Glutathione peroxidase, biochemical and hematological alterations among patients with *Falciparum* malaria

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A thesis submitted to University of Khartoum in partial fulfillment of the requirements for the degree of M. Sc Biochemistry

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November 2010
قال تعالى:

"أعوذ بالله من الشيطان الرجيم، وقل انعم الله علىầyكم وصلواته، ورسوله ومؤمنين، وسأتردون إلى عالم الغيب والشهادة، فنبئكم بما كنتم تعملون، مصدق الله العظيم، التوبة الأية 501."

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Dedication

To my Mother,

Who deserves a great deal of credit for any success

I have achieved.

She raised me to understand that

Nothing in life that is truly fulfilling is easy,

Brothers, sisters

&

All my friends….

Amar
Acknowledgements

I'm mostly indebted to my supervisor Dr. Osman M. Elsheikh willingly spending the time and energy for supervision, constructive discussion and correction of this study, patience and encouragement and a word of thanks alone are inadequate.

Thanks are extended to the Dr. Atif Hassan, Dr. Omar Eltype, Dr.Ogail and Ustaz Ashraf Naeem for their valuable help.

Great thanks are also extended to Ashraf elkinen, Dr. Khalid AbdelHafiz, Dr. Mohammed Ali, Dr.Rifga, Amna Hussein, Abdel Hafiz Elgilani and Mohammed Abdelazeem for technical assistance and to family of Sennar teaching hospital.

Finally I owe much to my family for their patience, support, and help I have received from them, can hardly be expressed in words.
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Abstract

This study was designed to investigate the activity of the enzyme glutathione peroxidase, level of malondialdehyde, biochemical parameters (total protein, albumin, uric acid, and conjugated and total bilirubin) and hematological parameters (red blood cells count, total white blood cells count, platelets count, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration and packed cell volume) in the blood of malaria patients. Forty patients infected with *Plasmodium Falciparum* malaria and ten healthy individuals (control group) were enrolled in this study in Sennar, Sudan, during 2010.

The results indicated that 25% of malaria patients were thrombocytopenic. The activity of the enzyme glutathione peroxidase was significantly different (*P* < 0.01) in platelets of the studied groups, the highest enzyme activity was observed for the control group (1979.0 U/L) followed by malaria non-thrombocytopenic (384.0 U/L) and the least was the malaria thrombocytopenic group (112.0U/L). On the other hand, the activity of the enzyme glutathione peroxidase was positively correlated with platelets count (*r* = 0.602; *P* < 0.01), and negatively correlated with malondialdehyde (*r* = -0.36; *P* < 0.02).

The level of malondialdehyde was significantly (*P*< 0.01) higher (7.3 μmol/L) in the platelets of malaria thrombocytopenic patients than that of malaria non-thrombocytopenic patients (3.9μmol/L) and control group (0.0μmol/L). Malondialdehyde was negatively correlated with platelets count (*r* = -0.7; *P*< 0.00).

There was a significant difference (*P* < 0.05) in total white blood cells between the studied groups, the highest count was observed for the non-thrombocytopenic patients (7.9 X10^3 cell/mm^3) compared to control
(6.1 X10³ cell/mm³) and malaria thrombocytopenic group (5.1 X10³ cell/mm³). The concentration of total bilirubin was significantly (P< 0.05) increased in all malaria patients compared to the control group. Other measured biochemical parameters (total protein, albumin and uric acid) showed no significant difference between the studied groups.

It is concluded that thrombocytopenia is associated with low activity of platelets glutathione peroxidase and high level of malondialdehyde.
المستخلص

صممت هذه الدراسة للتحقيق من نشاط خميرة جلوتاتيون بروكسيدز و المالون داي الديهايد
وغيرها من المعالم الكيميائية (البروتين الكلي، النزول، حمض البول، العصارة الصفراوية المقترنة
و الكلية) والمعالم الدموية (تعداد كرات الدم الحمراء، تعداد خلايا الدم البيضاء الكلي، تعداد خلايا
الخثريّن، خضاب الدم، متوسط حجم الخلية، متوسط خضاب الخلية، متوسط تركيز الخضاب للخلية،
حجم الخلايا المضغوطة) في دماء مرضى الملاريا.

سجل أربعون مريضًا مصابين بالملاريا و عشرة أشخاص أصحاء (شاهد) للمشاركة في
الدراسة وذلك بمنطقة سنار - السودان خلال العام 2010م.

أوضحت النتائج أن 25% من مرضى الملاريا يعانون من عوز خلايا الخثريّن. وأن نشاط
انزيم الجلوتاتيون بروكسيدز في خلايا الخثريّن في المجموعة المدرجة يختلف اختلافًا معنويًا (P > 0.01).
نلاحظ أن أعلى نشاط لانزيم كان في مجموعتي الشاهد (79.0 U/L) ومرضي الملاريا
الذين ليس لديهم نقص في خلايا الخثريّن (384.0 U/L) والذين يعانون من نقص خلايا الخثريّن (112.0 U/L).

ومع ناحية أخرى فقد ارتبط نشاط الانزيم الجلوتاتيون بروكسيدز ارتباطًا موجبًا مع عدد
خلايا الخثريّن (P > 0.02) ورتبط ارتباطًا سالبًا مع المالون داي الديهايد (r = -0.36).

مستوي المالون داي الديهايد كان أعلى معنويًا في خلايا الخثريّن لمرضي عوز خلايا
الخثريّن (7.3 μmol/L) مقارنة مع مرضى الملاريا الذين لا يعانون عوز خلايا الخثريّن
(0.0 μmol/L) ونوع الخثريّن (3.9 μmol/L). ورتبط المالون داي الديهايد ارتباطًا سالبًا
مع عدد خلايا الخثريّن (r = -0.7; P > 0.01).

أوضحت النتائج أن هناك اختلافاً معنويًا بين عدد الكلي لخلايا الدم البيضاء بين مجموعات
الدراسة، واعلى عدد الكلي كان لدى مرضى الملاريا الذين لا يعانون من عوز خلايا الخثريّن (7.9
خلية | م³) مقارنة بمجموعة الشاهد (6.1 خلية | م³) والمرضي الذين يعانون من عوز خلايا
الخثريّن (5.1 خلية | م³).

داد تركز العصارة الصفراوية الكلية في المجموعات المدرجة معنويًا مقارنة بمجموعة
الشاهد. كما لم تظهر المعالم الكيميائية التي تم قياسها ( البروتين الكلي، النزول، حمض البول)
فروقات معنوية في المجموعات المدرجة.
خلصت الدراسة إلى أن عوز خلايا الخثرتين يرتبط مع نقصان نشاط الأنزيم جلوتاثيون بيوركسيديد وزيادة تركيز المالون دايل الديهايد في خلايا الخثرتين لمرضى الملاريا.
1. Introduction and literature review

1.1. Introduction:

Malaria is a parasitic disease caused by the sporozoan protozoa plasmodium. It remains one of the most prevalent and serious diseases in the world. The endemic transmission occurs in about 120 countries; which together represent more than one half of the world population (WHO, 2002).

Malaria is transmitted to humans and animals by the bite of infected female anopheles mosquitos. Malaria is a very serious clinical problem as it affects normal growth, health and may cause many biochemical alterations in the blood, as it causes hypoglycemia and subclinical convulsion (Silamut et al., 1999). In tropical areas, malaria is one of the major causes of morbidity and mortality for children and women (Brian et al., 2005).

Snow et al (2005) reported that the epidemiology of malaria in adults who live in malaria endemic areas is a neglected area of research. Malaria control strategies have focused on children under the age of 5 years and pregnant women, as the majority of malaria-related sickness and death is seen in these two groups. However, early studies in West Africa showed that clinical attacks of malaria also occur in adults living in areas of high endemicity (Miller, 1958). Recent report points out the considerable contribution of malaria as a cause of death in adults (Adjuik et al., 2006).
Acute malaria is often associated with mild or moderate thrombocytopenia in non-immune adults and children in malaria endemic areas and is a sensitive but non-specific indicator of infection with malaria parasites. Profound thrombocytopenia is unusual, and thrombocytopenia is rarely associated with hemorrhagic manifestations or a component of disseminated intravascular coagulation either in non-immune adults or children in endemic areas (Kelton et al., 1983) (Wickramasinghe and Abdalla, 2000).

Reactive oxygen (ROS) or nitrogen species (RNS) are considered to play diverse roles in many aspects of physiological and pathological events (Akaike and Maeda, 2000). Oxidative stress can be measured in biological fluids by analysis of endogenous products of lipid peroxidation such as malondialdehyde (MDA) or by measurement of enzymes involved in antioxidant mechanisms.
1.2. Literature review:

1.2.1. Malaria etiology and life cycle:

Malaria is caused by obligate intra-erythrocytic protozoa of the genus Plasmodium. Humans can be infected with one (or more) of the following five species: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Plasmodia are primarily transmitted by the bite of an infected female Anopheles mosquito, but infections can also occur through exposure to infected blood products (transfusion malaria) and by congenital transmission. Among the five species, *P. falciparum* is the predominant one in Sudan, and is responsible for most of malaria-related morbidity and mortality (Brian et al., 2005).

When the infected anopheline mosquito takes a blood meal, sporozoites are inoculated into the bloodstream as shown in: Figure (1- 1). Within 2-30 minutes sporozoites enter hepatocytes and begin to divide into exo-erythrocytic merozoites (tissue schizogony). For *P. vivax* and *P. ovale*, dormant forms called hypnozoites may typically remain quiescent in the liver until a later time; *P. falciparum* does not produce hypnozoites. Once merozoites leave the liver, they invade erythrocytes and develop into early trophozoites, which are ring shaped, vacuolated and uninucleated. Once the parasite begins to divide, the trophozoites are called schizonts, consisting of many daughter merozoites (blood schizogony). Eventually, the infected erythrocytes are ruptured, releasing merozoites, which subsequently invade other erythrocytes, starting a new cycle of schizogony. The duration of each cycle in *P. falciparum* is about 48 hours. In non-immune humans, the
infection is amplified about 20-fold each cycle. After several cycles, some of the merozoites develop into gametocytes, the sexual stages of malaria, which cause no symptoms, but are infective for mosquitoes (Garcia, 2001). Within the mosquito gut, zygotes are formed, which develop further and eventually become sporozoites that reside in the salivary glands. These sporozoites are released into the blood stream of a new human victim when the female mosquito takes the blood meal.

1.2.2. Malaria symptoms:

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by hemolysis), hemoglobinuria, and convulsions. There may be the feeling of tingling in the skin, particularly with malaria caused by *P. falciparum*. The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae* (Aikawa, 1988).

*P. falciparum* can have recurrent fever every 36-48 hours or a less pronounced and almost continuous fever. For reasons that are poorly understood, but which may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (Idro et al., 2007). Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anemia during a period of rapid brain development and also direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable (Boivin, 2002).
Figure (1-1): the life cycle of *P. falciparum* in human host and mosquito vector.

Adopted by: (WHO, 2007)
Figure (1-2): the symptoms of malaria
Adopted by : (http://www.webmd.com)
1.2.3. *Malaria pathogenesis and complications:*

Pathogenesis relates to the various host and parasite factors that are responsible for causing pathology. The pathogenic process occurs only during the erythrocytic cycle. During this stage, there is a huge, periodic amplification of the size of parasite populations that may enhance the probability of differentiation to gametocytes, the stage infectious to mosquitoes. A peculiarity of *P. falciparum* is its ability to adhere to venular endothelium (cytoadherence) of erythrocytes infected with maturing parasites. The parasitized erythrocytes remain attached until merozoites are formed that are released to invade other erythrocytes. Thus, the predominant form seen in the peripheral circulation is the ring-infected erythrocyte, the young form of the parasite (Louis *et al.*, 1994).

Malaria in humans develops via two phases:

I. An exoerythrocytic (hepatic) and an

II. Erythrocytic phase.

When an infected mosquito pierces a person’s skin to take a blood meal, sporozoites in the mosquito’s saliva enter the bloodstream and migrate to the liver. Within 30 minutes of being introduced into the human host, they infect hepatocytes, multiplying asexually and asymptptomatically for a period of 6–15 days. Once in the liver these organisms differentiate to yield thousands of merozoites which, following rupture of their host cells, escape into the blood and infect red blood cells, thus beginning the erythrocytic stage of the life cycle (Bledsoe 2005). The parasite escapes from the liver undetected by wrapping itself in the cell membrane of the infected host liver cell (Sturm *et al*., 2006).
Within the red blood cells the parasites multiply further, again asexually, periodically breaking out of their hosts to invade fresh red blood cells. Several such amplification cycles occur. Thus, classical descriptions of waves of fever arise from simultaneous waves of merozoites escaping and infecting red blood cells (Aikawa, 1988).

Some *P. vivax* and *P. ovale* sporozoites do not immediately develop into exoerythrocytic-phase merozoites, but instead produce hypnozoites that remain dormant for periods ranging from several months (6–12 months is typical) to as long as three years. After a period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for long incubation and late relapses in these two species of malaria (Cogswell, 1992).

The parasite is relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating infected blood cells are destroyed in the spleen. To avoid this fate, the *P. falciparum* parasite displays adhesive proteins on the surface of the infected blood cells, causing the blood cells to stick to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation and the spleen (Chen *et al.*, 2000). This "stickiness" is the main factor giving rise to hemorrhagic complications of malaria. High endothelial venules (the smallest branches of the circulatory system) can be blocked by the attachment of masses of these infected red blood cells. The blockage of these vessels causes symptoms such as in placental and cerebral malaria. In cerebral malaria the sequestrated red blood cells can breach the blood brain barrier possibly leading to coma (Adams *et al.*, 2002).
Some merozoites turn into male and female gametocytes. If a mosquito pierces the skin of an infected person, it potentially picks up gametocytes within the blood. Fertilization and sexual recombination of the parasite occurs in the mosquito's gut, thereby defining the mosquito as the definitive host of the disease. New sporozoites develop and travel to the mosquito's salivary gland, completing the cycle. Pregnant women are especially attractive to the mosquitoes (Lindsay et al., 2000), and malaria in pregnant women is an important cause of stillbirths, infant mortality and low birth weight (Van Geertruyden et al., 2004), particularly in *P. falciparum* infection, but also in other species infection, such as *P. vivax* (Rodriguez et al., 2006).

Most complications attacking African children are severe anaemia and cerebral malaria, but respiratory distress is the most dangerous (Schellenberg et al., 1990).

Severe malarial anaemia also consists of a group of conditions with different causes, including direct destruction of parasitized red blood cells, indirect destruction of non-parasitized red blood cells by immune mechanisms, and bone-marrow suppression associated with imbalances in cytokine concentrations (Ekvall, 2003).

As a prelude to later sections on malaria pathogenesis, it is helpful to consider the multifaceted nature of the interaction between the host immune system and the parasite. Central to this interaction are cytokines that are released by immunocompetent cells in a highly regulated fashion. They participate in the control of all immunologically relevant events, whether they concern either activation, proliferation, or subsequent effector functions of recirculating immunocompetent cells or regulation of cells residing in tissues (Lyke et al.,
It has been established that cytokines not only participate in the qualitative (antibody isotype switch) and quantitative regulation of the immune response but also participate in many other complex processes such as hematopoiesis and pregnancy. During the erythrocytic cycle, soluble products of Plasmodium spp. known as malarial toxins direct systemic release of proinflammatory cytokines [for example, tumor necrosis factor-α (TNF-α)] which act on many other cellular systems such as endothelium.

Equally important are parasite antigens, which stimulate T cells to directly secrete or induce production of cytokines from other cells. Before *P. falciparum* infection, many individuals have *P. falciparum* reactive T cells, often at high frequency. Such parasite-reactive T cells have probably arisen as a result of antigenic cross reactivity between environmental organisms and parasite-derived molecules (Currier *et al.*, 1992).

The parasite also has other strategies for interacting with the immune system, including the following:

(i) Antigenic variation.

(ii) A still undefined, splenic dependent regulation of parasite genes encoding structural proteins and adhesive molecules on the erythrocyte surface that are involved in adherence to endothelium.

(iii) Low immunogenicity of conserved parasite peptides that are targets of antibodies able to interfere with parasite survival.
1.2.4. Malaria diagnosis:

The most economic, preferred, and reliable diagnosis of malaria is microscopic examination of blood films because each of the four major parasite species has distinguishing characteristics. Two sorts of blood film are traditionally used. Thin films are similar to usual blood films and allow species identification. Thick films allow the microscopist to screen a larger volume of blood (Warhurst and Williams, 1996).

In areas where microscopy is not available, or where laboratory staff are not experienced at malaria diagnosis, there are antigen detection tests that require only a drop of blood (Pattanasin et al., 2003).

Figure (1-3): Ring stage of *P. Falciparum*
Adopted by: (Cheesbrough, 2006)

Molecular methods are available in some clinical laboratories and rapid real-time assays (for example, QT-NASBA based on the polymerase chain reaction) (Mens et al., 2006) are being developed with the hope of being able to deploy them in endemic areas.
Thrombocytopenia:

Platelets, or thrombocytes are small, irregularly-shaped a nuclear cells (i.e. cells that do not have a nucleus containing DNA), 2-3 µm in diameter (Campbell, 2008), which are derived from fragmentation of precursor megakaryocytes. The average lifespan of a platelet is between 8 and 12 days. Platelets play a fundamental role in hemostasis and are a natural source of growth factors. They circulate in the blood of mammals and are involved in hemostasis, leading to the formation of blood clots (Campbell, 2008).

![Platelets picture stained by Giemsa stain](image)

Adopted by (cheesbrough, 2006)

When the number of platelets is too low, excessive bleeding can occur. However, if the number of platelets is too high, blood clots can form (thrombosis), which may obstruct blood vessels and result in such events as a stroke, heart attack, pulmonary embolism or the blockage of blood vessels to other parts of the body, such as the extremities of the arms or legs. An abnormality or disease of the platelets is called a thrombocytopathy (Maton et
which could be either a low number of platelets (thrombocytopenia), a decrease in function (thrombasthenia), or an increase in their number (thrombocytosis). There are disorders that reduce the number of platelets, such as heparin-induced thrombocytopenia (HIT) or thrombotic thrombocytopenic purpura (TTP) that typically causes thromboses, or clots, instead of bleeding (Kumar and Clark 2005).

Generally speaking, in humans, a normal platelet count ranges from 150,000 and 450,000 per mm$^3$. These limits, however, are determined by the 2.5th lower and upper percentile, and a deviation does not necessarily imply any form of disease (Correia et al., 2008) and (Cheung et al., 2005).

1.2.5. Thrombocytopenia and Malaria:

In most clinical studies, thrombocytopenia is associated neither with the severity of disease or death in malaria. Nevertheless, platelets have been implicated in animal, clinical and experimental studies of malaria pathogenesis. Platelets mediate a syndrome of cerebral malaria in an animal model of malaria infection (Van et al., 2005).

Histopathological studies of children who have died of severe malaria showed that platelet clumps with and without infected erythrocytes are frequently found in the vasculature (Grau et al., 2005). Also infected erythrocytes may adhere to platelets, and the clumps of infected erythrocytes and platelets have been associated with severe disease (Pain et al., 2001). Therefore, there remains a paradox that, while thrombocytopenia is associated with infection and platelets have been implicated in the pathogenesis of severe disease, most studies suggest that low counts of platelets are not associated
with an adverse outcome (Ladhani et al., 2002) (Newton et al., 2004). Without understanding this paradox, it is not possible to evaluate the true contribution of platelet to the pathogenesis of severe malaria. The causes of thrombocytopenia in acute malaria are poorly understood. Certainly, increased platelet destruction is significant during malaria infection. The platelet lifespan is reduced during malaria (Kreil et al., 2000), which seems to be associated with a diffuse pattern of platelet sequestration rather than a predominant splenic or hepatic clearance (Karanikas et al., 2004).

Thrombocytopenia is often associated with palpable splenomegaly (Kueh and Yeo, 1982) and circulating immune complexes. The contribution of abnormalities of platelet production to thrombocytopenia during malaria infection is less clear. Early studies in malaria patients described dysmorphic megakaryocytes in the bone marrow of patients with acute falciparum malaria (Knuttgen, 1987). Other investigators showed less lobulated and immature megakaryocytes in Gambian children with acute malaria (Abdalla, 1990). Moreover, the biosynthesis and regulation of thrombopoietin (TPO), the main growth factor for megakaryocytes and thrombopoiesis, seems to be normal in patients with malaria (Kreil et al., 2000).

1.2.6. Oxidative Stress:

Free radicals Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are considered to play significant and various roles in many aspects of physiological and pathological events. When pro-oxidants increase or antioxidants fall, Oxidative Stress (OS) ensues that leads to excessive molecular damage and tissue injury (Januel et al., 2006). However, OS has been defined as an imbalance of increased oxidants and decreased antioxidants. During metabolism, aerobic organisms form ROS, such as anion
radical superoxide (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid, hydroxyl (\'OH), hydroperoxyl or RNS such as nitric oxide (NO\'), nitrogen dioxide radical and anion peroxynitrite. These free radicals are constantly produced during normal aerobic metabolism and are safely removed by a variety of biological endogenous and exogenous antioxidants (Gutteridge, 1995).

Malondialdehyde (MDA) is an endogenous aldehyde produced by fatty acid oxidation and has been used as marker of OS. Glutathione peroxidase (GPx) is a selenium- dependent and lipid peroxide-scavenging enzyme that effectively reduces lipid peroxides with the concomitant oxidation of glutathione. Its activity can be altered under OS conditions (Gutteridge, 1995). Activated phagocytes produce ROS and RNS that help to kill some types of microorganisms. However, the method by which these species destroy microorganisms’ remain unclear; both direct oxidative damage and indirect damage (whereby reactive species promote the actions of other antibacterial agents) are involved, and no single mechanism is likely to account for the killing of all microorganisms. Neutrophils, monocytes, eosinophils and macrophages respond to appropriate stimuli by a marked increase in O$_2$ uptake, termed the respiratory burst (Halliwell, 2006).

The presence of OS during malaria infection is already known, although not totally understood. Either a protective or deleterious role of this OS seems to occur in patients with malaria (Pabón et al., 2003). Malarial infection induces the generation of \'OH radical in the liver, which may be responsible for the induction of OS and apoptosis (Guba et al., 2006). NO\’ is a molecule that has been proposed to have a crucial role in malaria pathogenesis (Sobolewscki et
The malaria parasite itself is reported to generate large quantities of 
$\text{H}_2\text{O}_2$ and $\text{O}_2$ (Hunt and Stocker, 1990), (Mishra et al., 1994).
The mechanism of thrombocytopenia in malaria is not clearly known and OS 
may play a role in this process. ROS species may have important functions in 
the structural and functional alterations of platelets and in the mechanism of 
thrombocytopenia in malaria (Erel et al., 2001).

1.2.7. *Glutathione peroxidase (GPx)*:

Glutathione peroxidase (EC 1.11.1.9) was discovered in 1957 by Gordon C.
Mills (Mills. 1957). It is the general name of an enzyme family with 
peroxidase activity whose main biological role is to protect the organism from 
oxidative damage. The biochemical function of glutathione peroxidase is to 
reduce lipid hydroperoxides to their corresponding alcohols and to reduce free 
hydrogen peroxide to water.

*Reaction:*

An example reaction that glutathione peroxidase catalyzes is:

$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS–SG} + 2\text{H}_2\text{O},$$

Where GSH represents reduced monomeric glutathione, and GS–SG 
represents glutathione disulfide.

Glutathione reductase then reduces the oxidized glutathione to complete the 
cycle (Valentina et al., 2009):

$$\text{GS–SG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+. $$
**Reaction mechanism:**

The mechanism is at the Selenocystein site, which is in a Se (-) form as resting state. This is oxidized by the peroxide to SeOH which is then trapped by a GSH molecule to Se-SG and by another GSH molecule to Se (-) again, releasing a GS-SG by-product (Forstrom *et al.*, 1978) and (Ceballos-Picot *et al.*, 1992).

**Structure:**

Mammalian GPx1, GPx2, GPx3, and GPx4 have been shown to be selenium-containing enzymes, whereas GPx6 is a selenoprotein in humans with cysteine-containing homologues in rodents. GPx1, GPx2, and GPx3 are homotetrameric proteins, whereas GPx4 has a monomeric structure. As the integrity of the cellular and subcellular membranes depends heavily on glutathione peroxidase, the antioxidative protective system of glutathione peroxidase itself depends heavily on the presence of selenium (Muller *et al.*, 2007).
1.2.8. Uric acid:

Uric acid is produced by xanthine oxidase from xanthine and hypoxanthine, which in turn are produced from purine. Uric acid is more toxic to tissues than either xanthine or hypoxanthine. Uric acid is released in hypoxic conditions (Baillie et al., 2007).

In humans and higher primates, uric acid is the final oxidation (breakdown) product of purine metabolism and is excreted in urine. In most other mammals, the enzyme uricase further oxidizes uric acid to allantoin (Angstadt and Carol, 1997). The loss of uricase in higher primates parallels the similar loss of the ability to synthesize ascorbic acid (Proctor, 1970). Both uric acid and ascorbic acid are strong reducing agents (electron donors) and potent antioxidants. In humans, over half the antioxidant capacity of blood plasma comes from uric acid (Baillie et al., 2007). In humans, about 70% of daily uric acid disposal occurs via the kidneys, and in 5-25% of humans impaired renal (kidney) excretion leads to hyperuricemia (Vitart et al., 2008).

Uric acid may be a marker of oxidative stress (Becker, 1993), and may have a potential therapeutic role as an antioxidant (Glantzounis et al., 2005). On the other hand, like other strong reducing substances such as ascorbate, uric acid can also act as a prooxidant (Proctor, 1972), particularly at elevated levels. Thus, it is unclear whether elevated levels of uric acid in diseases associated with oxidative stress such as stroke and atherosclerosis are a protective response or a primary cause (Proctor, 2008). For example, some researchers propose that hyperuricemia-induced oxidative stress is a cause of metabolic syndrome (Hayden and Tyagi, 2004). On the other hand, plasma uric acid levels correlate with longevity in primates and other mammals (Cutler, 1984). This is presumably a function of urate's antioxidant properties.
Uric acid is the most abundant aqueous antioxidant in humans, and contributes as much as two-thirds of all free radical scavenging capacity in plasma. It is particularly effective in quenching hydroxyl, superoxide and peroxynitrite radicals, and may serve a protective physiological role by preventing lipid peroxidation (Squadrito et al., 2000). In a variety of organs and vascular beds, local uric acid concentrations increase during acute oxidative stress and ischaemia, and the increased concentrations might be a compensatory mechanism that confers protection against increased free radical activity (Nieto et al., 2000). In animal models, local uric acid concentrations significantly increase in acute brain injury (Tayag, 1996). For example, in the rat, middle cerebral artery occlusion causes a significant increase in cerebral uric acid concentrations, which can persist for several days after the injury (Kanemitsu, 1988). These observations have prompted interest in the potential impact of raised local uric acid concentrations in the setting of acute ischaemic stroke.

1.2.9. Bilirubin:

Bilirubin (formerly referred to as hematoidin) is the yellow breakdown product of normal heme catabolism. Heme is found in hemoglobin, a principal component of red blood cells. Bilirubin is excreted in bile and urine (Chang et al., 2007), and elevated levels may indicate certain diseases. It is responsible for the yellow color of bruises, urine, and the yellow discoloration in jaundice.

Bilirubin consists of an open chain of four pyrrole-like rings (tetrapyrrole). In heme, by contrast, these four rings are connected into a larger ring, called a
porphyrin ring. Some of the double-bonds in bilirubin isomerize when exposed to light. This is used in the phototherapy of jaundiced newborns: the isomer of bilirubin formed upon light exposure is more soluble than the unilluminated isomer.

Bilirubin is created by the activity of biliverdin reductase on biliverdin, a green tetrapyrrolic bile pigment which is also a product of heme catabolism. Bilirubin, when oxidized, reverts to become biliverdin once again. This cycle, in addition to the demonstration of the potent antioxidant activity of bilirubin, has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant (Baranano et al., 2002).

1.2.10. **Total plasma protein:**

Also called serum proteins, are proteins found in blood plasma. Serum total protein in blood is 7g/dl, which in total makes 7% of total blood volume. They serve many different functions, including:

1. Circulatory transport molecules for lipids, hormones, vitamins and metals.
2. Enzymes complement components, protease inhibitors, and kinin precursors.
3. Regulation of a cellular activity and functioning and in the immune system.

All the plasma proteins are synthesized in liver except gamma globulins. 60% of plasma proteins are made up of the protein albumin, which are major contributors to osmotic pressure of plasma which assists in the transport of lipids and steroid hormones. Globulins make up 35% of plasma proteins and
are used in the transport of ions, hormones and lipids assisting in immune function. 4% is fibrinogen which is essential in the clotting of blood and can be converted into insoluble fibrin. Regulatory proteins which make up less than 1% of plasma proteins are proteins such as enzymes, proenzymes and hormones. Current research regarding blood plasma proteins is centered on performing proteomics analyses of serum/plasma in the search for biomarkers. These efforts started with two-dimensional gel electrophoresis (Anderson and Anderson, 1977).

The total thiol status in the body, especially thiol (-SH) groups present on protein are considered as major plasma antioxidants in vivo and most of the SH-groups are present over albumin and are major reducing groups present in our body fluids. (Himmelfarb et al., 2000),( Himmelfarb and McMongale, 2001).Ceruloplasmin (CP) is an alpha-2, acute phase-responsive, multicopper oxidase glycoprotein that contains>95% of the copper present in plasma. Antioxidant activity of CP can be ascribed mainly to its ferroxidase activity, which inhibits ferrous ion-stimulated lipid peroxidation and the formation of hydroxyl radicals in the Fenton reaction, and is also a scavenger of ROS (Osaki et a., 1966) and (Cunningham et al., 1995).

1.2.11. Plasma Albumin:

Serum albumin is the most abundant blood plasma protein and is produced in the liver and forms a large proportion of all plasma protein. It normally constitutes about 60% of human plasma protein. Serum albumins are important in regulating blood volume by maintaining the oncotic pressure (also known as colloid osmotic pressure) of the blood compartment. They also serve as carriers for molecules of low water solubility this way isolating their hydrophobic nature, including lipid soluble hormones, bile salts, unconjugated
bilirubin, free fatty acids (apoprotein), calcium, ions (transferrin), and some drugs like warfarin, phenobutazone, clofibrate & phenytoin. For this reason, it's sometimes referred as a molecular "taxi". Competition between drugs for albumin binding sites may cause drug interaction by increasing the free fraction of one of the drugs, thereby affecting potency. Low albumin (hypoalbuminemia) may be caused by liver disease, nephrotic syndrome, burns, protein-losing enteropathy, malabsorption, malnutrition, late pregnancy, artefact, genetic variations and malignancy (Ballmer, 2001).

High albumin (hyperalbuminemia) is almost always caused by dehydration. In some cases of retinol (Vitamin A) deficiency the albumin level can become raised to High-normal values. This is because retinol causes cells to swell with water (this is also the reason too much Vitamin A is toxic) (Gaull, 1986). In lab experiments it has been shown that All-trans retinoic acid down regulates human albumin production (Suzuki, 2006).

1.2.12. Red blood cells:

Red blood cells (also referred to as erythrocytes) are the most common type of blood cell and the vertebrate organism's principal means of delivering oxygen (O₂) to the body tissues via the blood flow through the circulatory system. A typical human erythrocyte has a disk diameter of 6–8 µm and a thickness of 2 µm, being much smaller than most other human cells. Women have about 4 to 5 million erythrocytes per microliter (cubic millimeter) of blood and men about 5 to 6 million. Human red blood cells take on average 20 seconds to complete one cycle of circulation (Hillman et al., 2005). As red blood cells contain no nucleus, protein biosynthesis is currently assumed to be absent in these cells, although a recent study
indicates the presence of all the necessary biomachinery in human red blood cells for protein biosynthesis (Kabanova et al., 2009).

The blood's red color is due to the spectral properties of the hemic iron ions in hemoglobin. Each human red blood cell contains approximately 270 million of these hemoglobin biomolecules, each carrying four heme groups; hemoglobin comprises about a third of the total cell volume. This protein is responsible for the transport of more than 98% of the oxygen (the remaining oxygen is carried dissolved in the blood plasma). The red blood cells of an average adult human male store collectively about 2.5 grams of iron, representing about 65% of the total iron contained in the body (Kenneth, 2007).

1.2.13. **White blood cells:**

White blood cells (WBCs), or leukocytes, are cells of the immune system involved in defending the body against both infectious disease and foreign materials. Five (LaFleur, 2008) different and diverse types of leukocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. Leukocytes are found throughout the body, including the blood and lymphatic system.

The number of WBCs in the blood is often an indicator of disease. There are normally between $4 \times 10^9$ and $1.1 \times 10^{10}$ white blood cells in a liter of blood, making up approximately 1% of blood in a healthy adult (Alberts, 2005). An increase in the number of leukocytes over the upper limits is called leukocytosis, and a decrease below the lower limit is called leukopenia. The physical properties of leukocytes, such as volume, conductivity, and granularity, may change due to activation, the presence of immature cells, or the presence of malignant leukocytes in leukemia. There are several different
types of white blood cells. They all have many things in common, but are all distinct in form and function. A major distinguishing feature of some leukocytes is the presence of granules; white blood cells are often characterized as granulocytes or agranulocytes.

**Granulocytes (polymorphonuclear leukocytes):**

Leukocytes characterized by the presence of differently staining granules in their cytoplasm when viewed under light microscopy. These granules are membrane-bound enzymes which primarily act in the digestion of endocytosed particles. There are three types of granulocytes: neutrophils, basophils, and eosinophils, which are named according to their staining properties (Witko-Sarsat, *et al.*, 2000).

**Agranulocytes (mononuclear leucocytes):**

Leukocytes characterized by the apparent absence of granules in their cytoplasm. Although the name implies a lack of granules these cells do contain non-specific azurophilic granules, which are lysosomes (Gartner and Hiatt, 2007) the cells include lymphocytes, monocytes, and macrophages.

Due to scare of information that relates the thrombocytopenia and oxidative stress in Sudan, therefore, this study aim to investigate the role of oxidative stress and some antioxidant markers in Sudanese patients with malaria infection.
1.3. Objectives:

1.3.1 General objective:

To investigate oxidative stress and antioxidant status in thrombocytopenia mediated by *Plasmodium falciparum* malaria.

1.3.2 Specific objectives:

1. To assay the activity of the enzyme glutathione peroxidase in platelets.
2. To evaluate the level of Malondialdehyde in platelets.
3. To measure the plasma level of total protein and albumin.
4. To measure the level of plasma uric acid.
5. To measure the level of plasma total and conjugated bilirubin in thrombocytopenic and non cytopenic malaria patients.
2. Materials and methods:

2.1 Subjects:
Forty patients with malaria participated in this study; twelve were male and twenty eight female, their ages ranged between (2- 75 years). They were admitted to Sennar Teaching Hospital. The patients were confirmed to have malaria through blood film examination and ten healthy individuals from Sennar area were selected as control group.

2.2 Instruments:

- Microscope (Olympus).
- U.V Spectrophotometer (P-D303S).
- Sysmex hematology analyzer (ORPHEE company; Switzerland, Id: 102405-000639).
- Bench centrifuge.
- Glassware.
- Water bath.

2.3 Chemicals and reagents:

The glutathione peroxidase kit and malondialdehyde kit were produced by Biodiagnostic Company (Giza - Egypt) and the total protein, albumin, uric acid, conjugated and un conjugated bilirubin kits were produced by Biosystems S.A. Company (Barcelona – Spain).
**Reagents storage:**

All reagents and standards were stored in refrigerator at 15 °C.

**2.3.1. Giemsa stain:**

Giemsa stain was prepared by the standard method adopted by Cheesbrough (2006).

**To prepare 500 ml of Giemsa stain:**

A. Giemsa powder . . . . . . . . . . . . . . . . . . . . . . . . . . . 3.8 g.
B. Glycerol (glycerin) . . . . . . . . . . . . . . . . . . 250 ml.
C. Methanol (methyl alcohol) . . . . . . . . . . . . . . 250 ml.

**Preparation of Giemsa stain:**

1. Giemsa powder was weighed, and transferred to a dry brown bottle of 500 ml capacity which contains a few dry glass beads.
2. Using a dry measuring cylinder, 250 ml methanol were measured, and added to the stain and mixed well.
3. Using the same cylinder, 250 ml glycerol was measured and added to the stain. The contents were mixed well.
4. The bottle of stain was placed in a water bath at 60 °C for one hour to dissolve. It was mixed well at intervals till well mixed.
5. The bottle was labeled, and marked as flammable and toxic. It was stored at room temperature in the dark.
2.3.2. *Glutathione peroxidase enzyme kits:*

**Reagent 1 (R1):**

- Assay Buffer, pH 7.0 (Phosphate Buffer) 50 mM.
- Triton X-100 0.1%.

**Reagent 2 (R2):**

- NADPH reagent (Lyopholized):
  - Glutathione (GSH) 24μmol.
  - Glutathione reductase ≥ 12 U.
  - Nicotinamide- adenine dinucleotide phosphate reduced (NADPH) 4.8 μmol.

**Reagent 3 (R3):**

Substrate (H₂O₂).

**Preparation of working reagents:**

Buffer reagent (R1): content ready for use.
- The content in the vial R2 was reconstituted with 5 ml buffer R1, (stable for one week at -20°C).
- R3 was diluted 100 times immediately before used (0.1 ml of H₂O₂ + 9.9 ml DW). It was discarded after use.

2.3.3. *Malondialdehyde assaying kit:*

- Malondialdehyde standard 10 nmol/ml.
- Chromogen (Thiobarbituric acid, Detergent and stabilizer) 25 mmol/L.
2.3.4. **Total protein estimation kits (Biuret reagent):**

*Composition of the reagent:*

**A. Reagent:**
- Copper (II) acetate 6 mmol/L.
- Potassium iodide 12 mmol/L.
- Sodium hydroxide 1.15mol/ L.
- Detergent.

**B. Protein standard:**
Bovine albumin 8g/dl.

2.3.5. **Albumin estimation kit (bromocresol green reagent):**

*Composition of the reagent:*

**A. Reagent:**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate buffer</td>
<td>100ml/ L.</td>
</tr>
<tr>
<td>Bromocresol green</td>
<td>0.27 mmol/L.</td>
</tr>
<tr>
<td>Detergent, pH4.1</td>
<td></td>
</tr>
</tbody>
</table>

**B. Albumin standard:**

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine albumin</td>
<td>6g/dl.</td>
</tr>
</tbody>
</table>
2.3.6. *Uric acid estimation kit:*

**Composition of the reagent:-**

A. Reagent:

- Phosphate buffer (pH 7.5) – 50 mmol/l.
- 4- Aminoantipyrine - 0.3mmol/l.
- 3, 5, Dichloro-2- hydroxybenzenesulphonate – 4.0 mmol/l.
- Uricase - 200 U/l.
- Peroxidase - 100 KU/l.
- Stabilize, detergents and preservatives –

B. Uric acid standard: 8mg/dl.

2.3.7. *Total bilirubin estimation kit:*

**Composition of the reagent:-**

- **AT reagent (reagent A):**
  - sulfanilic acid 29 mmol/L.
  - Hydrochloric acid 0.2mol/L.
  - Cetrimide 50mmol/L.

- **BT reagent (reagent B):**
  - Sodium nitrite 11.6mmol/L.
• **Reagent preparation:-**

  **Working reagent:**
  The content of BT reagent vial was transferred into AT reagent bottle and mixed thoroughly.

  **2.3.8. *Conjugated bilirubin Estimation kit (Diazotized sulfanilic reagent):***

  **Composition of the reagent:-**

  • **AD reagent (reagent A):**
    - Sulfanilic acid 35mmol/L.
    - Hydrochloric acid 0.24mol/L.
  
  • **BD Reagent (reagent B):**
    - Sodium nitrite 3.5mmol/L.

  **Reagent preparation:-**

  • **Working reagent:**

    The content of BD reagent vial was transferred into AD reagent bottle and mixed thoroughly.
2.4. Collection of blood samples:

Blood samples were collected by finger puncture technique to prepare thick and thin blood film (BF) for malaria investigation. Before any treatment 5 ml of venous blood were collected and transferred into Ethylene Diamine Tetra Acetic acid dipotassium salts (K₂ (EDTA)) test tubes and divided into two equal volumes, one was used for measuring the hematological parameters and platelets separation for assay the activity of platelet enzyme glutathione peroxidase and concentration of malondialdehyde. The other portion was centrifuged and plasma was separated, the plasma was used to measure the level of total protein, albumin, uric acid, total and conjugated bilirubin.

2.5. Malaria diagnosis:

Patients were examined for malaria, Thick and thin blood films were prepared, stained with Giemsa stain and were examined for malaria parasite. The patients were confirmed to have Plasmodium falciparum parasite.

2.6. Assay of glutathione peroxidase activity:

The glutathione peroxidase enzyme activity was measured according to the method adopted by Paglia and Valentine (1967).
1. **Principle of the method:**

   The assay is an indirect measure of the activity of cellular glutathione peroxidase (c-GPx).

   \[
   \text{R-O-O-H + 2GSH} + \text{H}_2\text{O} \rightarrow \text{R-O-H + GSSG}
   \]

   \[
   \text{GSSG + NADPH + H}^+ \rightarrow 2\text{GSH + NADP}^+
   \]

   The oxidation of NADPH to NADP\(^+\) is accompanied by a decrease in absorbance at 340 nm \((A_{340})\) providing a spectrophotometric means for measuring GPx enzyme activity.

**Platelets preparation for glutathione peroxidase and malondialdehyde:**

Platelets were prepared and lysed according to the method adopted by Claudio *et al* (2008).

2 ml of blood were centrifuged at 500 rpm for 5 min to obtain platelet-rich plasma (PRP). The platelets were washed three times by centrifugation at 2,000 rpm for 10 min. After each centrifugation, the supernatant was decanted and discarded, and the platelets pellet were resuspended into 500 μL of a sodium chloride solution (0.89%) and immediately kept in refrigerator at 4°C until the biochemical assays were performed. Platelet-poor plasma was separated from the remaining blood after the PRP separation by centrifugation at 3,500 rpm for 10 min.
2. **Procedure**:  

One ml of reagent 1 (R1) was taken into 10 ml test tube, 0.1 of reagent 2 (R2) was added, and then 0.1 ml of sample was added. They were mixed well. Absorbance was measured twice: directly after mixing and after being allowed to stand for 3 minutes at 340 nm/min against deionized water.

3. **Calculation**:  

I. The rate of decrease in absorbance ($A_{340}$) per minutes was determined by calculating the difference in $A_{340}$ nm between 60 and 120 second.

II. The net $A_{340}$ nm /min for the sample was converted to NADPH consumed (nmol/min/ml) the following relationship were used.

\[ 1\text{mU/ml} = 1\text{nmol/NADPH/min/ ml} = \frac{A_{340}/\text{min}}{0.00622^*} \]

III. The dilution of the sample was corrected:

- Dilution of the sample prior to adding to the cuvate.
- Dilution in the assay.

\[ \frac{\text{Total volume}}{\text{Volume of sample}} = \frac{1.21}{0.01} = 121. \]

IV. The unit of the activity was expressed in the original sample per liter or in relationship to the protein.

\[ \text{Enzyme activity (U/L)} = \frac{A_{340}/\text{min}}{0.00622} \times 121. \]

(*Extinction coefficient)
2.7. **Determination of malondialdehyde concentration:**

Malondialdehyde concentration was measured according to the method adopted by Ohkawa (1979).

1. **Principle:-**

   Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature 95°C for 30 min to form a complex product (pink colour) which can be measured colorimetrically.

2. **Procedure:-**

   One ml of chromogen reagent was pipetted into three test tubes which were labeled as blank, sample and standard. 0.2 ml of sample was added to the sample test tube, and 0.2 ml of standard was added to standard tube.

   Contents of each tube were mixed well, the test tube was covered by glass bead, and it was heated in water bath at 95 °C for 30 min, and it was allowed to cool. Then 0.2ml of sample blank was added to blank tube.

   Contents of each tube were mixed well, and the absorbances of the samples (A sample) and standard were read against the blank and standard at 534nm.
3. **Calculation:**

\[
\text{Malondialdehyde in sample} = \frac{A_{\text{sample}} \times 10}{A_{\text{standard}}}
\]

\[\text{nmol/l}\]

2.8. **Measurement of total protein concentration:**

Total protein was measured colorimetrically by Biuret method described by Tietz (1986).

1. **Principle of the method:**

Protein in the sample reacts with copper (II) ion in alkaline medium forming a coloured complex that can be measured by colorimeter.

2. **Procedure:**

I. One ml reagent was pipetted into three test tubes which were labeled as blank, sample and standard. 0.02 ml of sample was added to the sample test tube, and 0.2 ml of standard was added to standard tube, 0.02 ml distilled water was added to blank test tube.

II. Contents of each tube were mixed thoroughly and allowed to stand for 10 minutes at room temperature.

III. The absorbances (Abs) of the standard and the sample were read in a colorimeter at 545 nm against the blank.
3. **Calculation:-**

The protein concentrations in the samples were calculated using the following general formula:

\[
\text{Total protein (g/dl)} = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Concentration of standard.}
\]

2.9. **Estimation of albumin concentration:**

Albumin was measured by bromocresol green method (BCG) adopted by Young, (1995).

1. **Principle of the method:-**

Albumin in the sample reacts with bromocresol green in acid medium forming a coloured complex that can be measured by colorimeter.

**Procedure:**

1. One ml reagent was pipetted into three test tubes which were labeled as blank, sample and standard. 0.02 ml of sample was added to the sample test tube, and 0.2 ml of standard was added to standard tube, 0.02 ml distilled water was added to blank test tube.
II. Contents of each tube were mixed thoroughly and allowed to stand for 10 minutes.

III. The absorbances (Abs) of the standard and the samples were read in spectrophotometer at 545 nm against the blank.

2. **Calculation**: The albumin concentration in the samples was calculated using the following general formula:

\[
\text{Albumin (g/dl)} = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Concentration of standard.}
\]

2.10. **Estimation of uric acid concentration**: Uric acid was measured colorimetrically by (uricase/ PAP), the method adopted by Piero (2010).

1. **Principle of the method**: Uricase catalyzes the oxidation of uric acid to allantoin and H$_2$O$_2$. In the presence of peroxidase (POD), H$_2$O$_2$ react with 4-aminoantipyrine (4-AA) and 3, 5, dichloro-2-hydroxybenzenesulphonate (DHBS) to form a quinoneimine dye, the concentration of which at 545nm is directly proportional to the uric acid concentration.
Uric acid +O₂ + 2H₂O – uricase \[\rightarrow\] allantoin + CO₂ + H₂O₂.
2 H₂O₂ + DHBS + 4AA - POD \[\rightarrow\] Quinoneimine + 4H₂O.

2. **Procedure:**

   I. One ml reagent was pipetted into three test tubes which were labeled as blank, sample and standard. 0.02 ml of sample was added to the sample test tube, and 0.2 ml of standard was added to standard tube, 0.02 ml distilled water was added to blank test tube.

   II. Contents of each tube were mixed thoroughly and incubated for 10 minutes at room temperature.

   III. The absorbances (Abs) of the standard and the samples were read in colorimeter at 545 nm against the blank.

3. **Calculation:**

   The uric acid concentration in the samples was calculated using the following general formula:

   \[
   \text{Uric acid (mg/dl)} = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Concentration of standard.}
   \]
2.11. Estimation of total bilirubin concentration:

The total bilirubin was measured by Jendrassik and Grof, method adopted by Cheesbrough (2006).

1. **Principle of the method:**

   Bilirubin reacts with the diazotized sulphanilic acid (diazo reagent) to form azobilirubin. Caffeine is an accelerator and gives a rapid and complete conversion to azobilirubin. The pink colour of acidic azobilirubin is converted to blue azobilirubin by alkaline tartrate, which can be measured colourimetrically.

2. **Procedure:**

   I. One ml working reagent was pipetted into three test tubes which were labeled as blank, sample and standard. Fourth test tube label sample blank containing 0.1 ml sample and reagent (AD). 0.1 ml of sample was added to the sample test tube, and 0.1 ml of standard was added to standard tube, 0.01 ml distilled water was added to blank test tube.

   II. Content of each tube were mixed thoroughly and allowed to stand for 2 minutes at room temperature.

   III. The absorbances (Abs) of the samples and blank were read in colorimeter at 540 nm against the distilled water.
IV. The absorbances (Abs) of the samples and of the standard were read against the reagent blank.

3. **Calculation:-**

   The total bilirubin concentration in the samples was calculated using the following general formula:
   \[ \text{Total bilirubin (mg/dl)} = \frac{\text{Abs sample} - \text{Abs sample blank}}{\text{Abs standard}} \times \text{Concentration of standard} \]

2.12. **Measurement of conjugated bilirubin concentration:**

   The conjugated bilirubin was measured by Jendrassik and Grof, method adopted by Cheesbrough (2006).

1. **Principle of the method:-**

   Direct bilirubin is measured as the total bilirubin method but in absence of the caffeine- benzoate catalyst and at an acid pH. Under these conditions only the conjugated bilirubin will react.

2. **Procedure:**
   I. One ml working reagent was pipetted into three test tubes which were labeled as blank, sample and standard. Fourth test tube label sample blank containing 0.1ml sample and reagent (AD).
0.1 ml of sample was added to the sample test tube, and 0.1 ml of standard was added to standard tube, 0.01 ml distilled water was added to blank test tube.

II. The contents of each tube were mixed thoroughly and allowed to stand for 2 minutes at room temperature.

III. The absorbances (Abs) of the sample blank were read in colorimeter at 540 nm against the distilled water.

IV. The absorbance (Abs) of the sample and of the standard were read against the reagent blank.

3. **Calculation:-**

The total bilirubin concentration in the sample was calculated using the following general formula:

\[
\text{Conjugated bilirubin (mg/dl) = Abs sample – Abs sample blank} \times \frac{\text{Concentration of standard}}{\text{Abs standard}}
\]

2.13. **Hematological parameters:**

The hematological parameters measured in this study include: total white blood cell count (TWBCs), red blood cell count (RBCs), hemoglobin, mean cell volume (MCV), packed cell volume (PCV), mean cell
hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), thrombocyte count and differential count of white blood cells.

These parameters were measured using the cell counter Mythic-18, (ORPHEE company; Switzerland, Id: 102405-000639), which uses multi-techniques that can measure 18 blood parameters.

**Procedure:**

One ml venous blood was put in the measuring device.
Blood parameters were measured according to the following:

**A. Impedance:**

Total erythrocyte count, total leukocyte count and the differential count were measured by the impedance method, in which blood cells can create electric resistance according to their diameter, which then can be detected by the galvanometer.

**B. Colorimetric method:**

Hemoglobin was measured by colorimetric method. The method based on the conversion of the haemoglobin by means of drabkin's solution (0.2g potassium cyanide, 0.2 potassium ferricyanide, and 1g sodium bicarbonate per one liter of distilled water) to cyanomethaemoglobin concentration was measured by (cell counter Mythic-18) as g/dl of blood.
C. Centrifugation method:

Mythic cell counter provided with a microhematocrit centrifuge to measure the (PCV) percentage within few seconds.

D. Calculation method:

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC), were then calculated from the RBC count, Hb and PCV.

2.14. Statistical analysis:

Results of this study were statistically analyzed using (SPSS) program. Significant differences between groups were assessed by one-way ANOVA and t- test. Correlation matrix was done and the r values were obtained with level of significance.
3. Results

Data presented in the current study was obtained from a questionnaire and laboratory investigation of blood samples of Sudanese patients infected with malaria (40) and control group (10).

3.1. Malaria infection and thrombocytopenia:

Blood film examination indicated that the 40 patients admitted to Sennar Teaching Hospital were infected with the parasite Plasmodium Falciparum. The distribution of respondents according to thrombocyte count (> 150,000) revealed that 10 (25%) of the malaria patients were thrombocytopenic and 30 (75%) were without thrombocytopenia (Appendix I).

Because of the obvious differences between malaria patients with thrombocytopenia (10) and those without thrombocytopenia (30), the study groups were classified into malaria with thrombocytopenia, malaria without thrombocytopenia and control groups.

3.2. Activity of glutathione peroxidase:

Results presented in Table (1) and Figure (3-1) indicate that there was a significant difference (P >0.00) in the activity of glutathione peroxidase (GPx) between the studied groups, the highest enzyme activity was observed for the control group (1979 U/L) followed by malaria non thrombocytopenic (384 U/L) and the least was the malaria thrombocytopenic group (112 U/L).
The activity of the enzyme GPx was almost similar for male and female malaria patients, but the females of the control group showed higher activity of GPx relative to that of the male group Figure (3-1). Results shown in Figure (3-2) indicate that the age group (18 - 32 years) of the control group showed the highest activity of the enzyme GPx, compared to that of (2-18 years) and (> 32 years) groups, which was almost similar. On the other hand the various age groups of the malaria patients showed slight differences in the enzyme activity.

Results presented in Figure (3-3) and Table (2) indicated that the activity of the enzyme GPx was positively correlated with thrombocyte count \( r = 0.602; P < 0.001 \), and it was negatively correlated with malondialdehyde \( r = -0.36; P < 0.05 \) Figure (3-4). Whereas the activity of GPx enzyme was weakly correlated with total bilirubin, direct bilirubin, uric acid, total protein and albumin Table (2).

### 3.3. Malondialdehyde:

Results given in Table (1) indicated that TCP showed significantly higher \( P > 0.05 \) level of MDA (7.3 µmol/L) followed by NTCP (3.9 µmol/L) and the least was that of the control group (0.02 µmol/L).

The concentration of MDA was negatively correlated with platelet count \( r = -0.696; P > 0.001 \).

### 3.4. Hematological parameters:

Results of the hematological parameters Red Blood Cells (RBCs), Hemoglobin (Hb), hematocrit (HCT), Mean Cell Volume (MCV), Mean Cell Hemoglobin
(MCH) and Mean Cell Hemoglobin Concentration(MCHC) presented in table (1) indicated that the difference in these parameters between studied group was not significant, except HCT which was significantly higher (P <0.005) in TCP (42.9%) relative to other groups (37.9%). The hematological parameter are weakly correlated to GPx, except MCV which was negatively correlated with GPx enzyme (r = -0.32; P<0.05).

3.5. White blood cells and differential count:

Results presented in Table (1) indicate that there was a significant difference (P >0.05) in TWBC between the studied groups, the highest TWBC was observed for the NTCP (7.9X10^3 cell/cmm) followed by control (6.1X10^3 cell/cmm) and then the TCP group (5.1X10^3 cell/cmm).

3.6. Total and conjugated Bilirubin:

Results presented Table (1) show that the concentration of total bilirubin was slightly higher in blood of NCTP (0.7 mg/dl) and TCP (0.6 mg/dl) relative to that of the control group (0.47 mg/dl), whereas the level of conjugated bilirubin was almost similar for the studied groups.

3.7. Uric acid concentration:

Result presented in Table (1) showed that the concentration of uric acid in the plasma of the studied groups were similar.
3.8. Total protein and albumin:

Data presented in Table (1) indicated that the concentration of total plasma protein was similar for all studied groups; likewise the level of plasma albumin was similar for various groups.

Table (1): Some hematological parameters and markers of Oxidative stress in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.
Figure (3-1): Activity of glutathione peroxidase in platelets in males and females of Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

Figure (3-2): The activity of the platelets GPx in different groups of age of Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.
Figure (3-3): Correlation between blood platelet count and platelet glutathione peroxidase (GPx) ($r = -0.60; p < 0.001$) in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

Figure (3-4): Correlation between blood platelet count and platelet malondialdehyde (MDA) ($r = -0.69; p < 0.001$) in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.
Figure (3-5): The concentration of MDA in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.
Table (2): Correlation of platelets, GPX, and MDA with blood indices and some chemical parameters in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

|                | WBCs   | RBCs  | Hb     | Hct    | MCV    | MCH    | MCHC   | Plt    | Lymp  | Neu   | mix   | T. bilirubin | D. bilirubin | U.A    | Protein T | Albumin | gpx   | mda   |
|----------------|--------|-------|--------|--------|--------|--------|--------|--------|-------|-------|-------|-------|-------------|-------------|--------|-----------|---------|-------|-------|
| Platelets      | .359*  | -.079 | -.431**| -.491**| -.602**| -.468**| -.042  | -.205  | .210  | -.131 | .267  | .169  | -.248       | .254        | .173   | .602**    | -.696**  |       |       |
| Sig. (2-tailed)| .023   | .628  | .006   | .001   | .000   | .002   | .798   | .205   | .193  | .422  | .095  | .298  | .123        | .113        | .286   | .000      | .000     |       |       |
| N              | 40     | 40    | 40     | 40     | 40     | 40     | 40     | 40     | 40    | 40    | 40    | 40    | 40          | 40          | 40     | 40        | 40      | 40    | 40    |
| GPx            | .234   | -.056 | -.267  | -.274  | -.317  | -.278  | -.088  | -.109  | .117  | -.066 | .124  | .544  | .262        | .485        | .008   | -.364*    |         |       |       |
| Sig. (2-tailed)| .146   | .733  | .095   | .088   | .046   | .082   | .591   | .000   | .505  | .470  | .684  | .445  | .667        | .373        | .904   | .961      | .021     |       |       |
| N              | 40     | 40    | 40     | 40     | 40     | 40     | 40     | 40     | 40    | 40    | 40    | 40    | 40          | 40          | 40     | 40        | 40      | 40    | 40    |
| MDA            | -.253  | .037  | .297   | .371** | .507** | .361*  | -.038  | -.063  | -.096 | -.021 | .026  | .041  | -.279       | -.137       | -.364*  |          |         |       |       |
| Sig. (2-tailed)| .115   | .820  | .063   | .018   | .001   | .022   | .814   | .000   | .426  | .698  | .557  | .900  | .872        | .804        | .081   | .398      | .021     |       |       |
| N              | 40     | 40    | 40     | 40     | 40     | 40     | 40     | 40     | 40    | 40    | 40    | 40    | 40          | 40          | 40     | 40        | 40      | 40    | 40    |

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).
4. Discussion

The aim of this study was to investigate the levels of platelets GPx and MDA and their relation to other biochemical parameters in blood of thrombocytopenic patients mediate by *P. falciparum* infection.

Results indicate that the activity of the platelets enzyme glutathione peroxidase (GPx) was very low in thrombocytopenic malaria patients (TCP) compared to malaria non-thrombocytopenic (NTCP) and control subjects. These results agreed with those obtained by Claudio *et al* (2008) who found increased activity of GPx in platelets of NTCP compared to TCP.

The activity of the enzyme GPx was negatively correlated with platelets count. These results agreed with Claudio *et al* (2008) who studied GPx in platelets of patients with vivax malaria, the author stated that this may be due to increased lysis of platelets because of increased reactive oxygen species (ROS) and the low activity of GPx and low level of antioxidant.

Thrombocytopenia was observed in humans and animals infected with malaria parasites. There are several mechanisms that have been proposed for occurrence of thrombocytopenia, which include disseminated intravascular coagulation, immune mechanisms due to absorption of soluble malaria antigen by platelets and subsequent attachment of antibodies to such antigens. Other studies suggested are defective platelet formation and hypersplenism and reactive oxygen species (ROS).
However, the exact mechanism has not been elucidated yet (Abdalla 1990, Kumar and Shashirekha 2006).

The concentration of MDA was higher in the platelets of TCP relative to that of NTCP and control group. These results agree with study carried out by Sohail et al (2007) who found higher MDA levels in malaria patients infected with *P. vivax* patients.

The increased amount of MDA generated during malaria infection may due to activation of the immune system (Pabón et al., 2003). The increase in lipid peroxidation is probably due to production of ROS species by the immune cells and also due to the synchronized O$_2^-$ during hemoglobin degradation by the malarial parasites. It has been shown that infected RBCs by *P. falciparum* trophozoites produce H$_2$O$_2$ and OH$^-$ radicals twice as much as the normal erythrocytes (Sohail et al., 2007).

The concentration of malondialdehyde (MDA) is negatively correlated with GPx; these results suggest the direct role of enzyme GPx to prevent formation of free radicals in humans (Claudio et al., 2008).

The concentration of total bilirubin, conjugated bilirubin; total protein, albumin and uric acid were in normal range which may demonstrates an adequate capacity of plasma to protect its environment from free radical attack. Halliwell and Gutteridge (1999) found that the total oxidant status (TOS) of human plasma is mainly attributed to uric acid, protein and bilirubin.
Human protection against free radicals is by large distribution of free antioxidant with synergic reaction; the level of plasma antioxidant usually doesn’t reflect the antioxidant status (Claudio et al., 2008). It was reported that the total oxidative status activity of human is mainly ascribable to uric acid, albumin, total protein, and bilirubin (Halliwell and Gutteridge 1999).

The complete hemogram were in normal range except the thrombocyte count which was low in TCP.

Platelets membranes are less resistant to OS and the membranes of platelets are thinner than those of erythrocyte. Thus the lysis of platelets will be unavoidable, which may be increased by OS. Erel et al., (2001) found that platelet count, platelet superoxide dismutase and GPx activities of patients with P. vivax malaria infection were lower and platelets lipid peroxidation level were higher than normal controls, thus suggesting the OS as a possible cause of thrombocytopenia.

**Conclusion:**
The study concluded that the activity of glutathione peroxidase (GPx) is inversely relation to the infection of malaria and vice- versa for the malondialdehyde. Healthy females and (18 – 30 years) age group showed significantly higher (GPx) relative to other groups, whereas the difference between the same groups of the malaria patients was not significant.
The findings of this study support the suggestion that reactive oxygen species may play an important role in pathogenesis of thrombocytopenia caused by infection of malaria parasite.
**Recommendations:**

- Further studies are needed in order to clarify the association of OS and thrombocytopenia among Sudanese patients infected with *P. falciparum* malaria.

- To investigate thrombocytopenia in patients infected with other parasites that cause malaria.
References


http://www.webmd.com Malaria symptoms Last Updated: May 16, 2007


Lyke, K E. Burges, R. Cissoko. Sangare, Y. M. Dao, L. Diarra. Kone, A. Harley, R. Plowe, C V. Doumbo, O K and Sztein, M B. (2004) Serum Levels of the Proinflammatory Cytokines Interleukin-1 Beta (IL-1), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha, and IL-12(p70) in Malian Children with Severe Plasmodium falciparum Malaria and Matched Uncomplicated Malaria or Healthy Controls infection and immunity, 72: 10.


WHO. (2007), The Malaria Problem in South-East Asia Region . Regional office for the South East Asia. [http://www.searo.who.int/en/Section10/Section21/Section340_4269.htm](http://www.searo.who.int/en/Section10/Section21/Section340_4269.htm)


Appendix

Appendix (I): classification of individuals according to platelets counts.

<table>
<thead>
<tr>
<th>PLT group</th>
<th>Count</th>
<th>% within GROUP</th>
<th>GROUP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>low</td>
<td></td>
<td></td>
<td>control</td>
<td>malaria</td>
</tr>
<tr>
<td>normal</td>
<td>Count</td>
<td>% within GROUP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>25.0%</td>
<td>20.0%</td>
</tr>
<tr>
<td>high</td>
<td>Count</td>
<td>% within GROUP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.0%</td>
<td>72.5%</td>
<td>78.0%</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>% within GROUP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
Appendix (II): The mean of all parameters participate in the study.

<table>
<thead>
<tr>
<th>No</th>
<th>Parameter</th>
<th>Malaria</th>
<th>Control</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Glutathione peroxidase (U/l)</td>
<td>316.037</td>
<td>1979</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>Malondialdehyde (μmol/L)</td>
<td>4.8175</td>
<td>0.0230</td>
<td>0.021</td>
</tr>
<tr>
<td>3</td>
<td>TWBCs (X10^3 cell/ cmm)</td>
<td>7.203</td>
<td>6.060</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>RBCs (X10^6 cell/ cmm)</td>
<td>4.6267</td>
<td>4.4540</td>
<td>0.733</td>
</tr>
<tr>
<td>5</td>
<td>Hb (g/dl)</td>
<td>13.0025</td>
<td>12.5000</td>
<td>0.095</td>
</tr>
<tr>
<td>6</td>
<td>HCT (%)</td>
<td>39.163</td>
<td>37.850</td>
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</tr>
<tr>
<td>7</td>
<td>MCV (fl/cell)</td>
<td>84.500</td>
<td>85.200</td>
<td>0.046</td>
</tr>
<tr>
<td>8</td>
<td>MCH (pictogram/cell)</td>
<td>28.045</td>
<td>28.110</td>
<td>0.082</td>
</tr>
<tr>
<td>9</td>
<td>MCHC (g/dl)</td>
<td>33.113</td>
<td>32.980</td>
<td>0.591</td>
</tr>
<tr>
<td>10</td>
<td>Thrombocytes count (X10^3 cell/ cmm)</td>
<td>236.68</td>
<td>270.40</td>
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<tr>
<td>11</td>
<td>Lymphocytes %</td>
<td>36.893</td>
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<tr>
<td>12</td>
<td>Neutrophils %</td>
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<td>56.470</td>
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<tr>
<td>13</td>
<td>Mix%</td>
<td>9.135</td>
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</tr>
<tr>
<td>14</td>
<td>Total bilirubin (mg/dl)</td>
<td>0.697</td>
<td>0.470</td>
<td>0.445</td>
</tr>
<tr>
<td>15</td>
<td>Direct bilirubin (mg/dl)</td>
<td>0.155</td>
<td>0.160</td>
<td>0.667</td>
</tr>
<tr>
<td>16</td>
<td>Uric acid (mg/dl)</td>
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<td>5.57</td>
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</tr>
<tr>
<td>17</td>
<td>Total protein (g/dl)</td>
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<td>7.100</td>
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<tr>
<td>18</td>
<td>Albumin (g/dl)</td>
<td>4.030</td>
<td>4.040</td>
<td>0.961</td>
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Appendix (III): Total White Blood Cells in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group in different study ages.
Appendix (IV): Total White Blood Cells count in in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

Appendix (V): Concentration of total bilirubin in in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.
Appendix (VI): Concentration of direct bilirubin in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td>Direct bilirubin mg/dl</td>
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<tr>
<td>control</td>
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<td></td>
</tr>
<tr>
<td>malaria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>malaria+thrombocytopenia</td>
<td>.16</td>
<td>.15</td>
</tr>
<tr>
<td>control</td>
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<td>.14</td>
<td>.13</td>
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<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>malaria</td>
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<td></td>
</tr>
<tr>
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<td>.12</td>
<td>.11</td>
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Appendix (VII): Uric acid concentration in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

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<td>Uric Acid mg/dl</td>
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<tr>
<td>malaria</td>
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<td></td>
</tr>
<tr>
<td>malaria+thrombocytopenia</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
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<tr>
<td>malaria</td>
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<td>malaria+thrombocytopenia</td>
<td>5.4</td>
<td>5.2</td>
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Appendix (VIII): Total protein concentration in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group

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<tr>
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</tr>
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<td>Male</td>
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<tr>
<td>Malaria</td>
<td>6.9</td>
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Appendix (IX): Albumin concentration in Non-Thrombocytopenic, Thrombocytopenic malaria patients and healthy control group.

<table>
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<tr>
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<tr>
<td>Healthy controls</td>
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</tr>
<tr>
<td>Malaria</td>
<td>3.9</td>
</tr>
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