Original article

# The Effect of Haemozoin on Platelets in Malaria

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Chettinad Health City Medical Journal 2020; 9(3): 149 - 154

DOI: https://doi.org/10.36503/chcmj9(3)-02

## Abstract

Introduction: In India, after HIV/AIDS, the major disease that causes morbidity and mortality is malaria. Varying degrees of thrombocytopenia is noted in all cases of malaria. The mechanism of thrombocytopenia is still to be elucidated. Here, we performed an experiment to show that haemozoin (Hz) pigment maybe responsible for the platelet destruction.

Methods: Peripheral smear examination showed platelet aggregates with haemozoin pigment in the cytoplasm. Assuming that this haemozoin phagocytosis maybe responsible for platelet destruction an experiment utilizing Haemozoin pigment and platelet concentrate was done. 1 ml of platelet concentrate was mixed with 100 µl of Haemozoin solution in 9 tubes and incubated at room temperature in an orbital shaker. Each set had a control where distilled water was added instead of Haemozoin. The platelet count was done at 0 h, 1 h, 2 h, 4 h, and 24 h.

Results: The test sample containing haemozoin pigment showed a reduction in platelet count compared to the control in 7 out of 9 samples.

Conclusion: Our studies indicate that hemozoin pigment may contribute to the thrombocytopenia which is seen in malaria.

Keywords: Malaria, Haemozoin, thrombocytopenia

## Introduction

Malaria is considered as one of the major diseases that causes morbidity and mortality in India, after HIV/AIDS. The incidence of Plasmodium falciparum (P. falciparum)has also declined from 1.14 million in 1995 to 0.53 million in 2012. However,the percentage of P. falciparum infection percentage has increased from 39% in 1995 to 50.01% during 2012. The National Vector Borne Disease Control Program reported that the total number of cases of malaria was 190150 in 2013 (upto May) and of falciparum malaria was 115672. There were 51 deaths due to malaria. The mortality rate due to malaria was nearly 1000 per year. Between the years 2001 to 2012 the Annual Parasite Incidence (API) rate has gradually decreased from 2.12 per thousand to 0.88 per thousand. During this time the number of confirmed deaths caused by malaria fluctuated between 1707 to 519.[1] According to recent reports of WHO, in 2017 the worldwide malaria cases occurred was 219 million compared to 239 million cases in 2010 and 217 million cases in 2016. In this 219 million, India contributed 4 % cases.[2]

Hemozoin (Hz) is a toxic heme pigment formed after hemoglobin digestion and is stored in the food vacuoles of the intraerythrocytic parasites which are developing during malaria. The Hz is a dimer of hematin which is complexed with protein and lipid which exists in crystalline form. The crystals of Hz resemble β- hematin and consist of a ferric ion incorporated inside a protoporphyrin IX ring structure.[3] Hz forms a crystalline dimer of α hematin, in combination with lipid and protein. It appears as a black, brown, or amber pigment by light microscopy, and as a birefringent crystal under polarized light.[4-6] The lysis of infected RBCs(iRBC) releases Hz which is heterogeneous and usually gets phagocytosed by the patient's reticulo- endothelial system and is seen in the bone marrow macrophages and spleen.[7,8]

In the present retrospective study, we have isolated the Hz pigment from pooled whole blood of individuals positive for P.falciparum and characterized the pigment using different photophysical tools like infra-red spectroscopy and UV visible spectroscopy. The temporal effect of the isolated Hz has been studied on platelet count.

## Materials and methods

This retrospective experimental study was done in Chettinad Hospital and Research Institute in which 110 consecutive patients with smear positive malaria (both P.vivax and P.falciparum) over a period of 6 months (Jun 2010 to December 2010) were analyzed for the study. Out of these 113 samples 98 samples showed platelet aggregates and in 15 cases Hz pigmentwas seen within the platelet aggregates (Figure 1).

Chemicals like Sodium do Decyl Sulfate (SDS), TrisHCl, $CaCl_2$, Urea, Proteinase K, were purchased from Sigma Chemicals, USA. PBS and other chemicals were procured locally.

Hz pigment was isolated according to the Coban et al.,[9,10] and aliquoted in 27 tubes. Briefly, the parasite layer was aspirated from 2 ml of EDTA blood after centrifugation at 3000 rpm for 15 min at 4°C. Then the parasites were lysed using saponin and after washing with PBS (Phosphate Buffered Saline, pH= 7.2), the pellet was sonicated in 2 % SDS. The pellet was suspended in 1 mL of 10mMTrisHCl (pH= 8), 0.5 % SDS and 1 mM CaCl2 with 2 mg/mL proteinase k and incubated overnight at 37°C. The mixture was centrifuged at 3000 rpm at 4°C and the pellet obtained was washed with 2 % SDS and further incubated with 6 M urea for 3 h at room temperature on a shaker. Then the pellet was washed 1 time in 2 % SDS and resuspended in 5 ml of distilled water as done by Coban et al.[9] The Hz suspension was stored at 4°C and sonicated freshly before the experiments.

After dilution in distilled water the UV-visible absorption spectra was recorded against only distilled water as blank using Shimadzu (Japan) UV-1800 spectrophotometer. To find out the characteristic bonds corresponding to Hz structure, the attenuated reflectance spectra (ATR-IR) was measured using Bruker, Alpha –T FTIR at transmittance mode according to Girigoswami et al.[11]

The platelet concentrate was obtained from blood bank and thawed at room temperature and platelet count was done with and without Hz. The platelet count was done on cell counter Beckman Coulter LH 780. For studying the effect of Hz pigment on the platelet count, 1 ml of platelet plus 100 µl of Hz solution prepared as above was added in 9 tubes each and incubated at room temperature in an orbital shaker. The platelet count was done at 0 h, 1 h, 2 h, 4 h and 24 h. For control group distilled water was added instead of Hz and the platelet counts were done at the same time intervals and under same experimental conditions. The experiment was repeated thrice for each sample of isolated Hz.

## Results

The microscopic image of aggregated Hz pigment in platelet aggregates in the peripheral blood smear of

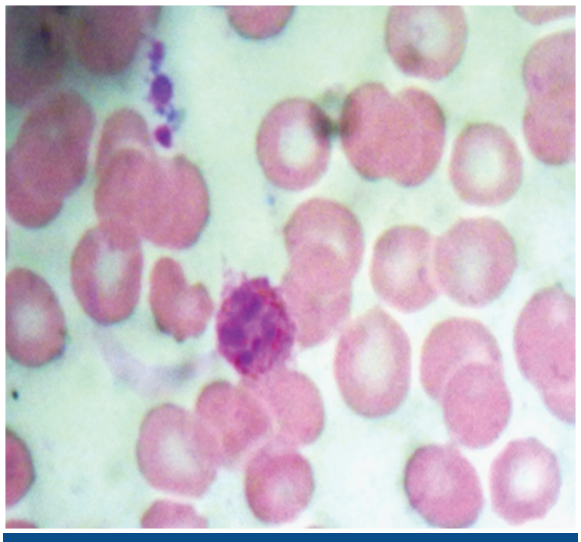
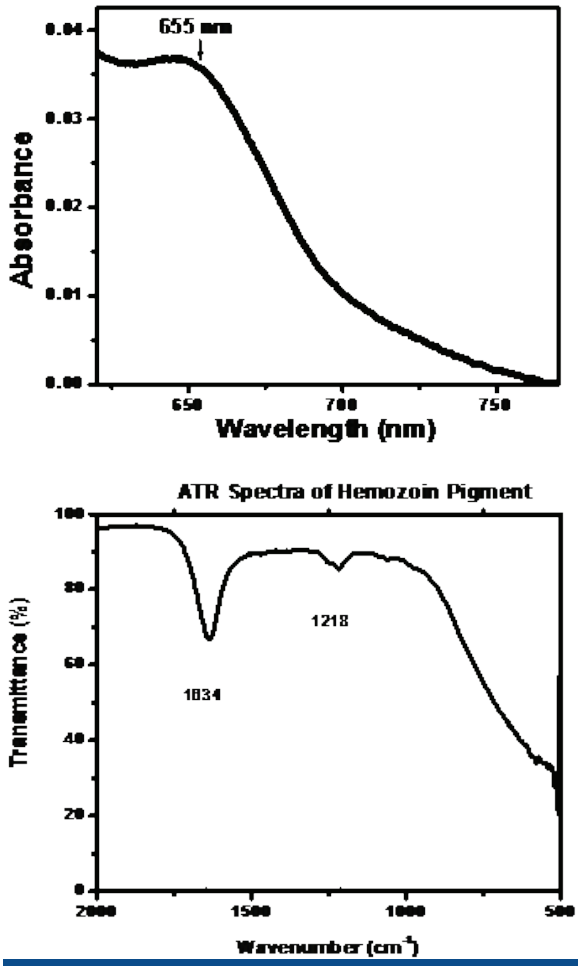


Figure 1: The peripheral smear stained by Leishman'stain showing malarial pigment in the platelet clumps at 10X× 100 X magnification.




Graph 1: a) The UV-visible spectrum isolated Hz pigment. b) The IR spectrum (ATRIR) of the of isolated Hz pigment.

malaria positive patients is shown in Fig 1, Graph 1 (a) and (b) shows the UV visible spectrum and Infra-Red (IR) spectrum of the isolated Hz pigment respectively.

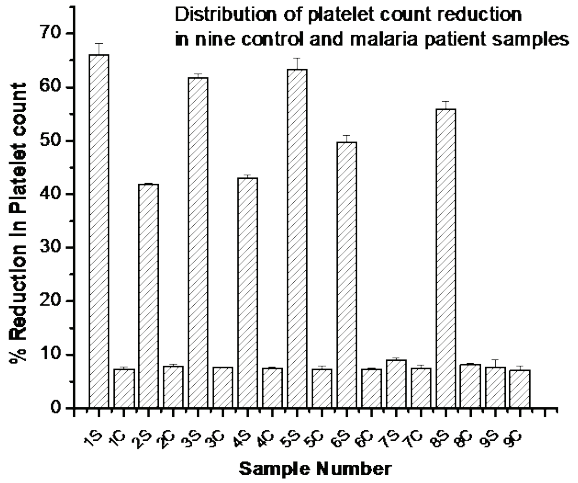
The test sample containing Hzpigment showed a reduction in platelet count compared to the control in 7 out of 9 samples (Table 1 in supplementary information, S1). In 2 out of 9 samples the reduction was not significant. In the positive group, 5 out of 7 samples the platelet count in the sample was reduced nearly 60% whereas in the other 2 samples the reduction was around 40%. The average and standard deviation of the results were plotted in Graph 2. The percent reduction of platelet count after incubation with distilled water was similar in all the control samples (around 7-8%).

To determine the significance difference between the control and the test samples, student's t-test was employed. The percentage of platelet destruction was found to be significant at 1% significance level (p value < 0.001).

| Sample No. | Set # | Platelet count (lakh/cu mm) | Time after incubation with Hz/distilled water | | | | | % reduction in platelet count |
|---|---|---|---|---|---|---|---|---|
| | | | 0 h | 1 h | 2 h | 4 h | 24 h | |
| 1 | Set I | Test | 9.6 | 7.0 | 4.9 | 3.0 | 3.0 | **68.75** |
| | | Control | 9.6 | 9.4 | 9.4 | 9.3 | 8.9 | **7.29** |
| | Set II | Test | 10.4 | 7.2 | 5.3 | 3.4 | 3.6 | **65.38** |
| | | Control | 10.4 | 10.3 | 10.0 | 9.8 | 9.6 | **7.69** |
| | Set III | Test | 11.1 | 8.5 | 5.6 | 4.2 | 3.9 | **64.86** |
| | | Control | 11.1 | 11.0 | 10.8 | 10.5 | 10.3 | **7.20** |
| 2. | Set I | Test | 12.86 | 11.0 | 10.9 | 8.0 | 7.5 | **41.67** |
| | | Control | 12.86 | 12.50 | 12.33 | 11.90 | 11.80 | **8.24** |
| | Set II | Test | 12.11 | 12.0 | 9.9 | 8.0 | 7.0 | **42.19** |
| | | Control | 12.11 | 11.60 | 11.55 | 11.30 | 11.21 | **7.43** |
| | Set III | Test | 12.52 | 11.85 | 10.12 | 8.22 | 7.30 | **41.69** |
| | | Control | 12.52 | 12.11 | 12.10 | 11.80 | 11.57 | **7.58** |
| 3. | Set I | Test | 10.6 | 8.9 | 7.6 | 5.0 | 4.0 | **62.26** |
| | | control | 10.6 | 10.4 | 10.2 | 10.0 | 9.8 | **7.54** |
| | Set II | test | 11.56 | 8.5 | 7.2 | 5.6 | 4.50 | **61.07** |
| | | control | 11.56 | 11.01 | 11.0 | 10.9 | 10.80 | **7.60** |
| | Set III | test | 12.12 | 8.8 | 7.5 | 5.4 | 4.6 | **62.04** |
| | | control | 12.12 | 11.6 | 11.5 | 11.4 | 11.2 | **7.59** |
| 4. | Set I | test | 11.55 | 10.55 | 8.53 | 7.0 | 6.5 | **43.72** |
| | | control | 11.5 5 | 11.10 | 10.8 | 10.9 | 10.7 | **7.35** |
| | Set II | test | 12.35 | 10.2 | 8.9 | 7.5 | 7.1 | **42.51** |
| | | control | 12.35 | 11.90 | 11.6 | 11.5 | 11.4 | **7.69** |
| | Set III | test | 12.23 | 9.9 | 8.6 | 7.4 | 7.0 | **42.76** |
| | | control | 12.23 | 12.0 | 11.7 | 11.5 | 11.3 | **7.60** |
| 5. | Set I | test | 9.0 | 8.0 | 6.33 | 4.0 | 3.1 | **65.55** |
| | | control | 9.0 | 8.7 | 8.6 | 8.5 | 8.3 | **7.77** |
| | Set II | test | 11.9 | 7.4 | 6.2 | 4.9 | 4.6 | **61.34** |
| | | control | 11.9 | 11.5 | 11.4 | 11.2 | 11.1 | **6.72** |
| | Set III | test | 13.21 | 9.1 | 7.2 | 5.5 | 4.9 | **62.90** |
| | | control | 13.21 | 12.92 | 12.6 | 12.44 | 12.23 | **7.41** |
| 6. | Set I | test | 11.7 | 9.0 | 8.9 | 8.45 | 6 .0 | **48.71** |
| | | control | 11.7 | 11.2 | 11.1 | 10.9 | 10.8 | **7.69** |
| | Set II | test | 13.33 | 11.20 | 8.5 | 7.3 | 6.5 | **51.23** |
| | | control | 13.33 | 12.9 | 12.7 | 12.5 | 12.4 | **6.97** |
| | Set III | test | 12.17 | 10.25 | 8.77 | 7.12 | 6.2 | **49.05** |
| | | control | 12.17 | 11.86 | 11.52 | 11.41 | 11.32 | **6.98** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 7. | Set I | test | 8.90 | 8.70 | 8.45 | 8.23 | 8.12 | **8.76** |
| | | control | 8.90 | 8.60 | 8.52 | 8.30 | 8.21 | **7.75** |
| | Set II | test | 11.5 | 11.32 | 11.10 | 10.75 | 10.40 | **9.56** |
| | | control | 11.5 | 11.10 | 11.00 | 10.85 | 10.52 | **8.52** |
| | Set III | test | 13.15 | 12.20 | 11.95 | 11.74 | 12.00 | **8.74** |
| | | control | 13.15 | 12.7 | 12.6 | 12.3 | 12.10 | **7.98** |
| 8. | Set I | Test | 9.45 | 7.0 | 6.9 | 5.2 | 4.1 | **56.61** |
| | | control | 9.45 | 9.0 | 8.9 | 8.8 | 8.7 | **7.93** |
| | Set II | test | 14.17 | 10.5 | 8.9 | 7.2 | 6.5 | **54.13** |
| | | control | 14.17 | 13.52 | 13.41 | 13.11 | 12.98 | **8.39** |
| | Set III | test | 10.21 | 8.52 | 7.23 | 6.12 | 4.4 | **56.90** |
| | | control | 10.21 | 9.91 | 9.63 | 9.54 | 9.4 | **7.93** |
| 9. | Set I | test | 14.05 | 13.90 | 13.54 | 13.72 | 13.20 | **6.04** |
| | | control | 14.05 | 14.01 | 13.9 | 13.46 | 13.13 | **6.54** |
| | Set II | test | 12.26 | 11.85 | 11.56 | 11.32 | 11.15 | **9.05** |
| | | control | 12.26 | 11.91 | 11.78 | 11.52 | 11.39 | **7.09** |
| | Set III | test | 13.78 | 13.20 | 12.90 | 12.85 | 12.72 | **7.69** |
| | | control | 13.78 | 13.32 | 12.96 | 12.85 | 12.69 | **7.91** |

Table 1: The platelet count of samples (after incubation with Hz pigment) and respective controls (after incubation with distilled water) after different time of incubation.




Graph 2: The reduction in platelet count for nine patient samples (1S to 9S) and their respective controls (1C to 9C).

## Discussion

Malaria is associated with varying degrees of thrombocytopenia. The mechanism is still not clear. In our experiment, based on the observation of Haemozoin pigment in platelet clumps, Hz isolate was incubated with platelet concentrate. Significant reduction in platelet count in 7 out of 9 samples were found. The characterization of isolated Hz pigment was executed using different photo physical tools. Graph 1 (a) shows the visible light absorption of the Hz pigment and typical absorption peak of Hz at 655 nm. Previous studies have shown that the crystalline malarial pigment Hz shows a typical absorption peak at 655 nm.[12,13] The structural conformation was further verified by using infra red (ATR-IR) spectroscopy. The FTIR peaks of the isolated Hz pigment were found at 1634 cm-1 and 1218 cm-1as shown in Graph 2 (b). The IR spectrum shows the corresponding peaks of Hz at 1634 cm-1 and 1218 cm-1 and is related to C=O and C-O stretching vibration of carboxylate group that is coordinated with the Fe (III) center, respectively. The FTIR peaks found in our experiment is identical to that found by previous researchers.[14,15] Thus, the characterization of Hz using UV-visible spectroscopy and FTIR analysis shows that Hz was isolated properly and was used for further studies.

Platelets phagocytosing latex particles, polymorphs and monocytes have been seen.[16-18] We propose that Hz pigment maybe phagocytosed using a similar mechanism. On schizogony free haemoglobin, Hz pigment and merozoites are released into the circulation. Monocytes, polymorphs, macrophages, endothelial cells and dendritic cells phagocytose the Hz.[16,17]

Once phagocytosed, the Hz pigment may cause platelet destruction by the release of reactive oxygen species. Prato et al., also observed the release of free oxygen species, in an ex-vivo experiment, where reactive oxygen species were found in monocytes.[18] High levels of immunoglobulin capable of binding infected platelets have been seen in thrombocytopenic patients. The infected platelets are sequestered and removed by the spleen.[19-23] Other studies have shown that platelets are capable

of phagocytosis and degranulation.[16,24] Ultrastructural study of platelets showed centralization of dense granules, glycogen depletion and formation of pseudopods and platelet clumps, leading to activation of platelets, which may lead to its destruction.[21] Platelets in patients suffering with acute malaria are highly sensitive to Adenosine DiPhosphate (ADP) addition in vitro,[25] and it is believed that ADP release following haemolysis could contribute to higher platelet aggregation.[26] Platelets engulfing malarial parasite and subsequently getting destroyed has also been proposed by other studies.[16, 19, 23, 24, 26]

Other investigators have proposed the invasion of platelets by the parasites themselves. Fajardo & Tallent found a P.vivax trophozoite in a platelet.[23] In another study, plasmodia were found in platelets of 2 patients with naturally acquired, acute P. vivax infections. Even after clinical cure in one of these patients, the parasite was seen in a platelet. In mice infected with P berghei, 50% of them showed parasites within platelets. In all these studies, the megakaryocytes seem to be non-parasitized and hence the proposal that the parasitization occurs in the peripheral circulation. Parasitization of the platelets may be a cause of malarial thrombocytopenia.[23, 27]

The outcome of our present study showed that the presence of Hz significantly decreased platelet count. Our studies indicated that phagocytosis of Hz pigment by platelets may contribute to the thrombocytopenia seen in malaria. Future studies exploring the mechanism of platelet destruction mediated by Hz needs to be performed.

## References


1. National Vector Borne Disease Control Programme. Directorate General of Health Services Ministry of Health & Family Welfare, Govt. of India. Trends of malaria. 2001-2013.
2. World Malaria Report, Published by World Health organization. 2018, ISBN: 978 92 4 156565 3.
3. Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. The structure of malaria pigment beta-haematin. Nature. 2000;404(6775): 307–310.
4. Olivier M, Van Den Ham K, Shio MT, Kassa FA, Fougeray S. Malarial pigment hemozoin and the innate inflammatory response. Front Immunol. 2014;5:25.
5. Sullivan Jr DJ. Hemozoin: a biocrystal synthesized during the degradation of hemoglobin. Biopolymers Online. 2005;9:129-163.
6. Kodandapani Y, Srinivas P, Ramya P, Venu K. Association of leucocytosis and hemozoin pigment in leucocytes with disease severity of malaria in children. IOSR-J. Dent. Med. Sci. 2015;14(11):44-51.
7. Urban BC, Hien TT, Day NP, Phu NH, Roberts R, Pongponratn E et al. Fatal Plasmodium falciparum malaria causes specific patterns of splenic architectural disorganization. Infect Immun. 2005; 73(4): 1986–94.
8. Wickramasinghe SN, Abdalla SH. Blood and bone marrow changes in malaria. Baillieres Best Pract. Res. Clin. Haematol. 2000;13(2): 277–99.
9. Coban C, Ishii KJ, Sullivan DJ, Kumar N. Purified malaria pigment (hemozoin) enhances dendritic cell maturation and modulates the isotype of antibodies induced by a DNA vaccine. Infect. Immun. 2002;70(7):3939-43.
10. Sultan S, Irfan SM. Intraleukocytic hemozoin pigments in complicated Plasmodium falciparum cerebral malaria. Blood Res. 2015;50(2):72.
11. Girigoswami K, Viswanathan M, Murugesan R, Girigoswami A. Studies on polymer-coated zinc oxide nanoparticles: UV-blocking efficacy and in vivo toxicity. Mater.Sci. Eng. C. Mater Biol. Appl. 2015; 56:501-10.
12. Frosch T, Koncarevic S, Becker K, Popp J. Morphology-sensitive Raman modes of the malaria pigment hemozoin. Analyst. 2009;134(6):1126-32.
13. Serebrennikova YM, Patel J, Garcia-Rubio LH. Interpretation of the ultraviolet-visible spectra of malaria parasite Plasmodium falciparum. Applied optics. 2010;49(2):180-8.
14. Pershina AG, Saltykova IV, Ivanov VV, Perina EA, Demin AM, Shevelev OB et al. Hemozoin "knobs" in Opisthorchisfelineus infected liver. Parasit. Vectors. 2015;8(1):459.
15. Slater AF, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A et al. An iron-carboxylate bond links the heme units of malaria pigment. Proc.Natl.Acad.Sci. 1991;88(2):325-9.
16. Lewis JC, Maldonado JE. Phagocytosis in human platelets: localization of acid phosphatase-positive phagosomes following latex uptake. Blood. 1976;47(5):833-40.
17. Male R, Vannier WE, Baldeschwieler J.D. Phagocytosis of liposomes by human platelets. Proc. Natl. Acad. Sci. 1992;89(19):9191-5.
18. Prato M, Giribaldi G, Polimeni M, Gallo V, Arese P. Phagocytosis of hemozoin enhances matrix metalloproteinase-9 activity and TNF-alpha production in human monocytes: role of matrix metalloproteinases in the pathogenesis of falciparum malaria. J. Immunol. 2005; 175(10):6436-42.
19. Kotepui M, PhunphuechB, Phiwklam N, Chupeerach C, DuangmanoS.Effect of malarial infection on haematological parameters in population near Thailand-Myanmar border. Malar J. 2014; 13: 218.

20. Ladhani S, Lowe B, Cole AO, Kowuondo K, Newton CR. Changes in white blood cells and platelets in children with falciparum malaria: relationship to disease outcome. Br J Haematol. 2002; 119(3):839-47.

21. Mohanty D, Marwaha N, Ghosh K, Sharma S, Garewal G, Shah S, Devi S, Das KC. Functional and ultrastructural changes of platelets in malarial infection. Trans R Soc Trop Med Hyg. 1988; 82(3):369-75.

22. Skudowitz RB, Katz J, Lurie A, Levin J, Metz J. Mechanisms of thrombocytopenia in malignant tertian malaria. Br Med J. 1973;2(5865):515-8.

23. Fajardo LF, Tallent C. Malarial parasites within human platelets. J Am Med Assoc. 1974; 229:1205–9.

24. Movat HZ, Weiser WJ, Glynn MF, Mustard JF. Platelet phagocytosis and aggregation. The J. Cell Biol. 1965; 27(3):531-43.

25. Essien, E. M. &Ebhota, M. I. Platelet hypersensitivity in acute malaria (Plasmodium falciparum) infection in man. Thrombosis and Haemostasis. 1981;46,:547-9.

26. Bashawri LA, Mandil AA, Bahnassy AA, Ahmed MA. Malaria: hematological aspects. Ann. Saudi Med. 2002; 22(5-6):372-376.

27. Lacerda MVG, Mourão MPG, Coelho HCC, Santos JB. Thrombocytopenia in malaria: who cares? MemInstOswaldo Cruz, Rio de Janeiro, 2011;106(I): 52-63.