanoparticles against *Plasmodium berghei*

Abstract

The nanotechnology has arisen as a high-tech and avant-grade technology with diverse applications in a wide array of fields. Among all the nanomaterials, the research has widely held on nanoparticles because they can be easily prepared and manipulated. The green synthesis of nanoparticles is environment friendly, non-toxic and safe method. The silver and magnetic nanoparticles are an important class of inorganic nanoparticles having diverse applications and unique properties. Many iron metallodrugs have been approved for human or veterinary supplements based on the association of iron to a reducing sugar coating. The resistance against existing antimalarial drugs led to the discovery of new medications.

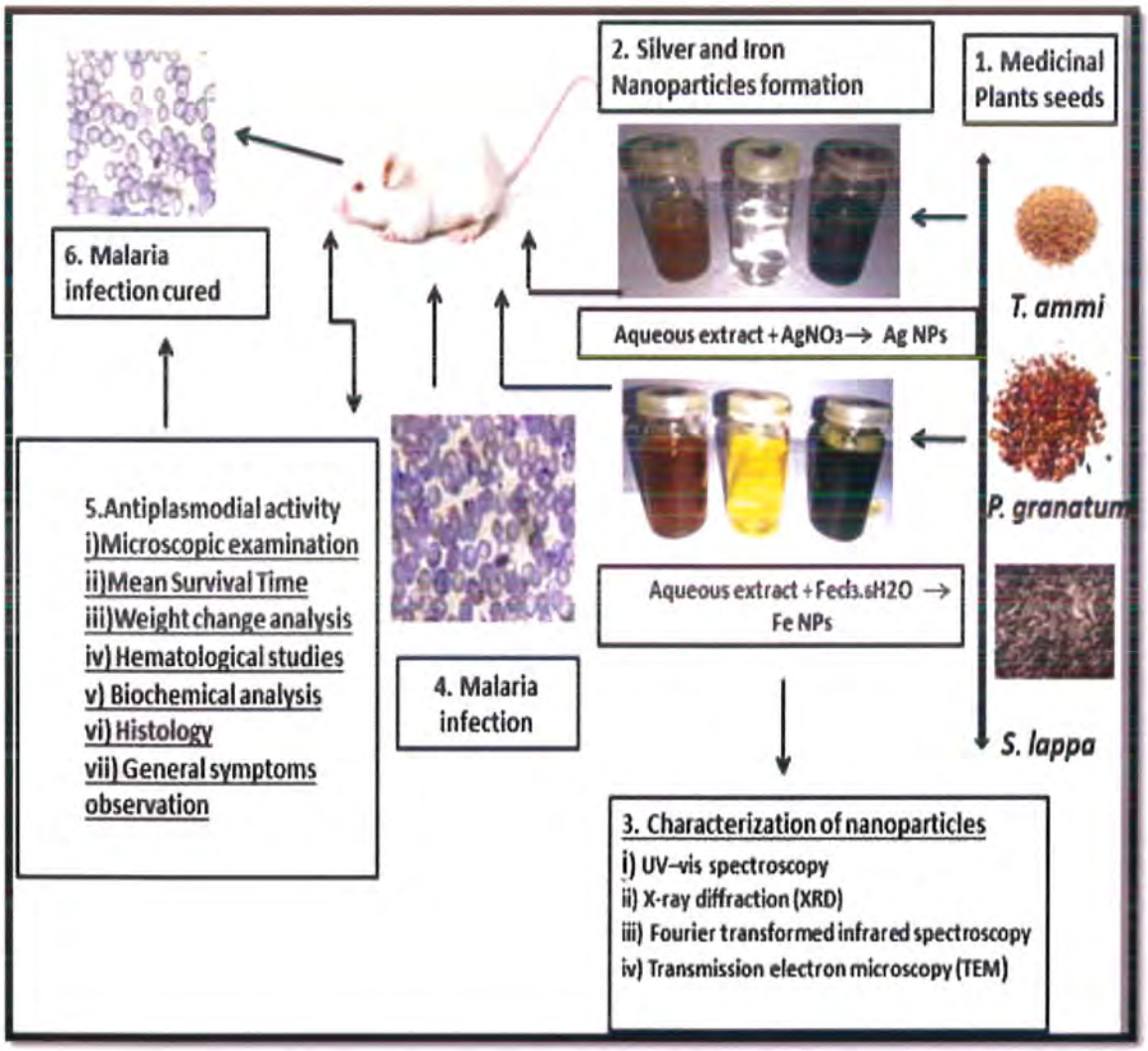
The Ag NPs and Fe NPs were synthesized from $AgNO_3$ and $FeCl_3.H_2O$ respectively through a simple green route using the aqueous seed extracts of *Trachyspermum ammi*, *Punica granatum* and *Saussurea lappa*. The obtained nanoparticles were characterized by UV-vis (UV-visible spectroscopy), FTIR (Fourier transformed infrared spectroscopy), XRD (X-ray diffraction analysis), and TEM (Transmission electron microscope) analysis. The antiplasmodial activity of Ag NPs and Fe NPs were determined using BALB/c mice infected with *Plasmodium berghei*. After seventy two hours post infection; mice were treated with (i) green synthesized Ag NPs of different concentrations, i.e., 50, 100 and 150 mg/kg and green synthesized Fe NPs at different concentrations i.e., 100, 200 and 300 mg/kg (ii) water and (iii) Chloroquine. The effect of Ag NPs and Fe NPs were studied by general symptom observation, parasite count, chemosupression, survival time measurement, histopathology, biochemical and hematological analysis. High resolution imaging through scanning electron microscopy of red blood cell interacting with parasite and NPs were also conducted.

The UV-vis spectroscopy indicated the maximum absorbance at 420 and 402 nm for Ag NPs and Fe NPs respectively. The FTIR spectrum of Ag NPs and Fe NPs suggested that Fe NPs were surrounded by different organic molecules such as alcohols, ketones, aldehydes and carboxylic acids. The XRD and TEM analysis

showed the average particle size of Ag NPs of *T. ammi*, *P. granatum* and *S. lappa* as 73 nm 10 nm, 46 nm respectively. The shapes of the Ag NPs of *T. ammi* were face centered cubic while the Ag NPs of *P. granatum*, and *S. lappa* were spherical in shape. However the Fe NPs of *T. ammi*, *P. granatum* and *S. lappa* had an average particle size as 12 nm, 10nm, and 9 nm respectively and revealed the spherical shape. The maximum antiplasmodial activity was observed for the Ag NPs of *P. granatum* (85.92 ±0.5), > *S. lappa* (85.32 ±0.82) > than *T. ammi* (83.50 ±0.65) that was treated at the dose of 150 mg/kg. The maximum antiplasmodial activity was observed for the Fe NPS of *S. lappa* (82.51±4.68), > *P. granatum* (81.46±3.10) > than *T. ammi* (77.14±4.18) that was treated at the dose of 300 mg/kg. The results revealed highly non-significant difference (P>0.5) when the chemosupression was compared with chloroquine treated group. The mean survival time of Ag NPs treated groups at the dose of 100 and 150 showed non-significant differences between normal and drug treated groups. The mean survival time of Fe NPs of *S. lappa* at all dose and Fe NPs of *P. granatum* at the dose of 200 mg/kg showed non-significant difference with normal and drug treated groups. The biochemical, hematological and histopathological analysis also showed a marked difference in infected, but the untreated group compared to groups treated with Ag NPs and chloroquine. A progressive decrease in the weight was also observed in untreated groups as compared to the treated ones. The scanning electron microscopy of red blood cells of mice showed that the nanoparticles were present and interacting with the parasite.

The significant outcome of the present study is the development of value added products from medicinal plants, i.e. Ag NPs and Fe NPs and its low quantity effectively reduced the malarial infection since it did not show adverse effects on the hematological, histological and biochemical parameters.

Graphical Abstract



Graphical representation of Green synthesis Silver and Iron Nanoparticles and their *in vivo* antiplasmodial activity

Introduction

The nanotechnology has arisen as a high-tech and advent-grade technology with diverse applications in a wide array of fields. It is a very vast field, including nanomaterials, nano tools, and nano devices. Nanoparticles are small sized particles having a size range of 1-100 nanometers. It has been observed that the physical and chemical properties of matter are different in the Nano range as compared to the same material in bulk amount (Navaladian *et al.*, 2007). Among all the nanomaterials, the research has widely held on NPs because they can be easily prepared and manipulated.

There are many applications of NPs due to the distinctive physicochemical properties, e.g. drug delivery and targeting (Hu *et al.*, 2009), cancer therapy (Peer et al., 2007), biosensors (Lord and Kelley, 2009), food additives and cosmetics (Nohynek et al., 2007). Generally three methods are used to prepare the NPs such as, Chemical (Starowicz *et al.*, 2006), Physical (Sreeram *et al.*, 2008) and Biological methods (Begum *et al.*, 2007).

The most prevalent method used for the synthesis of NPs is a chemical method (Starowicz *et al.*, 2006; Zhang *et al.*, 2002).

The use of bacteria, fungi and plant extracts, for the synthesis of NPs has many benefits including low cost, environmentally friendly and quick (Gopinath *et al.*, 2013). They are suitable for medicinal purpose because they do not involve toxic chemicals in the synthesis process (Sharma *et al.*, 2009).

The comparison of biological material that can be used for the synthesis of NPs, botanical materials have advantages over other biological processes since tedious effort is required for the maintenance of bacterial culture and the very low yield of NPs (Thakkar *et al.*, 2010).

The extracellular enzymes of the fungi can also be used for the synthesis of firm metal ion NPs. The main limitation of this process is the over expression of various enzymes causing complications in the chemical process (Sastry *et al.*, 2003). NPs that involved plant materials is known as green synthesis method because it does use any damaging chemicals (Husen and Siddiqi, 2014 a).

The NPs can be synthesized by using different metals e.g. iron, platinum, gold, sodium and silver, etc. Metal NPs that are widely used for humans are gold, silver and platinum. However the Ag is widely used in medical devices, textiles, electronics, food packaging materials, household appliances, food supplements, cosmetics, and water disinfectants.

The nanoparticles are also used in the health care sector, for imaging, drug transport, therapeutics and diagnosis as nano medicine (Mishra *et al.* 2010). The iron, gold and platinum has been widely applied as Nanomedicine, but the application of iron oxide NPs in medical and other sectors is increasing rapidly (Makarov *et al.*, 2014).

The Ag NPs are broadly used for its antibacterial activity in medical and other products because of its wide range of activity with low cost and toxicity as compared to other bactericides (Das *et al.,* 2013). The toxicity of Ag NPs are generally low on humans (Ray *et al.*, 2009).

The NPs of Au and Ag have been synthesized from many microorganisms, such as bacteria, yeasts and other fungi (Huang *et al.*, 2007). However the plant based biological synthesis of NPs is getting importance in recent years, due to its cost effective, simple and ecofriendly procedure. Although biosynthesis of Ag NPs by plant leaves of *Andrographis paniculata* (Panneerselvam *et al* 2011), aqueous and ethanolic extracts of leaves of *Solanum tricobatum, Syzygium cumini* and peel powder of *Citrus sinensis* (Logeswari *et al.*, 2013) aqueous extracts of leaves of *Catharanthus roseus* (Ponarulselvam *et al.*, 2012), aqueous extract of leaves of *Pelargonium graveolens* (Shankar *et al.*, 2003) have been reported.

The NPs and spherical gold nanoparticles have been synthesized using sundried mass of the leaves of *Cinnamomum cashora* (Huang *et al.*, 2007). Similarly *Aloe vera* leaf extract also used for the production of gold and Ag NPs (Chandran *et al.,* 2006). Thus, there is a need to explore plants potential for the synthesis of NPs (Panneerselvam *et al.*, 2011).

The magnetic NPs are an important class of inorganic NPs having various applications and unique properties that is why in the recent past the synthesis of NPs it has become a stuff of abundant attention. The magnetic NPs have wide range of physicochemical properties depending on the size including magnetic storage media

(Sun *et al.*, 2000), ferro fluids (Jeyadevan *et al.*, 2003), biosensor (Miller *et al.*, 2002), catalysts (Zhang *et al.*, 2005), separation processes, and environmental remediation (Mahdavi *et al.*, 2013).

The common iron oxides NPs are magnetite (Iron) that has a cubic inverse spinel shape (Huang *et al.*, 2014). These magnetic iron oxide NPs have exclusive electric as well as magnetic properties. It transfers electrons between Fe^{2+} and Fe^{3+} in octahedral sites (Revati and Pandey, 2011).

These NPs have vast applications in many biomedical fields e.g. drug delivery, cellular therapy, physical, mechanical, thermal and chemical properties. Superparamagnetic NPs play an important role, MRI detection and in imaging through electron microscope to locate and measure binding or as the drug carrier for certain anti-cancer drugs (Gupta and Gupta, 2005).

Other medical applications of Fe NPs include immunoassay, tissue repair, hyperthermia, purification of biological fluids and cell isolation. But it is necessary that the NPs have size smaller than 100 nm and high magnetization values in order to achieve biomedical applications, (Gupta and Gupta, 2005).

The surface coating of NPs is necessary for the targeted delivery. The material that can be coated on the magnetic NPs include drugs, nucleotides, antibodies, proteins or any enzyme and can be targeted to an organ, tissue, or tumor using an external magnetic field (Chastellain *et al.*, 2004).

The combination of a metal ion to an organic molecule is results in metallodrugs that have the synergistic activity due to the combined effect of the metal or the organic molecule. They are formed by the combination of metals and sugars or amino acids. Metal based compounds or drugs have attained the systematic attention (Guo *et al*, 2012).

There is a mounting need to develop NPs through ecologically supportive processes. Biological methods for NPs synthesis using, enzyme, microorganism and plant extract have been suggested as possible ecofriendly that avoid the toxic chemicals used in chemical and physical methods (Song and Kim, 2009).

The present study was based upon the synthesis of Ag NPs and Fe NPs from a fast, easy green synthesized method using the aqueous extracts of seeds of *Trachyspermum ammi*, *Punica granatum* and *Saussurea lappa* in order to evaluate their antiplasmodial activity against *Plasmodium berghei* in the BALB/c mice. These seeds were used to prepare Ag NPs and Fe NPs on the basis of cost effectiveness, easy availability and its medicinal properties.

Materials and Methods

Preparation of plant extracts

The seeds of T. ammi, ***P. granatum*** and ***S. lappa*** were collected on the basis of presences of phytochemical constituents e.g. flavonoid, alkaloids, phenols and carbohydrate. The 20 g powdered seeds of T. ammi, ***P. granatum*** and ***S. Lappa*** were taken in a beaker containing 200 ml deionized water and simply heated for 30 min. However, for Fe NPs 25 g powdered seeds of these three experimented plant were kept in a beaker containing 100 ml deionized water and heated for 20 min at 80 °C. The extract was filtered with Whatman filter paper no.1 and cooled down.

Green synthesis

Silver nanoparticles (Ag NPs)

The extracts of seeds of plants T. ammi, ***P. granatum*** **and** ***S. lappa*** and 3-5mM solution of $AgNO_3$ were mixed in 1: 8 with the constant stirring at 350 rpm and 37 ^{0}C for two hours. The reduction of silver was indicated by a color change from yellowish to brown of the aqueous solution of the plant extracts immediately or one to two hours after the reaction. The mixture was kept for 24-48 hours at room temperature. The whole procedure was conducted and mixture was kept in dark chamber in order to avoid the photo-activation of silver nitrate at room temperature.

Iron nanoparticles (Fe NPs)

The extracts experimented plant parts were mixed with a 0.5 M solution of ($FeCl_3$. $6H_2O$) at 1:1. The 0.5 M solution of (FeCl3. 6H2O) was prepared in deionized water. The reduction of iron was indicated by a color change from yellowish to dark green to black of the aqueous solution of the plant extracts immediately or one to two hours after the reaction. The mixture was kept for 24-48 hours at room temperature.

pH analysis of iron nanoparticles

The pH of Fe NPs was determined by using pH paper. It is a basic indication for the formation of Fe NPs. The pH of the extract and $FeCl_3.6H_2O$ mixture solution was found to be acidic. The pHs of synthesized Fe NPs of three experimented plants was decrease and give the acidic range. The 50 mg/L of Malachite green was prepared. The Fe NPs (0.01 g) were added to solution containing 8 ml of Malachite

Green at the concentration of 50.0 mg/l this mixture was shake constantly and the color change was observed.

Purification and annealing of nanoparticles

The Ag NPs and Fe NPs were purified from the solution by a simple process of centrifugation at 4000 rpm, for 10 min. The supernatant was decanted. The pellet was washed five times with deionized water for Ag NPs and with absolute ethanol for Fe NPs. Then the pallet was air dried in the china dish and annealed in the furnace at 500 ◦C and 300 ◦C for three hours for Ag NPs and Fe NPs respectively. The powder obtained was used for more characterization studies.

Characterization of nanoparticles

i) UV–vis spectroscopy

The formation of Ag NPs and Fe NPs was also confirmed by using UV-visible spectroscopy. The 1:10 was prepared and the synthesis of Ag NPs and Fe NPs were monitored by UV–vis spectroscopy (300–600 nm) using UV spectrophotometer (Mecasys Optizen 3220) operated at a resolution of 1 nm.

ii) X-ray diffraction (XRD)

The size and nature of the Ag NPs and Fe NPs were determined by XRD. The XRD analysis was conducted using (Shimadzu XRD-6000/6100). The X-ray diffraction analysis is a fast analytical technique primarily used for identification of a crystalline material and its phase. It can provide information on unit cell dimension. The Debye Sherrer's equation ($D = 0.94\ \lambda / B \cos\theta$) is used to find out the particle size. In Debye Sherrer's equation λ (is the specific wavelength of X-ray source and is equal to 1.54 A), B= FWHM (full breadth at half maxima) θ = diffraction angle D= particles diameter size.

iii) Fourier transformed infrared spectroscopy

The FTIR analysis was done using an IR spectrophotometer (Bruker Tensor 27) in order to confirm the functional groups of biomolecules present. The IR spectrum was recorded in the range of middle infrared of 400-4000 cm^{-1}at the resolution of 4 cm^{-1} in the absorption mode.

iv) Transmission electron microscopy (TEM)

The particle sizes and shape of the Ag NPs and Fe NPs were determined by transmission electron microscopy. The lean films of the sample were set on a carbon coated copper grid by taking the minimum amount of sample; the superfluous solution was removed with the help of blotting paper. It was dried for 5 minutes. The TEM analysis was done using a JEOL 2010F field-emission gun operating at 120 Kv.

The antiplasmodial activity

The *in vivo* propagation of strains and animal rearing was done as described in the chapter two. The experimental animals from the rare stalk were divided into twenty one groups, including controls, each having fifteen individuals as described in (Table 4.1). Each mouse of all experimented groups except (A) was inoculated intraperitoneally with the 0.2 ml diluted *P. berghei* strains. The drug and Ag NPs and Fe NPs were administered after the 72 hours of the post infection for the five days. The microscopic examination, monitoring of Parasitemia, antiplasmodial activity, survival time, haematological studies, liver function test, kidney function test, histological studies, general symptoms along with the statistical analysis were done as described in the chapter number 2.

SEM imaging of red blood cell interacting with parasite and Nanoparticles

The surface morphology of RBCs of *P. berghei* infected mice and treated with nanoparticles was studied through SEMs.

Table 4.1: Different experimental groups of mice for antiplasmodial activity

Groups	Dose
A	Non infected non treated (normal control)
B	0.5 ml distilled water (infected control)
C	5 mg/kg chloroquine (chloroquine control)
I1	50 mg/kg Ag NPs of *T. ammi*
I 2	100 mg/kg Ag NPs of *T. ammi*
I 3	150 mg/kg Ag NPs of *T. ammi*
J 1	50 mg/kg Ag NPs of *P. granatum*
J 2	100 mg/kg Ag NPs of *P. granatum*
J3	150 mg/kg Ag NPs of *P. granatum*
K1	50 mg/kg Ag NPs of *S. lappa*
K2	100 mg/kg Ag NPs of *S. lappa*
K3	150 mg/kg Ag NPs of *S. lappa*
L1	100 mg/kg Fe-NPs of *T. ammi*
L 2	200 mg/kg Fe-NPs of *T. ammi*
L 3	300 mg/kg Fe-NPs of *T. ammi*
M 1	100 mg/kg Fe-NPs of *P. granatum*
M 2	200 mg/kg Fe-NPs of *P. granatum*
M 3	300 mg/kg Fe-NPs of *P. g ranatum*
N 1	100 mg/kg Fe-NPs of *S. lappa*
N 2	200 mg/kg Fe-NPs of *S. lappa*
N 3	300 mg/kg Fe-NPs of *S. lappa*

Results

(i) Silver nanoparticles

Confirmation of Green synthesis

The Ag NPs were synthesized using three different plant extracts i.e. *T. ammi, P. granatum, and S. lappa*. One of the criteria for NPs formation is the reduction of metals resulting in color change of the solution. The reduction of silver ions into Ag NPs was indicated by a color change from yellowish to bright yellow, then into dark brown when mixed with the aqueous solution of the plant extracts (Figure 4.1).

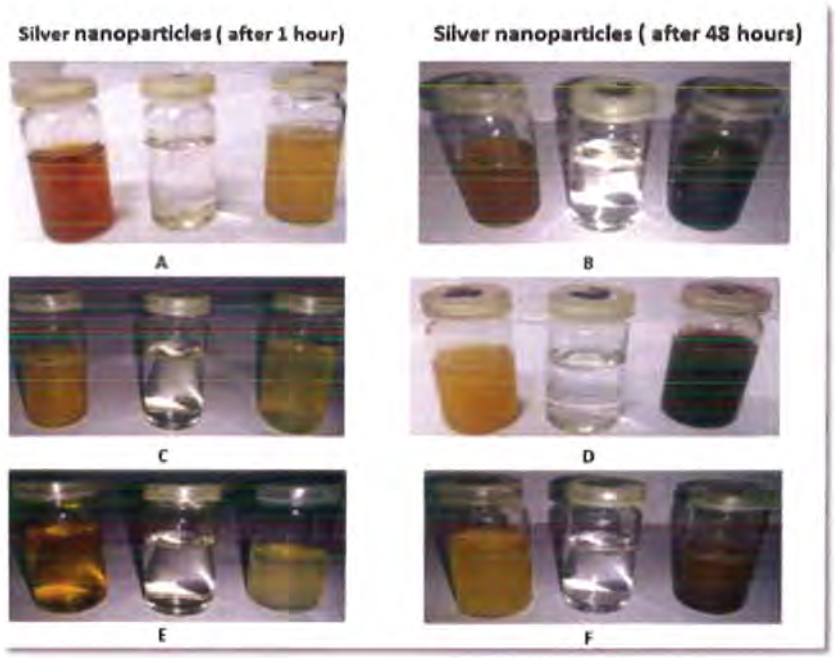


Figure 4.1: Physical observation of Ag NPs synthesized by using aqueous seed extracts of: *T. ammi*, *P. granatum* and *S. lappa*.

A, C, E at 1st hours and B, C, and D after forty eight hours of synthesis. Transparent solutions indicatae the solution of Silver nitrate. The yellow color solutions indicate the plant extracts and dark brown solutions indicate the Ag NPs.

UV–Vis spectrophotometer analysis

The formation and stability of Ag NPs in aqueous solution were confirmed by UV–vis spectrophotometer analysis. The absorbance of the solution was measured at 1, 24 and 48 hrs intervals with different wavelengths. Maximum absorption peak for *T. ammi, P. granatum and S. lappa* was observed at 420 nm. The absorbance increased as the reaction time was increased, i.e. the absorbance of NPs of *T. ammi* at 320 nm was 0.3 then 0.5 and 2 at 1, 24 and 48 hours, respectively (Figure 4.2).

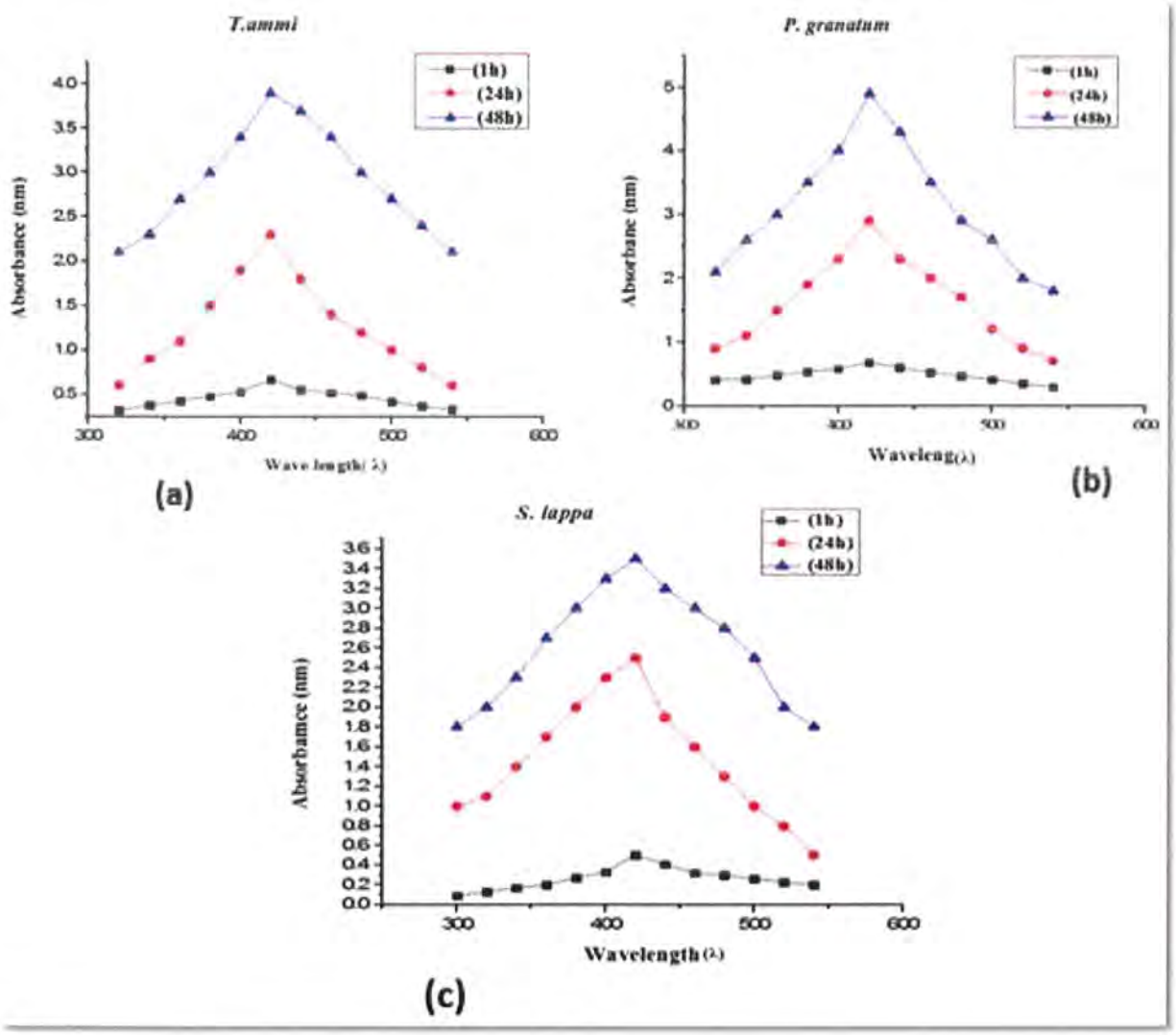


Figure 4.2: UV-vis absorption spectra of aqueous silver nitrate solution with aqueous seed extracts of (*a) T. ammi (b) P. granatum and (c) S. lappa* at different time intervals

Similarly the absorbance at 320 nm of NPs of *P. granatum* was observed as 0.5, 0.9 and 2 and for *S. lappa* it was recorded as 0.2, 0.9, and 1.8 at reaction hour in 1, 24 and 48 respectively. Similar absorbance increased pattern was observed at other wavelength i.e. at 340, 360, 380, 400, 420, 440, 460, 480, 500, 520, 540, 560, 580, and 600 nm. The absorbance of Ag NPs was recorded in a quartz cuvette using UV-spectrophotometer (Mecasys Optizen 3220) with silver nitrate solution as blank (Figure 4.2).

Fourier transformed infrared spectroscopy

The FTIR studies were done in order to determine and identify the possible biomolecules in the seed extract which may be responsible for synthesis and stabilization of Ag NPs. The FTIR spectrum of Ag NPs of *T. ammi P. granatum,* and *S. lappa* showed the presence of many functional compounds (Figure. 4.3) and (Table 4.2). The FTIR spectrum of Ag NPs suggested that Ag NPs were encapsulated by different organic molecules such as alcohols, ketones, aldehydes and carboxylic acids etc.

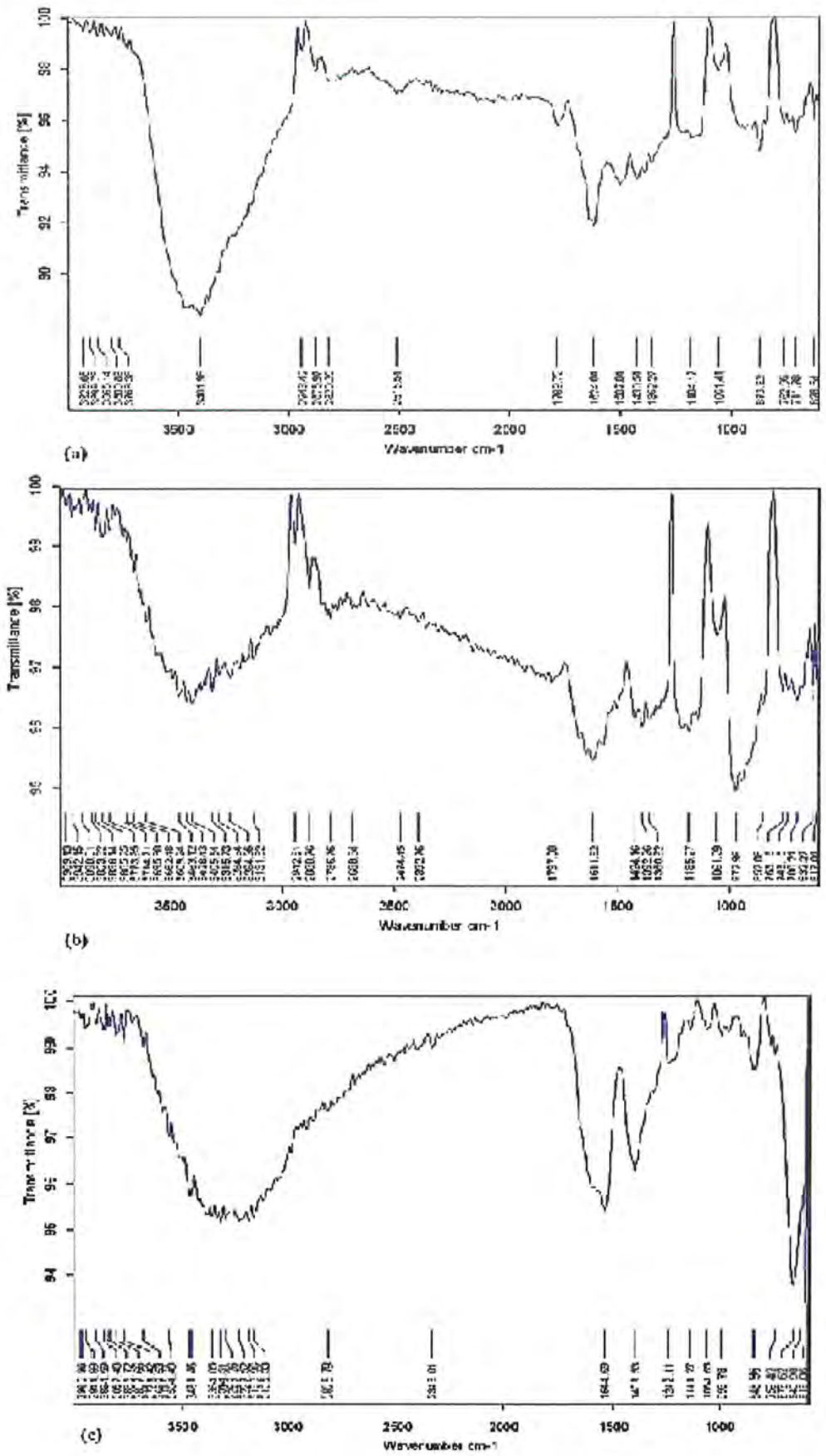


Figure 4.3: FTIR analysis of Ag NPs of (a) *T. ammi,* (b) *P. granatum, and* (c) *S. lappa*

Table 4.2: FTIR analysis of possible organic compounds on the surface of Ag NPs of *T. ammi, P. granatum, and S. lappa*

Sr.No	IR spectrum peak of Ag NPS of *T. ammi*	IR spectrum peak of Ag NPS of *P. granatum*	IR spectrum peak of Ag NPS of *S. lappa*	Types of stretching	Functional groups
1	-	617.01 (B)	616.05 (B)	C≡C-H:C-H	Alkynes
2	628.64 (S)	632.27 (B)	640.98 (B)	C≡C-H:C-H	Alkynes
3	711.78 (S)	700.21 (B)	676.62 (B)	C≡C-H:C-H	Alkynes
4	762.96 (S)	743.15-763.16 (B)	759.42 (B)	C≡C-H:C-H	Alkynes
5	873.92 (S)	852.08 (B)	848.96 (S)	C-C-H	Aromatics
	-	972.99 (S)	993.78 (S)		
	1061.41 (S)	1061.29 (S)	1064.65 (S)	C-N	Aliphatic amines
6	1184.17 (S)	1185.27 (S)	1141.27 (S)	C-N	Aliphatic amines
	-	-	1242.11 (S)	C-O-C	Ether
7	1362.27 (S)	1360.92-1392.26 (B)	-	N-O	Nitro compounds
8	1430.51 (S)	1424.16 (S)	1409.93 (S)	C-C (Ring)	Aromatics
9	1507.01 (S)		1544.69 (S)	C-C (Ring)	Aromatics
10	1624.01 (S)	1611.82 (S)	-	N-H	Primary amines
11	1789.71(S)	1797.08 (S)	-	C=O	Aldehyde
	-	2392.76-2474.4 (S)	2343.01(S)	C≡C	Alkynes
	2515.15 (S)	2688.34-2786.76 (S)	-	H-C=O:C-H	Aldedydes
12	2820.20 (S)	2880.76 (S)	2825.79 (S)	C-H	Alkanes
	2943.42 (S)	2942.81(S)	-	C-H	Alkanes
13	-	3131.82-3284.34 (B)	3165.33-3289.26 (B)	C-O-O-H	Carboxylic acid
14	-	3315.73 (B)	3314.51-3364.05 (S)	O-H	Phenol/Alcohol
15	3401.93 (S)	3463.12 (B)	3461.46-3564.49 (S)	O-H	Phenol/Alcohol
16	3769.36-3899.7 (B)	3609.24-3942.15 (B)	3689.53-3941.8 (B)	O-H	Phenol/Alcohol
17	3929.66 (S)	3969.13 (S)	3960.39(S)	O-H	Phenol/Alcohol

- S = stretch B= bends

XRD Analysis

The crystal structure and chemical nature of the Ag NPs can be studied through the XRD. The XRD analysis of *T. ammi* showed the broad peak at 38.84 °, 44.90°, 64.49° and 77.74° which indicated the miller indices of a faced centered cubic to the crystallographic planes of 110, 200, 220 and 311 respectively. The XRD investigation of *P. granatum* showed the broad peak at 28.12° ,32.32°, 44.45°, and 64.49° which indicated the miller indices of a faced centered cubic to the crystallographic planes of 110, 200, 220 and 311 respectively.

The X-ray pattern of *S. lappa* showed the broad peak at 28.14°, 32.34°, 44.39°, 64.39°, and 78.04° which indicated the miller indices of a faced centered cubic to the crystallographic planes 110, 200, 220 and 311respectively. The mean particle diameter of Ag NPs was calculated from the XRD pattern in order to verify the results of UV-vis spectral analysis, according to the line width of the plane, refraction peak, using the Scherrer equation.

The size of Ag NPs of *T. ammi, P. granatum* and *S. lappa* were calculated as 67.32, 11.4, and 46.86 nm respectively. The XRD pattern thus clearly demonstrated that the Ag NPs formed were crystalline in nature. The additional unassigned peaks were also observed, suggesting that the crystallization of Bioorganic phase occurred on the surface of the Ag NPs (Figure 4.4).

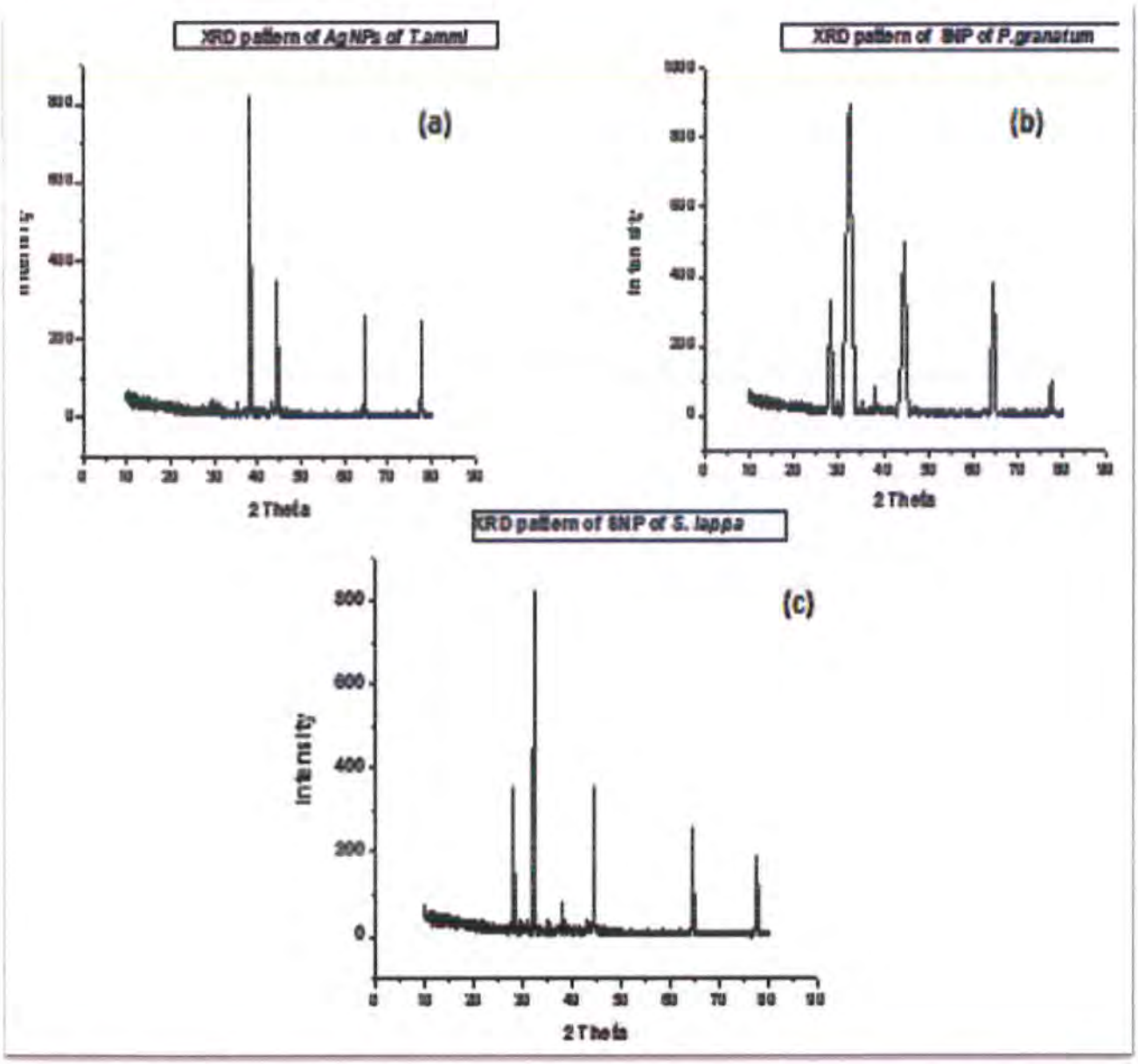


Figure 4.4: XRD analysis of biosynthesized Ag NPs of (a) *T. ammi,* (b) *P. granatum* and (c) *S. lappa*

Transmission electron microscopy (TEMs)

The TEM image showing the high density Ag NPs synthesized by the seeds extracts of *T. ammi, P. granatum,* and *S. lappa* further confirmed the development of silver nanostructures by the plant extracts. The TEM showed that the average particles size of Ag NPs of *T. ammi, P. granatum,* and *S. lappa* were 65, 10 and 45 nm respectively and particles were stable as single crystal (Figure 4.5). The shapes of the Ag NPs of *T. ammi* were face centered cubic however the Ag NPs of *P. granatum,* and *S. lappa* were spherical in shape.

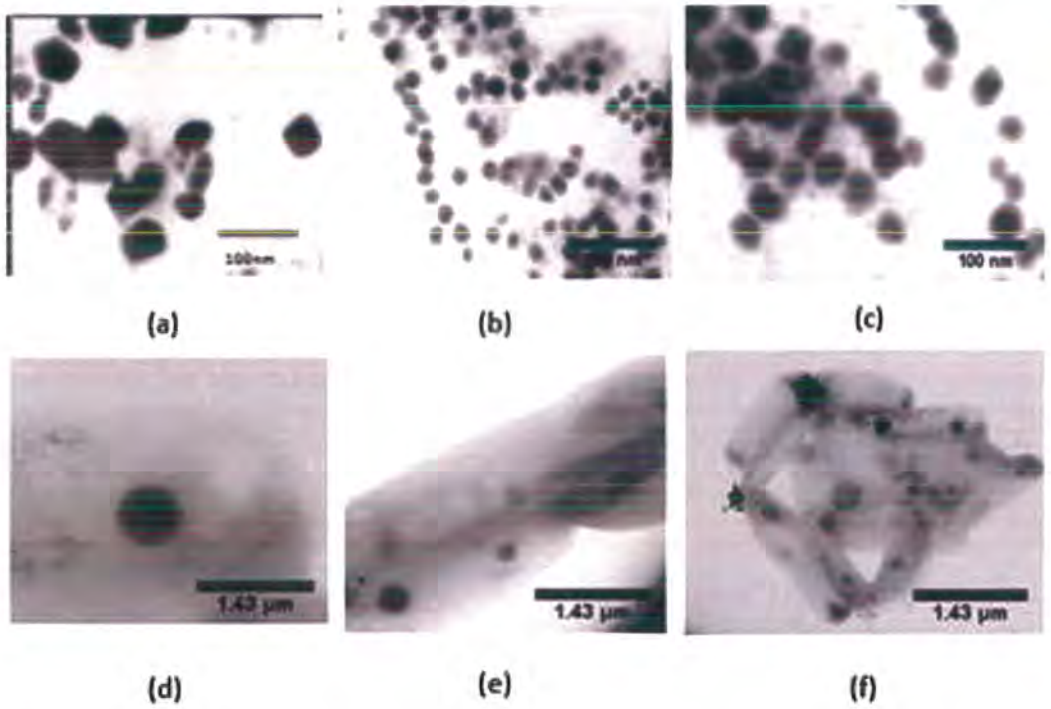


Figure 4.5: Transmission Electron Microscopy (TEM) images of Ag NPs of (a) *T. ammi* (b) *P. granatum and* (c) *S. lappa.* The biosynthesized Ag NPs of *T. ammi* (a), *P. granatum* (b) and *S. lappa* (c)

The bar showing the 100 nm scale and (d), (e) and (f) showing NPs of *T. ammi, P. granatum and S. lappa* at 1.43 µm resolution depicting that Ag NPs are surrounded by some phytochemical constituents that stabilizing them as a single particle.

General Symptoms

The ruffled fur was observed in all the groups from 3rd to 5th day of post infection except in the normal control group A and in Group B it was observed up to 10th day of the infection. Limb paralysis was observed only in two mice of infected and non-treated group B. No sign of convulsion and coma was observed among all groups.

Qualitative analysis of parasites in infected erythrocytes

Different life cycle stages of *Plasmodium berghei* were studied in the erythrocytes during microscopy. Early Trophozoites and late shizont stages were detected (Figure 4.6).

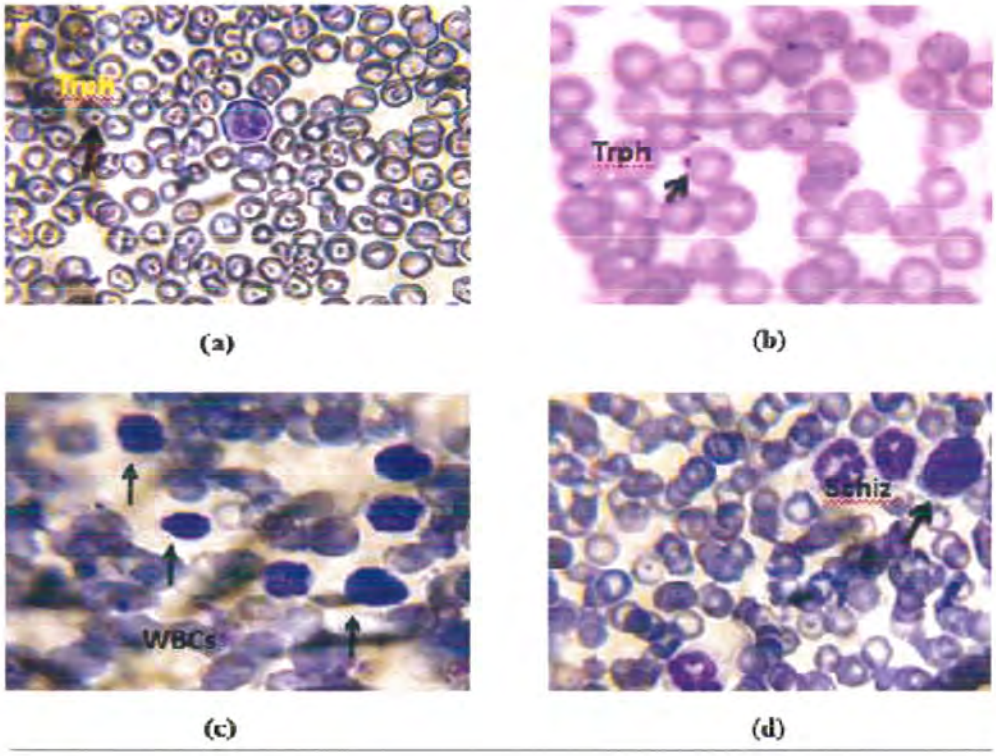


Figure 4.6: The different stages of *P. berghei* observed in erythrocytes.

(a) Early trophozoites stage (b) late stage trophozoites (multiple trophozoites infect the same cell), (c) the no of white blood cell increased in the late stage of infection (d) late stage schizonts. Observed under light microscope at 10 X 100 except (a) magnify at 10 X 40 X.

After the treatment with Ag NPs the Parasitemia was reduced and could be seen by the thin smear observation (Figure 4.7).

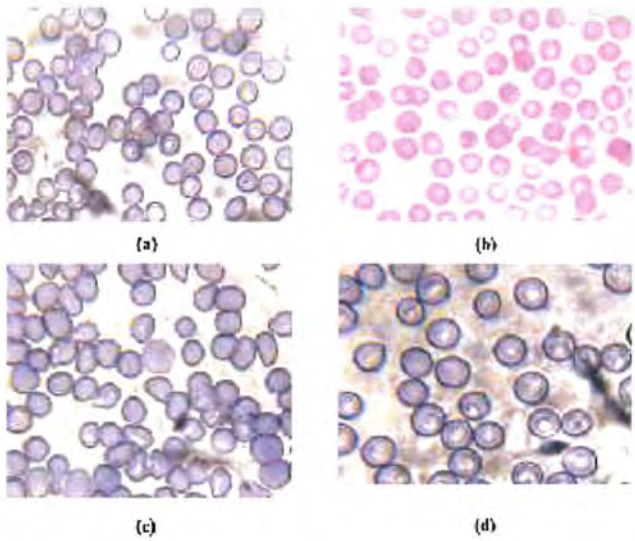


Figure 4.7: Images of thin blood smears of infected mice treated with Ag NPs of different plant extracts. No red blood cells are infected. Observed under light microscope at 10 x 10 magnification.

Parasitemia

The maximum percentage Parasitemia was observed in the negative control group B that was significantly difference (P=0.000) when compared with the drug treated group C. The Parasitemia in Ag NPs of *T. ammi*, *P. granatum* and *S. lappa* treated all groups showed highly significantly difference (P=0.000) high when compared to the non-treated group B, on the fifteenth day of the experiment.

The Parasitemia in Ag NPs of *T. ammi* treated groups I2, and I3, Ag NPs of *P. granatum* treated group, J1 and J2 were depicted the highly non significantly (P≥0.5) and NPs of *P. granatum* treated group J3 and NPs of *S. lappa* treated group K3 were showed only non-significantly (P≤0.1) when compared to the drug treated group C (3.32±0.67). But there was the significant difference between the remaining two groups of NPs of *S. lappa* (K1) and one group of nanoparticle of *T. ammi* (I1) showed significant difference (P≤0.05) as compared to the drug treated group C, on the fifteenth day of the experiment. The highest level of percentage age parasitemia was observed on the day four to six for almost all groups and continuously increased in group B and gradually decreased in Ag NPs and chloroquine treated groups (Figure. 4.8 a).

Chemosuppression

Maximum antiplasmodial activity was observed for the group treated with nanoparticle of *P. granatum, S. lappa and T. ammi* J3 (85.92±0.10), K3 (85.32 ± 0.82), I3 (83.50 ±0.65) at the dose 1500 mg/kg respectively and it was highly non-significant (P≤0.5) when it was compared with the chemosupression of the drug treated group C. The group treated with NPs of *P. granatum* J2 (83.47± 0.10), JI (82.47 ±0.34) at the dose of 100 and 50 mg/kg also indicated the significant high difference (P≥0.5). The groups I2 (80.97 ±0.54) K2 (80.82 ±0.72), andI1 (80.47 ±0.15) showed only non-significant difference (P≤0.1). The antiplasmodial of K1 treated with the 50 mg/kg of Ag NPs of *S. lappa* showed significant difference (P≤0.05) when compared to the group C (Figure. 4.8 b) and (Table 4.3).

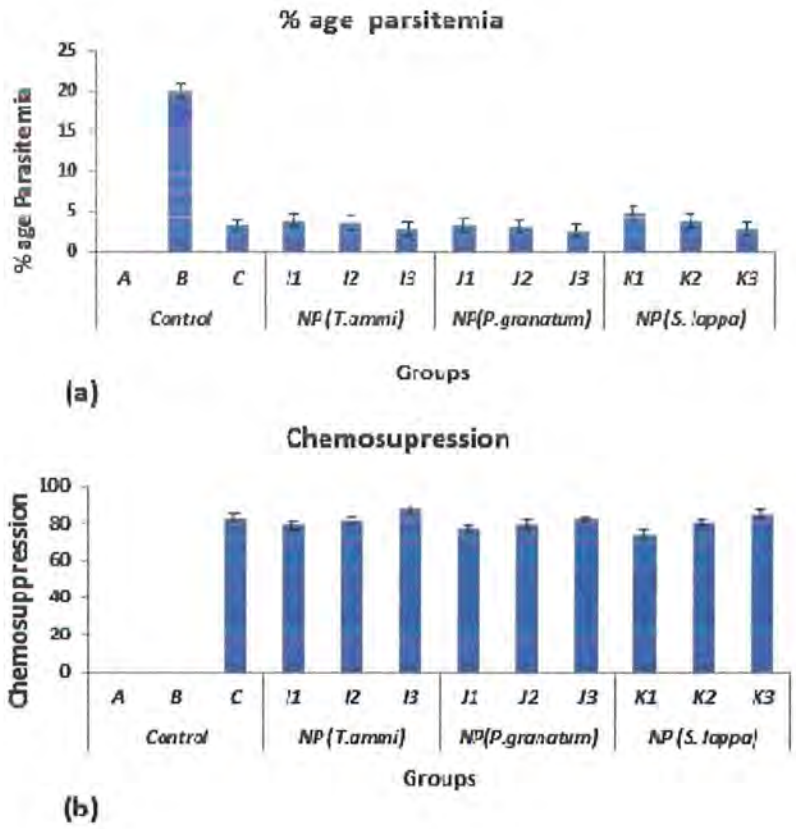


Figure 4.8: The figure shows the (a) Percentage Parasitemia, (b) Chemosuppression of control and green synthesized Ag NPs of different plant extract treated groups.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, $P<0.001$ for graph (a) and 0.07 for graph b.

Survival time

The mean survival time was highest for normal control group A mice i.e., (25.6 ±1.64), the difference was significant (P=0.006) when compared with the non-treated group B (10.4±1.064) and the difference was non-significant (P= 0.096) when compared with the drug treated group C (16.2±1.1).

Table 4.3 Antiplasmodial activity of Ag NPs of different plants against *P. berghei* VS control

Group Names	Treatment	Dose mg/kg	Parasitemia (%) Mean ±SE	Chemosuppression (%) Mean ± SE	Survival Time Mean±SE (days)
(A)	None	-	-	-	>25.6 ±1.64 (a)[b]
(B)	Water	-	19.97±0.74	-	10.4±1.064 {a}[c]
(C)	Chloroquine	5	3.32±0.67 (a)	83.48±2.15	16.2±0.4 {b}(c)
(I1)	Ag NPS of *T. ammi*	50	3.9± 1.457 (a) [c]	80.47 ±0.15 [d]	16.30 ± 1.0 {d}(c) [e]
(I2)		100	3.8±1.944 (a) [e]	80.97 ±0.54 [d]	16.40±1.6 {d}(c) [e]
(I3)		150	3.29±1.36 (a) [e]	83.50 ±0.65 [e]	17.75 ± 1.3 {d}(c) [e]
(J1)	Ag NPS of *P. granatum*	50	3.5±1.621 (a) [e]	82.47 ±0.34 [e]	15.20 ±1.9 {c}(b) [e]
(J2)		100	3.3 ± 1.687 (a) [e]	83.47 ±0.10 [e]	17.80 ±2.3 {e}(c) [e]
(J3)		150	2.81±1.6.2 (a) [d]	85.92 ±0.5 [e]	19.00 ± 1.6 {e}(c) [d]
(K1)	Ag NPS of *S. lappa*	50	4.87±1.75 (a) [b]	75.13 ± 0.67 [c]	17.00±1.1 {e}(c) [e]
(K2)		100	3.83±1.71 (a) [e]	80.82 ±0.72 [e]	17.20 ±1.2 {e} (c) [e]
(K3)		150	2.93±1.70 (a) [d]	85.32 ±0.82 [e]	18.20 ± 3.5 {e} (c) [d]

Values are presented as mean ± SE (n = 5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. ^{a} =P ≤ 0.001, ^{b} =P≤0.01 ^{c} =P≤0.05, ^{d} =P≤0.1 ^{e} = P≤0.5.

The NPs treated groups highest survival time was observed for group J3 (19.0 ± 1.6), K3 (18.20 ± 3.5), I3 (17.75 ± 1.3), J2 (17.80 ±2.3), K2 (17.20 ±1.2), K1 (17.00±1.1), I2 (16.40±1.6), I1 (16.30 ± 1.0) and J1 (15.20 ±1.9). When these values were compared with the normal control group A, all the Ag NPs treated group showed non-significant difference ($P \leq 0.5$) except for JI where the difference was significant ($P \leq 0.05$). The survival time of all NPs treated groups revealed the significant difference ($P \leq 0.05$) when compared with the infected but not treated group B. The survival time of all Ag NPs treated groups were highly non-significant ($P \leq 0.5$) when compared to the chloroquine treated group C (Figure 4.9 a).

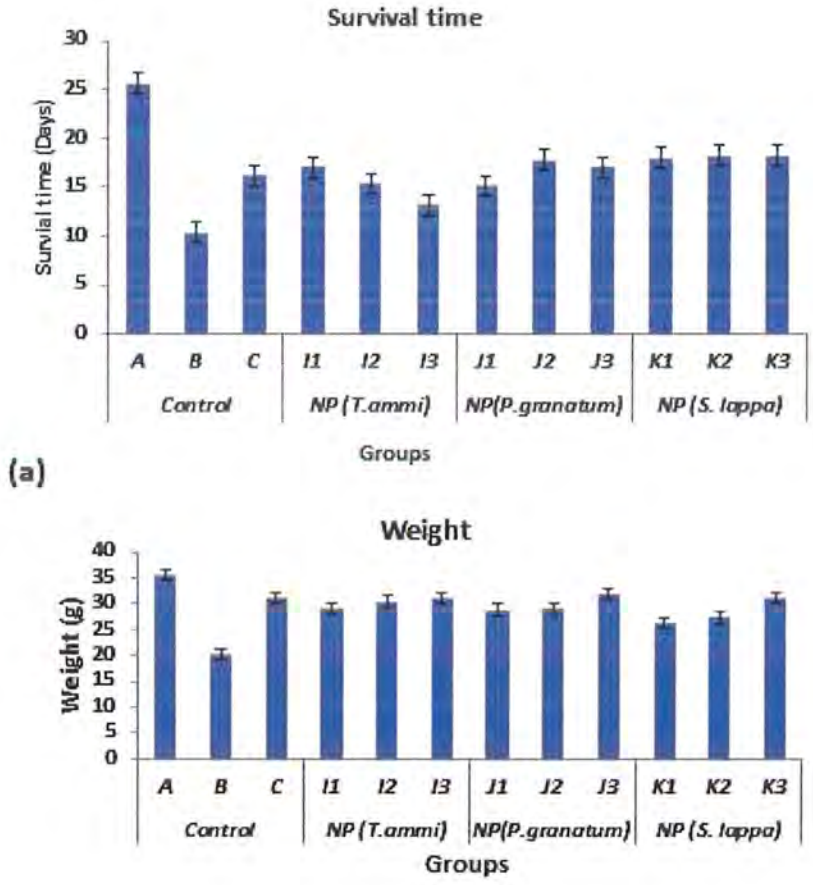


Figure 4.9: The figure shows the (a) Survival time (b) weight change of control and Ag NPs of different plant extracts treated groups.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, P = 0.08 for (a) and P= 0.125 for (b)

Weight analysis

There was a gradual increase in weight of the control group A (P=0.000) till the fifteenth day of the experiment, whereas a progressive decrease in body weight was observed in infected but treated with water group B although both groups differ significantly (P =0.006). But the change in weight between group A and group C treated with chloroquine was highly non-significant (P = 0.33). The all groups treated with the Ag NPs showed the non-significant difference (P≤0. 5) in weight change when compared with the normal control group A. The highest non-significant difference was observed for the I2, I3, J3, K2, and K3 groups. The groups treated with Ag NPs of *T. ammi*. *P. granatum* and *S. lappa* showed significant difference (P≤0.05) when compared with non-treated group B. However, percentage weight change for all Ag NPs was highly significant (P≤0.001) when compared to the infected but non-treated group B.

The change in weight among Ag NPs of *T. ammi* treated groups I1, I3, Ag NPs of *P. granatum*J1, J2 and J3 and Ag NPs of *S. lappa* at the dose of 50 mg/kg K1showed the non-significant difference (P≤0.1). The Ag NPs of *S. lappa* at the dose of 100 mg/kg (K2 group), and 1500 mg/kg (K3 group) and I2 group treated with the Ag NPs at 100 mg/kg and J2 group treated with the Ag NPs at 100 mg/kg indicated the highest non-significant difference (P≤0.5) when compared with the chloroquine treated group C. However percentage weight change for all Ag NPs treated groups were non-significant (P≤0.1 and P≤0.5) when compared with the normal control group (A) and chloroquine treated group (C) (Figure 2.6 d and 2.8 b).

The highest non-significant difference was observed for K3, J3 and I2. The treated groups showed a gradual loss in weight till the day third and some up to the day fourth as compared to the day first of infection after that a steady gain in weight occurred in the treated groups (Figure. 4.9 b) and (Table 4.4).

Table 4.4. Comparison of body weight and percentage weight change among Ag NPs treated groups Vs controls

Groups	Treatment	Body weight (g) Mean±S.E		Percentage weight change (%) Mean±S.E
		Day 0	Day 15	
A	No	35.83±0.764	40.31±0.153 [a][c]	12.51±1.02 [a][c]
B	Distilled water	33.90±0.265	19.87±3.87 [a][b]	-41.38±1.65 [a][a]
C	Chloroquine	34.40±0.107	37.60±0.393 [e][a]	9.30±0.85 [e][a]
I1	50 mg/kg Ag NPs of *T. ammi*	33.40 ±0.32	34.89± 0.48 [d] [c] [d]	4.46±0.34 [d] [a] [d]
I2	100 mg/kg Ag NPs of *T. ammi*	33.42 ±0.32	36.192 ±1.121 [e] [c] [e]	8.29±1.02 [d] [a] [e]
I3	150 mg/kg Ag NPs of *T. ammi*	32.85 ± 0.5	36.880 ± 1.11 [e] [c] [d]	12.26±1.67 [e] [b] [d]
J1	50 mg/kg Ag NPs of *P. granatum*	33.25±0.43	35.95 ± 1.24 [d] [c] [d]	8.12±0.92 [d] [a] [e]
J2	100 mg/kg Ag NPs of *P. granatum*	33.43±0.55	35.86 ± 1.98 [d] [c] [d]	7.26 ±0.87 [d] [a] [e]
J3	150 mg/kg Ag NPs of *P. granatum*	33.24± 0.471	36.172 ± 1.15 [e] [c] [e]	7.68 ±1.23 [d][a][e]
K1	50 mg/kg Ag NPs of *S. lappa*	32.83 ± 0.56	35.226 ± 1.6 [d] [c] [d]	7.28 ±0.85 [d] [a] [e]
K2	100 mg/kg Ag NPs of *S. lappa*	32.80 ± 0.43	36.388 ± 1.54 [e] [c] [e]	10.93±1.11 [d] [a] [e]
K3	150 mg/kg Ag NPs of *S. lappa*	33.49 ± 0.71	37.178 ± 1.32 [e] [c] [e]	10.98±0.91 [d] [a] [e]

Values are presented as mean ± SEM (n = 5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a] =P ≤ 0.001, [b] =P≤0.01 [c] =P≤0.05, [d] =P≤0.1 [e] = P≤0.5.

Hematological analysis

The **Red Blood Cell (RBC) count of** normal control group (A) when compared with infected but untreated group (B) and infected but treated with chloroquine group (C) showed highly significant (P=0.008) and less significant difference (P=0.033) respectively. The RBC in the I2 was higher that was treated with 100 mg/kg of Ag NPs of *T. ammi* than the I1 and I3 when compared with normal mice of group A and the difference was non-significant 0.232, 0.113 and 0.09 respectively. The non-significant difference (P≤0.5) was observed when the Ag NPs of *P. granatum* groups J2, J3 and J1 compared with normal mice of group A. The Ag NPs of *S. lappa* treated groups K1, K2 and K3 also depicted the non-significant difference (P≤0.5) compared with normal mice of group A. The highest non-significant difference was observed for the K3 than for and J2 and I2 in contrast to normal control group A.

There was the significant difference (P= 0.03) of RBC number of B and C group. The significant difference (P≤0.05) was observed for Ag NPs treated groups I1, I2, I3, J1, J2, J3 and K1. The Ag NPs of *S. lappa* treated groups K2 and K3that was treated at the dose of 100, and 150 mg/kg revealed the very highly significant difference (P≤0.01) when compared with infected but not treated group B.

The RBC count of chloroquine treated group C and Ag NPs treated groups of all plants indicated the non-significant difference (P≤0.1). Highest non-significant difference was observed for J2, J3, K1, and K3.It means that the RBC numbers in these treated groups was compatible with chloroquine treated group. The Red blood cells are high in K2 and K3 groups as compared A and C group.

The normal control group (A) showed significant difference (P=0.005) of **Hemoglobin** with infected but untreated group (B) and highly non-significant difference (P=0.332) with the group (C) treated with chloroquine. The Ag NPs treated groups of all plants showed the very high non-significant difference (P≤0.5) when compared with normal control group A.

The highest significant difference (P= 0.004) was noted for **Hemoglobin** between B and C group. The difference was highly significant (P≤0.01) for all NPs treated groups and Chloroquine treated group when compared with infected but non-treated group B.

The significant difference for Hemoglobin was detected for groups I1, I2, I3, J1, J2, J3, K1, K2 and K3 (P≤0.5). The highest non-significant difference was observed for I3, J3, K2 and K3.

The group (A) showed a highly significant difference (P=0.003) of **Hematocrit (packed cell volume)** with infected, but non-medicated group (B) and less significant difference (P=0.038) with the group (C) treated with Chloroquine. The Ag NPs treated groups I3, J2 and J3 showed the low non-significant difference (P≤0.1) and I1, I2, J1, K1, K2 and K3 indicated that there was highly non-significant (P≤0.5) as compared with the normal control group A.

There was a significant difference (P= 0.012) between B and C group. The Ag NPs treated groups I2, I3, J1, J2, K1, K2, and K3 revealed highly significant difference (P≤0.01) and I1 and J3 showed the low significant difference (P≤0.05) when compared with the infected but non treated group B.

There was the non-significant difference (P≤0.5) for **Hematocrit** between drug treated group C and Ag NPs of all experimented plant treated groups I1, I2, I3, J1, J2 , J3, K1, K2, and K3 groups.

The group (A) revealed highly significant difference (P=0.001) of **Mean cell volume (MCV)** with infected, but non medicated group (B) and significant difference (P=0.01) with the group (C) treated with Chloroquine. The Ag NPs treated groups of *T. ammi, P. granatum*, and *S. lappa* at all dose levels showed non-significant difference (P≤0.5) except for the J2 which depicted the significant difference as compared with the normal control group (A).

There was a highly significant difference (P= 0.005) between B and C group. The Ag NPs treated groups I1 I2 I3, J1 J2 J3 K1, K2 and K3 exhibited the high significant difference (P≤0.01) when compared with the infected but treated with water group B.

There was significant difference (P≤0.05) for MCV between drug treated group C and Ag NPs treated groups I1, I2, I3, J1, J3 K1, K2 and K3 except for J2 that showed the non-significant difference (P≤0.5).

The group (A) showed highly significant difference (P=0.000) of **Mean cell hematocrit (MCH)** with infected, but treated with water group (B) and significant

difference (P=0.066) with the group C treated with Chloroquine. The Ag NPs treated groups I1, I2, I3, J1, J2, J3, K1, K2, and K3 exhibited non-significant difference (P≤0.5) except the J2 that showed the significant difference (P≤0.05) when compared with normal group of mice (A). Highest significant difference was observed for I1, I3, J1, and K3.

There was a highly significant difference (P= 0.036) between B and C group. The Ag NPs treated groups I1, I3, J1, J3, K1, K2 and K3 revealed the low significant difference (P≤0.05) however I2 (P= 0.008) and J2 (P= 0.001) revealed the high significant difference when compared with the infected but treated with water group (B). The MCH value of drug treated group C and Ag NPs treated group I1, I3, J1, and K3 groups showed the non-significant difference (P≤0.1) and remaining Ag NPs treated groups I2, J2, J3, K1, K2 indicated the significant difference (P≤0.05).

The group (A) showed a highly significant difference of **Mean cell hematocrit concentration (MCHC)** with infected, but treated with water group (B) (P=0.02) and no significant difference (P=0.052) with the group (C) treated with Chloroquine. The Ag NPs treated I2, J1, J2, J3 and K2 groups showed high non-significant difference (P≤0.5) and I1, I3, K1 and K3 groups revealed the when compared with the normal control group (A).

There was a highly significant difference (P= 0.009) between B and C group. The Ag NPs treated groups I1, I2, J1, J3, K2 and K2 showed the low significant difference (P≤0.05) and remaining groups I3, J2, K1 and K3 showed the high significant difference (P≤0.01) of MCHC when compared with the infected but treated with water group (B).

There was non-significant difference (P≤0.5) for MCHC between drug treated group C and Ag NPs treated groups I1, J1, J2, J3 and K1 groups remaining Ag NPs treated groups I2, I3, K2 and K3 revealed the significant difference (P≤0.05).

The group (A) showed the highly significant difference (P=0.001) of **Platelets** with infected, but treated with water group (B) and significant difference (P=0.009) with the group (C) treated with Chloroquine. The Ag NPs treated groups I1, I2, I3 J1, J3, K2, and K3 showed the high non-significant difference (P≤0.5) except the J2 and K1 group which indicated the significant difference (P≤0.05) when compared with the normal control group (A).

There was highly significant difference(P= 0.003) between B and C group, Ag NPs treated groups I2, I3, J2, J3, K1, K2, and K3 exhibited highly significant difference (P≤0.01) and remaining Ag NPs treated I1, J1, indicated the low significant difference (P≤0.05) when compared with the infected but treated with water group (B).

There was significant difference (P≤0.05) between the drug treated C group and Ag NPs of three experimented plants treated groups I1, I2, I3, J1, J3, K2 and K3 groups only J2 and K1 showed the non-significant difference (P≤0.5) when there their Platelets count was compared with the drug treated group C.

The group (A) showed a highly significant difference of **neutrophils** with infected, but treated with water group (B) (P=0.008) and non-significant difference (P=0.263) with the group (C) treated with Chloroquine. The Ag NPs treated groups of *T. ammi, P. granatum,* and *S. lappa* I1, I2, I3, J1, J2, J3, K1, K2 and K3 indicated the non-significant level (P≤0.5) when compared with the normal control group (A). The highest non-significant difference was observed for I3, J2, K2 and K3.

There was a significant difference (P= 0.034) among neutrophils of infected but non treated group B and drug treated group C. The all Ag NPs treated groups three experimented plants of also indicated the significant difference (P≤0.01) when compared with infected but non treated group B. There was high non-significant difference (P≤0.5) in the neutrophils of Chloroquine treated C and all Ag NPs of three experimented plants treated groups.

The group (A) showed the highly significant difference (P=0.006) of **lymphocytes** with infected but treated with water group (B) and non-significant difference (P=0.694) with the group (C) treated with Chloroquine. The Silver NPs treated groups I1, I2, I3, J1, J2, J3, K1, K2 and K3 also showed the non-significant difference (P≤0.5) when compared with the normal control group A.

There was a significant difference among the lymphocytes of group B and C (P≤0.05) as well as Ag NPs of all experimented treated groups.

There was non- significant difference (P≤0.5) between drug treated group C and Ag NPs treated group I1, I2, I3, J1, J2, J3, K1, K2, and K3 groups (Table 4.5).

Biochemical analysis

The liver function test was done by determining the level of ALT, ALP and AST of all treated groups and control groups. The ALT level was increased in infected but non-treated group B as compared to the normal control group A (P = 0.005). The Ag NPs of *T. ammi*, and *S. lappa* at the dose of 100 mg/kg (I2 and K2 group) and 150 mg/kg (K3) showed the non-significant difference (P≤0.5) and remaining Ag NPs of *T. ammi*, *P. granatum* and *S. lappa* treated groups, i.e. I1, I3, J1, J2, J3, K1 showed significant difference (P≤0.05) when compared to the normal group A.

There was a high Significant difference (P≤0.001) in the in the ALT range between Ag NPs treated groups of all experimented plants at the dose of 1000 and 1500 mg/kg however the dose of 50 mg/kg of these plants showed the low significant difference (P≤0.05) when compared to the infected but non treated group B.

The Ag NPs of *T. ammi and P. granatum* treated at the dose of 50 mg/kg (I1 and J1), 150 mg/kg (I3 and J3), and *S. lappa* treated at the dose of 50 mg/kg (K1) revealed a significant difference (P≤0.05) however the Ag NPs of *S. lappa* treated group at the dose of 100 mg/kg and 150 mg/kg and *P. granatum* and *T. ammi* at the dose of 100 mg/kg exhibited non-significant difference (P≤0.5) when compared chloroquine treated group C.

Table 4.5: Comparison of Hematological parameters of Ag NPs Treated groups VS controls

Haem-para	Groups											
	A	B	C	I1	I2	I3	J1	J2	J3	K1	K2	K3
RBCs	8.51±0.4 [c][c]	3.97±0.16 [c][b]	6.0±0.48 [c](b)	6.82±0.21 [e](c)[d]	7.03±0.29 [e](c)[d]	6.76 ±0.33 (d)(c)[d]	6.93±0.08 [e](c)[d]	6.28±0.43 [e](c)[e]	6.503 ±0.34 (e)(c)[e]	6.03±0.28 [e](b)[e]	7.20±0.55 [e](b)[d]	7.03±0.29 [e](b)[e]
HB	11.3±0.50 [b][e]	5.43±035 [b][a]	10.5±0.9 [e](a)	11.5±0.28 [e](c)[e]	11.53±0.49 [e](b)[d]	11.03±0.29 [e](b)[e]	11.36±0.44 [e](b)[e]	11.5±0.57 [d](b)[e]	10.43 ±0.34 (d)(c)[e]	11.21±0.59 (e)(b)[d]	10.4±0.305 [e](b)[e]	11.26± 0.64 (e)(b)[d]
PCV	38.50 0.55 [b][d]	26.87 ±1.1 [b][c]	34.53±0.72 [d](c)	36.7±0.25 [e](c)[e]	37.2±0.47 [e](b)[e]	36.03±0.60 [d](b)[e]	36.167±0.56 [e](b)[e]	35.8 ± 0.69 [d](b)[e]	33.33 ±0.16 [e](c)[e]	30.66 ±0.88 [e](b)[e]	38.23 ±0.64 [e](b)[d]	37.53±0.7 [e](b)[e]
MCV	53.43± 0.62 [a][c]	43.23± 0.66 [a][a]	48.07± 1.10 [c](a)	53.4±0.68 [e](a)[e]	54.83±0.75 [e](b)[c]	54.7 ±0.88 (c)(b)[b]	54.23±0.53 [e](a)[c]	46.63±0.66 [c](c)[e]	50.63 ±0.43 (e)(b)[e]	52.23±0.75 (e)(b)[e]	54.83±0.76 (e)(a)[b]	56.5 ±0.90 (e)(a)[b]
MCH	16.9±0.2 [a][b]	12.9±0.20 [a][b]	15.23±0.41 [b](b)	17.1±0.58 [e](c)[d]	18.2±0.43 [d](b)[c]	17.03±0.57 [e](c)[d]	17.2±0.47 [e](c)[d]	18.73 ±0.37 (e)(a)[b]	17.26±0.53 (e)(c)[d]	17.93 ±0.49 (e)(c)[e]	18.1 ± 0.51 (d)(c)[e]	17.2 ± 0.47 (e)(c)[d]
MCHC	32.13±0.94 [a][e]	26.16±1.48 [a][a]	31.86±0.95 [c](a	32.63±0.63 [e](c)[e	34.2± 0.72 (e)(c)[e]	35.03± 0.60 (d)(b)[b]	33.8±0.60 [e](c)[d]	32.8 ± 0.41 (e)(b)[e]	32.7 ± 0.83 (e)(c)[e]	32.06±0.51 (e)(b)[e]	35.26± 0.53 (d)(c)[e]	35.5 ± 0.76 (d)(b)[e]
PLATELETS	325000±27.9 [a][b]	178000±18.1 [a][b]	27400± 23.6 [b](b)	30500±19.8 [e](c)[e]	306000±11.1 [e](b)[e	319000±17.82 [e](a)[b]	306000±13.22 [e](c)[e]	299000±14.89 [c](b)[e]	336000±11.03 [e](b)[e]	286000±11.79 [b](b)[e]	326000 ± 16.8 [e](a)[e	339000±15.4 [e](a)[b]
NEUTRO-PHILS	24±0.882 [b][e]	33±1.5 [b][b]	26±1.2 [e](b)	22.33±0.45 [e](b)[e]	21.33±0.88 [e](b)[d]	20.33±0.88 [d](b)[e]	25.66±0.88 (e)(b)[e]	24.01 ±0.25 (e)(b)[e]	22.33±0.88 [e](b)[d]	28.333±0.33 (d)(c)[e]	24.33±0.78 (e)(b)[e]	23.66 ±0.20 (e)(b)[e]
LYMPH-OCYTES	65±1.1 [b][e]	83±071 [b][b]	66±1.23 (e)(b)	63.33±0.12 [e](c)[e]	64 ± 1.57 (d)(b)[e]	63.5 ± 1.15 (e)(c)[e]	65.667±1.72 (e)(c)[e]	70.333 ±1.9 (e)(c)[d]	66.33±1.342 (e)(c)[e]	69.667±1.33 (e)(c)[e]	68.667 ± 1.52 (d)(b)[e]	67.333±1.56 (e)(c)[e]

Difference between the **ALP** level was highly significant (P = P=0.007, 0.002) in the A and C group as compared to the B. The Ag NPs treated groups I1, J1 showed significant difference (P≤0.05) and only I2, I3, J2, J3 K1, K2 and K3 indicated non-significant difference (P≤0.5) when compared to the normal group A. All Ag NPs treated groups of *T. ammi, P. granatum,* and *S. lappa* showed significant difference (P≤0.05) when compared with the non-treated group B. It was highly significant for I2, I3, J1, K1, and K3 and only significant for the remaining groups.

The Ag NPs of *T. ammi* treated with 50 mg/kg (I1), and *P. granatum* treated at the dose of 100 and 150 mg/kg (J2 and J3) *S. lappa* treated with at the dose of 50, 100 mg/kg (K1 and K2) indicated the high significant difference (P≤0.01) the remaining groups of Ag NPs of three experimented plants i.e. I1, I3, J1 and K3 depicted the highly non-significant difference (P≥0.05) when compared to the chloroquine treated group C.

The elevated level of AST, was observed in infected but non-medicated group B as compared to the normal control group A and chloroquine treated group C. Statistically it was highly significant (P = 0.002, and 0.000). The AST level of Ag NPs of all three experimented plants revealed the significant difference (P≤0.05) when compared to normal group A and highly significant difference (P≤0.1) when compared with the non- treated group B. There was a highly non-significant difference (P≤0.5) for I1, I2, I3, J1, J2, J3, K1, K2 and K3 respectively when compared to the chloroquine treated group C (Figure 4.10 a)

Kidney function test was determined by **Creatinine** and **uric acid** level. There was the Increased levels of Creatinine in the non-treated group B as compared to the normal group A (P=0.001) and drug treated group C (P= 0.002). the Ag NPs treated groups I2, I3, J2 K2 and K3 revealed non-significant difference (P≤0.1) and remaining groups I1, J1, J3, K1 and K3 showed significant difference (P≤0.01) when compared to the normal group A.

The groups treated with Ag NPs of all three experimented plants showed the highly significant difference in Creatinine level when compared with non-treated group B (P≤0.001). The Ag NPs s of *T. ammi, P. granatum* and *S. lappa* treated group I1, I2, I3, J2, K1, K2 and K3 Showed non-significant difference (P≤0.5). However the

groups J1 and J3 differ significantly (P≤0.05) when compared to the chloroquine treated group C (Figure 4.10 b).

Uric acid level in the non-treated group B was significantly high (P= 0.018) and (P= 0.002) as compared normal group A and drug treated group C respectively. There was the highest non-significant difference (P≤0.5) for the groups treated with Ag NPs i.e. I2, J2, J3 and K3 and significant difference (P≤0.1) for the I3 and K2 group. But the remaining groups I1, J1, K1, and K3 differ significantly (P≤0.05) when compared to the normal group A (Figure 4.10 c).

The all Ag NPs treated groups of three experimented plants differed significantly (P≤0.01) when compared to the infected but non treated group B. There was non-significant difference (P≤0.5) between the treated groups I1, I2, I3, J3, K1, K2 and K3. But J1 and J2 showed significant difference (P≤0.01) when compared to the drug treated group C (Figure 4.10 c and Table 4.6).

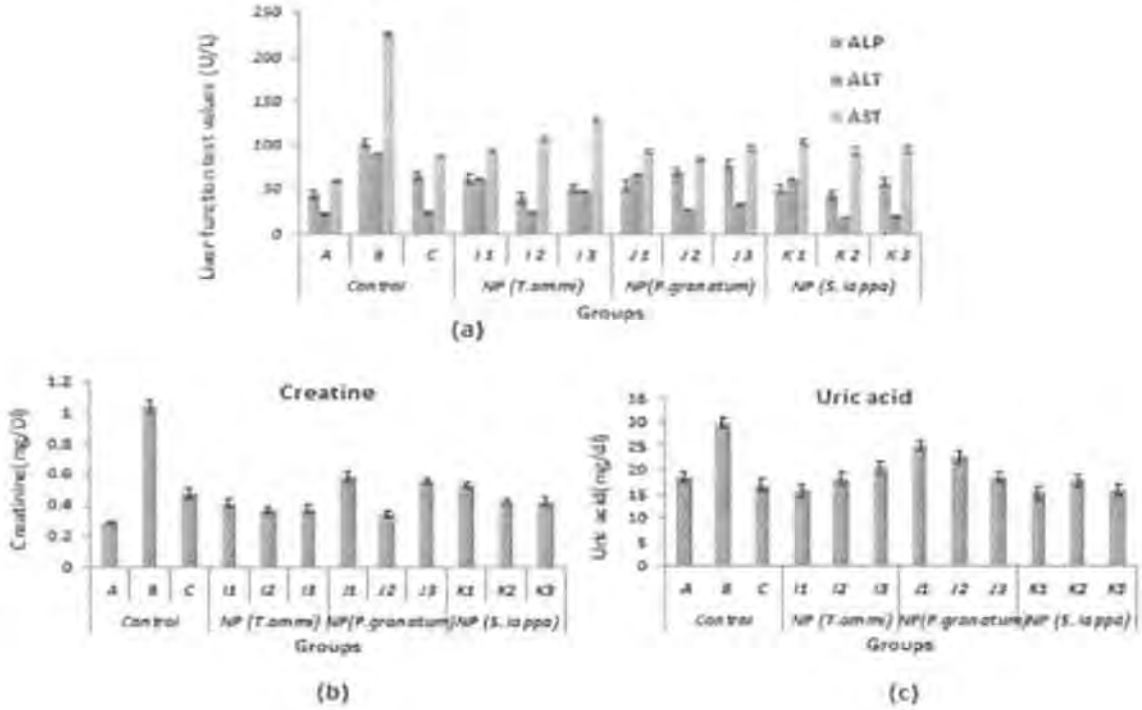


Figure 4.10: Plasma concentrations of (a) ALP, ALT, AST, (b) Creatinine and (c) Uric acid of control and Ag NPs of different plant extracts treated groups.

A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is shown as Mean ± SE with N=5. P = 0.06 for (b) and P= 0.08 for (c)

Table 4.6: Comparison of liver and kidney function test of Ag NPs treated groups VS control

Treatment	Dose mg/kg	Liver function test (U/L)			Kidney function test (mg/dl)	
		ALT	ALP	AST	Creatinine	Uric acid
Normal Control	-	20.36±1.01 (b)[e]	43.63±2.73 (b)[b]	57.59 ±1.36 (a)[a]	0.29±0.02 (a)[a]	18.5±0.81 (b)[c]
Infected control	-	89.62±2.61 {b}[a]	108.35±2.3 {b}[b]	250.15± 3.17 {a}[a]	1.04±0.04 {a}[b]	29.78±0.44 {b}[b]
Chloroquine control	5	23.56±2.3 {e}(a)	62.84±2.03 {b}(b)	91.66 ± 2.9 {d}(b)	0.48±0.03 {a}(b)	16.84± 0.59 {e}(b)
T. ammi	**50**	62.9±2.5 {b}(c)[c]	61.81±1.15 {c}(c)[e]	93.41 ±2.84 {c}(b)[e]	0.42±0.02 {b}(a)[e]	14.67±0.71 {c}(b)[c]
	100	24.44± 2.31 {d}(a)[e]	40.90±2.73 {e }(b)[b]	108.5 ±3.5 {{c}(b)[e]	0.376 ±0.025 {d}(a)[e]	18.20±1.74 {e}(b)[e]
	150	48.30±2.00 {b}(a)[b]	51.81± 2.21 {e}(b)[d]	128.33± 2.59 {b}(b)[d]	0.373± 0.021 {{d}(a)[e]	20.44±1.13 {d}(b)[e]
P. granatum	**50**	67.51± 1.62 {b}(c)[c]	54.54± 2.73 {e}(b)[d]	93.12± 3.04 {e}(b)[e]	0.586± 0.0321 {{b}(a)[c]	25.00± 1.62 {e}(c)[c]
	100	25.897± 0.508 {e}(a)[e]	70.90± 2.46 {c}(c)[b]	84.39± 2.11 {e}(b)[e]	0.3467±0.020 {d}(a)[e]	22.72± 1.17 {e}(c)[c]
	150	33.75± 1.96 {b}(a)[c]	78.17± 2.33 {{b}(c)[b]	97.19 ± 2.53 {c}(b)[e]	0.560± 0.017 {{b}(a)[c]0	18.45± 0.66 {e}(b)[e]
S. lappa	**50**	62.85± 1.67 {b}(c)[c]	50.90± 2.86 {e}(b)[c]	103.86± 2.72 {{c}(b)[e]	0.543 ± 0.040 {{b}(a)[e]]	15.37± 0.94 {{c}(b)[e]
	100	18.33 ± 2.31 {e}(a)[e]	44.90±2.07 {e}(a)[b]	93.03± 1.20 {{c}(b)[e]	0.433± 0.092 {{d}(a)[e]	17.95± 0.63 {d}(b)[e]
	150	20.08± 2.31 {e}(a)[e]	58.17± 2.15 {{e}(b)[e]]	96.61± 1.00 {c}(b)[e]	0.436± 0.035 {d}(a)[c]	15.87± 1.17 {{c}(b)[e]

Values are presented as mean ± SEM (n = 5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. ^{a} =P ≤ 0.001, ^{b} =P≤0.01 ^{c} =P≤0.05, ^{d} =P≤0.1 ^{e} = P≤0.5.

Postmortem Examinations of organs

The postmortem examinations of the internal organs like liver, spleen, lungs and the kidneys of the malaria-infected and Ag NPs treated groups could be observed by (Table 4.7, Figure 4.11 and 4.12)

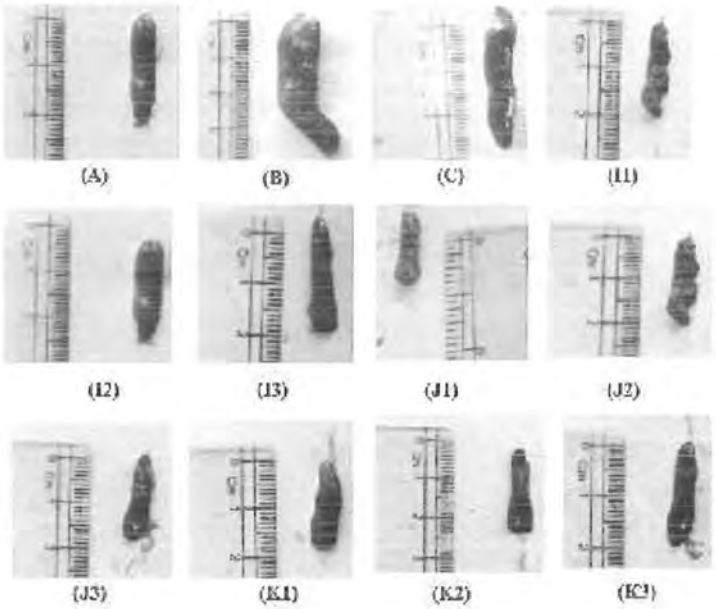


Figure 4.11: Post mortem observations of spleen of control and Ag NPs treated groups

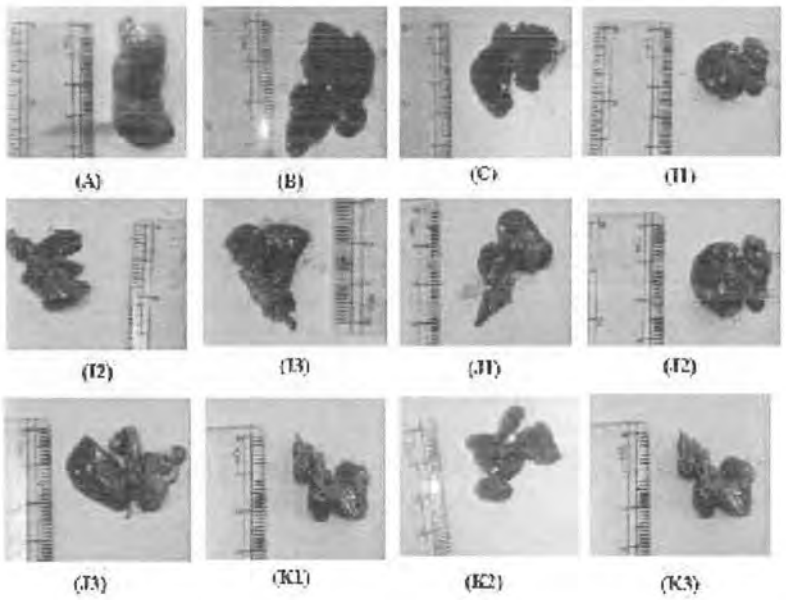


Figure 4.12: Post mortem observations of liver of control and Ag NPs treated groups

Table 4.7: Post mortem observations of major organs in Ag NPs treated groups' VS Controls

Observation	A	B	C	I1	I2	I3	J1	J2	J3	K1	K2	K3
Enlargement of the size of spleen (splenomegaly)	–	+++	+	–	+	+	–	–	–	+	–	+
Discoloration of the spleen	–	++	+++	–	–	+	+	+	–	+	–	++
Enlargement of the size (hepatomegaly)	–	++	+	+	+	+	+	+	+	–	–	–
Discoloration of the liver	–	++	+++	++	–	+	+	–	+	–	+	–
Discoloration of the kidneys	–	+++	++	–	–	+	+	–	+	+	–	+
Discoloration of the lungs	–	+++	+	+	–	+	–	+	+	+	–	+

Indicator: (-) absent; (+): mild; (++): moderate; (+++): sever

Histopathological observations

The histopathological studies revealed the marked differences between the control and NPs treated groups. The sequestrations of Parasitized RBCs in the microvasculature were observed in the liver, spleen and kidney of non-treated group as compared to the NPs and chloroquine treated groups. The liver of infected but non-treated mice atrophy of hepatocytes, hypertrophy and hyperplasia of the kupffer cells was observed. Exoerythrocytic forms in liver in the malarial infected but non-treated was obvious.

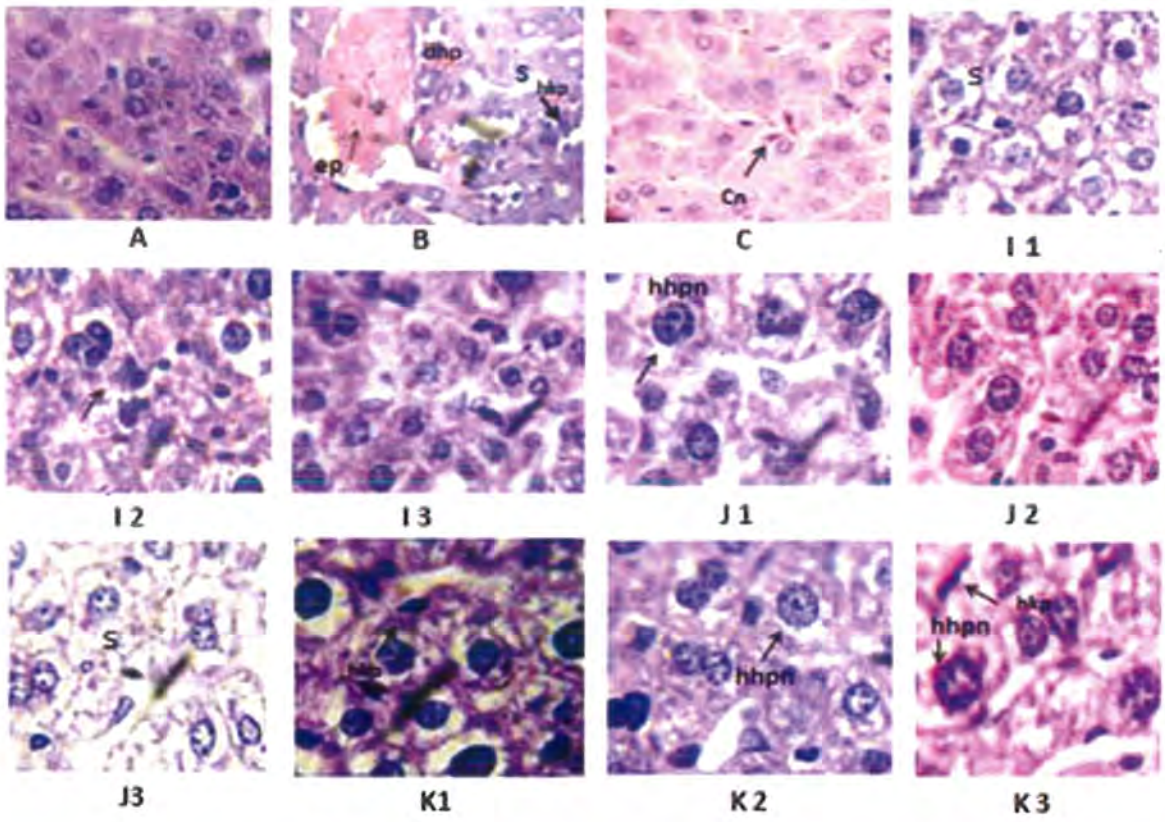


Figure 4.13: The histological study of Liver of experimental animals treated with Ag NPs VS Controls.

The liver of malaria-infected mice, exoerythrocytic form of *P. berghei* was observed in B (ep), hyperplasia and hypertrophy of kupffer cells (hkc) and vacuolar degeneration, atrophy of hepatocytes (dhp) and wide sinusoidal space (s) as compared to normal A and other treated groups. Hyperplasia and hypertrophy of some hepatocytes and its nucleus (hhpn) as well as hypertrophy of some Kuffers cells (hkp) were also observed in I1, K1, and K3. Atrophy of hepatocytes was observed in I1, I2, J1 and J3 group. Viewed under light microscopy at 10 x100 X magnifications.

The hyperplasia and hypertrophy of some kupffer cells were also observed in I1, K1, and K3. The atrophy of hepatocytes was observed in I1, I2, J1 and J3 group (Figure 4.13). The Malaria pigments **haemozoin** was seen in the pulp histiocytes and sinusoidal lining cells and in the microvasculature of spleen tissue. Congestion of blood vessels was also observed in the spleen of infected but non treated group as compared to the Ag NPs treated groups. The Hemozoin was also observed in the I1, I2, K2 and K3 (Figure 4. 14).

The kidney tissues were presented with abundant of sequestrations of Parasitized RBC and haemozoin in the microvasculature and interstitium Congestions were seen in the infected, but non treated group as compared to the Ag NPs treated group (Figure 4.15).

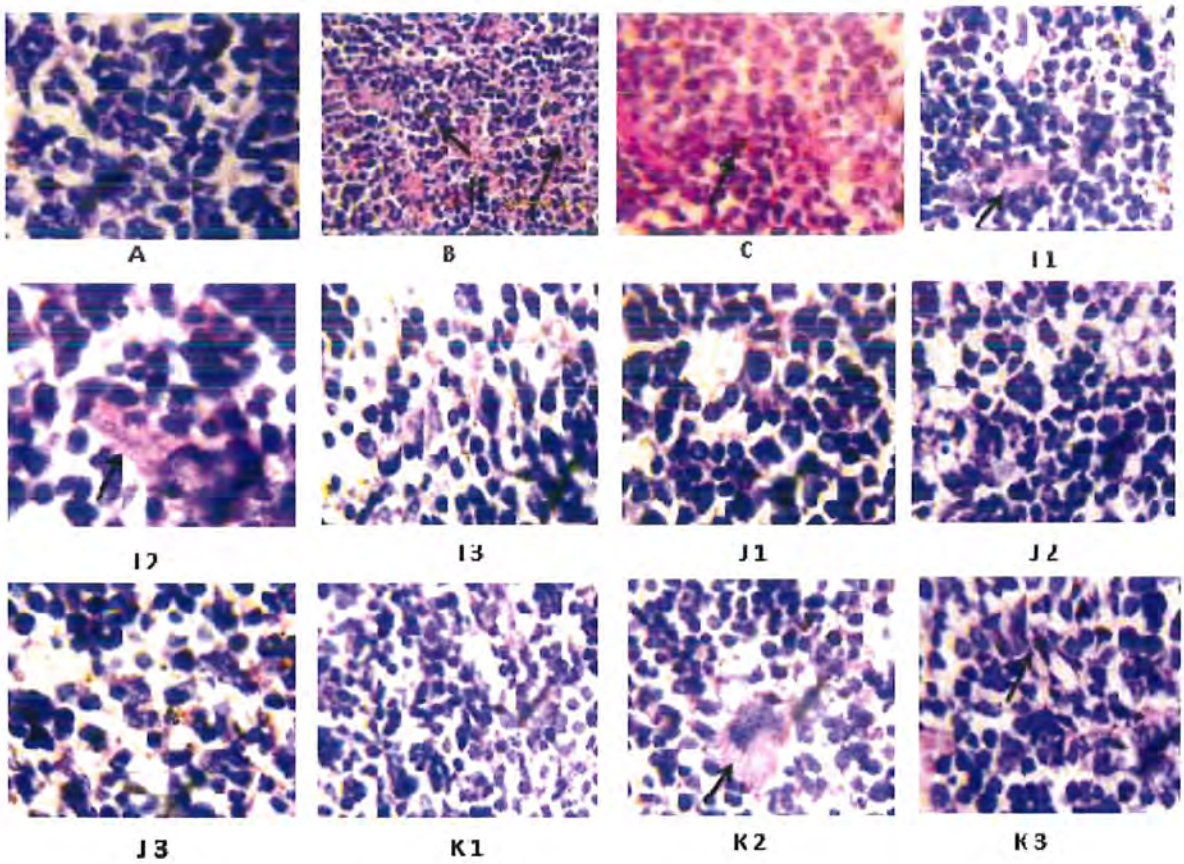


Figure 4.14: The histological study of the spleen of experimental animals treated with AgNPs VS Controls. The Accumulation of more malarial pigments, haemozoin, was more in the pulp histiocytes and sinusoidal lining cells of the spleen in the B group (i) as compared to a normal control group A and other treated group. Hemozoin was also observed in the I1, I2, K2 and K3. Microvascular sequestrations of PRBCs were also present B (ii) and a little bit in I1, J1, J3 and K3. Viewed under light microscopy at 10 x 100 magnifications

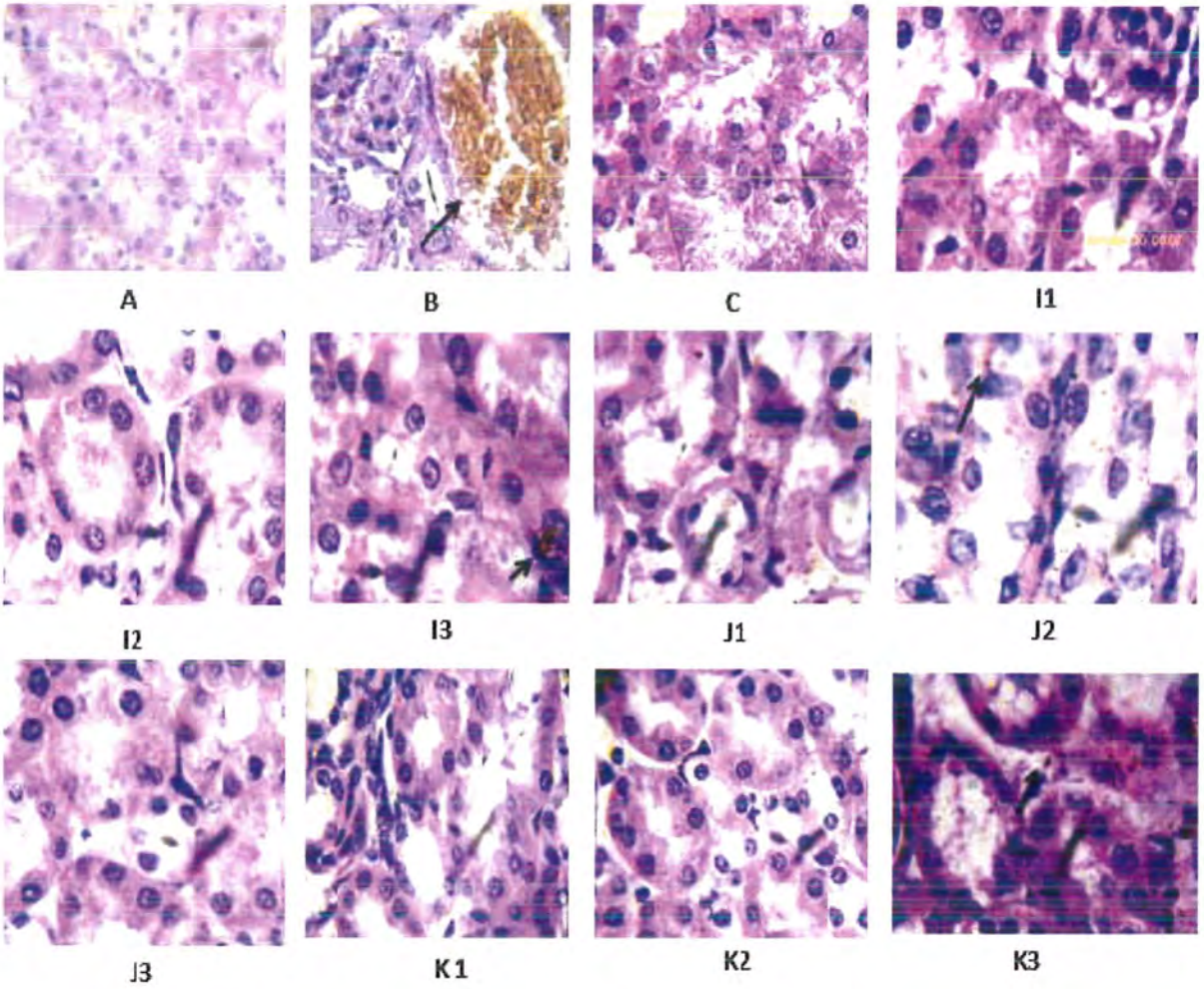


Figure 4.15: The histological study of kidney of experimental animals treated with Ag NPs VS Controls.

The kidney from infected mice was presented with widespread sequestration of PRBCs, Haemorrhages and congestion of blood vessels (B), as compared to the to normal control group A and other treated groups. Viewed under light microscopy at 10 x100 X magnifications

Results

ii) Iron nanoparticles (Fe NPs)

Synthesis of iron nanoparticles

When the aqueous seeds extract of *T. ammi, P. granatum and S. lappa* was mixed with the 0.5 M solution of $FeCl_3.6H_2O$ the color of the plant seed extracts was changed from brown and yellow to black and dark green as described in the (Figure 4.16 and Table 4.8).

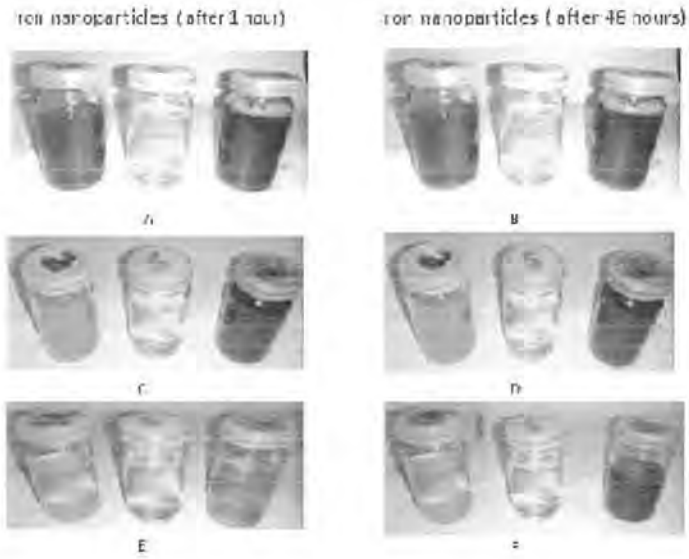


Figure 4.16: Physical observation of green synthesis of Fe NPs using aqueous seed extracts of: *T. ammi*, *P. granatum* and *S. lappa*.

A, C, E at 1st hours and B, C, and D after twenty four hours of synthesis. Bright Yellow solutions in the middle of each pic indicate the solution of $FeCl_3.6H_2O$. The brown and turbid yellow color solutions indicate the plant extracts and black and dark green solutions indicate the formation of NPs.

pH observation of iron nanoparticles

The pH of synthesized Fe NPs of three experimented plants was observed to decrease and move towards the acidic range. The malachite green was degraded by the synthesized by green synthesized Fe NPs (Figure 4.17 and Table 4.2).

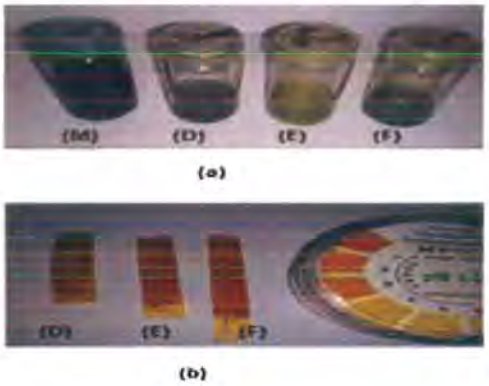


Figure 4.17: The figure shows the (a) Degradation of malachite green by the Fe NPs, (a) pH analysis of Fe NPs of (D) *T. ammi,* (E) *P. granatum* and (F) *S. lappa.* (M) Shows the malachite green

Table 4.8: Indication of color and pH change of green synthesized Fe NPs

Iron Nanoparticle	Color change		Color intensity Time	Time	pH Change	
	Before	After			Before reduction	After reduction
Fe NPs of *T. ammi*	Brown	Black	+++	Immediate	6.00	1.00
Fe NPs of *P. granatum*	Turbid yellow	Black	+++	Immediate	5.00	2.00
Fe NPs of *S. lappa*	Bright yellow	Dark Green	++	After24 hours	5.00	2.00

Characterization of iron NPs

UV–Vis spectrophotometer analysis

The formation and stability of Fe NPs in aqueous solution were confirmed by UV–vis spectrophotometer analysis. The absorbance of the solution was measured after twenty four hours. The maximum absorption peak of Fe NPs of *T. ammi, P. granatum and S. lappa* were observed at 402 nm. The absorbance of iron NPs was decreased as the wave length increased. The absorbance was recorded in quartz cuvette using UV-spectrophotometer (Mecasys Optizen 3220) with $FeCl_3.6H_20$ solution as standard (Figure 4.18).

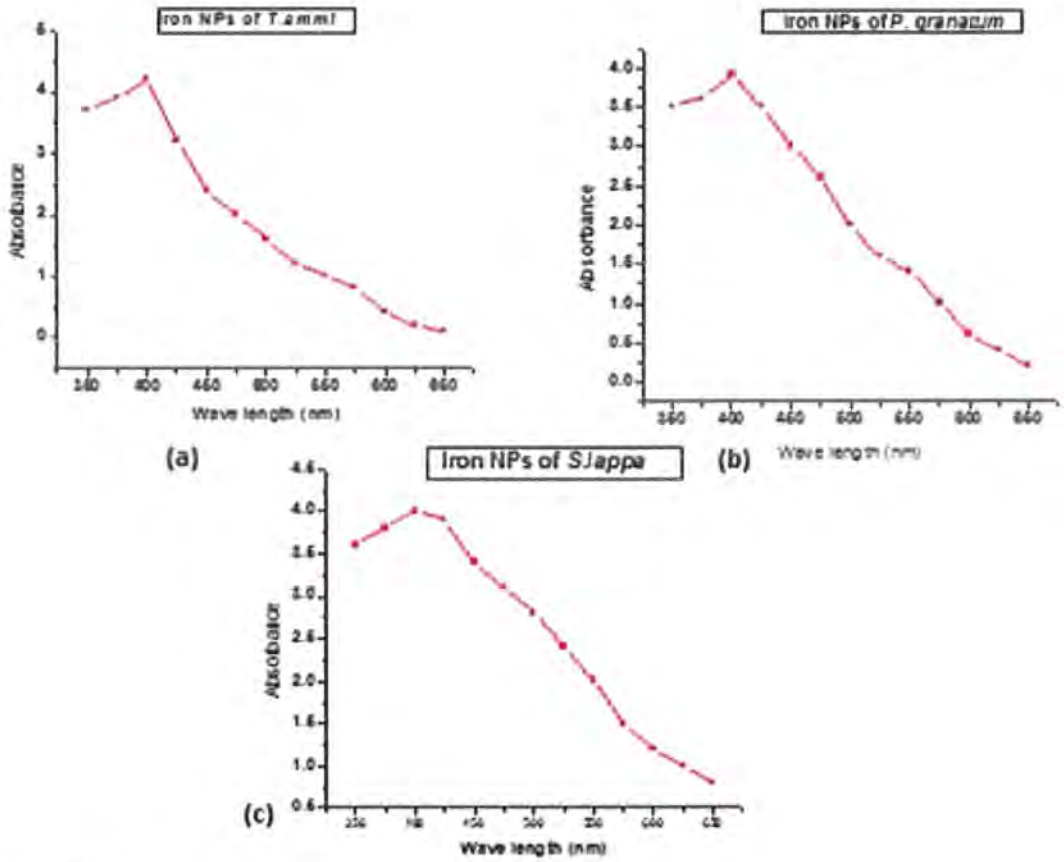


Figure 4.18: UV-

vis absorption spectra of aqueous $FeCl_3.6H_2O$ solution with aqueous seed extracts of *(a) T. ammi (b) P. granatum and (c) S. lappa*

Fourier transformed infrared spectroscopy

The FTIR studies were done in order to find out the possible organic molecules in the seed extract which may be responsible for synthesis and confirmation of Fe NPs. The FTIR spectrum of Fe NPs of *T. ammi, P. granatum,* and *S. lappa* showed the presence of many functional compounds (Figure 4.19). The detailed of a band stretching and bending of biomolecules are given in the (Table 4.9). The FTIR spectrum of Fe NPs suggested that Fe NPs were surrounded by different organic molecules such as, alcohols, ketones, aldehydes and carboxylic acids etc.

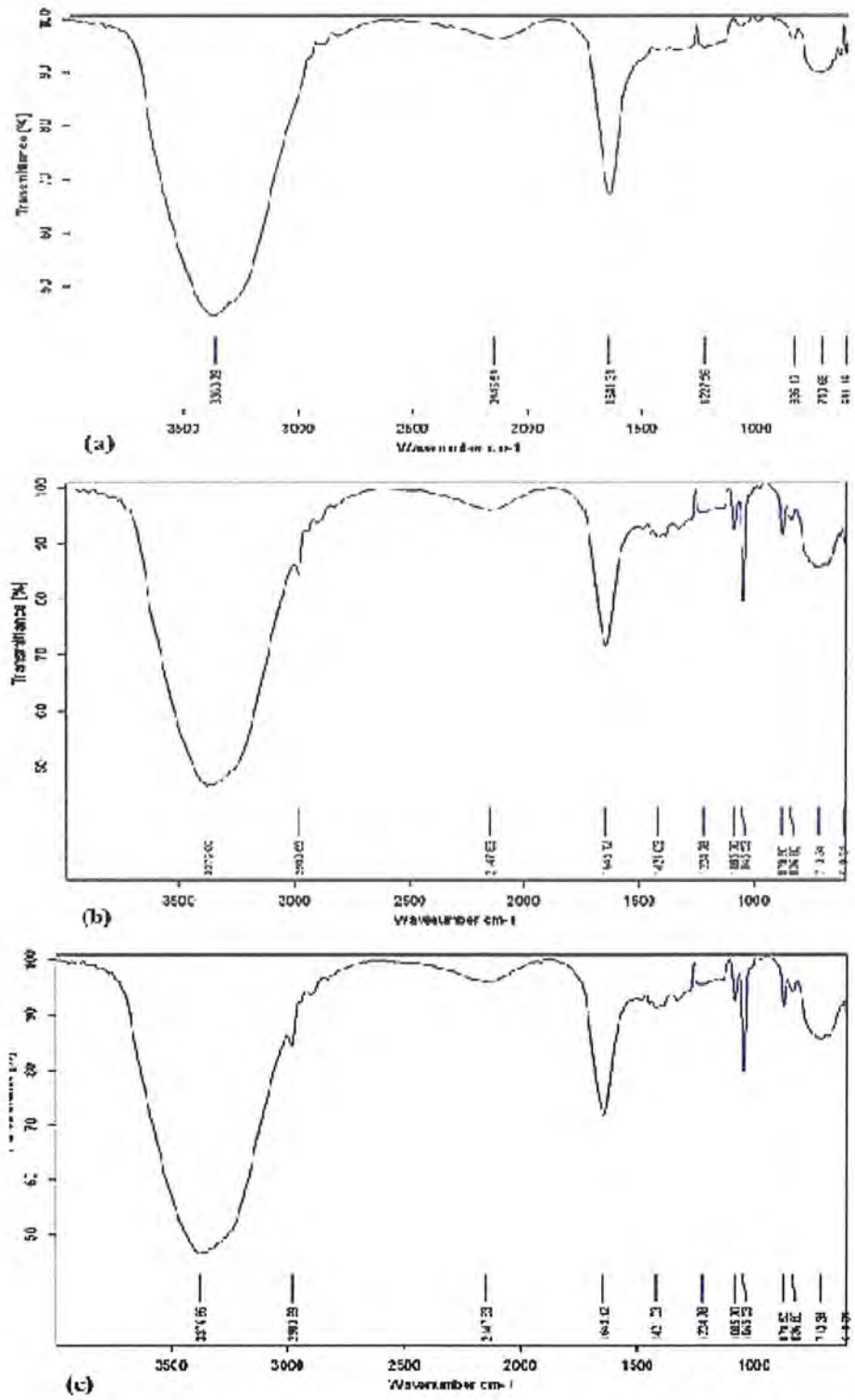


Figure 4.19: FTIR analysis of Iron nanoparticles of (a) *T. ammi,* (b) *P. granatum, and* (c) *S. lappa*

Table 4.9: FTIR analysis of possible organic compound on the surface of Fe NPs of *T. ammi, P. granatum, and S. lappa*

Sr no	IR spectrum peak of Fe NP of *T. ammi*	IR spectrum peak of Fe NP of *P. granatum*	IR spectrum peak of Fe NP of *S. lappa*	Types of stretching	Functional groups
1	611.18 (S)	610.31 (S)	-	C≡C-H:C-H	Alkynes
2	710.68 (S)	713.34 (S)	716.18 (S)	C≡C-H:C-H	Alkynes
3	836.17 (S)	836.80 (B)	839.01 (B)	C-C-H	Aromatics
4	-	878.50 (S)	878.57 (S)		Aromatics
5	-	1045.53 (B)	1045.49 (B)	C-N	Aliphatic amines
6	-	1085.70 (S)	1085.86 (S)	C-N	Aliphatic amines
7	1227.56 (S)	1224.78 (S)	1235.71(S)	C-O-C	Ether
8	-	1421.03 (S)	1420.38 (S)	C-C (Ring)	Aromatics
9	1641.31(S)	1645.12(S)	1645.97(S)	N-H	Primary amines
10	2146.51 (S)	2147.83 (S)	2146.60 (S)	C≡C	Alkynes
11	-	2983.89 (S)	2983.25 (S)	C-H	Alkanes
12	3360.79 (S)	3376.66 (S)	3361.67 (S)	O-H	Phenol/Alcohol

- B= Bends, S= Stretch

X-Ray Diffraction Analysis

The crystal structure and chemical nature of the Fe NPs can be studied through the XRD. The XRD analysis of *T. ammi* showed the broad peak at 30.96 °, 35.56 °, 43.23 ° and 54.10 ° which indicated the crystallographic planes of (200), (311), (511) and (434) respectively. The XRD investigation of *P. granatum* showed the broad peak at 30.40°, 35.02°, 43.09°, 54.10° and 57.18 which indicated the crystallographic planes of (200), (311), (511) and (440) respectively. The X-ray pattern of *S. lappa* showed the broad peak at 30.26°, 35.00°, 42.95°, 53.96°, and 57.30° which showed

crystallographic planes of (200), (311), (511) and (440) respectively. The XRD analysis showed the number of Bragg's reflections that may be indexed on the basis of the cubic structure of Fe NPs. The mean particle diameter of Fe NPs was calculated from the XRD pattern in order to verify the results of UV-vis spectral analysis, according to the line width of the plane, refraction peak, using the Scherrer equation.

The size of Fe NPs of *T. ammi, P. granatum* and *S. lappa* were calculated as 12.34 nm, 10.12 nm, and 9.89 nm respectively. The XRD pattern thus clearly demonstrated that the Fe NPs formed were crystalline in nature. The Bragg peaks were the representative of the cubic structure of Fe NPs; additional unassigned peaks were also observed, suggesting that the crystallization of bioorganic phase occurred on the surface of the Fe NPs (Figure 4.20).

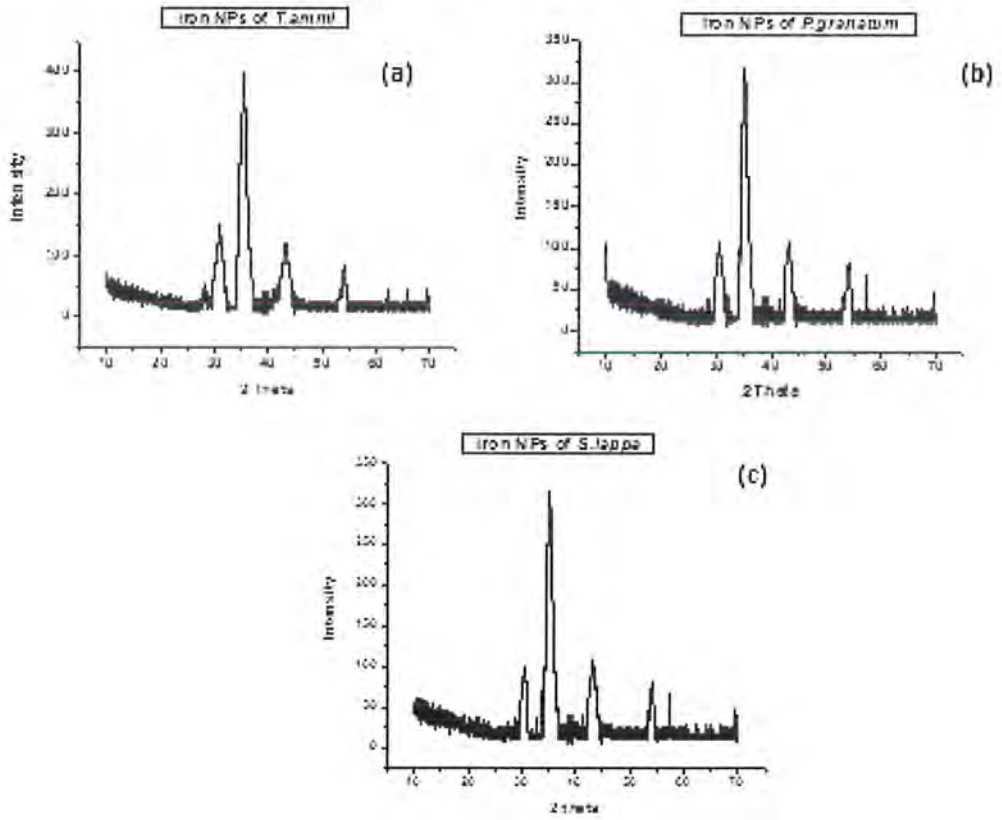


Figure 4.20: XRD analysis of biosynthesized Iron nanoparticle *T. ammi* (a), *P. granatum* (b), and *S. lappa* (c)

Transmission electron microscopy (TEMs)

The TEM image showing the high density Fe NPs synthesized by the seeds extracts of *T. ammi, P. granatum* and *S. lappa* further confirmed the development of Fe NPs by the seed extracts. The TEM showed that the average particles size of Fe NPs of *T. ammi, P. granatum,* and *S. lappa* were 12, 10 and 9 nm respectively and particles were stable as single crystal (Figure 4.21). The shapes of the Fe NPs were spherical in shape.

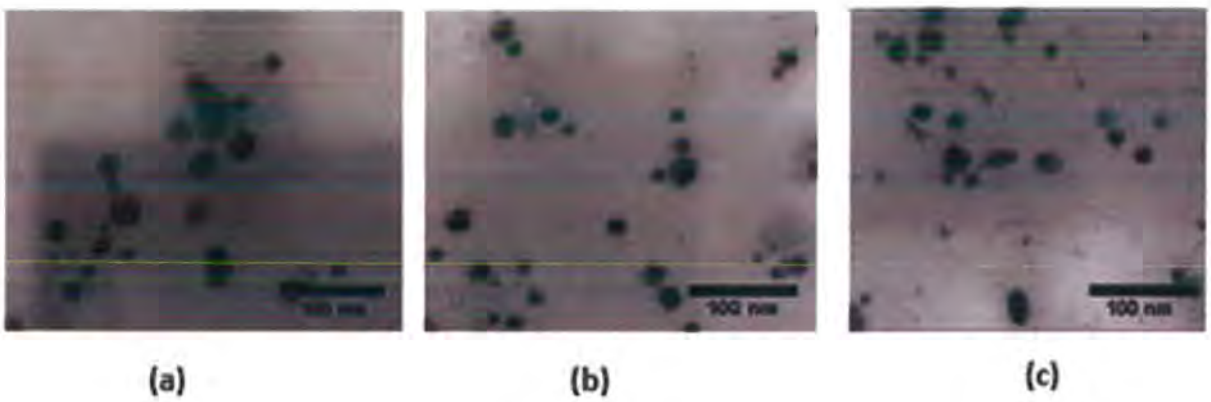


Figure 4.21: Transmission Electron Microscopy (TEM) images of green synthesized Fe NPs of (a) *T. ammi,* (b) *P. granatum* and (c) *S. lappa*

General Symptoms

The ruffled fur was observed in all the groups from third to six day of post infection except in the normal group of mice A. It was observed up to the 10^{th} day of the infection in the group B. There were no limb paralysis, convulsion and coma observed among all groups.

Qualitative analysis of parasites in infected erythrocytes

After the treatment with Fe NPs the Parasitemia was reduced and could be seen by the thin smear observation (Figure 4.22).

Parasitemia

The maximum percentage Parasitemia was observed in the negative control group B that was significantly difference (P=0.000) when compared with the drug

treated group C. The Parasitemia in Fe NPs of *T. ammi*, *P. granatum* and *S. lappa* treated all groups showed highly significantly difference (P=0.001) when compared to the non-treated group B, on the fifteenth day of the experiment.

The Parasitemia in all Fe NPs of *T. ammi*, *P. granatum* and *S. lappa* depicted the highly non significantly (P≥0.5) except the one group L3 of Fe NPs of *T. ammi* treated at the dose of 300 mg/kg showed only non-significantly (P≤0.1) when compared to the drug treated group C. The highest level of%age Parasitemia was observed on the day four to six for almost all groups and continuously increased in group B and gradually decreased in Fe NPs and chloroquine treated groups (Figure. 4.23 a).

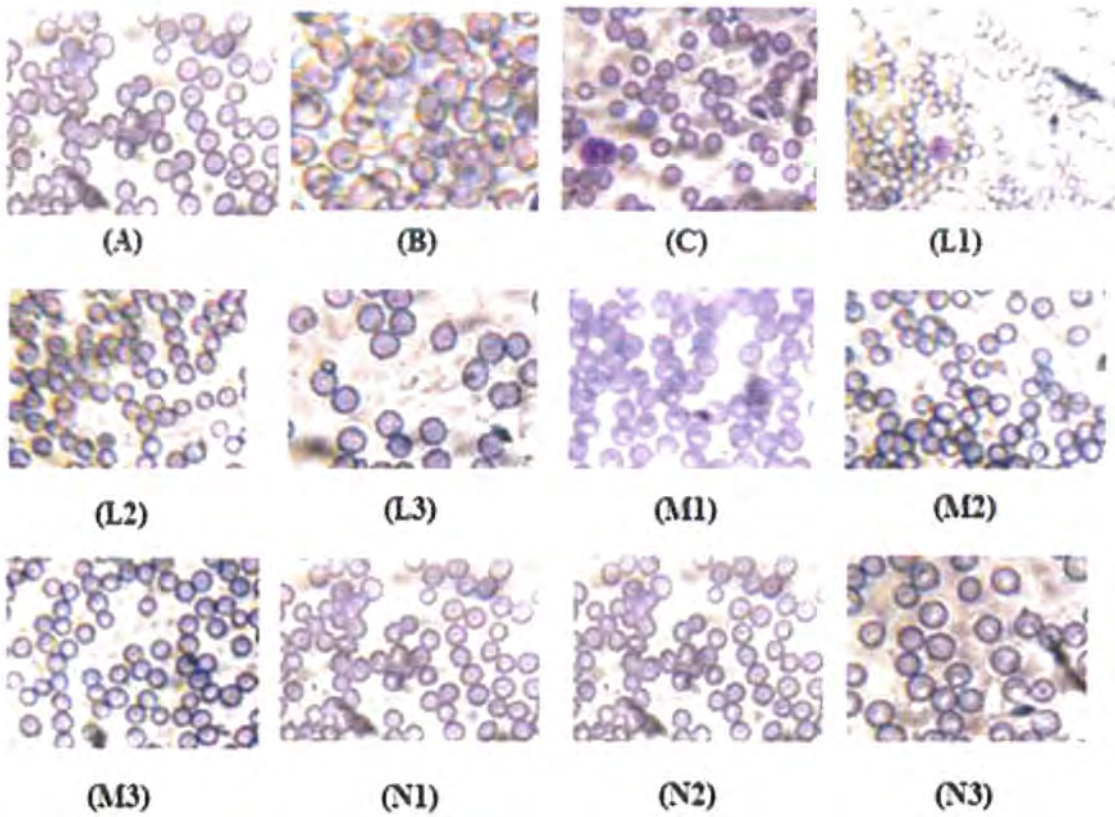


Figure 4.22: Thin blood smear of infected but non treated mice and Fe NPs treated groups. No red blood cells are infected at 15th day of infection in the treated group as compared to the non-treated group. Observed under light microscope at 100 x magnification

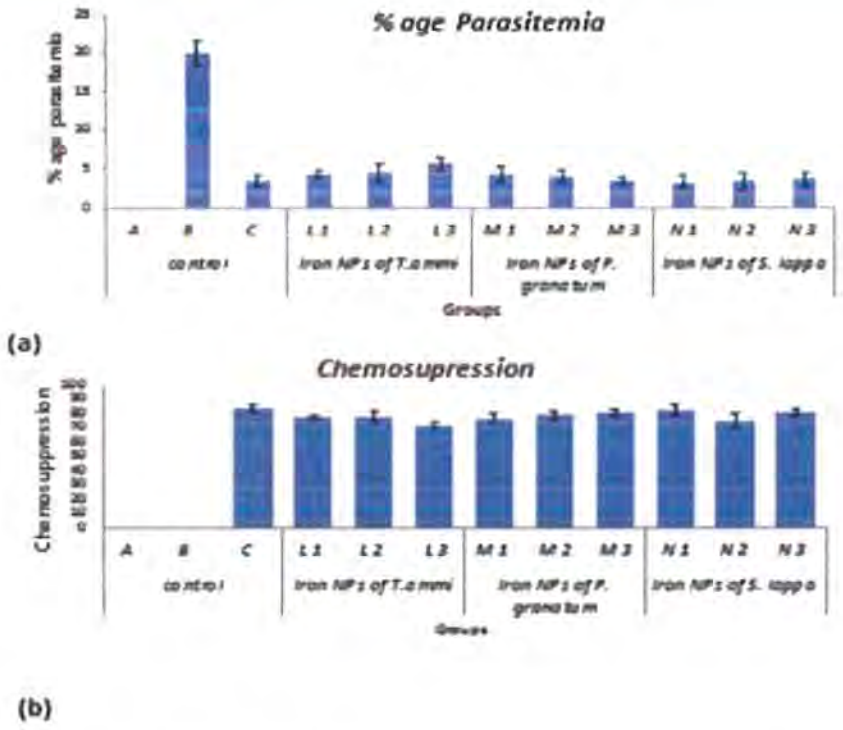


Figure 4.23: The figure shows the (a) Percentage Parasitemia, (b) Chemosupression of control and green synthesized Fe NPs treated groups.

A= normal control, B = infected but non-treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, $P<0.001$ for graph (a) and 0.8 for graph b.

Antiplasmodial activity

Maximum antiplasmodial activity was observed for the group treated with Fe NPs of *S. lappa, P. granatum and T. ammi* N3> M3> L3 treated at the dose 300 mg/kg and it was highly non-significant ($P\leq0.5$) when it was compared with the chemosupression of the drug treated group C. The remaining two dose of Fe NPs treated groups of *S. lappa* N1, and N2 also showed highly non-significant ($P\leq0.5$). The group treated with Fe NPs of *T. ammi* and *P. granatum* at the dose of 100 and 200 mg/kg indicated the non-significant difference ($P\leq0.1$).The antiplasmodial of L1 treated with the 100 mg/kg of Fe NPs of *T. ammi* showed significant difference ($P\leq0.01$) when compared to the group C (Figure. 4.23 b and Table 4.10).

Mean survival time

The mean survival time was highest for normal control mice of group A the difference was significant (P=0.006) when compared with the non-treated group B and the difference was non-significant (P= 0.096) when compared with the drug

treated group C. Among the Fe NPs treated groups highest survival time was observed for group N3> N2, and N1 >M2, When their survival time was compared with the normal group of mice A. They showed non-significant difference (P≤0.1).

Table 4.10: Antiplasmodial activity of Fe NPs of different plants against *Plasmodium berghei* VS control

Group Names	Treatment	Dose mg/kg	Parasitemia (%) Mean ±SE	Chemosuppression (%) Mean ±SE	Survival Time (days) Mean ±SE
(A)	None	-	-	-	>25.6 ±1.64 [a][b]
(B)	Water	-	19.97±0.74	-	10.4±1.064 [a][c]
(C)	Chloroquine	5	3.32±0.67 [a]	83.48±2.15	16.2±0.4 [b][c]
(L1)	*Fe NPs of T. ammi*	100	4.296±0.43 [a] [e]	71.48±2.75 [b]	16.4±2.10 [b][c] [e]
(L2)		200	4.438±1.18 [a] [e]	76.75±1.81 [c]	18.8±3.27 [c][c] [e]
(L3)		300	5.567±0.74 [a] [d]	77.14±4.18 [d]	18.0±4.45 [c][c] [e]
(M1)	*Fe NPs of P. granatum*	100	4.26±1.04 [a] [e]	76.19±2.37 [c]	16.6±3.12 [b][c] [e]
(M2)		200	4.01±0.77 [a] [e]	78.75±2.86 [d]	19.0±3.36 [d][b] [d]
(M3)		300	3.47±0.49 [a] [e]	81.46±3.10 [e]	18.2±3.68 [c][b] [e]
(N1)	*Fe NPs of S. lappa*	100	3.50±0.82 [a] [e]	80.12±2.39 [e]	20.4±5.07 [d][b] [d]
(N2)		200	3.274±1.03 [a] [e]	81.23±3.83 [e]	20.0±4.24 [d][b] [d]
(N3)		300	3.557±0.99 [a] [e]	82.51±4.68 [e]	23.0±4.34 [e][a] [d]

Values are presented as mean ± SEM (n = 5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a] =P ≤ 0.001, [b] =P≤0.01 [c] =P≤0.05, [d] =P≤0.1 [e] = P≤0.5.

The survival time of all Fe NPs treated groups revealed the significant difference (P≤0.05) when compared with the infected but not treated group B. The survival time of all Fe NPs treated groups were highly non-significant (P≤0.5) when compared to the chloroquine treated group C (Figure 4.24a). The remaining groups of Fe NPs of *T. ammi* and *P. granatum* revealed the significant difference (P≤0.05) compared with the normal group of mice A.

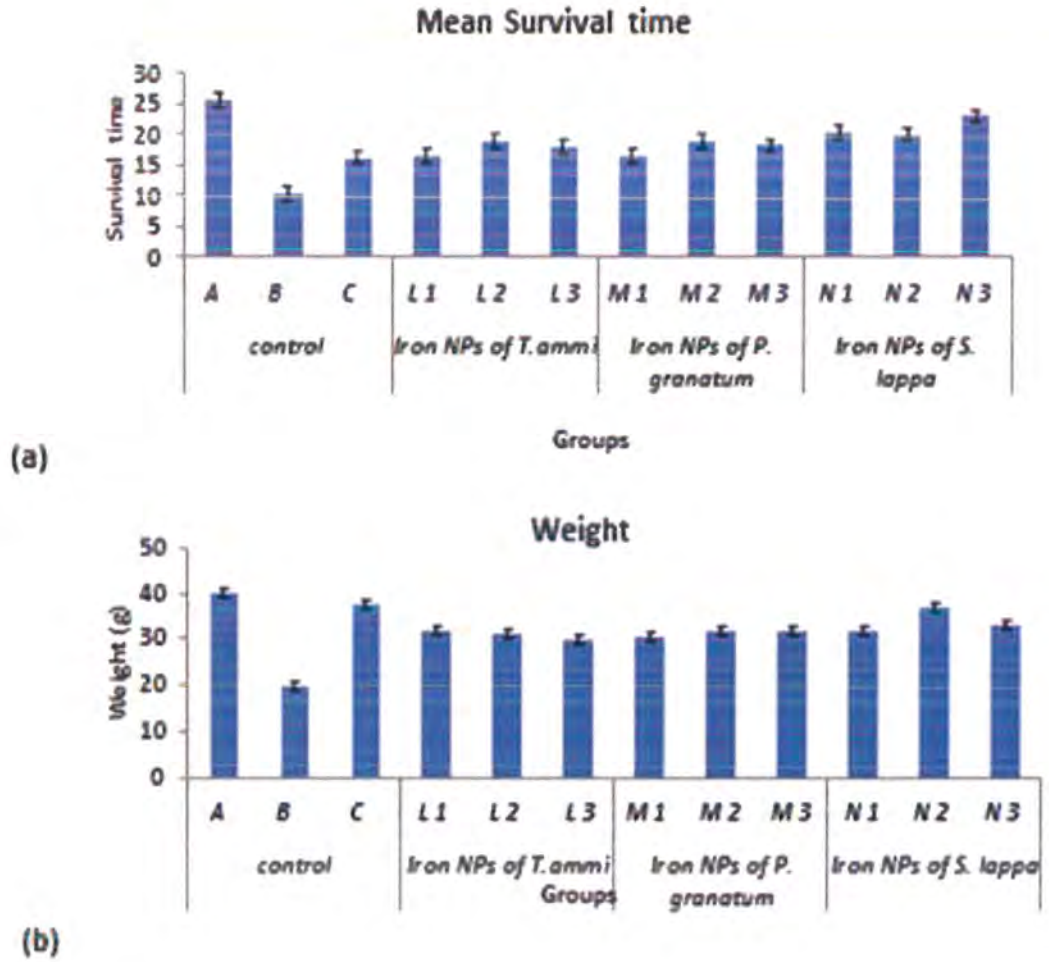


Figure 4.24: The figure shows the (a) Survival time (b) weight change of control and Fe NPs treated groups VS Control.

A= normal control, B = infected, but non treated, C = infected but treated with chloroquine. Data is presented as Mean ± SE with N=5, P = 0.5 for (a) and P= 0.01 for (b)

Weight analysis

There was a gradual increase in weight of the control group A (P=0.000) till the fifteenth day of the experiment, whereas a progressive decrease in body weight was observed in infected but treated with water group B although both groups differ significantly (P =0.006). But the change in weight between group A and group C treated with chloroquine was highly non-significant (P = 0.33). Almost all groups treated with the Fe NPs showed the significant difference (P≤0.05) except the N2 and N3 that was treated with the 200 mg/kg and 300 mg/kg of Fe NPs of *S. lappa* that revealed the non- significant difference (P≤0.5) when compared with normal control group A.

The groups treated with Fe NPs of *T. ammi*. *P. granatum* and *S. lappa* showed high significant difference (P≤0.01) when compared with non-treated group B. Almost all groups treated with the Fe NPs showed the significant difference (P≤0.05) except the N2 and N3 that was treated with the 200 mg/kg and 300 mg/kg of Fe NPs of *S. lappa* that revealed the non- significant difference (P≤0.5) when compared with the chloroquine treated group C. Similar, observation was noted for percentage weight change of all Fe Nps treated groups when compared with three control groups. The treated groups showed a gradual loss in weight till the day third and some up today fourth as compared to the day first of infection after that a steady gain in weight occurred in the treated groups (Figure. 4.24 b

and Table 4.11).

Hematological analysis

The **Red Blood Cell (RBC) count of** normal control group (A) when compared with infected but untreated group (B) and infected but treated with chloroquine group (C) showed highly significant (P=0.008) and less significant difference (P=0.033) respectively. The RBC of all Fe NPs showed the non-significant difference (P≤0.5) except the L1 and M1 that was treated with the 100 mg/kg of Fe NPs of *T. ammi* and *P. granatum* that revealed the significant difference (P≤0.05) compared with normal mice of group A.

There was the significant difference (P= 0.03) of RBC number of B and C group. There was the significant difference (P≤0.05) between all Fe NPs treated groups and infected but not treated group B.

The RBC count of chloroquine treated group C and Fe NPs treated groups of all Fe NPs indicated the high non-significant difference (P≤0.5) when compared with the chloroquine treated group C.

Table 4.11: Comparison of body weight and percentage weight change among Fe NPs treated groups VS controls

Group Names	Treatment	Dose mg/kg	Body weight (g) Mean±SE		Percentage weight change (%) Mean±SE
			Day 1	Day 15	
(A)	None	-	35.83±0.764	40.31±0.153 [a] [e]	12.51±1.02 [a] [e]
(B)	Water	-	33.90±0.265	19.87±3.87 [a] [b]	-41.38±1.65 [a] [a]
(C)	Chloroquine	5	34.40±0.107	37.60±0.393 [e] [a]	9.30±0.85 [e] [a]
(L1)	*Fe NPs of T. ammi*	100	31.6±0.932	31.6±1.94 [c] [a] [c]	0±0.00 [a] [a] [b]
(L2)		200	31.2±0.43	31.6±1.58 [c] [a] [c]	1.24±0.87 [b] [a] [c]
(L3)		300	31.2±0.23	31.14±1.79 [c] [a] [c]	-0.64±0.95 [a] [a] [c]
(M1)	*Fe NPs of P. granatum*	100	31.2±0.34	30.54±0.35 [c] [a] [c]	-2.16±0.43 [b] [a] [c]
(M2)		200	32.04±0.23	31.87±0.96 [c] [a] [c]	-0.53±0.56 [a] [a] [c]
(M3)		300	33.02±0.21	31.64±0.67 [c] [a] [c]	-0.04±0.33 [a] [a] [c]
(N1)	*Fe NPs of S. lappa*	100	31.08±0.32	31.72±1.22 [c] [a] [c]	2.05 ±0.98 [b] [a] [c]
(N2)		200	30.08±0.14	33.13±1.07 [d] [b] [d]	9.20 ±1.12 [d] [a] [e]
(N3)		300	31.08±0.23	33.03±1.23 [d] [b] [d]	6.27 ±1.43 [c] [a] [d]

Values are presented as mean ± SEM (n = 5). The Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. [a]=P ≤ 0.001, [b] =P≤0.01 [c]=P≤0.05, [d] =P≤0.1 [e] = P≤0.5.

The normal control group (A) showed significant difference (P=0.005) of **Hemoglobin** with infected, but the untreated group (B) and highly non-significant difference (P=0.332) with the group (C) treated with chloroquine. The Fe NPs treated

groups of all plants showed the very high non-significant difference (P≤0.5) when compared with the normal control group A.

The highest significant difference (P= 0.004) was noted for **Hemoglobin** between B and C group. The difference was highly significant (P≤0.01) for all Fe NPs treated groups and chloroquine treated group when compared with infected but non-treated group B.

The non-significant difference for Hemoglobin was detected for groups L1, L2, L3, M1, M2, M3, N1, N2 and N3 (P≤0.1). The highest non-significant difference was observed for L1, M1, M2, M3 and N1 when compared with drug treated group C.

The group (A) showed highly significant difference (P=0.003) of **Hematocrit (packed cell volume)** with infected, but non-medicated group (B) and less significant difference (P=0.038) with the group (C) treated with chloroquine. The Fe NPs treated groups of all experimented plants showed the non-significant difference (P≤0.5) except the L3 that was treated with the 300 mg/kg of Fe NPs of *T. ammi* indicated that there was significant (P≤0.01) as compared with the normal control group A.

There was a significant difference (P= 0.012) between B and C group. The Fe NPs treated groups revealed a highly significant difference (P≤0.01) when compared with the infected but non treated group B.

There was the non-significant difference (P≤0.5) for **Hematocrit** between drug treated group C and Fe NPs of all experimented plants treated groups except for the N3 group that revealed the significant difference (P≤0.05) when compared with the drug treated group C.

The group (A) revealed a highly significant difference (P=0.001) of **Mean cell volume (MCV)** with infected, but non medicated group (B) and significant difference (P=0.01) with the group (C) treated with Chloroquine. The Fe NPs treated groups of *T. ammi, P. granatum*, and *S. lappa* at all dose levels showed non-significant difference (P≤0.5) except for the L2 and M1 which depicted the significant difference (P≤0.01) as compared with the normal control group (A).

There was a highly significant difference (P= 0.005) between B and C group. The Fe NPs treated groups of all experimented plants exhibited the high significant difference (P≤0.01) when compared with the infected but treated with water group B.

There was significant difference (P≤0.05) for MCV between drug treated group C and all Fe NPs treated groups except the L1, L2, and N1 showed non-significant difference (P≤0.5).

The group (A) showed highly significant difference (P=0.000) of **Mean cell hematocrit (MCH)** with infected but treated with water group (B) and significant difference (P=0.066) with the group C treated with chloroquine. The Fe NPs treated groups of all experimented plants exhibited the high non-significant difference (P≤0.5) when compared with normal group of mice (A).

There was a highly significant difference (P= 0.036) between B and C group. The Fe NPs treated groups of all experimented plants revealed the significant difference (P≤0.05) when compared with the infected but treated with water group (B). The MCH value of drug treated group C and Fe NPs treated groups showed the non-significant difference (P≤01). The highest non-significant difference was observed for L1, N2 and N3 groups that was treated with 100 mg/kg of Fe NPs of *T. ammi* and 200 and 300 mg/kg of Fe NPs of *S. lappa* respectively.

The group (A) showed a highly significant difference of **Mean cell hematocrit concentration (MCHC)** with a infected, but treated with water group (B) (P=0.02) and no significant difference (P=0.052) with the group (C) treated with chloroquine. The Fe NPs of all experimented plants showed high non-significant difference (P≤0.5) when compared with the normal control group (A).

There was a highly significant difference (P= 0.009) between B and C group. The Fe NPs treated groups revealed the high significant difference (P≤0.01) of MCHC when compared with the infected but treated with water group (B).

There was non-significant difference (P≤0.5) for MCHC between drug treated group C and Fe NPs treated groups of all experimented plants.

The group (A) showed a highly significant difference (P=0.001) of **Platelets** with infected, but treated with water group (B) and significant difference (P=0.009) with the group (C) treated with chloroquine. The Fe NPs treated groups of all experimented plants showed the significant difference (P≤0.05) except the N1 group indicated the non-significant difference (P≤0.1) that was treated with the 100 mg/kg of iron NP of *S. lappa* when compared with the **Platelets count of** normal control

group (A). There was a highly significant difference (P= 0.003) between B and C group. The Fe NPs treated groups of all experimented plants i.e. *T. ammi, P. granatum and S. lappa* showed the significant difference (P≤0.01) when compared with the infected, but treated with water group (B).

There was non-significant difference (P≤0.5) between the drug treated C group and Fe NPs of three experimented plants treated groups L1, M1, M2, M3, N1, N2, and N3 groups only L1 and L2 showed the non-significant difference (P≤0.05) when there their Platelets count was compared with the drug treated group C.

The group (A) showed a highly significant difference of **neutrophils** with infected but treated with water group (B) (P=0.008) and non-significant difference (P=0.263) with the group (C) treated with chloroquine. The Fe NPs treated groups of *T. ammi, P. granatum,* and *S. lappa* indicated the non-significant level (P≤0.5) except the M2 and M3 groups that revealed the non-significant level (P≤0.5) that were treated with the Fe NPs of *P. granatum* when compared with the normal control group (A). There was a significant difference (P= 0.034) among neutrophils of infected but non treated group B and drug treated group C. The all Fe NPs treated groups three experimented plants of also indicated the significant difference (P≤0.01) when compared with infected but non treated group B. There was high non-significant difference (P≤0.5) in the neutrophils of chloroquine treated C and all Fe NPs of three experimented plants treated groups except the N3 that depicted the significant difference (P≤0.05).

The group (A) showed a highly significant difference (P=0.006) of **lymphocytes** with infected but treated with water group (B) and non-significant difference (P=0.694) with the group (C) treated with chloroquine. The Fe NPs treated groups I1, I2, I3, J1, J2, J3, K1, K2 and K3 also showed the non-significant difference (P≤0.5) except the N3 that showed the significant difference (P≤0.05) when compared with the normal control group A. There was a significant difference among the lymphocytes of group B and C (P≤0.01) as well as Fe NPs of all experimented plants treated groups. There was non- significant difference (P≤0.5) between drug treated group C and Fe NPs of all experimented plants treated groups (Table 4.12).

Table 4.12: Comparison of Hematological parameters of Fe NPs treated groups Vs control

Hematol-ogical Para	Groups											
	A	B	C	L1	L2	L3	M1	M2	M3	N1	N2	N3
RBCs	8.510±0.4 {c}{c}	3.97±0.16 {c}{b}	6.00±0.480 {c}{b}	6.34±0.34 {c}{c}{e}	7.73±0.67 {e}{e}	7.23±0.56 {e}{b}{e}	6.216±0.19 {c}{c}{e}	7.65±0.78 {e}{c}{e}	7.16±0.56 {e}{c}{e}	5.948±0.24 {d}{c}{e}	7.10±0.65 {e}{c}{e}	7.383±0.45 {e}{c}{e}
Hb	11.3±0.5 {b}{e}	5.43±0.35 {b}{a}	10.5 ±0.9 {e}{a}	12.12±0.74 {e}{b}{e}	13.02±0.78 {e}{b}{d}	13.43±0.67 {e}{b}{d}	12.02±0.87 {e}{a}{e}	14.01±0.56 {e}{a}{e}	12.04±0.67 {e}{b}{e}	10.8 ±0.68 {e}{b}{e}	13.93±0.83 {e}{a}{d}	13.05± 0.28 {e}{a}{d}
PCV	38.50± 0.55 {b}{d}	26.87 ±1.1 {b}{e}	34.53±0.72 {d}{c}	37.12±0.51 {e}{b}{d}	35.56±0.86 {e}{b}{e}	31.46±0.55 {b}{c}{d}	35.67±0.98 {e}{c}{e}	37±0.30 {e}{b}{d}	37.33±0.39 {e}{b}{d}	36.12±0.3 {d}{b}{d}	38.40±0.56 {e}{b}{d}	40.2±1.12 {e}{b}{e}
MCV	53.4±0.62 {a}{c}	43.2±0.66 {a}{a}	48.0±1.10 {c}{a}	50.2±0.68 {e}{b}{d}	48.6±0.89 {b}{b}{e}	51.4±0.89 {e}{a}{c}	49.06±2.23 {c}{b}{e}	53.85±0.56 {e}{a}{b}	52.8±0.87 {e}{a}{b}	53.13± 1.23 {e}{a}{b}	55.88±1.21 {e}{a}{b}	55.01±1.34 {e}{a}{b}
MCH	16.9±0.2 {a}{b}	12.9±0.20 {a}{b}	15.23±0.41 {b}{b}	16.53±0.90 {e}{c}{e}	17.45± 0.56 {e}{c}{d}	17.43 ± 0.45 {e}{c}{d}	17.23± 0.78 {e}{c}{d}	18.45± 0.28 {e}{b}{d}	17.23± 0.78 {e}{c}{d}	18.90± 0.28 {e}{b}{d}	16.99±0.67 {e}{b}{e}	17.87± 0.54 {d}{b}{e}
MCHC	32.13±0.94 {a}{c}	26.16±1.48 {a}{a}	31.86±0.95 {c}{a}	32.85±0.84 {e}{b}{e}	34.34± 0.85 {e}{b}{e}	34.21±0.23 {e}{b}{e}	32.04±0.85 {e}{b}{e}	35.7±0.84 {d}{a}{d}	31.6±0.56 {e}{b}{e}	35.2±0.84 {d}{a}{d}	32.4±0.34 {e}{b}{e}	33.6± 0.15 {e}{b}{e}
Platelets	325000± 27.9 {a}{b}	178000±18.1 {a}{b}	27400± 23.6 {b}{b}	256000±12.12 {b}{b}{d}	240000±5.43 {b}{b}{e}	230000±13.64 {b}{b}{b}	250000±20.81 {b}{c}{d}	240000±11.43 {b}{c}{e}	25000±14.23 {b}{b}{e}	270000± 16.49 {d}{c}{e}	280000±13.04 {b}{b}{d}	286000±16.13 {b}{b}{d}
Neutro-phils	24±0.882 {b}{e}	33±1.5 {b}{b}	26±1.2 {e}{b}	25±0.58 {e}{b}{e}	27.01±0.57 {d}{b}{e}	26.03± 2.20 {d}{b}{d}	25.13±1.23 {e}{b}{e}	28.66±0.98 {e}{e}{e}	29.33±0.58 {c}{c}{d}	26.24±1.23 {e}{b}{e}	25.12±0.54 {e}{b}{e}	20.667±0.63 {d}{a}{e}
Lympho-cytes	65±1.1 {b}{e}	83±071 {b}{b}	66±1.23 {e}{b}	64.12±1.11 {e}{b}{e}	68±0.57 {d}{b}{e}	64.43±0.45 {e}{b}{e}	65.67±0.34 {e}{b}{e}	63.12±1.23 {d}{b}{d}	70±1.12 {c}{b}{e}	63.01±1.15 {d}{b}{d}	64.23±1.45 {d}{b}{e}	60.21±0.67 {d}{b}{e}

Biochemical analysis

The liver function test was done by determining the ALT, ALP and AST of all treated groups and control groups. The **ALT** level was increased in infected but non-treated group B as compared to the normal control group A ($P = 0.005$). The Fe NPs of all experimented plants showed significant difference ($P \leq 0.05$) except for M3 group that was treated with the 300 mg/kg of Fe NPs of *P. granatum* when compared to the normal group A.

There was a high Significant difference ($P \leq 0.01$) in the in the ALT range between Fe NPs treated groups of all experimented plants treated groups when compared to the infected but non treated group B. The Fe NPs of all experimented plants exhibited the significant difference ($P \leq 0.05$) when compared chloroquine treated group C.

The difference between the **ALP** level was highly significant ($P = P=0.007$, 0.002) in the A and C group as compared to the B respectively. The Fe NPs of *P. granatum* and *S. lappa* treated groups M2, M3 and N2that was treated at the dose of 200, 300 mg/kg and 200 mg/kg showed non-significant difference ($P \leq 0.5$) and remaining groups L1, L2, L3, M1, N1 and N3 indicated the significant difference ($P \leq 0.05$) when compared to the normal group A.

All Fe NPs treated groups of *T. ammi, P. granatum,* and *S. lappa* showed significant difference ($P \leq 0.01$) when compared with the non-treated group B. It was highly significant for L1, L2, M1 and M2 groups.

The Fe NPs of all *T. ammi*, *P. granatum* and *S. lappa* treated groups indicated the non- significant difference ($P \leq 0.5$) except the L3 group that showed the significant difference ($P \geq 0.05$) and treated with 300 mg/kg of *T. ammi* when compared to the chloroquine treated group C.

The elevated level of **AST**, was observed in infected but non-medicated group B as compared to the normal control group A and chloroquine treated group C. Statistically it was highly significant ($P = 0.002$, and 0.000). The AST level of Fe NPs of *T. ammi* and *S,lappa* revealed the non-significant difference ($P \leq 0.5$) for all doses and significant difference ($P \leq 0.01$) for Fe NPs of *P. granatum* treated groups at the all dose level when compared to normal group A. The Fe NPs of all experimented treated

groups revealed the significant difference (P≤0.01) when compared with the non-treated group B.

There was a highly non-significant difference (P≤0.5)for L3, M1, M2, M3, N1, and N2 Fe NPs treated groups except the L1, L2 and N3 groups that depicted the significant difference (P≤0.05)when compared to the chloroquine treated group C (Figure 4.25 a)

Kidney function test was also determined by **Creatinine** and **Uric acid** level. There was the Increased levels of **Creatinine** in the non-treated group B as compared to the normal group A (P=0.001) and drug treated group C (P= 0.002). The Fe NPs treated groups of all experimented plants showed significant difference (P≤0.01) except the N3 group that was treated with the 300 mg/kg of Fe NPs of *S. lappa* when compared to the normal group A.

The groups treated with Fe NPs of all three experimented plants showed the highly significant difference in creatinine level when compared with non-treated group B (P≤0.001). The Fe NPs of *T. ammi*, *P. granatum* and *S. lappa* treated group non-significant difference (P≤0.5) when compared to the chloroquine treated group C (Figure 4.25 b).

Uric acid level in the non-treated group B was highly significant (P= 0.018) and (P= 0.002) as compared normal group A and drug treated group C respectively. There was the highest non-significant difference (P≤0.5) for the groups treated with Fe NPs of all experimented plants except for the L3 and M1 groups that indicated the significant difference (P≤0.05)when compared to the normal group A (Figure 4.25 c).

The Fe NPs treated groups L1, L2, L3, M1 and M3, showed non-significant difference (P≤0.5) and M2, N1, N2, and N3 depicted the significant difference (P≤0.01) when compared to the infected but non-treated group B. There was non-significant difference (P≤0.5) between the Fe NPs treated groups M2, N1, N2, and N3 but other groups L1, L2, L3 and M1 and M3 showed significant difference (P≤0.01) when compared to the drug treated group C (Figure.4.25 c) and (Table 4.13).

Table 4.13: Comparison of liver and kidney function tests of Fe NPs treated groups Vs controls

Group Names	Treatment	Dose mg/kg	Liver function test (U/L)			Kidney function test (mg/dl)	
			ALT	ALP	AST	Creatinine	Uric acid
(A)	None	-	20.36± 2.04 {b}[e]	43.63±2.73 {b}[b]	57.59 ±1.36 {a}[a]	0.29±0.02 {a}[a]	18.49±0.81 {b}[e]
(B)	Water	-	89.62± 4.12 {b}[a]	108.35±9.3 {b}[b]	250.1±2.17 {a}[a]	1.04±0.04 {a}[b]	29.789±0.44 {b}[b]
(C)	Chloroquine	5	23.56 ± 1.9 {e}(a)	62.84±7.03 {b}(b)	91.66 ±3.9 {d}(b)	0.48±0.03 {a}(b)	16.84±0.593 {e} (b)
(L1)	*Fe NPs of T. ammi*	100	42.96 ±3.15 {c}(b)[c]	57.26±3.01 {b}(a)[e]	63.14±3.55 {e}(a)[c]	0.701±0.06 {b}(b)[d]	23.01±2.44 {d}(d)[b]
(L2)		200	43.96±3.34 {c}(b)[c]	59.9±4.25 {b}(a)[e]	65.49±2.41 {d}(a)[c]	0.89±0.02 {b}(c)[d]	22.02±2.75 {d}(d)[b]
(L3)		300	48.43±4.67 {c}(b)[a]	69.9±2.86 {b}(b)[c]	67.14±2.45 {d}(a)[c]	0.787±0.03 {b}(c)[d]	25.05±2.11 {c}(e)[b]
(M1)	*Fe NPs of P. granatum*	100	50.03±4.98 {b}(b)[a]	57.4±3.34 {b}(a)[e]	86.53±3.12 {b}(a)[d]	0.726±0.01 {b}(b)[d]	26.09±2.34 {a}(e)[b]
(M2)		200	41.77±3.34 {c}(b)[c]	49.2±2.12 {e}(a)[d]	85.88±2.34 {b}(a)[d]	0.690±0.04 {b}(b)[d]	19.01±2.57 {e}(b)[e]
(M3)		300	30.81±2.18 {d}(b)[c]	47.6±1.23 {e}(b)[d]	90.01±2.12 {b}(b)[e]	0.614±0.01 {b}(a)[d]	21.23±2.69 {d}(e)[b]
(N1)	*Fe NPs of S. lappa*	100	35.31±2.88 {c}(b)[c]	53.2±4.32 {c}(b)[e]	62.12±2.56 {e}(b)[d]	0.55±0.04 {c}(a)[e]	17.92±2.117 {e}(b)[e]
(N2)		200	32.96±3.34 {c}(b)[c]	50.9±3.59 {d}(b)[d]	68.11±2.34 {d}(b)[d]	0.531±0.03 {c}(a)[d]	16.23±1.512 {e}(b)[e]
(N3)		300	39.37±2.12 {c}(b)[c]	62.9±2.88 {b}(b)[e]	56.7±1.45 {e}(a)[c]	0.44±0.022 {d}(a)[e]	14.45±1.90 {e}(b)[e]

Values are presented as mean ± SEM (n = 5). Probability value in bracket { }, ( ) and [ ] indicate the comparison with normal group (A), infected but treated with water group (B), drug treated group (C) respectively. a =P ≤ 0.001, b =P≤0.01 c =P≤0.05, d =P≤0.1 e = P≤0.5.

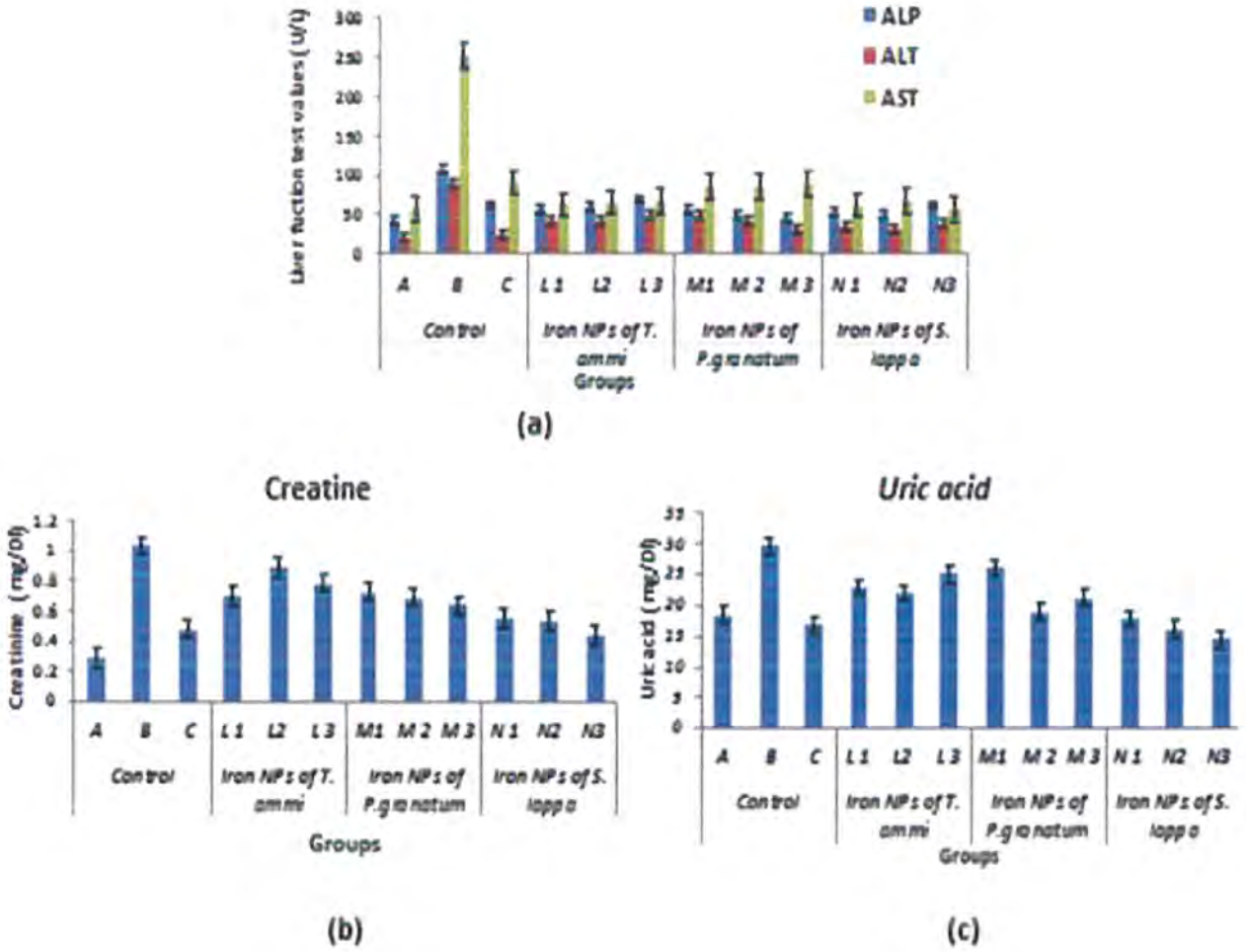


Figure 4.25: Plasma concentrations of ALP, ALT, AST (a), Creatinine (b), and Uric acid (c) of control and Fe NPs treated groups are shown in this graph. A= normal control, B = infected but non treated, C = infected but treated with chloroquine. Data is shown as Mean ± SE with N=5 P = 0.08 for (b) and P = 0.09 for (c)

Postmortem Examinations of organs

The postmortem examinations of the internal organs like liver, spleen, and the kidneys of the malaria-infected and Fe NPs treated groups can be observed by (Table 4.14, Figure 4.26 and 4.27)

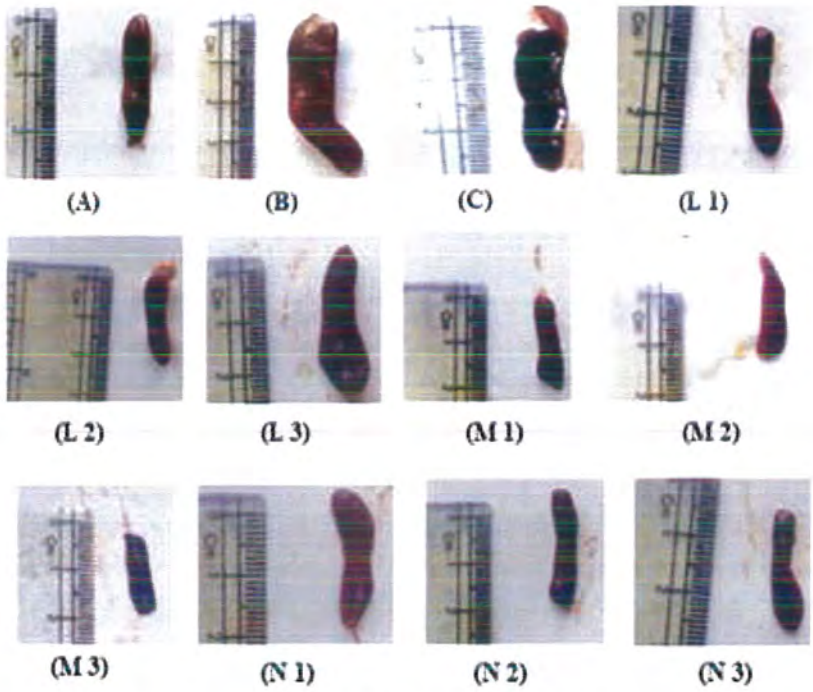


Figure 4.26: Post mortem observations of spleen of control and Fe NPs treated groups

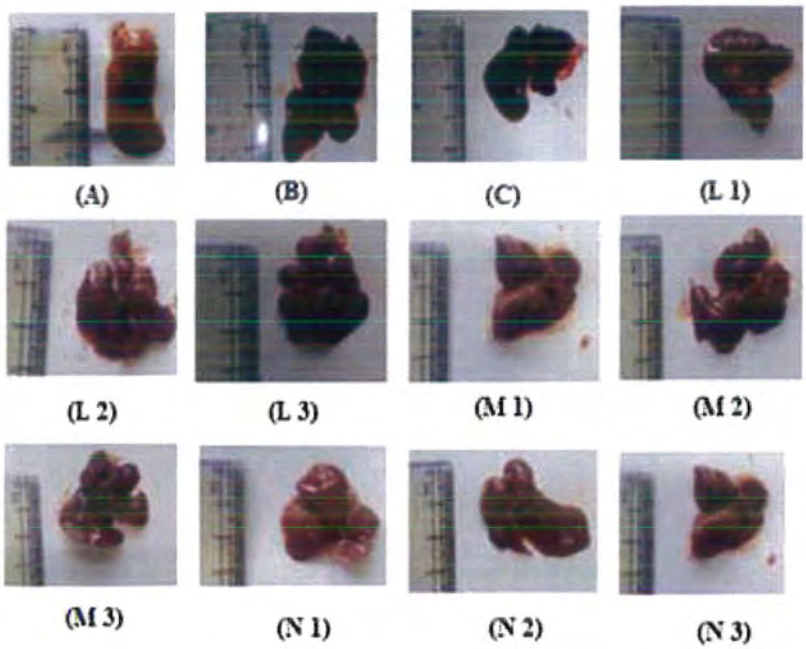


Figure 4.27: Post mortem observations of liver of control and green synthesized Fe NPs treated groups

Table 4.14: Post mortem observations of major organs in Fe NPs treated groups' VS Controls

Observation	A	B	C	L1	L2	L3	M1	M2	M3	N1	N2	N3
Enlargement of the size of spleen (splenomegaly);	–	+++	+	+	–	+++	–	–	–	++	–	+
Discoloration of the spleen	–	++	+++	+	+	+	+	–	–	+	+	++
Enlargement of the size (hepatomegaly)	–	++	+	–	+	+	–	–	–	–	–	–
Discoloration of the liver	–	++	+++	–	–	+	–	+	+	–	–	–
Discoloration of the kidneys	–	+++	++	–	–	+	+	–	–	–	–	–

Indicator: (-) absent; (+): mild; (++): moderate; (+++): sever

Histological observation

The histopathological studies showed the marked differences between the control and Fe NPs treated groups. The Parasitized RBCs were sequestered in the microvasculature of liver, spleen and kidney of non-treated group as compared to the Fe NPs treated and chloroquine treated groups.

The sequestration of Parasitized RBCs were observed in the microvasculature in the liver of L2 and little bit in the L3 groups that was treated with the 200 mg/kg and 300 mg/kg of Fe NPs of *T. ammi* respectively. The liver of infected but non-treated mice atrophy of hepatocytes, hypertrophy and hyperplasia of the kupffer cells was observed. Exoerythrocytic forms in the liver in the malarial infected, but non-treated was obvious. The hyperplasia and hypertrophy of some hepatocytes were in L2 group. The remaining Fe NPs treated groups showed the normal shaped hepatocytes and

kupffer cells (Figure 4.28). The Malaria pigments **haemozoin** was seen in the pulp histiocytes and sinusoidal lining cells and in the microvasculature of spleen tissue. Congestion of blood vessels was also observed in the spleen of infected but non treated group as compared to the Fe NPs treated groups. The Hemozoin was also observed in the L1, L2 and little bit in N3 (Figure. 4.29). The kidney tissues were presented with abundant of sequestrations of Parasitized RBC and haemozoin in the microvasculature and interstitium. The Congestions were seen in the infected but non treated group as compare to the Fe NPs treated groups (Figure. 4.30).

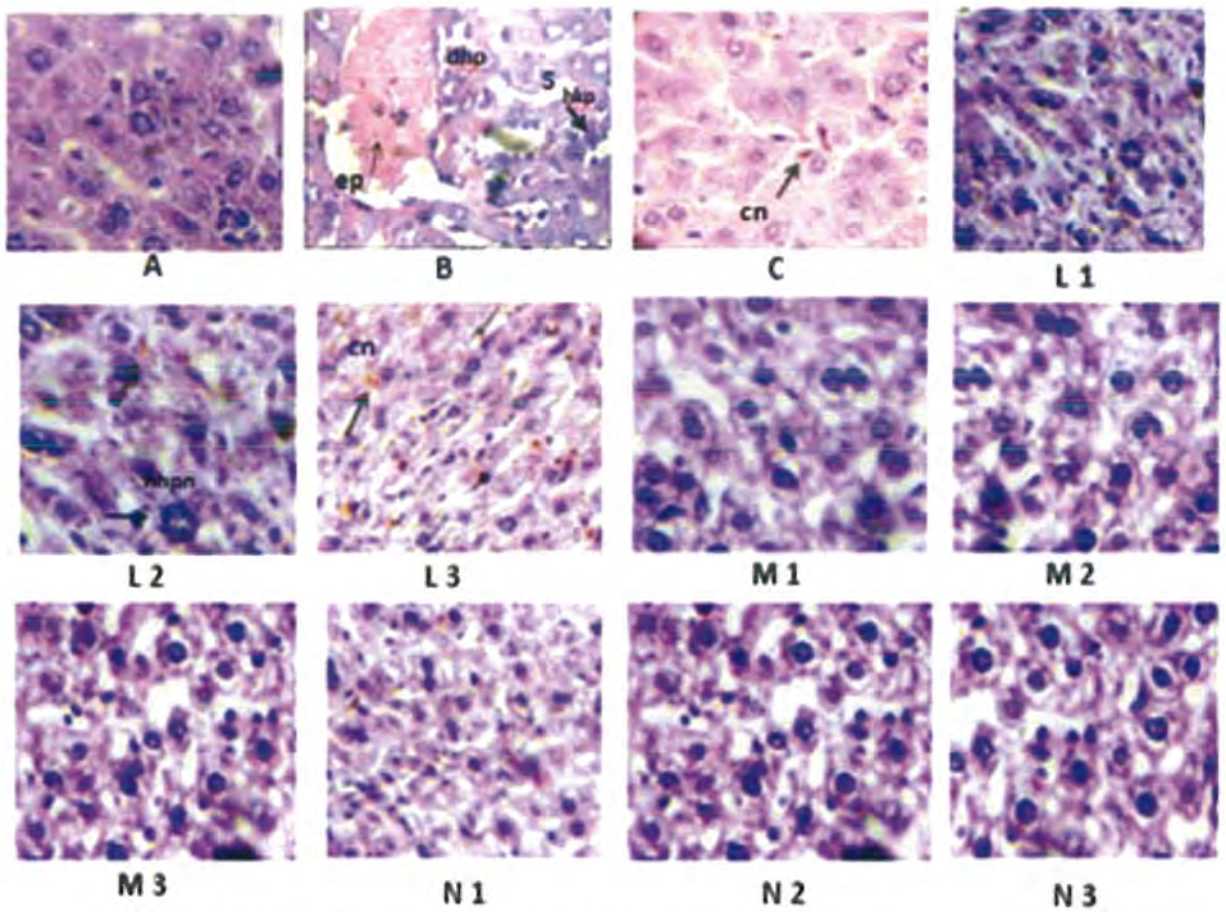


Figure 4.28: The histological study of Liver of experimental animals treated with Fe NPs Vs Controls. The liver of malaria-infected mice, exoerythrocytic form of *Plasmodium berghei* was observed in B (ep), hyperplasia and hypertrophy of kupffer cells (hkc) and vacuolar degeneration, atrophy of hepatocytes (dhp) and wide sinusoidal space (s) as compared to normal A and other treated groups. Hyperplasia and hypertrophy of some hepatocytes were observed in L2 group. The sequestrations of Parasitized RBCs were observed in the microvasculature in L2, L3 and little bit in N1 group. Viewed under light microscopy at 10 x100 X magnifications

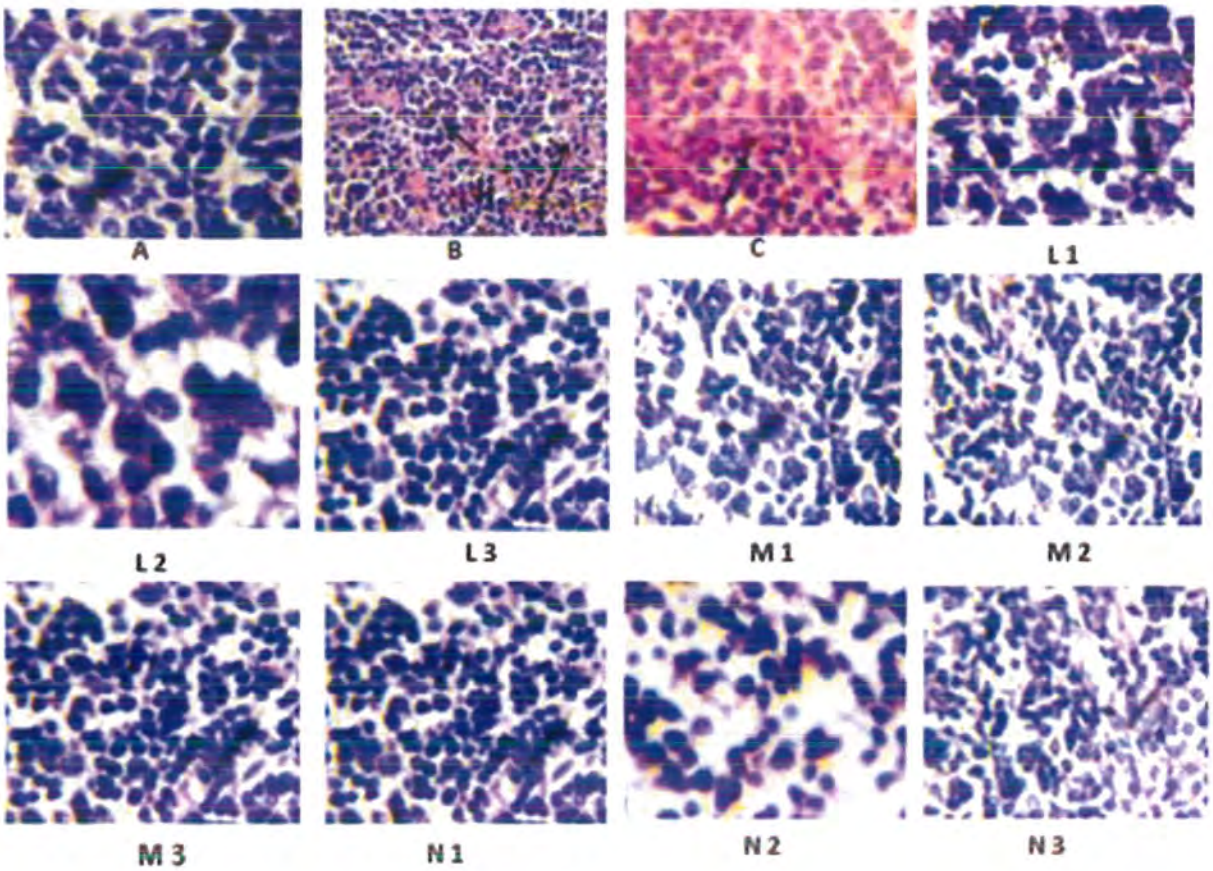


Figure 4.29: The histological study of Spleen of experimental animals treated with Fe NPs Vs Controls.

Accumulation of more malarial pigments, haemozoin, was more in the pulp histiocytes and sinusoidal lining cells of the spleen in the B group (i) as compared to normal control group A and other Fe NPs treated group. The Hemozoin was also observed in the L1, L2 and little bit in M3. Microvascular sequestrations of PRBCs were present B group (ii). Viewed under light microscopy at 10 x100 X magnifications

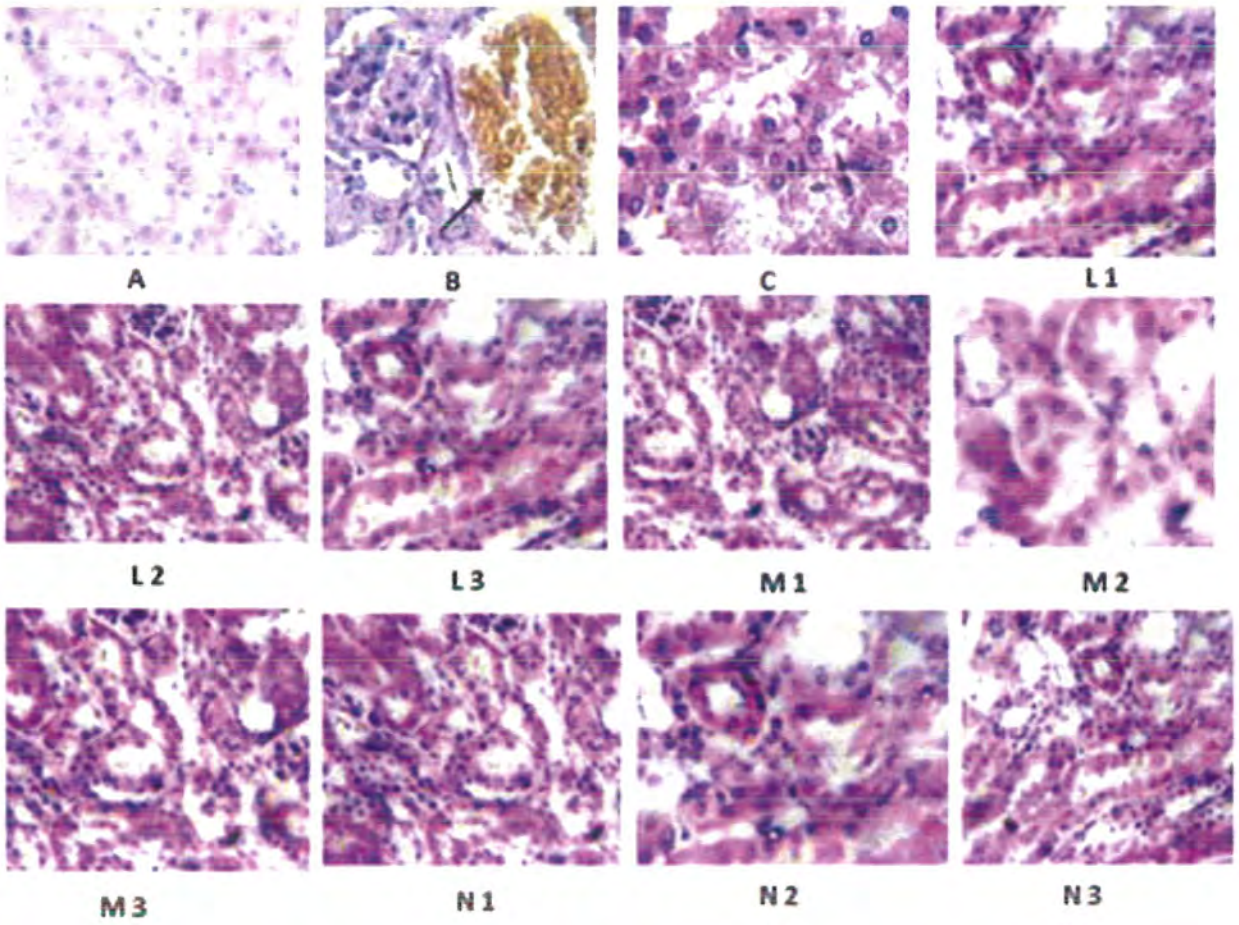


Figure 4.30: The histological study of Kidney of experimental animals treated with Fe NPs Vs Controls.

The kidney from infected mice was presented with widespread sequestration of PRBCs, Haemorrhages and congestion of blood vessels (B), as compared to the normal control group A and other Fe NPs treated groups. Viewed under light microscopy at 10 x100 X magnifications

High resolution imaging of red blood cells interacting with parasite and nanoparticles

The surface morphology (SEM) of RBCs of *P. berghei* infected mice treated with Fe NPs of *S. lappa* and *P. granatum* at dose of 300 mg/ kg were studied because they were more effective and safer than Ag NPs. These groups showed the presences of nanoparticles in the blood smears. The treated RBCs were not damaged (Figure 4.31 a-f). The interaction of nanoparticles with the parasite in the RBCs was also apparent (Figure 4.31 f).

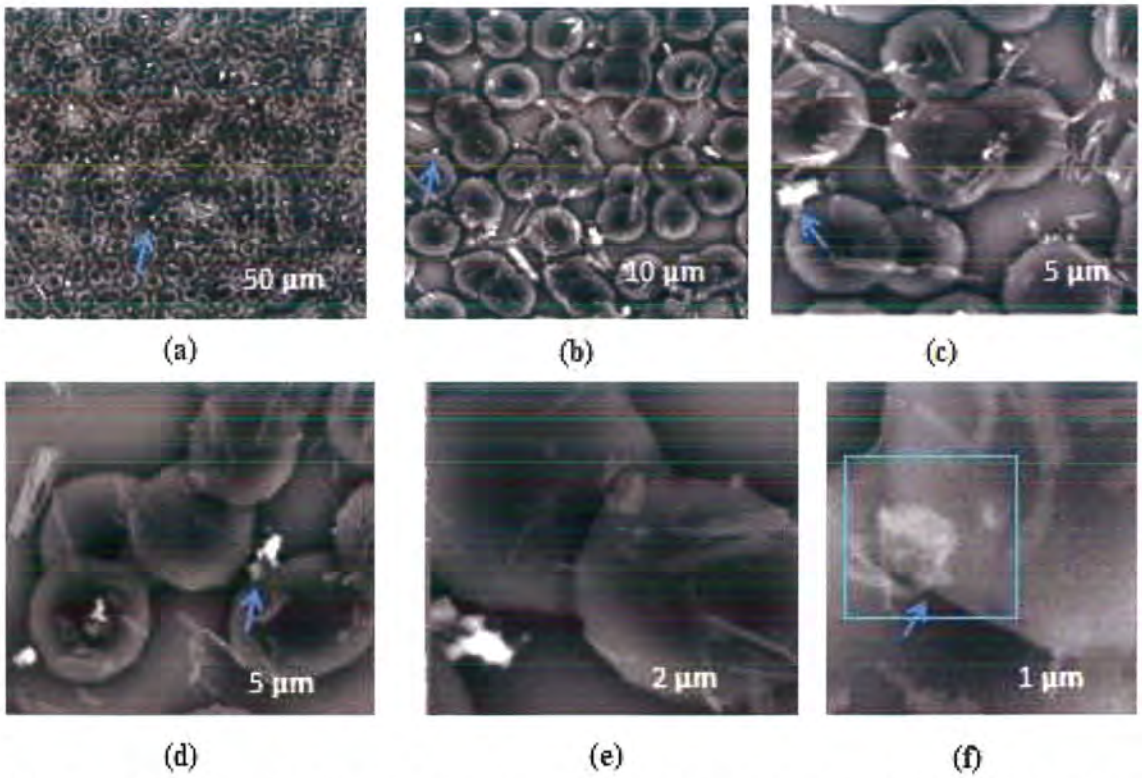


Figure 4.31: Scanning electron microscopy images of malaria infected RBCs treated with nanoparticles.

Figure (a) to (f) showing malaria infected RBCs treated with nanoparticles at different resolution. The blue arrows point the nanoparticles on the infected RBC surface, however the rectangle in the figure (f) indicating the interaction of RBCs parasite and nanoparticle.

Discussion

Currently malaria is cured with drugs commonly used are chloroquine, mefloquine and artemisinin unfortunately becoming resistant. New anti-malarial drugs are required to overcome the current scenario of resistance. The plants are the crucial source to discover new drugs because they have been used for the treatment of various diseases since ever (Tshibangu *et al.*, 2002).

The biological methods of NPs synthesis using, enzyme, microorganism and plant extract have been suggested as possible ecofriendly that avoid the toxic chemicals used in chemical and physical methods (Song and Kim 2009). Plants contain chemical constituents like flavonoids, phenols and alkaloids that have great potential to reduce the metals for the formation of their NPs and their stabilization (kasthuri *et al.*, 2009).

The flavonoids, phenols and alkaloids present in the *T. ammi, P. granatum*, as well as in *S. lappa.* These phytochemical constituents may be the act as a reducing and alleviating agent for the synthesis of Ag NPs and Fe NPs.

The Ag NPs are used in the health care sector, for imaging, drug delivery, therapeutics and diagnosis (Mishra *et al.* 2010). The Iron metallodrugs have used as mineral supplements, anti-hypertensive agents and as magnetic nanomaterials with both therapeutic and diagnostic roles (Vitorino *et al.*, 2015).

The potential metal-based anti-malarial drugs have become available. There are many metal complexes used as effective anti-malarial include metals such as iron, copper, cobalt, zinc, osmium gold, ruthenium, cobalt, rhodium, iridium, and palladium. The most likely anti-malarial mechanism involving metal complexation occurs by heme aggregation inhibition.

The promising therapeutic that can be used for the treatment of *P. falciparum* are metal chelating agents. They also have activities against resistant parasites due to the structural modification. The ferroquine is the metal based anti-malarial that has been found active against both chloroquine-resistant and chloroquine-susceptible *P. vivax and P. falciparum* strains. The Ru (II) chloroquine complexes are more potent against resistant parasites than the chloroquine diphosphate (Biot *et al.*, 2012).

The color change was the indication of reduction of silver into Ag NPs when the plant extracts were combined with 5 mM solution of $AgNO_3$. When the extracts of *T. ammi, P. granatum and S. lappa* were mixed with the 5mM solution of $AgNO_3$ the color changed from yellow to bright yellow than into dark brown. This dark brown color was developed after 24 hours for *T. ammi and P. granatum* and 48 hours after for *S. lappa*. This color change is due to the resonance of surface Plasmon (Shankar *et al.*, 2004).

Similarly, when the extracts of *T. ammi, P. granatum and S. lappa* were mixed with the 0.5M solution of $FeCl_3.6H_2O$ the color of the extract was changed from yellow to dark green or black these indications are according to (Mahdavi *et al.*, 2013: (Pattanayak and Nayak, 2012). One of the criteria for NPs formation is the reduction of metals resulting in color change of the solution (Shankar *et al.*, 2004). All NPs prepared showed color change.

However the confirmatory test for NPs formation is through UV-visible spectroscopy. When the metal is reduced by the plant extract in to NPs it could easily be related to the UV-visible spectrum. When the peak of UV-visible spectrum is broad it indicated that the Ag NPs are poly dispersed. Broad UV-visibles spectrums of *T. ammi and P. granatum* and *S. lappa* were observed, like the silver NPs was generated by the leave extract *Catharanthus roseus* by Ponarulselvam *et al* in 2012.

The UV–Vis spectrophotometer analysis of *S. tricobatum, S. cumini, C. asiatica and C. sinensis* showed the nearly similar UV-visible spectrum as observed in this study. In this study the peak match to the surface Plasmon resonance of Ag NPs was observed at 420 nm. It is described previously that absorbance at 420 nm is a characteristic of this Ag NPs (Nestor *et al.*, 2008). Mishra *et al.*, (2013) synthesized the Ag NPs with aqueous leaf extracts of Neem and Ashoka reported the Plasmon Resonance at 425 nm and after 30 hours Plasmon Resonance was 430 because the absorbance intensity decreased.

However the Fe NPs synthesized by *T. ammi, P. granatum* and *S. lappa* showed the maximum absorption at 402 nm like the Fe NPs generated by the *Sargassum muticum* by Mahdavi *et al* (2013).

Moreover (Pattanayak and Nayak, 2012) synthesized Fe NPs using ten different medicinal plant like black tea, green tea leaves, clove buds, nango leaves,, coffee seeds etc. observed the highest peak at 268nm. The FTIR analysis was used to recognize the functional groups of the dynamic compound that may play a role as capping agents and preventing the clustering of nanoparticles in solution.

The FTIR spectra of the extract showed strong bands at 1061.41cm−1, 1061.29 cm−1 and 1064.65 cm−1 correspond to C-N stretching of aliphatic amines, 1624 cm−1, 1611 cm−1, 1632cm−1 respond N-H stretching of Amides 3401.93 cm−1, 3463.12 cm−1, and 3461.46 cm−1 assigned to O-H stretching of Alcohols and Phenols for Ag NPs of *T. ammi, P. granatum* and *S. lappa* respectively.

Isaac *et al*, (2013) synthesized the Ag NPs using *Averrhoa bilimbi* Fruit extract observed the strong peaks at 3285 cm−1, 1633 cm−1, and 1025 cm−1 assigned to O–H stretching vibration of alcohols and phenols, C=O stretching vibration of Tertiary amides, and C–OH stretching of primary alcohols. The FTIR analysis of the present study is also accordance to the Ag NPs synthesized by commercially available plant powder of S. cumini, C. sinensis, S. tricobatum and C. asiatica (Logeswari *et al.*, 2013).

The FTIR spectrum of Fe NPs of *T. ammi, P. granatum and S. lappa* showed the characteristic stretching in the range of 800 cm−1, 1020, 1200 cm−1, 1400 cm−1, 1600 cm−1, 2100 cm−1, 2900 cm−1, and 3300 cm−1. These bands are assigned to different to different compounds like at 800 cm−1can be assigned to aromatic amines. The peaks at 3,300 cm−1 (OH stretching) and 2,929 cm−1 (CH stretching) were also observed. The lowering in intensity at 3,200 cm−1 after reduction of FeCl3 implies the involvement of the OH group in the reduction process (Mohan *et al* 2014).

The FTIR analysis is in accordance with Mahdavi *et al.*, 2013. They synthesized the Fe NPs by using aqueous extracts of *Sargassum muticum* and observed the characteristic stretching at 305 cm^{-1}, 535 cm^{-1}, 1018 cm^{-1}, 1416 cm^{-1}, 1604 cm^{-1}, and 3217 cm^{-1}.

The crystal structure and chemical nature of the NPs can be studied through XRD analysis. The XRD analysis of *T. ammi* showed the broad peak 38.84 °, 44.90 °,

64.49 ° and 77.74 at plane100, 200, 220, and 311 respectively. The XRD investigation of *P. granatum* showed the broad peak at 28.12°,32.32°, 44.45°, and 64.49°at plane100, 200, 220, and 311 respectively. Similarly the X-ray pattern of *S. lappa* showed the broad peak at 28.14°, 32.34°, 44.39°, 64.39°, and 78.04°at plane 100, 200, 220, and 311 respectively.

Govindaraju *et al*, (2010) synthesized the silver nanoparticles synthesized using *Solanumtorvum* leaf extract obtained the broad peak at 38°, 44° and 64° at 111, 200, 220 crystallographic plan. Similar peaks were observed by Logeswari *et al.*, (2013). The XRD pattern thus clearly demonstrated that the Ag NPs formed were crystalline in nature. These Bragg peaks representative of Face Centred Cubic Ag NPs.

The size of Ag NPs synthesized were calculated by Debye Sherrer's equation and observed as e 67.32, 11.4, and 46.86 nm for *T. ammi, P. granatum* and *S. lappa* respectively. According to a study by Logeswari *et al*, (2013) Ag NPs were prepared using the leave extract of *S. tricobatum, S. cumini, C. asiatica and C. sinensis and their* particle size was observed as 53 nm, 41 nm, 52 nm and 42 nm respectively.

The XRD pattern reported by the Panneerselvam *et al.* (2011) of Ag NPs synthesized by *Andrographisp aniculata* showed a number of Bragg reflections observed in the XRD pattern at 2θ = 28.0, 32.4 and 46.4 correspond to the (111), (200) and (311) sets of lattice planes were indexed as face-centered-cubic (FCC) structure of silver.

Mishra *et al*, (2013) synthesized the Ag NPs with aqueous leaf extracts of Ashoka and Neem and described the XRD Peaks as 38.35 at (1 1 1) at 44.352 at (2 0 0) at 64.537 at (2 2 0), 77.473 at (3 1 1) and 81.785 at (2 2 2). The silver nanoparticles synthesized from *T. ammi, P. granatum*, and *S. lappa* in the present study showed the XRD peak according to the above mentioned studies. Shameli *et al.*, (2012) also synthesized the Ag NPs *Curcuma longa* tuber powder observed similar XRD peaks as described by the above mentioned study.

The XRD analysis of Fe NPs of *T. ammi* showed the broad peak at 30.96 °, 35.56°, 43.23° and 54.10° which showed the crystallographic planes of (200), (311),

(511) and (440) respectively. The XRD investigation of Fe NPs of *P. granatum* showed the broad peak at 30.40°, 35.02°, 43.09°, 54.10° and 57.18 which revealed the crystallographic planes of (200), (311), (511) and (440) respectively. The X-ray pattern of *S. lappa* showed the broad peak at 30.26°, 35.00°, 42.95°, 53.96°, and 57.30° which showed crystallographic planes of (200), (311), (511) and (440) respectively.

The XRD analysis showed the number of Bragg's reflections that may be indexed on the basis of the cubic structure of Fe NPs (Shahwan *et al*, 2011). The size of Fe NPs synthesized were calculated by Debye Sherrer's equation and observed 12.34nm, 10.12nm, and 9.89 nm for *T. ammi, P. granatum* and *S. lappa* respectively.

The Transmission electron microscopy technique was used to confirm the size and shape of NPs. According to the present study the shape and size of Ag NPS of *T. ammi, P. granatum and S. lappa* were observed as 65, 10 and 45 nm respectively. The Ag NPs generated by Ponarulselvam *et al* (2012) reported that the TEMs showed the NPs size of 15 to 35 nm when it was reduced with the leaves extract of *Catharanthus roseus.*

According to the present study size of Fe NPs of *T. ammi, P. granatum and S. lappa* was observed as 12, 10 and 9 nm respectively. The Fe NPs generated by Shahwan *et al.*, (2011) using green tea leaves reported that the TEMs showed the nanoparticle size of 40 to 60 nm. The Mahdavi *et al.*, 2013 reported the size of Fe NPs synthesized by *Sargassum muticum* as 18 ± 4 nm by TEM analysis. The size of Fe NPs was observed as 21 nm and 32 nm by using the *Lawsoni ainermi s*and *Gardenia jasminoides* leaves and $FeSO_4$ salt solution (Naseem and Farrukh, 2015).

The Ag NPs have robust anti-bacterial activity. This therapeutic value led the scientist to explore it more medicinal activities like malaria. The NPs were synthesized from Neem and Ashoka gave the hypothesis that every plant extract could give mount to a widespread variety of distinctive microenvironments that can affect the biological and physicochemical properties of the Ag NPs formed (Mishra and Sardar, 2012). There was no vivo study on the antiplasmodial activity of the green

synthesized Ag NPs Although there are the in-vitro assays of antiplasmodial activity of Ag NPs synthesized with different plant extracts.

Mishra *et al*, (2013) synthesized the silver nanoparticles with aqueous leaf extracts of Ashoka and Neem and reported that it had antiplasmodial activity as 68.02% and IC_{50} as 0.5 µg/ml. Different antiplasmodial activity was showed by of Ag NPs that was formulated by *Catharanthus roseus* (Ponarulselvam *et al.*, 2012). The maximum antiplasmodial activity of Ag NPs of *T. ammi, P. granatum and S. lappa* was observed at the dose of 150 mg/kg (83.50 ±0.65), (85.92 ±0.5) and (85.32 ±0.8) respectively.

There was no in-vivo as well as in-vitro study on the antiplasmodial activity of the green synthesized Fe NPs. However, other medical applications of iron NPs include detoxification of biological fluids, detoxification of biological fluids, hyperthermia, tissue repair, and cell separation (Gupta and Gupta, 2005). The Fe Ag NPs have strong anti-bacterial activity (Naseem and Farrukh, 2015).

This therapeutic value led the scientist to explore it more medicinal activities like malaria. The maximum antiplasmodial activity of Fe NPs of *T. ammi, P. granatum and S. lappa* was observed at the dose of 300 mg/kg (77.14±4.18), (81.46±3.10) and (82.51±4.68) respectively. The NPs are administered via intra venous injection in the present study. Thus, systemic administration is also a potential route of entry.

The Nano silver products and materials are getting highest degree of commercialization especially in the healthcare sectors and medicine (Drake and Hazelwood, 2005). Subsequently, silver as Ag NPs has gained an increasing access to tissues, cells and biological molecules within the human body.

The harmful effects of the silver only occur among the workers who have a prolonged history of silver exposure. According to a study by Drake and Hazelwood (2005) metallic silver depicted the minimal health risk.

The present study revealed the low quantity of the Ag NPs and Fe NPs effectively reduced the malaria infection and did not show the adverse effects on the hematological and biochemical parameters, and different organs as depicted by histological studies.

The antiplasmodial activity of green synthesized Ag NPs is greater than the green synthesized Fe NPs. However the effect of iron on the tissue and hematological parameter is more progressive. However, more studies are required for the implementation of present research.

General Discussion

Malaria is still a severe public health risk, especially in third world countries. A majority of the world's population is at risk. Approximately 214 million cases of malaria are reported annually (WHO, 2015). For instance, in sub-Saharan Africa, 10% of child deaths is caused due to malaria. Malarial parasites are spread throughout the world mainly through mosquito vector or blood transfusion (WHO, 2015).

Pakistan is one of the major victim of malaria as it is serious health issue commonly prevalent in Pakistan. Out of 214 million cases, about 4.5 million suspected cases and approximately 2.6 million positive cases of malaria are reported annually in Pakistan (Khatoon *et al.*, 2009; Qayum *et al.*, 2012). Among the major provinces of Pakistan, Sindh and Baluchistan have a high number of incidences of malaria compared to Punjab. However Baluchistan and Federally Administered Tribal Areas (FATA) were detailed with the highest incidence of malaria, and Sindh and Khyber Pakhtunkhwa were reported with a moderate number of incidences (Kondrachine, 2008).

It is difficult to get accurate statistical data regarding the prevalence of malaria in different geographical areas of Pakistan due to the variations in temperature and the variation in the genes of *Plasmodium* species that causes malarial infection (WHO, 2012). In many areas, correct estimation of whether the malaria endemicity is rising, declining or stable is necessary because it will enable the implementation of an effective control measure program and new strategies for its diagnosis and treatment. According to old surveys, both *P. vivax* and *P. falciparum* are prevalent in Pakistan. Approximately 60% of Pakistan's population is at the menace of malaria (Khattak *et al.*, 2013). In fact, among all the cases of malaria, 64% are due to the *P. vivax* and 36% due to *P. falciparum* (WHO, 2006).

The accurate identification of the *Plasmodium* species is also necessary for drug formulation against its infection. The basic tool of identification is microscopy although it is cost effective, rapid diagnosis and simple however it needs high expertise and leads to the misdiagnosis in low Parasitemia and mixed infection (Singh *et al.*, 2010).

The immunodiagnostic tests for the detection of malarial parasite antigen is considered to have advantages our microscopy especially in the rural areas of developing and underdeveloped countries due to lack of electricities in such localities (Endeshaw *et al.,* 2012 and Kyabayinze *et al.,* 2012). Sometimes the RTDs provides high false-positive results due to the long persistence of histidine rich protein 2 (HRP2) in the circulation even after parasitological clearance (Mason *et al.,* 2002). The comparatively molecular techniques provide more accurate results at species level identification thus it should be preferred during low parasitemia and mixed infection.

Regarding seasonal variations the malarial infection increases the incident of malaria began to increase in the spring season. The highest cases were recruited in summer and post monsoon season. The winter season demonstrated lowest cases. Mahmood (2005) and Farogh *et al.*, (2009) also described the similar findings from Karachi and Bahawalpur, Pakistan respectively. The high incidence in these months may be due to the post monsoon period as climatic conditiond in those months are more favorable for breeding of mosquitoes. The *P. malariae and P. ovale* infection was not observed during present and likewise by previous studies (Aijaz *et al.*, 2010).

The graph of malaria incidence fluctuates according to the season the transmission peak is high in post monsoon i.e. from August to October. The finding of the present study is in accordance with the study by Dutta *et al.*, (2010).

The high incidence of malarial parasites was observed in school going children at the age between 5-10 years in Larkana (Soomro *et al.*, 2010). In the present study it is reported that the group of children and youngsters having age between the 1 to 20 years was more infected as compared to the group of the middle age and old age group. This may be due to the greater movement and outdoor activities of these age groups people hence there is more chances of exposure to mosquitos' bite that lead to malaria.

The male were more infected as compared to the females this finding is supported by many previous studies. The prevalence study conducted by Sahar *et al.*, (2010) from Muzaffargarh, Punjab-Pakistan also reported that more males were infected as compared to females. The appropriate reasons may be social and religious

norm. Moreover the females don't get ehough medical attention as compared to male due to least access to medical centers and might not seek medical attention as compared to the males (Khattak *et al.*, 2013).lessened

The incidence on the basis of molecular analysis conducted by Khattak *et al* (2013) showed the distribution of human infection in Pakistan, 76% *P. vivax* infection, 18% *P. falciparum* and 6% mixed infection result. But in Punjab *P. vivax* infection rate was 82% and *P. falciparum* was 18%. However in this study 66.70% *P. vivax* and 23.67% *P. falciparum* infection was reported. The ratio of mixed infection found to be 9.72% by microscopy. The comparison of these two studies showed the frequency of mixed infection is increasing with time. However according to the PCR analysis the percentage infection of *P. vivax* was 62.19% and that of *P. falciparum* was 22.45%. The frequency of mixed infection was observed to be 15.35%. Total 15.02% samples did not amplify with the PCR analysis. However the prevalence on the basis of molecular analysis showed the overall prevalence in a Punjab was 4.8% but according to Khattak *et al* (2013) it was 3.3%.

The outbreak of malaria in Pakistan is because of the many fundamental risk factors for example the population in the low endemic areas has low immune status due to malnutrition. There was irregular transmission patterns, the movement of population from one place to another within and across the country especially across Iran and Afghanistan borders, the poor socioeconomic status, and natural disasters including heavy rain fall and flood (Kebede *et al.*, 2014).

Malarial transmission time increase with increase in sowing and harvesting of crops like wheat, rice and suger However there are still substantial variations in the epidemiology of malaria between different districts of the Punjab. The situations need to be improved and malaria control program needs to be accelerated (DMC, 2007)

The first line of defense against malaria is the antimalarial drugs because there is no commercially available vaccine for malaria infection. The main target of most of antimalarial drugs is the erythrocytic stage of *Plasmodium* which is the symptomatic stage. It is suggested that the antimalarial drugs used for the hypnozoite stage of *P. ovale* and *P. vivax,* should be used for the terminal prophylaxis because once they

enter in the liver they reside for months and years in some cases (Taylor and White, 2004).

The treatment of malaria has been changed in the last few decades due to the reinforcement of the disease in tropical regions and the variations in drug sensitivity for *P. falciparum* infection. Malarial medication is mostly based on targeting various life cycle stages, chemical structure, half life and cost effectiveness (Schlitzer, 2007).

The Quinoline derivatives include chloroquine, quinine, primaquine, lumefantrine, halofantrine, quinidine, mefloquine, primaquine, amodiaquine, lumefantrine, and halofantrine. All these drugs are used against the symptomatic stage (erythrocytic stage) of the *Plasmodium*. However the primaquine also kills gametocytes and exoerythrocytic stages of the *Plasmodium* species but these prime drugs are becoming resistant in the different parts of the world.

However keeping in view about the current scenario of resistance of malarial parasites against existing antimalarial drugs, and plants as a potential source for the new antimalarial agents there is a need to explore new plants for their antiplasmodial potential. The commonly used antimalarial drugs such as **quinine** derived from the bark of cinchona tree and **artemisinin** from the Artemisia annua/Qinghao plant (sweet wormwood) (CDC, 2005).

The only way besides the development of vaccine to save lives from malaria today and tomorrow, there is the need to focus on the two wide areas i.e. the maximize use of current malarial medicines and developing next-generation medicines to support elimination and eventual eradication. The medicinal chemistry is grooming through plant derived compounds since they offer an approach to chemotherapy with specific clinical activity.

The world's one of the oldest medical tradition is the Tibetan medical system. It is an important part of Tibetan culture and has been developed through many centuries. Its origin is as old as civilization itself. The survival and existence of mankind mostly depends on their relay on nature in order to find the solution of their problems especially related to the health. This accumulated knowledge built the steps by discovering certain remedies for common ailments from natural sources (TMH, 2016). Thus five plants i.e. *T. ammi, P. granatum, C. oblonga, B. hispida,* and *S.*

lappa were selected due to their medicinal importance to explore their antiplasmodial activity. The present study applied different concentrations of five selected plants after calculating their LD_{50} in mice infected with *P. bergei* parasitic species.

The *T. ammi* showed the maximum antiplasmodial activity at 500 mg/kg. *P. granatum, C. oblonga,* and *B. hispida* also revealed the highest antiplasmodial activity at 500 mg/kg. Antiplasmodial activities of three doses used for *T. ammi* differ from each other but for *P. granatum, C. oblonga, B. hispida* the difference between three doses like 250, 500, 750 mg/kg did not differ so much especially between 500 and 750 mg/kg. The antiplasmodial activity was found to be dose dependent. The antiplasmodial activity of *P. granatum* at the dose of 500 mg/kg and *S. lappa* at the dose of 500 mg/kg and 750 mg/kg showed highest non-significant ($P \leq 0.5$) when compared with the chloroquine treated group.

The in-vivo antiplasmodial activity of *T. ammi* seed extract against malarial parasite is first time reported in the present study however there are number of reports of in-vitro studies. Kamaraj *et al* (2012) reported that Seed extract of *T. ammi* prepared in ethyl acetate showed the antiplasmodial activity up to 25±0.7 against *P. falciparum* in in-vitro culture.

The Crude methanol extracts of fruit *P. granatum* showed antiplasmodial activity in in-vitro cultures against *P. falciparum* (Al-Musayeib *et al.*, 2012). It is used as anti-malarial home remedy in India. A study conducted by Dell'agli *et al*, (2010) fruit rind of the immature fruit of this plant is used as an herbal formulation in India, for the treatment and prophylaxis of malaria. They used the methanolic extract that inhibited the parasitemia in vitro.

The present finding on the antiplasmodial activity of *P. granatum* is in accordance to the research by Al-Musayeib *et al.*, (2012). However in the present study the extracts were taken in water instead of methanol and experiments are based upon *in vivo* practice. There is no study reported on the antiplasmodial activity of *C. oblonga.* Although other biological activities have been reported like antidiabetic and antioxidant etc. The Hydroalcoholic extract of the fruits of *C. oblonga* Miller (quince) increased the aphrodisiac activity in wistar rats. It is regarded as a strong libido invigorator in Unani System and Tib-e-Nabvi medicine (Aslam and Sial, 2014).

The antiplasmodial activity of *B. hispida* was first time reported in the present study. However its antidepressant, in the treatment of Alzheimer's disease, anti-inflammatory, analgesic, antiasthmatic, antidiabetic, hypolipidemic, diuretic, nephroprotective, and antimicrobial effects were studied (Al-Snafi, 2013).

The *S. lappa* is also used usually for the treatment of skin diseases such as scabies, ringworm bruises and cuts (Sun *et al.*, 2003). It possesses the anti-parasitic activities but its antiplasmodial activity is not reported up to the date. The antiplasmodial activity has been reported in the present study and aqueous extracts of the seeds of this plant exhibit the maximum antiplasmodial activity as compared to the other plants used in the present study.

An in-vivo study on the solvent fraction of methanolic root extract of *Dodonaea angustifolia* against *P. berghei* infected mice revealed that LD_{50} was observed above than 2000 mg/kg and antiplasmodial activity as 67.51% at an oral dose of 600 mg/kg of n-butanol fraction. Lower dose 200 mg/kg and 400 mg/kg produce the activity as of the fraction also resulted in parasitemia suppression of 38.02% and 55.85%, respectively. Similarly in this study the *P. granatum, C. oblonga, B. hispida, and S. lappa* although showed antiplasmodial activity but of the medium and high doses did not differ significantly although in both cases the antiplasmodial activity was higher than n-butanol fraction of root extract of *Dodonaea angustifolia*as studied by Amelo *et al.*, 2014.

A study conducted by Hafiz *et al*, (2016) in order to evaluate the activity of methanolic peel extract of *P. granatum* against malaria-induced hepatic injury and oxidative stress in *P. chabaudi* infected mice. The *P. granatum* extract successfully reduced the anemia, hepatic injury and oxidative stress. The Methanolic extract of *P. granatum* reduced parasitemia oxidative stress and inflammation of spleen in the *P. chabaudi* infected mice (Mubaraki *et al.*, 2016) similarly in this study the experimented plants reduced the inflammation of liver and spleen. The parasite burdon during malaria infection is associated with the complete blood picture. So *the* hallmark of malaria is Hematological abnormalities especially during *P. falciparum* infection (Perrin *et al.*, 1982). The severe malarial anemia is considered as main cause of mortality in animals. The mechanism of anemia due to malaria is similar to those of human anemia which is due to the loss or damage of infected RBCs or may be due to

loss of non-infected RBCs (Lamikanra *et al.* 2007). There was a marked reduction in hemoglobin (HB), mean cell hemoglobin (MCH), red blood cell (RBC) count, hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC) and Platelet count of infected but non treated group as compared to the normal and treated groups.

The malaria infects multi-organs and drug may also leave side effects on many organs. So the histopathological studies were carried out on the three majorly infected organs like liver, kidneys and spleen. The confiscation of PRBCs, in the microvasculature was observed. In the liver, macrophages swallow up the PRBCs resulted in the lyses of blood cells the haemozoin were remained there.

The spleenomegaly and discoloration of the spleen of mice infected with *P. berghei* copy the conditions of *P. falciparum* infection in human as splenomegaly is a common symptom in which spleen becomes enlarged due to the accumulation of malaria pigment haemozoin caused by an increase of the pulp element (Basir *et al.*, 2012). The sequestrations of PRBC were also observed in the kidney cells.

The results of histological studies were comparable with histopathological study on ICR mice infected with *P. berghei* ANKA strains, as a model of cerebral malaria (CM). He observed the splenomegaly, hepatomegaly and discolorations of major organs and severe anemia due to the high parasitemia (Basir *et al.*, 2012). Similarly all the symptoms were observed for the infected but not treated group. The histological results were also similar to Hafiz *et al.*, (2016) worked on liver and Mubarak *et al.*, (2016) on spleen of mice infected with malaria. Among these serum ALT, ALP, and AST are the bio markers for liver health and urea and creatinine concentration were used for the evaluation of renal efficiency.

According to previous reports, chloroqunine was found to increase enzymatic activities and also a mark increase in autophagy of exposed cells. These results supports our fingings of increased enzymatic activity of kidney and liver cells of mice treated with cloroqunine A significancant change in alanine amino transferase ALP, serum aspartate aminotransferase, ALP have been reported due to administration of artemisinine (Udobre *et al.*, 2009). The elevated level of liver bio mark i.e., ALT, ALP, and AST in chloroquine treated group was observed as compared to the group

treated with *P. granatum* (500 mg/kg) and *S. lappa* treated groups (500 and 750 mg/kg) was also observed in the present study.

Now a day the pharmaceutical science is diverging towards the nanotechnology. The nanoparticles of metals and their oxides are extensively used in drug delivery, cosmetics, agriculture, food supplements, paints, catalysis, and material science. The Nanoparticles of different metals e.g. iron, platinum, Gold, and silver has been synthesized (Mishra *et al.*, 2010). The silver and Iron nanoparticles were prepared in the present study because the silver is widely used as nanomedicine. They are used in the health care centers, for imaging, drug delivery, therapeutics and diagnosis (Mishra *et al.* 2007). They have been added in many consumers' products like industrial and food products (Krutyakov *et al.*, 2008).

The Ag and Fe NPs were confirmed by UV-visible Spectroscopy, XRD, FTIR spectroscopy and Transmission electron microscopy. The present studies revealed that maximum antiplasmodial activity of Ag NPs of *T. ammi, P. granatum and S. lappa* was observed at the dose of 150 mg/kg was (83.50 ±0.65), (85.92 ±0.5) and (85.32 ±0.8) respectively. The antiplasmodial activity of low doses used for the Ag NPs e.g. 50 and 100 mg/kg did not differ significantly with the high dose except for the Ag NPs of *S. lappa.*

The Maximum antiplasmodial activity of Fe NPs of *T. ammi, P. granatum and S. lappa* was observed at the dose of 300 mg/kg and was (77.14±4.18), (81.46±3.10) and (82.51±4.68) respectively. The antiplasmodial activity of low doses used for the Fe NPs e.g. 100 and 200 mg/kg did not differ significantly with the high dose except for the Fe NPs of *T. ammi* similar to Chandel & Bagai, (2010).

The antiplasmodial activity was found to be greater for Ag NPs than of Fe NPs in the present study. The survival time of the Ag NPs of *T. ammi, P. granatum, and S. lappa* treated groups showed between 15 to 19 days and that of Fe NPs of *T. ammi and P. granatum* was 16 to 19 days and three dose of *S. lappa* treated groups showed the survival rate between 20 to 23 days that was comparable to the normal control group this finding was in accordance with the Amelo *et al.*, (2014).

The percentage weight change among the Ag NPs treated groups was maximum in the *T. ammi* treated group (12.26 g) at dose of 150 mg/kg than in the *S.*

lappa group (10.93 g) and (10.98 g) treated at the dose of 100 and 150 mg/kg respectively. The minimum percentage weight change was observed in the Fe NPs treated groups. Among all the experimented plants the maximum weight change (9.20 g) was observed in the Fe NPs treated group of *S. lappa* at the dose of 200 mg/kg. The weight change in the Fe NPs was in agreement with Amelo *et al.*, (2014) who determine the antiplasmodial activity of different solvent fractions of *Dodonaea angustifolia* extract prepared in the methanol.

There Hematological parameters (RBC), (HB), (HCT), (MCV), (MCH), (MCHC) and Platelet count of plant treated group was high in the *P. granatum, B. hispida and S. lappa* treated groups infected but non treated group as compared to the treated groups. These parameters were high for all Ag NPs of *S. lappa* treated groups and it was comparable to the normal group of mice. The remaining Ag NPs treated groups showed these values were in compatible to chloroquine treated group that did not differ so much from the normal groups.

The Fe NPs treated groups showed that these Hematological parameters were well-matched with the normal group values. The monocyte and lymphocytes value was high in the non-treated groups as compared to the drug and plants and NPs treated groups. There was significant increase in serum ALT, ALP and AST, urea and creatinine in infected control as compared to the normal ones. The ALT, ALP, and AST values in the Ag NPs of *T. ammi* and *P. granatum* treated at the dose of 100 mg/kg was in agreement with the chloroquine treated groups however the Ag NPs of *S. lappa* indicated that ALT, ALP, and AST values were compatible to the normal group. The ALT value in the Fe NPs treated group was high but an ALP and AST values were in comparable to drug treated group. The creatinine and uric acid values of all plant treated and NPs treated group was compatible to chloroquine treated group but less than infected but non treated groups. The elevate level of ALP and creatinine was observed in the infected group as compared to the treated steroidal alkaloid conessine isolated from the bark of *Holarrhena antidysenterica* treated groups (Dua *et al.*, 2013) and against the use of homeopathic compound Nosode 30 and 200 (Bagai *et al.*, 2012).

The mechanism by which the nanoparticles interacts with cell and initiates any biological response is very important and this understanding can reduced or overcome

the possible toxic effect of nanoparticles. Banerji and Hayes, (2007) reported that some nano particles diffuse through the cell by non edocytic pathways. They also reported that gold particle capped with citrate having diameter of 7-15 cannot pass through the lipid membrane and are surrounded by small vesicles composed of phosphatidylcholine, cholesterol and phosphatidic acid.

On the other hand hydrophobic alkane-thiol coated gold nanoparticles (2mm) become locked inside of the membrane (lipid bilayer). Similar results have also been reported whole using nanoparticles made up of amorphous silica (Grigor'eva *et al.*, 2013).

The nanoparticles having diameter of 5 nm were found to have good biocompability, can easily enter the cytoplasm of cell and have haemolytic activities (Rasch et al., 2010). The Ag-NPs can produce the cytotoxicity through oxidative stress. The mode of action between Ag-NPs and cell is not clear yet (Carlson *et al.*, 2008).

For malaria control various metal-based anti malarial drugs are used. Many metal complexes that are in use as antimalarial use metals such as iron, gold, rhodium, copper, cobalt, zinc, ruthenium, cobalt, and palladium. The possible mechanism of inhibition of malaria is heme aggregation inhibition by metal ion complex. The metal chelating agents is the strong agent that can cure severe *P. falciparum* infection. The activity against resistant parasites may be due to the structural modification.

The active constituents in the plant or plant extract reduce it and make it effective for different medicinal use. The coated Fe_3O_4 nanoparticles would showed the superior antiplasmodial activity (Sekhon, 2016: Biot *et al.*, 2012).

The green synthesis of nanoparticles has many advantages including economic viability, easy to handle and synthesize. Therefore the development of new metal based anti-malarial drugs is strongly recommended. There is a need to explore more plant resources. The experimental conditions for the preparation of plant extract, namely, plant mass: solvent ratio, extraction temperature, and time, along with the mixing ratio of plant extract and metal salt solution, should always be optimized.

The antiplasmodial activity of green synthesized Ag NPs found to be greater than the green synthesized Fe NPs. However the effect of iron on the tissue and

hematological parameter are more progressive. The green synthesis of NPs are more effective and cost effective than chemical methods so the current study was also focused on the use of medicinal plants for the formation of Ag and Fe NPs in order to analyses their antiplasmodial effect.

This study contributes to a whole heap in the nanomaterials as an alternative medicine for the future. The green synthesis of nano particles contributed to the development of least toxic material having high biocompatibility. These are synthesized by using biomaterial such as lipids, proteins and plants extract. This green synthesize helped to overcome one of the challenge i.e. toxicity of todays used nanprticles during drug delivary. However, further studirs are required for the development of safer nanoparticles used in pharmaceutical industry.

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Appendix A

Parental Approval Form

(Parent or Guardian Must Complete)

I hereby give my consent to have my son/daughter_____ Participate in a study about malaria control. I also authorize the physician or technician to share blood sample of my children to Ms. Huma Fatima from Parasitology Lab of Quaid-e-Azam university for research purpose only.

_____ _____

Signature of parent **Date**

_____ _____

Relationship to Child **Telephone**

Address_____

City_____ **State**_____ **Zip code**_____

Mom/Dad/Guardian's work phone_____

Son/Daughter's birth date_____

Name of child's physician_____

Medical History (if any)

_____

_____

_____

Appendix B

Performa of sample collection

Date: _____ **Month:** _____ **Year :**_____				
	Subject 1	**Subject 2**	**Subject 3**	**Subject 4**
Personal				
Field visit (s)				
Performa No.				
Sample I.D				
Slide collection				
Origin of subject				
Gender				
Age				
Language				
Education				
Occupation				
Contact				
Symptoms				
Shivering				
Temperature				
Coma				
Convulsion				
Specie Identification (Through Microscopy)				
P .viavx				
P. falciparum				
P. malariae				
P. ovale				
Mixed infection				

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IN VIVO ANTIPLASMODIAL POTENTIAL OF AQUEOUS EXTRACTS OF *TRACHYSPERMUM AMMI* AND *PUNICA GRANATUM* SEEDS IN MALE BALB/C MICE

HUMA FATIMA[1], NAVEEDA AKHTER QURESHI[1]*, NARGIS SHAHEEN[1], UMER RASHID[2] and MUHAMMAD FIAZ QAMAR[3]

[1]Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[2]Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan
[3]University of Vetinery Sciences, Lahore, Pakistan

Abstract: Natural products are the rich source of drug discovery especially in the case of malaria. To explore the new antimalarial agent, present study was conducted. In the present study, antiplasmodial activity of the aqueous extracts of *Trachyspermum ammi* and *Punica granatum* seeds were studied using *Plasmodium berghei* infected male BALB/c mice. After seventy two hours post infection; mice were treated with (i) extracts of different concentrations i.e., 250, 500 and 750 mg/kg of both experimented plants (ii) water and (iii) with chloroquine. The effect of seed extracts was studied by general symptoms observation, parasite count, chemosupression, survival time measurement, histopathology, biochemical and hematological analysis. The maximum antiplasmodial activity (77.58 ± 2.09) for *P. granatum* and (68.76 ± 2.01) for *T. ammi* was observed at the dose of 500 mg/kg for both plants. The results revealed significant decrease ($p < 0.001$) when the parasitemia was compared with water treated group.The mean survival time was increased up to 1.5-1.7 fold for *T. ammi* and *P. granatum* that confirmed their curative activity. The biochemical and histopathological analysis also showed a marked difference in infected untreated group compared to groups treated with extracts or chloroquine. A progressive decrease in the weight was also observed in untreated groups as compared to the treated ones. The qualitative phytochemical analysis of experimented plants revealed the presences of a number of chemical compounds such as tanins, flavonoides, alkaloides, phenols and reducing sugar etc. It is concluded from the present study that the seeds of *T. ammi* and *P. granatum* exhibit antiplasmodial activity and the active compounds can be identified and isolated by using HPLC and GC-MS.

Keywords: medicinal plants, aqueous extract, antiplasmodial activity, *Plasmodium berghei*, BALB/c mice

Malaria, a major tropical disease is responsible for significant morbidity and mortality worldwide especially in the Third World countries. Among the global prevalence in 2015, 214 million cases were estimated. Most of the cases were from African Region (88%) followed by South-East Asia Region (10%) and Eastern Mediterranean Region (2%). It is the fourth main cause of death, accounting for 10% of child deaths in sub-Saharan Africa (1). In 2010, it was considered as neglected tropical disease along with other protozoan infection. These diseases may cause adverse impacts on the socio-economic status of the country as well as on the infected people (2).

Estimated 4.5 million suspected cases of malaria has been reported in Pakistan. Pakistan is one of the 6 countries within WHO Eastern Mediterranean Region with high malaria transmission of which 64% cases were reported by *P. vivax* and 36% by *P. falciparum* (3). The *Plasmodium* species responsible for producing infection in human are *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae* and *P. knowlesi* (4). Among all these *Plasmodium* species, the *P. falciparum* infection is the most virulent and is characterized by long-term, persistent contagions that can last for many months (5).

Currently, many drugs are in use for curing malarial infection like quinine, resochin, chloroquine, sontochin, proguanil, sulfones and sulfonamides: proguanil or pyrimethamine, mefloquine artemisinin (6). These drug therapies are associated with parasite resistance and side effects (7). Thus, there is a dire need of alternative antimalarial chem-

* Corresponding author: nqureshi@qau.edu.pk; naveedaqresh@gmail.com; phone: +92 (51) 90643201

icals to combat the resistance of the existing drugs used to control malaria. One of the most promising is the use of natural products.

Natural products are the rich source of medicinally important substances. Their contribution towards the drug discovery is impressive. Use of phytochemicals/botanical sources are the starting points for many drug discovery programs. The medicinal plants have provided very important lead compounds against various biological targets including infectious diseases, cancer, pain, HIV/AIDS. Biologically active compounds isolated from natural source would be easily degradable and safer to man (8). The present study is based upon the exploration of antiplasmodial activities of *Trachyspermum ammi* (Ajwain) and *Punica granatum* (Pomegranate, Anar).

Trachyspermum ammi (family Apiaceae) also known as Ajwain, is an important seed spices, cultivated in Pakistan, Afghanistan, India, Iran and Iraq. It has medicinal importance and is used for various diseases in human as carminative, anthelmintic, antispasmodic and laxative (9). *P. granatum* (Pomegranate, Anar) is an ancient fruit that cultivated all over the Mediterranean regions. Antioxidant properties of pomegranate are elaborated through various studies. Pomegranate comprises on anthocyanins and flavonoids that show potent antioxidant activity. It is used for diarrhea, microbial infections, hemorrhage, acidosis, dysentery respiratory pathologies and protozociadal properties (10).

Keeping in view the medicinal importance of *T. ammi*, and *P. granatum* plants, antiplasmodial activity of the aqueous extracts of seeds of these two plants was studied using *Plasmodium berghei* infected male BALB/c mice.

METHODOLOGY

Experimental design

The present study was conducted with the approval of Ethical Review Committee of the Biological Sciences Quaid-i-Azam University, Islamabad. The selected plants *Trachyspermum ammi* (Ajwain) and *Punica granatum* (Pomegranate, Anar) were identified with the help of taxonomists of the Department of Plant Sciences Quaid-i-Azam University Islamabad. The aqueous seed extracts of plants were obtained with Soxhlet apparatus (SYD-260) and percentage yield was calculated. The BALB/c mice six to eight week old were taken from National Institute of Health, Islamabad. The model animals were acclimatized for four weeks in the animal house at 26-29°C provided with Pellet diet and water at *libitum*. The animals with average weight of 30 to 36 g from the reared stock were infected with chloroquine-sensitive *Plasmodium berghei* NK 65 strains (attained from MR4/American Type Culture Collection). Serial passage of *P. berghei* was initiated according to the Product Information Sheet no. MRA-268. Growth of parasites was monitored daily by tail vein blood sampling and Giemsa-staining of thin blood smear under light microscopy, starting on the day 3, post-inoculation until the parasitemia reaches 10-30%. Malaria parasites were collected at the ring or early trophozoite stage. The cell concentration of the stock was determined and diluted with phosphate buffer saline such that 0.2 mL of the final inoculums contained 10^6 parasitized red blood cells (RBCs).The experimental animals were divided into nine groups, including controls, in triplets each having five individuals (Table 1). Each mouse was inoculated intraperitoneally with 0.2 mL diluted infected

Table 1. Division of experimental animal in different groups.

Groups	Dose
A	Non infected non treated (control 1)
B	0.5 mL distilled water (control 2)
C	5 mg/kg chloroquine (control 3)
D 1	250 mg/kg of *T. ammi*
D 2	500 mg/kg of *T. ammi*
D 3	750 mg/kg of *T. ammi*
E 1	250 mg/kg of *P. granatum*
E 2	500 mg/kg of *P. granatum*
E 3	750 mg/kg of *P. granatum*

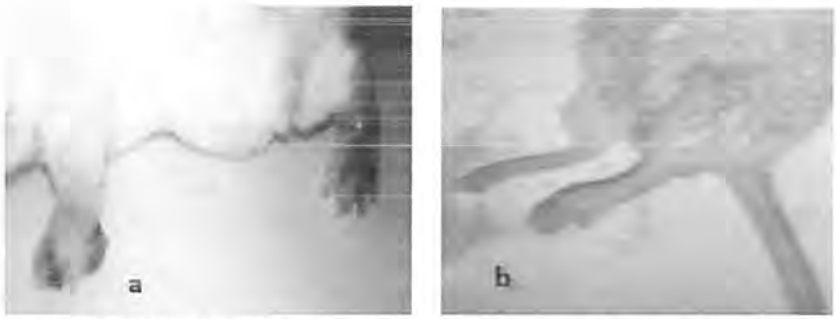

Figure 1. Limb paralysis (a) Two limbs of a mouse were paralyzed in B group. (b) One limb was paralyzed in D 1 group

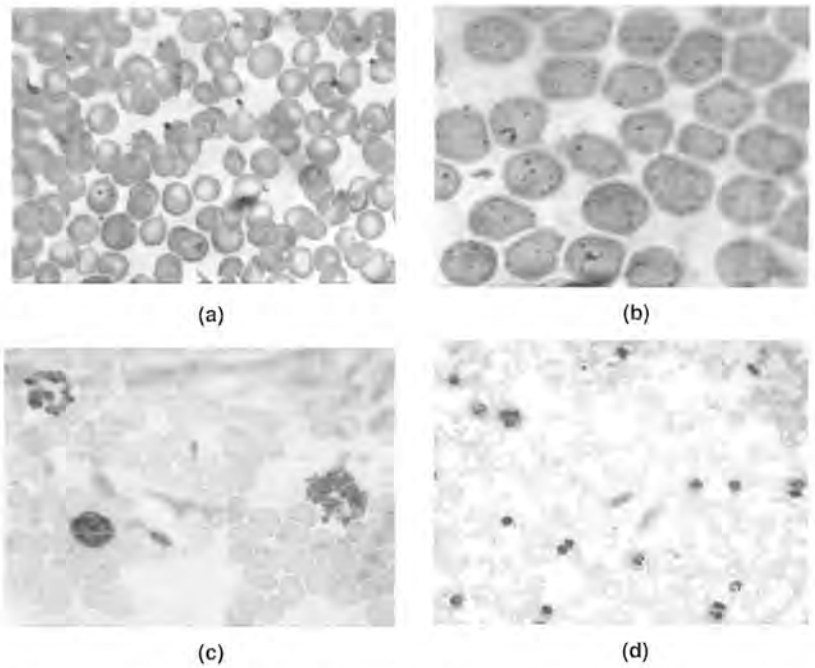

Figure 2. The life cycle stages of *Plasmodium berghei* observed in erythrocytes. (a) Early trophozoites stage (b) late stage trophozoites (multiple trophozoites infect the same cell), (c) late stage schizonts. Rupturing schizonts can be seen indicated by arrow. (d) Multiple schizonts in the late stage of infection. Observed under light microscope at 10 × 100 except (d) magnification at 40 ×

blood except the five mice in group A. Drug and extracts were administered after the 72 h of the post infection for five days.

The effect of plant extracts against *P. berghei* infection

The antiplasmodial activity of *T. ammi* (Ajwain) and *P. granatum* was confirmed by following tests.

Acute toxicity test

A trial study was conducted to identify the acute toxicity of the plant extracts. There were five mice in each group and each group was taken in replicate. The animals were observed for toxicity signs like changes in physical appearance, motor and feeding activities, behavioral change, and other signs of acute toxicity and mortality after administration of the dose. Death rates of the mice in each group were observed. The LD_{50} value of each plant was calculated with the help of probit analysis test on the mini tab version 16. Acute toxicity test was conducted according to the guidelines provided by Organization for Economic Cooperation and Development (OECD).

Microscopic examination

Blood sampling from the tail vein of each mouse, for microscopy, from experimented animals was started after 72 h of inoculation of parasite

strains, taken after each 48 h and continued up to 30th day of the experiment. The slides were stained according to the procedure as described by Sathpathi et al. (11). The prepared slides were examined under 10 × and 100 × magnification with the light microscope (B350 Optika, Italy).

Monitoring of parasitemia and antiplasmodial activity

The numbers of parasitized erythrocytes were counted out of 500 total red blood cells at random fields from prepared slides under light microscope. The mean survival time for each group was determined by finding the average survival time (days) of the mice (post-inoculation) in each group over a period of 30 days (D0–D29). The % age parasitaemia, chemosupression and survival time were calculated by using formulas suggested by Amelo et al. (12).

Morphology, hematology, biochemical and histopathology analysis

Morphological symptoms of malaria like body weight, ruffled fur, wobbly gait, limb paralysis, convulsion, coma, and survival rate were recorded from first day up to 30th day of the experiment after each 24 h interval for all the groups. Approximately 13 mL of blood from each group was collected in EDTA Vacutainers® by cardiac puncture for hematological analysis e.g., anemia, thrombocytopenia, and reduced blood count as in malaria and CBC using BC-3200 Auto Hematology Analyzer. The Histopathological study was performed on liver, spleen and kidneys, collected from the animals on tenth day of post-infection, preserved in 10% buffered formalin. Permanent slides were prepared using stains hematoxylin and eosin (H&E). The morphological changes within the tissues were observed under light microscopy (B350 Optika, Italy) at 10 × and 100 × magnifications and compared with control. The liver function test (LFT) i.e. aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and kidney function tests e.g., creatinine and uric acid levels were analyzed by using their respective standards of Randox diagnostic kits with chemistry analyzer (Motenu MTN-658C).

Qualitative phytochemical analysis

The qualitative phytochemical analysis of the seeds of both plant were analyzed by different chemical tests using standard procedure of Sofowara to identify constituents (13).

Statistical analysis

The statistical analysis was performed by Minitab version 16. The values of the different groups were compared using one-way ANOVA (analysis of variance) and multiple comparison tests (Tukey's test). The comparison of each sample with control was made by t-test. All results were

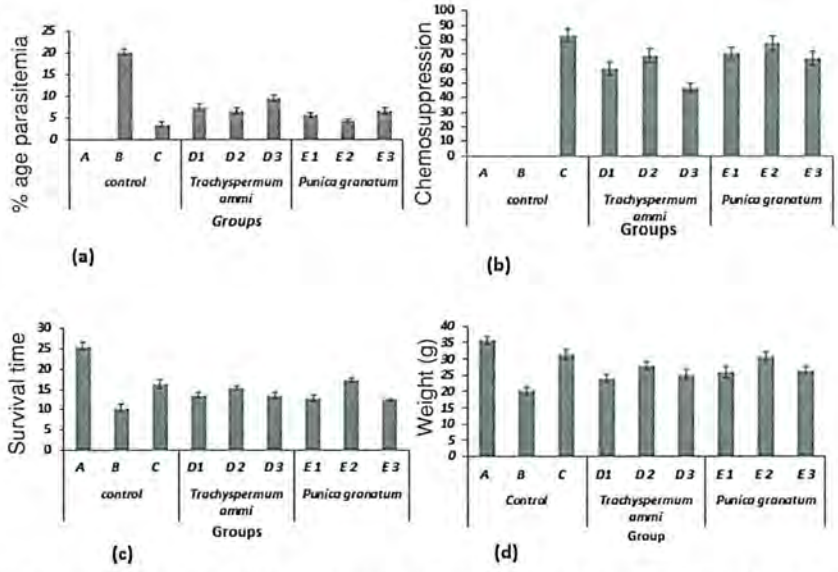

Figure 3. Shows (a) percentage parasitemia, (b) chemosuppression (c), survival time (d) change in weight of control and treated groups. Data is presented as the mean ± SE with n = 5, $p < 0.0001$ except for graph d where $p < 0.05$.

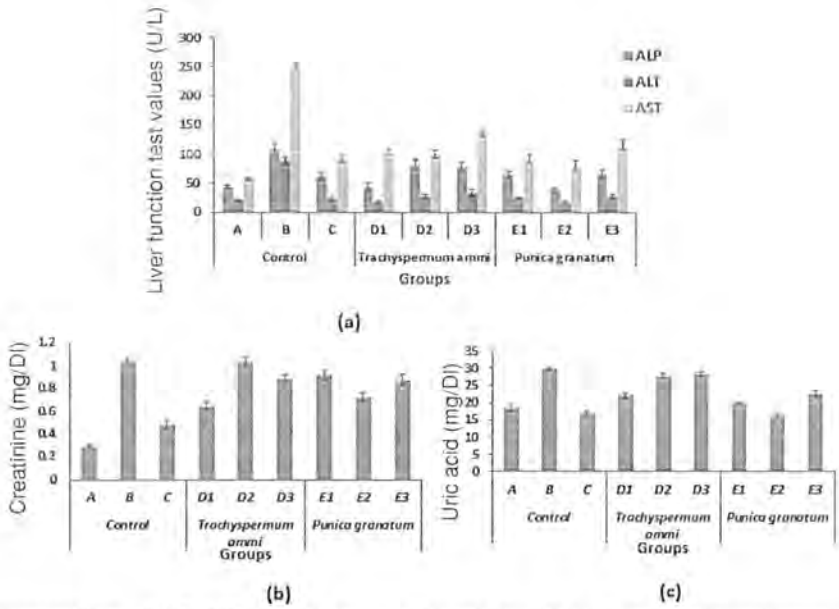

Figure 4. Plasma concentrations of ALP, ALT, AST (a), creatinine (b), and uric acid (c) of control and treated groups are shown in this graph. Data are shown as the mean ± SE with n = 5, $p < 0.001$

expressed as the mean + S.E.M (standard error of the mean). $p < 0.05$ was taken as statistically significant.

RESULTS

Antiplasmodial activity of *T. ammi*, *P. granatum* seed extracts

The antiplasmodial activity was confirmed by: (i) percentage yield of extract, (ii) acute toxicity, (iii) general symptoms, (iv) percentage parasitaemia, (v) chemosupression, (vi) mean survival time (MST), (vii) weight analysis, (viii) hematology, (ix) liver and kidney function tests, (x) morphologycal study of organs and (xi) histopthalogy.

The percentage yield for 25 grams of *T. ammi* and *P. granatum* seeds was calculated as 4.8, and 7.2 as calculated by formula suggested by Zhang et al. (14) and lethal doses LD_{50} were observed as 831 and 1989 mg/kg, respectively, for the two experimented plants. The minimum effective lethal dose (ED_{50}) was observed as 143.02 of *T. ammi* and 124.45 of *P. granatum*. The ruffled fur was observed in all the groups from third to fifth day of infection except in the normal control group A, while in Group B it was observed up to 10^{th} day of the infection. Limb paralysis was observed in infected and non-treated group (B) and two animals from D1 group and D3 group that were treated with 250 mg/kg and 750 mg/kg of *T. ammi*, respectively (Fig. 1). No convulsion and coma was observed among all groups. The early trophozoites and late schizont stages of parasites were observed in liver cells of infected animals (Fig. 2).

The maximum % age parasitemia was observed in the negative control group B (19.97 ± 0.74) that was significantly differ ($p = 0.001$) when compared with the chloroquine treated group C (3.32 ± 0.67). The groups treated with plant extracts like D2 (6.496 ± 0.76), E1 (5.53 ± 0.60) and E2 (4.26 ± 0.26) showed very high significant difference (p = 0.001, 0.000 and 0.000), respectively, when compared with the infected but non treated group B (19.97 ± 0.74). Maximum parasitemia in plant treated group was observed in the following descending order D3 (9.33 ± 2.19) > D1 (7.294 ± 0.782) > E3 (6.44 ± 1.22) > D2 (6.450 ± 1.27) > E1 (5.53 ± 0.71) > E2 (4.26 ± 0.26). Other plant treated groups D1, D3 and E3 also indicated the significant difference (p = 0.001, 0.013 and 0.001), respectively, when compared to the non-treated group B on the fifteenth day of the experiment. Highest level of % age parasitemia was observed on the day four to six for almost all groups and continuously increased in group B and gradually decreased in treated groups (Fig. 3a).

The maximum antiplasmodial activity was observed for the group treated with chloroquine (83.48 ± 2.15), which differ non-significantly (p = 0.057, and 0.163) for the groups E1 (70.89 ± 2.1) and E2 (77.58 ± 2.09) that were treated with concentrations of 250 and 500 mg/kg of *P. granatum*,

respectively. While groups D2 and D3 treated with 500 and 750 mg/kg of *T. ammi* also showed non-significant difference ($p = 0.154$ and 0.062) in antiplasmodial activity i.e., (68.76 ± 2.01) and (46.90 ± 1.15), respectively. The mice of group D1 (59.95 ± 2.15) and E3 (67.10 ± 1.5) showed significant difference ($p = 0.030$ and 0.043), respectively when compared with group C. Highest non-significant difference was observed for groups E2 and D2 indicating maximum chemosupression when compared with group C (Fig. 3b).

The mean survival time (MST) was maximum for normal control mice group A i.e., (25.6 ± 1.64) days and it differed significantly ($p = 0.006$) when compared with the non-treated group B (10.4 ± 1.064). The MST of the chloroquine treated group C was 16.2 ± 0.4 days and was significantly different ($p = 0.05$), when compared with infected but non-treated group B (10.4 ± 1.064). In the treated groups highest survival time was observed for E2 group (17.2 ± 0.385) then for chloroquine treated group C (16.2 ± 1.1days), D2 (15.4 ± 0.57) D1 (13.60 ± 0.98), E3 (12.14 ± 0.99) on 15th day of experiment. The survival time was significantly different ($p = 0.05$) for E2, C, D2, and D1, respectively, and low non-significant difference ($p = 0.07$) for E3 group as compared with non-treated group B (10.4 ± 1.064) (Fig. 3c).

There was a gradual increase in weight of the control group A up to the fifteenth day of the experiment whereas a progressive decrease in body weight was observed in infected but non treated group B ($p = 0.005$). However, a non-significant difference was observed among group A (non-infected) and C (treated with chloroquine) ($p = 0.330$). The groups treated with plant extracts E2, D2 and D1 showed significant difference ($p = 0.009$, 0.043 and 0.018) and E1, E3 and D3 depicted the non-significant ($p = 0.060$, 0.160 and 0.145) weight change when compared with non-treated group B. The change in weight among plant treated groups,D2, D3, E1, E2 and E3 was non-significant ($p = 0.5$) except for the D1 when compared with the chloroquine treated group C. The treated groups showed a gradual loss in weight till the day third and up to day five in some groups as compared to the day first of infection after that a steady gain in weight occurred in the treated groups (Table 2, Fig. 3d).

The hematological studies were based upon the red blood cell (RBC) count of normal control group (A) compared with infected but untreated group (B) and infected but treated with chloroquine group (C) demonstrated highly significant ($p = 0.005$) and less significant ($p = 0.033$) difference, respectively. Probability was equal to 0.009, 0.01 and 0.011 for D1, D2, and D3 groups, respectively, indicating that the RBC in the D3 group treated with 750 mg/kg of *T. ammi* was higher than the D1 and D2 when compared with normal mice of group A. There was highest non-significant difference ($p = 0.162$, 0.049 and 0.014) for the E2 group than for E1 and E3, respectively, in contrast to normal control group A. The significant difference of RBC count was observed between B and C groups ($p = 0.003$). Among the D1 ($p = 0.147$), D2 ($p = 0.049$) and D3 ($p = 0.012$), only the D2 and D3 groups showed the significant difference. But the highest significant difference i.e., ($p = 0.006$, 0.003 and 0.004) was detected for E1, E2 and E3 groups respectively. Highest significant difference was observed for the E2 group. The non-significant difference of RBC count was observed between C group and D1, D2, D3, E1, E2, and E3 groups illustrating the RBC

Table 2. Comparission of body weight change.

Groups	Treatment	Body weight		% age change
		Day 1	Day 15	
A	No	35.83 ± 0.764	40.31 ± 0.153	12.51
B	Distilled water	33.90 ± 0.265	19.87 ± 3.87	-41.38
C	Chloroquinine	34.40 ± 0.107	37.60 ± 0.393	9.30
D1	250 mg/kg *T. ammi*	33.25 ± 0.274	32.40 ± 0.841	2.40
D2	500 mg/kg *T. ammi*	33.86 ± 0.399	36.99 ± 1.09	9.24
D3	750 mg/kg *T. ammi*	36.33 ± 0.322	39.03 ± 0.313	7.45
E1	250 mg/kg *T. ammi*	34.11 ± 0.495	36.88 ± 0.168	8.12
E2	500 mg/kg *T. ammi*	35.27 ± 0.255	39.9 ± 0.470	11.67
E3	750 mg/kg *T. ammi*	33.82 ± 0.371	36.42 ± 0.540	7.68

numbers in the plant extract treated groups was compatible with chloroquine treated group.

The normal control group (A) showed significant difference (p = 0.002) of hemoglobin with infected but untreated group (B) and highly non-significant difference (p = 0.312) with the group (C) treated with chloroquine. Different dose groups of plant *T. ammi* D1, D2, and D3 groups. D2 showed the non-significant difference (p = 0.408) and significant difference (p = 0.007 and 0.043) for D1 and D3, respectively, when compared with control group (A). Highest non-significant difference (p = 0.950 and 0.087) was observed for the E2 and E3 groups, respectively, and in the E1 there was significant difference (p = 0.023) and when compared with normal control group A. The highest significant difference was observed for hemoglobin between B and C group (p = 0.001). The plant treated groups D1 exhibited the non-significant difference (p = 0.158) while the D2 and D3 groups indicated the significant difference (p = 0.002) (p = 0.025) respectively when compared with the non-treated group B.

There was the non-significant difference (p = 0.871, 0.072, 0.377, and 0.175) for hemoglobin among the D2, D3, E2, and E3 groups, respectively. But there was significant difference for D1 and E1 (p = 0.007 and 0.032) when compared with the chloroquine treated group C.

The group (A) showed highly significant difference (p = 0.006) of hematocrit (packed cell volume) with infected but non medicated group (B) and significant difference (p = 0.038) with the group (C) treated with chloroquine. The groups treated with the different doses of *T. ammi* i.e., D1, D2, and D3 groups, which showed significant difference (p = 0.027, 0.009, and 0.017), respectively, for hematocrit as compared with normal control group (A).

There was significant difference between B and C group (p = 0.023). Groups treated with the *T. ammi* plant extract D1, D2 and D3 showed non-significant (p = 0.080, 0.089 and 0.059) respectively when compared with the infected but treated with water group B. The significant difference p = (0.093, 0.017 and 0.017) was observed for *P. granatum* treated groups E1, E2 and E3, respectively, when compared with the group B. Non-significant difference (p = 0.132, 0.080, 0.421, 0.098, 0.251 and 0.314) for hematocrit was observed between C group and D1, D2, D3, E1, E2, and E3 groups, respectively.

The group (A) showed highly significant difference (p = 0.000) of MCV with infected but non medicated group (B) and significant difference (p = 0.018) with the group (C) treated with chloroquine. The groups D1 and D3 showed significant difference (p = 0.014, 0.018) while D2, E1, E2 and E3 indicated the non-significant difference (p = 0.079, 0.748 0.099 and 0.079), respectively, as compared with normal control group (A) (Table 2).

There was a highly significant difference (p = 0.005) between infected but non treated group B and chloroquine treated group C. The plant treated groups D1, D2 and D3 depicted the significant difference (p = 0.001, 0.002 and 0.008), respectively, of MCV when compared with the infected but treated with water group B. Similarly, the highly significant difference p = (0.002, 0.000 and 0.005) was also observed for the *P. granatum* treated groups E1, E2 and E3 group, respectively, when compared with the infected but treated with water group B. There was non-significant difference (p = 0.638, 0.064, 0.819, 0.093, 0.186 and 0.137) for MCV of chloroquine treated C group and plant treated groups D1, D2, D3, E1, E2, and E3, respectively.

The group (A) showed highly significant difference (p = 0.000) of MCH with infected but treated with water group (B) and non-significant difference (p = 0.066) with the group (C) treated with chloroquine. Groups D3 and E3 showed significant difference (p = 0.024, 0.004). While D1, D2, E1 and E2 indicated the non-significant difference (p = 0.064, 0.139, 0.881 and 0.502), respectively, for MCH when compared with normal group of mice (A) (Table 2).

There was significant difference (p = 0.036) between B and C group. The plant treated groups D1, D2, D3, E1, E2 and E3 indicated the highly significant difference (p = 0.032, 0.005, 0.005, 0.008, 0.003, and 0.016), respectively, of MCH when compared with the infected but treated with water group (B). There was non-significant difference (p = 0.906, 0.578, 0.827, 0.185, 0.072 and 0.263) for MCH between C group and D1, D2, D3, E1, E2, and E3 groups, respectively.

The group (A) showed highly significant (p = 0.000) difference of MCHC with infected but treated with water group (B) and only significant difference (p = 0.05) with the group (C) treated with chloroquine. Groups treated with plants seeds extract D1, D2, D3, E1, E2, and E3 showed the significant difference (p = 0.016, 0.228, 0.06, 0.183, 0.21 and 0.23), respectively, for MCHC as compared with normal control group (A). There was highly significant difference (p = 0.000) between infected but treated with water group B and treated with chloroquine C group. Comparison of plant treated groups D1, D2, D3, E1, E2, and E3 illustrated the significant difference (p = 0.014, 0.009,

Table 3. Comparison of hematological parameters of different groups.

Hematology parameters	Groups								
	A	B	C	D1	D2	D3	E1	E2	E3
RBC	8.51 ± 0.41	3.97 ± 0.16	6.00 ± 0.48	4.86 ± 0.48	5.34 ±0.58	5.73 ± 0.37	6.63 ± 0.46	7.36 ± 0.52	6.16 ± 0.52
HB	11.3 ± 0.497	5.43 ± 0.34	10.5 ± 0.87	6.83 ± 0.62	10.6 ± 0.46	8.53 ± 0.73	8.3 ± 0.32	11.37 ± 0.80	9.20 ± 0.589
Hematocrit	38.50 ± 0.54	26.87 ± 1.1	34.53 ± 0.72	30.80 ± 0.44	32.03 ± 1.09	33.70 ± 1.50	32.50 ± 8.12	36.37 ± 1.10	35.3 ± 1.00
MCV	53.43 ± 0.62	43.23 ± 0.66	48.07 ± 1.09	47.57 ± 0.92	50.87 ± 0.82	48.33 ± 1.05	51.57 ± 0.46	53.70 ± 2.11	50.57 ± 1.18
MCH	16.9 ± 0.2	12.9 ± 0.20	15.23 ± 0.41	15.13 ± 0.55	15.90 ± 0.70	15.36 ± 0.38	17.03 ± 0.81	17.43 ± 0.29	14.50 ± 0.62
MCHC	32.13 ± 0.93	26.16 ± 1.48	31.86 ± 0.94	29.97 ± 0.95	32.76 ± 0.96	30.4 ± 1.25	33.3 ± 1.64	31.36 ± 1.42	31.6 ± 0.871
Platelets	325000 ± 27.9	178000 ± 18.1	274000 ± 23.6	183000 ± 30.00	225000 ± 32.4	179000 ± 13.45	352000 ± 21.23	3750000 ± 24.34	267000 ± 19.56
Neutrophils	24 ± 0.882	33 ± 1.5	26 ± 1.2	27 ± 0.58	24 ± 0.88	33 ± 0.57	28 ± 1.227	25 ± 0.75	29 ± 1.1
Lymphocytes	65 ± 1.1	83 ± 0710	66 ± 1.23	69 ± 1.15	60 ± 0.756	69 ± 1.227	67 ±0.84	62 ± 0.30	67 ± 1.53

0.019, 0.006, 0.012, and 0.013), respectively, when compared with the infected but treated with water group (B). There was no significant difference (p = 0.332, 0.311, 0.109, 0.611, 0.343 and 0.786) for MCHC between chloroquine treated group C and plant seeds treated groups D1, D2, D3, E1, E2 and E3, respectively (Table 3). The group (A) showed highly significant difference (p = 0.000) of platelets of infected but treated with water group (B) and significant difference (p = 0.004) with the group (C) treated with chloroquine as well as D1, D2, D3 (p = 0.000) for all three E1, E2 and E3 and (p = 0.040, 0.006 and 0.002), respectively, with normal control group (A).

There was highly significant difference (p = 0.000) for platelets count of infected but non treated group B and chloroquine treated group C. Plants treated groups D1, D2, E1, E2, and E3 showed significant difference (p = 0.044, 0.008, 0.001, 0.000 and 0.004), respectively, except for D3 which indicated the non-significant difference (p = 0.401), for platelet counts when compared with the infected but treated with water group (B). There was significant difference (p = 0.000, 0.006, 0.000, 0.002 and 0.001) for platelet count for group D1, D2, D3, E1, and E2, respectively, except for E3 it showed the non-significant (p = 0.366) difference when compared with the chloroquine treated group C. The group (A) showed highly significant (p = 0.008) difference of neutrophils with infected but treated with water group (B) and non-significant difference (p = 0.263) with the group (C) treated with chloroquine. The plants treated group D1, D2, E2 indicated the non-significant difference (p = 0.135, 0.773 and 0.111). The group D3, E1 and E3 did differ significantly (p = 0.004, 0.033, 0.021) when compared with normal control group (A) (Table 3).

There was a significant difference (p = 0.034, 0.018, 0.018, 0.037 and 0.024) among neutrophils of group B infected but treated with water and chloroquine treated group, and other plants treated groups like D1, D2, E1 and E2, respectively. The groups D3 and E3 indicated the non-significant difference (p = 0.667 and 0.073), respectively when compared with infected but treated with water group B. The non-significant difference in neutrophil number between drug treated control group C and plant treated groups was observed. The group (A) showed highly significant difference (p = 0.002) of lymphocytes with group (B) and non-significant difference (p = 0.687) with the group (C). The plant treated groups D1, D2, D3, E1, E2 and E3 also showed the non-significant difference (p = 0.09, 0.074, 0.128, 0.330, 0.223 and 0.435), respectively, when compared with

the normal control group A. There was a significant difference ($p = 0.002$, 0.003, 0.000, 0005, 0.002 and 0.001) among the lymphocytes of group B and C, D1, D2, D3, E1, E2 and E3 groups, respectively. There was no significant difference between drug treated group C and plant treated groups D1, D2, D3, E1, E2 and E3 groups, respectively (Table 3).

The analysis of ALT, ALP and AST in the liver function tests showed that the level of ALT, was significantly high in infected but non-treated group B as compared to the normal control group A ($p = 0.005$). The ALT range differ significantly ($p = 0.000$) for all groups, like drug treated group C and plant extracts treated groups D1, D2, D3, E1, E2, and E3 groups when compared with B group. Comparison of C, D1, D2, D3, E1, E2 and E3 groups with normal group A showed following level of significance ($p = 0.093$, 0.083, 0.047, 0.047, 0.017, 0.101 and 0.023), respectively. The difference between the ALP level was highly significant ($p = 0.007$, 0.002) in the A and C group as compared to the infected but treated with water group B. In plant treated groups the significant level was observed ($p = 0.015$, 0.045, 0.047, 0.006, 0.006 and 0.009) for D1 D2, D3, E1, E2 and E3, respectively, in comparison with the group B. It was highly significant for E1, E2 and E3 and less significant for three sub groups of D group. The comparison of treated groups with normal group A, D1 and E1 showed high significant difference ($p = 0.021$, 0.025) and low significant difference for D2, D3 and E3 ($p = 0.046$, 0.041, 0.048), respectively. Chloroquine treated group C and E2 showed non-significant difference ($p = 0.873$, 0.070), respectively. The elevated level of AST, was observed in infected but non-medicated B group as compared to the normal control group A and chloroquine treated group C. Statistically it was highly significant ($p = 0.002$ and

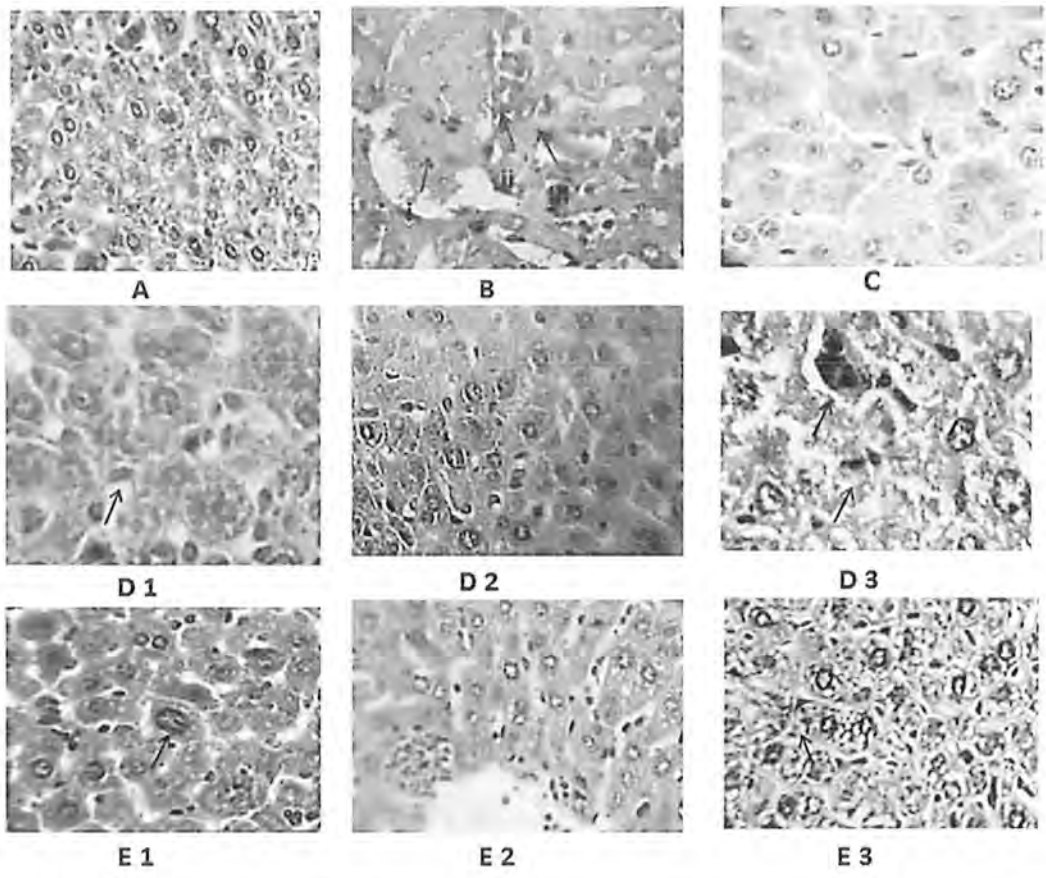

Figure 5. In the liver of malaria-infected mice, exoerythrocytic form of *Plasmodium berghei* was observed in B (I), hyperplasia and hypertrophy of kupffer cells and vacuolar degeneration and atrophy of hepatocytes (ii) and (iii) as compared to normal A and other treated groups. Hyperplasia and hypertrophy of some kupffer cells were also observed in C, D1, and E 1 (along with atrophy of hepatocytes) group. Viewed under light microscopy at 100 × magnification

0.000). It was also differ significantly (p = 0.004, 0.001, 0.019, 0.001, 0.003 and 0.004) for different plants treated groups (D1, D2, D3, E1, E2 and E3), respectively, with comparison with non-medicated control group B. AST value of treated groups D1, D2, D3, E1, E2 and E3 did not differ greatly from normal value of Group A (p = 0.034, 0.013, 0.015, 0.076, 0.048, 0.044 and 0.009), respectively (Fig. 4). Kidney function test was determined by creatinine and uric acid level. Increased levels of creatinine were observed in the non-treated group B as compared normal group A the difference was statistically very high (p = 0.001). The drug treated group C also indicated the elevated level of creatinine and showed the highly significant (p = 0.002) when compared with normal group A. Groups treated with plant extracts like D1, D2, D3, E1, E2 and E3 showed the significant difference (p = 0.001, 0.004, 0.028, 0.015, 0.000 and 0.000) in creatinine level when compared with non-treated group B. The treated groups C, D1, D2, D3, E1, E2 and E3 comparisons with normal group A indicated the following level of significance (p = 0.041, 0.011, 0.049, 0.009, 0.014, 0.070 and 0.030). Non-significant difference was observed only for E2 and D2 (Fig. 4).

Uric acid level in the non-treated group B was significantly high as compared normal group A (p = 0.018) and drug treated group C (p = 0.002). In the treated groups highly significant difference was observed for D2 (p = 0.009). E1 (p = 0.003), E2 (p = 0.001), and E3 (p = 0.017), it was non-significant for D1 (p = 0.290) and D3 (p = 0.492) as compared to the non-treated group B. The comparisons with normal control group A, plant extract treated groups D1, D2, D3, E1, E2, and E3 showed significant difference (p = 0.013, 0.001, 0.001, 0.066, 0.020, and 0.022) respectively but non-significant difference was observed for drug treated group C (p = 0.255) (Fig. 4). Postmortem examinations of the internal organs revealed the darkening of the liver, spleen, lungs and the kidneys of the malaria-infected mice as compared to the other treated groups (Table 4).

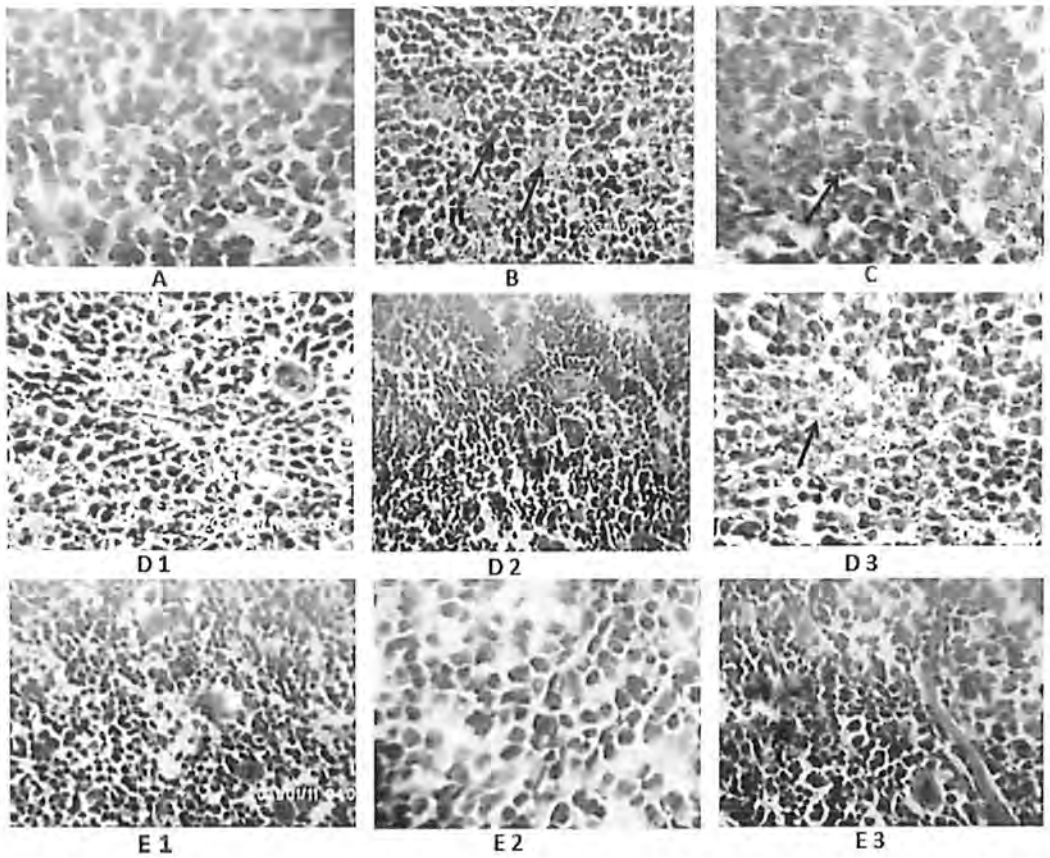

Figure 6. Accumulation of more malarial pigments, hemozoin, was more in the pulp histiocytes and sinusoidal lining cells of the spleen in the B group (i) as compared to normal control group A and other treated group. Microvascular sequestrations of PRBCs were also present B (ii) and little bit in C, D 1 D 3. Viewed under light microscopy at 10 × 100 magnifications

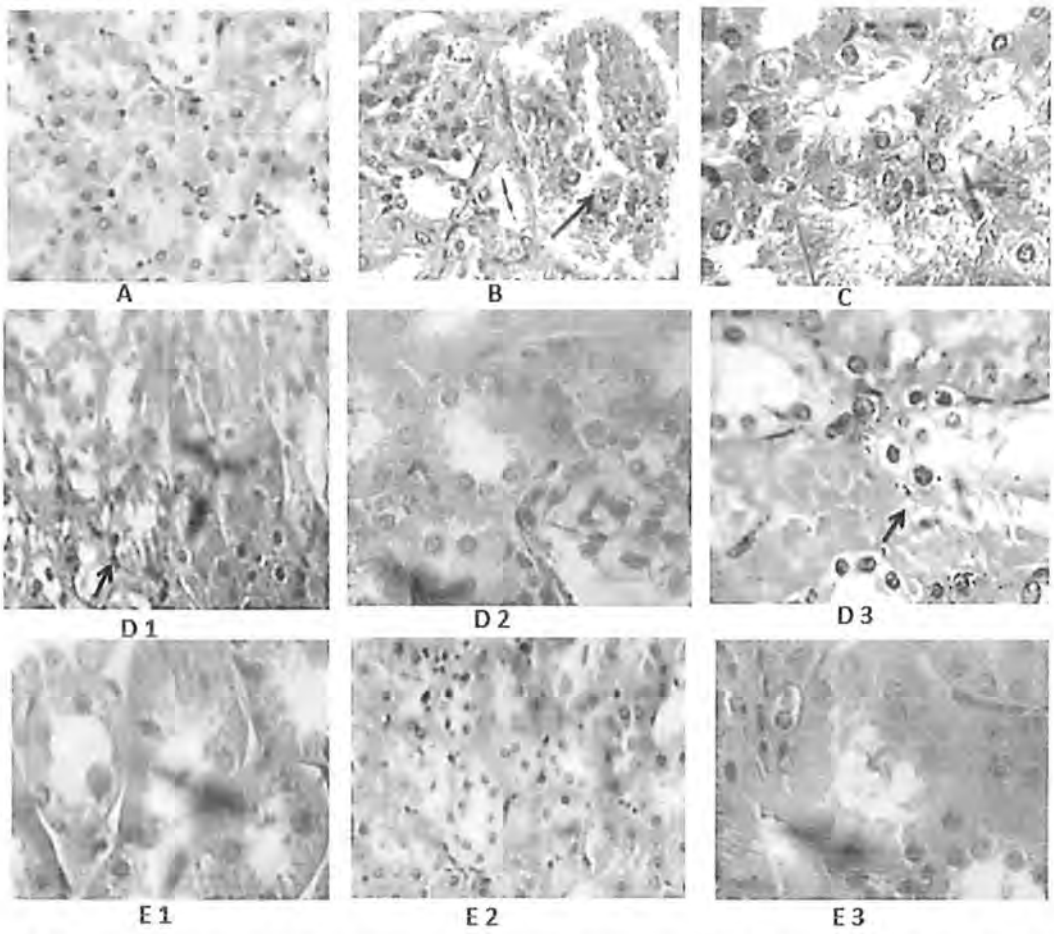

Figure 7. The kidney from infected mice was presented with widespread sequestration of PRBCs, Hemorrhages and congestion of blood vessels (B), as compared to the to normal control group A and other treated groups. Viewed under light microscopy at 100 × (A,B, C, D1, E2) and 200 × (D2,D3, E1 & E3) magnification.

Histopathological studies revealed the marked differences between the control and treated groups. The sequestrations of parasitized RBCs in the microvasculature were observed in the liver, spleen and kidney of infected but non-treated group as compared to the treated groups. In the liver of infected but non-treated mice atrophy of hepatocytes, hypertrophy and hyperplasia of the kupffer cells was observed. Exoerythrocytic forms in liver in the malarial infected but non-treated were observed (Fig. 5). The malaria pigments hemozoin was seen in the pulp histiocytes and sinusoidal lining cells and in the microvasculature of spleen tissue. Congestion of blood vessels was also observed (Fig. 6). The kidney tissues were presented with abundant of sequestrations of parasitized RBC and hemozoin in the microvasculature and interstitium. Congestions and hemorrhages were seen (Fig. 7).

Phytochemical analysis of *T. ammi, P. granatum* seed extracts

The experimented plants were subjected for qualitative phytochemical analysis and found to constituted as tannins, alkaloids, cardiac glycosides saponins, phenols, anthraquinone, reducing sugar, carbohydrates, volatile oils, steroids and amino acids (Table 5).

DISCUSSION

Malaria is caused by a protozoan parasite *Plasmodium*. Among all five species of *Plasmodium* that infect the humans *P. falciparum* is the most lethal infection. But the *P. falciparum* and *P. vivax* both are prevalent in Pakistan. Currently, no vaccine is available up to date. The only treatment of malaria is through drugs. Owing to the current scenario of

Table 4. Post mortem observations of major organs in different groups.

Observation	A	B	C	D1	D2	D3	E1	E2	E3
Enlargement of the size of spleen(splenomegaly);	-	+++	+	++	-	++	+	-	-
Discoloration of the spleen	-	+++	+++	+	-	++	+	-	-
Enlargement of the size (hepatomegaly);	-	++	+	+	-	++	++	-	+++
Discoloration of the liver	-	++	+++	++	++	++	+	-	+
Discoloration of the kidneys	-	+++	++	++	+	++	+	-	+
Discoloration of the lungs	-	+++	+	++	+	+	+	+	+

Indicator: (-) absent; (+): mild; (++): moderate; (+++): severe

Table 5. Qualitative phytochemical analysis of seeds of *T. ammi* and *P. granatum*.

Sr.#	Name of constituent	Reagents added	Color change	Aqueous extract of seeds of *T. ammi*	Aqueous extract of seeds of *P. granatum*
1	Tannins	0.5 g of the extract 1 mL of distilled water 10% $FeCl_3$	Bluish green	+	+
2	Alkaloids	0.5 g of the extract 0.5 mL of 1% HCL Few drops of distilled water Few drops of Wagners reagent.	Reddish brown ppts	+	+
3	Cardiac glycosides	0.5 g of the extract 2 mL of glacial acetic acid Few drop of $FeCl_3$ solution 2 mL of concentrated H_2SO_4	Ring of brown color	+	+
4	Flavonoids	2 mL of extract 2 mL of diluted NaOH	Yellow	+	+
5	Saponins	0.5 g of the powdered leaves 12 mL of distilled water	Foam produced persist for 10 min	+	–
6	Phenols	Equal volume of plant extract and $FeCl_3$ (1 : 1)	Deep bluish green	+	+
7	Anthraquinone	0.5 g of the extract 10 mL of benzene 10% of ammonia	Pink, red or violet color	–	–
8	Reducing sugar	3 ml of extract 5 mL of distilled water Fehling solution A and B	Red precipitate	+	+
9	Carbohydrates	0.5 g of the extract Few drops of distilled water Few drops of Molish reagent 1 mL of conc. H_2SO_4	Brown ring	+	+
10	Volatile oils	0.5 g of the extract 90% ethanol Few drops of $FeCl_3$	Green color	+	–
11	Steroids	0.5 g of the extract 3 mL of chloroform Few drops of conc. H_2SO_4	Reddish brown color	+	+
12	Amino acids	1 mL of extract few drops of ninhydrin reagents	Purple color	–	–

+ = Presence of constituent; – = Absence of constituent

resistance against the existing antimalarial drugs new chemicals are required to cure it. The studies revealed that plants are the crucial source of medicines since they have been used for the treatment of various diseases (15). Medicinal chemistry is flourishing through plant derived compounds because they offer an approach to chemotherapy with specific clinical activity. Plants contain chemical constituents that have great potential for medicinal use.

Disease pathogenesis and its treatment can be understandable by the *in vitro* studies but for proper physiological conditions and full chemistry of the body, *in vivo* model is required (16). In BALB/C mice malaria is caused by *P. berghei* and this infection is very similar to human *P. falciparum* infection. The *P. berghei* infection cause anemia, hypoglycemia, weight loss, changes in body temperature and occasional death and these symptoms are very similar to human malaria infection (17). There is no other animal model except *Aoutus* monkey (available in USA) and C57BL/6 mice that are the carrier of human *Plasmodium* species. Another advantage of using this model animal is that mice have 99.9% similarity with human genome. So this *in vivo* model is effective to explore the new antimalarial drugs.

The recent study further supports the idea that medicinal plants can be promising sources of potential antiplasmodial agents. In the established infection *T. ammi*, and *P. granatum* produced dose-dependent chemosupression causing significant reductions in parasitemia. *T. ammi*, and *P. granatum* produced anti-plasmodial activity up to 68.76 ± 4.99 and 77.58 ± 5.09, respectively.

A seed extract of *T. ammi* prepared in ethyl acetate showed the anti-plasmodial activity up to 25 ± 0.7 against *P. falciparum* in *in vitro* culture (18). In a study, the fraction of essential oil of *T. ammi* was evaluated for possible antibacterial and antifungal activities (19).

Medicinally, it has been recognized to possess different pharmacological activities for example antifungal, antioxidant, antimicrobial, cytotoxic activity, broncho-dilating actions, nematicidal, anthelmintic, abortifacient, antitussive, antilithiasis, diuretic and antifilarial activity (20). Its nutritional and medicinal properties have been verified through lots of papers.

In another study, crude methanol extracts of fruit *P. granatum* showed antiplasmodial activity (IC_{50} 4.68 µg/mL) but in *in vitro* cultures against *P. falciparum* (21).

A study conducted by Hafiz et al., in order to evaluate the activity of methanolic peel extract of *P. granatum* against malaria-induced hepatic injury and oxidative stress in *P. chabaudi* infected mice (22). The *P. granatum* extract successfully reduced the anemia, hepatic injury and oxidative stress by reducing hepatic oxidative markers, glutathione, nitric oxide and malondialdehyde. The methanolic extract of *P. granatum* reduced parasitemia oxidative stress and inflammation of spleen in the *P. chabaudi* infected mice (23).

In a study conducted by Dell'Agli et al., fruit rind of the immature fruit of this plant is used as an herbal formulation in India, for the treatment and prophylaxis of malaria (24). They used the methanolic extract that inhibited the parasitemia *in vitro*. The tannins fraction enriched with in punicalagins (29.1%), punicalins, ellagic acid (13.4%) and its glycoside are more active than other fractions. The present study confirmed the findings of previous studies conducted on the same plant but in this study seeds are explored to have antiplasmodial activity in the aqueous extracts and the active components can be isolated under the information of above mentioned qualitative phytochemical analysis. Aqueous extracts are economical as compared to the other organic solvents extracts. Sánchez-Lamar et al. in 2008 confirmed it's following properties microbial infections, acidosis, dysentery, diarrhea, helminthiasis, respiratory pathologies, and hemorrhage (10). However, in the present study, aqueous extracts of this plant showed highest antiplasmodial activity at 500 mg/kg. *T. ammi*, and *P. granatum* exhibited the LD_{50} as831 and 1989 mg/kg, respectively.

Usually, if the lethal dose (LD_{50}) of any test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies of drug formulation (25). Minimum effective median lethal dose ED_{50} was observed for *P. granatum and T. ammi* as 124.45 mg/kg and 143.02 mg/kg, respectively. But the toxicity of *P. granatum* was low as compared to the *T. ammi* because LD_{50} of *P .granatum* was calculated as 1989 mg/kg as compared to LD_{50} of *T. ammi* 831 mg/kg. In another study (26), anti-malarial activity of 6-(8'Z-pentadecenyl)-salicylic acid from *Viola websteri* was determined in mice and LD_{50} was calculated as 500 mg/kg while the highest antiplasmodial activity 82.1 was observed at 25 mg/kg. The n-butanol fraction of *Dodonaea angustifolia* was given to the mice infected with *P. berghei* and found that LD_{50} was greater than 2000 mg/kg but the highest antiplasmodial activity 67.51 was observed at 600 mg/kg dose (12).

Serum ALT. ALP and AST are the bio markers for liver health and urea and creatinine concentra-

tion are used for the evaluation of renal efficiency. In earlier studies, chloroquine has also been reported to induce secondary increase in the certain enzymatic activities along with the increase in autophagy of cells exposed to chloroquine (27). Hence, this can probably be justifying the observed elevation in enzyme activity of liver and kidney of chloroquine treated mice in this study. The administration of artemisinin is also reported to cause a significant increase in serum aspartate aminotransferase, ALP and alanine amino transferase ALP (28). In the present study, elevated level of liver bio mark i.e. ALT, ALP and AST in chloroquine treated group was observed as compared to the group treated with *P. granatum* (500 mg/kg). There was significant increase in serum ALT, ALP and AST, urea and creatinine in infected control as compared to normal. Elevated level of ALP and creatinine was observed in the infected group as compared to the treated steroidal alkaloid conessine isolated from the bark of *Holarrhena antidysenterica* treated groups (29) and homeopathic compounds Nosode 30 and 200 (30).

The parasite Burdon in malaria infection is associated with the peripheral blood. So the hallmark of malaria is hematological abnormalities especially during *P. falciparum* infection (31). The severe malarial anemia is considered a main cause of mortality in animals. The mechanism of anemia due to malaria is similar to those of human anemia which is due to the loss or damage of infected RBCs or may be due to loss of non-infected RBCs (32).There was a marked reduction in red blood cell (RBC) count, hemoglobin (HB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) of infected but non treated group as compared to the treated groups. Our result are comparable with (33). A study conducted by Hafiz et al., (2016) on the methanolic peel extract of *P. granatum* significantly reduced the anemia in mice infected by *P. chabaudi* (22). The present study also revealed the protective effects on the hemoglobin concentration and erythrocyte count in the plant treated groups as compared to the infected but non treated groups.

Hence the malaria infects multi-organs and drug may also infect the many organs. So the histopathological studies were conducted on the three majorly infected organs during malaria i.e., liver, spleen, and kidneys. The sequestration of PRBCs in the microvasculature was observed. In the liver, macrophages engulfed the PRBCs causing lysis of the blood cells leaving behind the hemozoin deposited in the parenchyma. Splenomegaly observed on the spleen of infected mice in this model also mimic the conditions of *P. falciparum* infection in human, in which splenomegaly is a common phenomenon with the spleen becomes congested with malaria hemozoin caused by an increase of the pulp element rendering trabeculae and follicles unclear. Sequestrations of PRBC were observed in the kidney. These histological changes were clearly observed in the infected but non-treated group as compared to normal and treated with plant groups and these results are compatible with histopathological study conducted on liver, spleen and kidney only on liver and on spleen of mice infected with malaria (16, 22, 23).

CONCLUSION

The plant seeds experimented for antiplasmodial activity is frequently used in our daily life, even the simple use of these seed can cure the malaria. So, the aqueous extracts of these plants might contain potential molecules for the development of a new drug for treatment of malaria.

Conflict of interests

The authors declared that there is no conflict of interest in this paper.

Acknowledgments

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