EVALUATING *PLASMODIUM BERGHEI* INFECTION INFLUENCE AND ASIATIC ACID ADMINISTRATION EFFICACY IN SPRAGUE DAWLEY MALE RATS: EFFECTS ON PARASITAMIA, GLUCOSE HOMEOSTASIS AND RENAL ELECTROLYTE HANDLING

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(Posthumously)

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Preface:

The continued malaria disease scourge on the human population, the resources spent on control measures against infection, current antimalarial multi-drug resistance and the inadequate anti-disease remedies for malaria pathophysiology has motivated the conception of this work. By observing the influence of Asiatic acid on other conditions similar to malaria, differing only in aetiological agent, we hypothesized that the epitome of malaria treatment should lie in amelioration of the disease pathophysiology. Our results, in part, vindicate our conjecture that Asiatic acid when administered per oral or applied as an Asiatic acid-amidated pectin hydrogel patch before or after infection with *Plasmodium berghei* in Sprague Dawley male rats supresses parasitaemia and ameliorated malaria pathophysiology. However, more work still needs to be done.

PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

Doctor of Philosophy (PhD) in Human Physiology 2016

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.

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THESIS DECLARATION

I, Mr Greanious Alfred Mavondo, declare as follows:

1. that the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. that my contribution to the project was as follows: a) I conceived and designed the concepts of the research project; b) Designed the research protocol; c) Designed the application and applied for the ethical clearance certificate for animal research work; d) Was granted the ethical clearance by the animal ethics committee of the University of KwaZulu Natal South Africa; e) Carried out the experiments, obtained, analysed data and interpreted the data; f) Prepared the manuscripts, submitted them for publication and wrote final thesis;

3. that the contributions of others to the project were as follows:

a) Dr Mkhwananzi, B.N. gave a critical appraisal and editorial review of the work

b. Dr Mabandla M.V. supervised part of the work and approved the project for submission to the examiners

c. Professor Musabayane, C.T. gave guidance during inception of the work and proposal writing, gave guidance on protocol development, introduction, methods and results of the work, gave guidance on the ethical clearance application, problem statement writing and supervised a greater part of the animal experiments.

4. that the following articles and manuscript are part of the work formulating the current thesis:

a. Chapter 2 Part I Article 1: G. A. Mavondo, B. N. Mkhwananzi, M. V. Mabandla and C. T. Musabayane.ˆ Asiatic acid influences parasitaemia reduction and ameliorates malaria anaemia in P. berghei infected Sprague–Dawley male rats BMC Complementary and Alternative Medicine (2016) 16:357 DOI 10.1186/s12906-016-1338-z

b. Chapter 2 Part II Article 2: Alfred Mavondo, Blessing Nkazimulo Mkhwananzi and Musa Vuyisile Mabandla. Pre-infection administration of asiatic acid retards parasitaemia induction in Plasmodium berghei murine malaria infected Sprague-Dawley rats 2 Greanious Malar J (2016) 15:226 DOI 10.1186/s12936-016-1278-6

c. Chapter 2 Part III Article 3: Greanious Alfred Alfred Mavondo, Musabayane Cephas Tagumirwa. Asiatic acid-pectin hydrogel matrix patch transdermal delivery system influences parasitaemia suppression and inflammation reduction in P. berghei murine malaria infected

Sprague-Dawley rats. Asian Pacific Journal of Tropical Medicine 2016; 9(under printing) www.elsevier.com/locate/apjtm

d. Chapter 3 Article 4: Mavondo Greanious Alfred,* Mkhwananzi Blessing Nkazimulo, Mabandla Musa Vuyisile, Musabayane Cephas Tagumirwa. Asiatic acid influences glucose homeostasis in P. berghei murine malaria infected Sprague-Dawley rats. Afr J Tradit Complement Altern Med. (2016) 13(5):91-101 Doi:10.21010/ajtcam.v13n5.13

e. Chapter 4 Article 5 Greanious Alfred Mavondo, Musa Vuyisile Mabandla, Cephas Tagumirwa Musabayane. Transdermal drug delivery of Asiatic acid influences renal function and electrolyte handling in Plasmodium berghei-infected Sprague-Dawley male rats

4. Student: Mavondo Greanious Alfred **Signature:** 

Date: 16 September 2016

5. Supervisor: Dr MV Mabandla **Signature:** 

Date: 16 September 2016

Dedication

I dedicate this work to my GOD who made it possible for me to embark on this study, carry out this work in spite of voices to the contrary, complete this work, clothed me with HIS grace that has been always more than sufficient for me and above all given me a HOME GUARD keeping the fort from intrusion in my years of absence. **Prof Cephas Tagumirwa Musabayane**, you live on though you are no more. I will NEVER disappoint you. I salute you. Your purpose and assignment for me is clear and I will carry it through. **My Wife Joy Govero and Director of Imagegate Holdings,** you are truly special, courageous, beautiful, spiritual, temperate, resolute, loving, amorous, resourceful, aptly blessed and tender hearted. You became the reason why I pushed on despite the torrid time I received. The nights alone were long but the thought of you waiting made me endure it all. The days were hard but the imagination of you saying well done brought a smile on my face and could face another day up and down the physical hills of UKZN and the academic steep ascents to the lofty top. You are the world to me. I love you more than life itself. **Greanaldean Sheunesu, Director of Imagegate Mining**, and the strength of my youth and the gentle giant of a man you have become. You put the wind in my wings to fly. I always look forward to the bear hug you gave me each time I came home and the genuine "I love you daddy" coming so easily form you made me to work harder so that I could be home with you. Your brilliant scientific mind enthrals me and seeing your work make me want to be an example for you to follow after. I know you will do greater than me for you have become several times more what I was at the same time point you are already. Greatness you create and Greatness is your name. **Greanaldeanine Mufarowashe, Director of Imagegate Farming Technology**, my Mother. Your beauty and heart-rending smile has been a push in my steps when weariness get the best of me. The bridge between the two mountains, strong and relentless ever inspiring and inspired to achieve greatness. You withstood it all and emerged golden, charming and attractive like never before. Thinking of you and the multi-talents you have, made me want to make the time I was away from you count. For you, I have gone these extra miles so that you can see that you have it all laid out and up for you. I love you to bits. **Greandenver Anesuishe, Director of Imagegate Transport Technology,** the gate keeper and ultimate goal getter. To you nothing is impossible. The man of faith. The one whom God hears when he calls. I am overly delighted with the success you have become, all around. Your precision, accuracy, dedication and intuition made me want to come home and be equipped for the next stint during my studies. I could stand the time away knowing that you will be there when I come home successfully completed my course and you to furnish me with the many exploits you will have achieved. You will always be better than me and so this bar I set is a walk in the park for you. **I love you my FAMILY my World and I love you my GOD. Long live the gargantuan Elephant from Dzimbabwe, Prof CTM.**

Acknowledgements:

Professor Musabayane Cephas Tagumirwa will remain engrained in my mind and thoughts as the man who helped shape my destiny by his incisive, timely and insightful guidance of my research work. Professor Chimbari Moses, indeed "everything is possible with Moses", makandigonera, rambai makadaro. Makandiitira musa nengoni. Ndinofara nokupembera nokuda kwenyu. Ngazivisa guma neni chete. Asi saMoses, nunurai vakawanga vanouya neronga renyu. Professor Mukaratirwa Samson, never dreamt our meeting in Masvingo could stretch to this end, but your impact is unmatched. National University of Science and Technology, Faculty of Medicine Deans (previous: Dr. N. Ndiweni; current: Dr R. Gwini) your support has always been outstanding and my gratitude knows no boundary to you. I am highly indebted to Professor Fanie R Van Heerden for the initial Asiatic acid that allowed us to embark on this project. My thanks goes to the Discipline of Physiology Endocrinology Group. Mr. M. Luvuno is highly appreciated for the tremendous contributions in animal and laboratory experiments. Though you are part of the authorship, Dr B.N. Mkhwananzi, it was serendipity and providence that you came in at the time point you did- forever together. Special appreciation goes to Ms Sibiya HP and Mbatha B for the invaluable inputs into the preliminary animal handling work and procedures you walked me through and for the continued efforts to build the Malaria Group conceived by **Professor Cephas Tagumirwa Musabayane**. Last but not least, I would like to appreciate **Dr. M.V. Mabandla** (Professor) for his sterling contribution towards the successful finalization of this work after the passing on of Prof. May the Good Lord, who is my God, sufficiently provide for **ALL** of your wants and **ALL** of your needs.

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List of acronyms

AA Asiatic acid

AC	absolute control
ADH	antidiuretic hormone
ALD	aldosterone
ANOVA	analysis of variance
AVP	arginine vasopressin
BRU	Biomedical Research Unit
CA	*Centella asiatica*
CHQ	chloroquine
CRP	C-reactive protein
DMSO	dimethyl sulphoxide
ELISA	enzyme linked immunosorbent assay
GPx	glutathione peroxidase
IC	infected control
ip	intraperitoneal
LDH	lactate dehydrogenase
OGTR	oral glucose tolerance response
MDA	malonyldialdehyde
NIC	non-infected treated control
npRBC's	Non-parasitized red blood cells
po	per oral
PbANKA	*Plasmodium berghei* Antwerp-Kasapa
pRBC's	parasitized red blood cells
RI	reference interval
TNF-α	tumour necrosis factor-α
SD	Sprague Dawley
SEM	standard error of the mean
SOD	superoxide dismutase
UKZN	University of KwaZulu Natal

Annexes

Abstract

Introduction: Five human-infecting *Plasmodium* species orchestrate varied pathophysiology culminating in malaria. Despite being treatable, malaria has high mortality and morbidity in pregnant women and children under five years. Current treatment is hampered by drug resistance, toxicity and failure to address malaria induced pathology directly. Asiatic acid has antioxidant, pro-oxidant, antihyperglycaemiac renoprotective qualities which may be anti-disease properties in malaria. Very little is currently known or reported about these asiatic acid anti-disease perspectives in malaria and therefore require further investigations. The aim of this study was to investigate the influence of asiatic acid administration and malaria infection on parasitaemia suppression, glucose homeostasis, renal function and electrolyte handling in *Plasmodium berghei*-infected Sprague Dawley male rats.

Methods: Three sub-chronic studies and one acute study were conducted. The sub-chronic protocol involved per-oral pre- and post-infection administration of asiatic acid (5, 10, 20 mg/kg) and post-infection transdermal drug delivery system application of asiatic acid (5, 10, 20 mg/kg-amidated hydrogel matrix pectin patch). Acute studies included post-infection oral glucose tolerance response to asiatic acid. Influence of asiatic acid on %parasitaemia changes, physicochemical changes, immunological effects of malaria, haematological results of malaria, antioxidant capacity, glucose homeostasis, renal function and renal electrolyte handling were investigated.

Results: Asiatic acid suppressed parasitaemia to varying extents with asiatic acid 10mg/kg and 5mg/kg emerging as the most efficacious amongst the three doses by per oral administration and transdermal delivery drug delivery system, respectively. Malaria suppression occurred in both pre-infection and post-infection administration of asiatic acid. Asiatic acid preserved physicochemical parameters, ameliorated haematological effects of malaria, influenced immunological effects of malaria, modulated glucose homeostasis in malaria, protected renal function and electrolyte handling in malaria. Asiatic acid improved glucose tolerance response in acute malarial states. Antioxidant status was also improved in malaria.

Conclusions: Asiatic acid displayed chemoprophylactic and chemotherapeutic effects in malaria. Asiatic acid has both glucose homeostatic and renoprotective properties in malaria. The antioxidant characteristics of the amphiphilic asiatic acid seem to exert anti-disease and anti-parasitic effects in malaria. Asiatic acid may be used as an antimalarial compound with ability to ameliorate malaria associated pathophysiology.

Chapter 1:
Introduction

1.0 Introduction:

The orientation to the thesis and the study as a whole is done in this introduction. The thesis approach and lay out are also described here. The problem statement is given showing why it is necessary to conduct this study as well as the significance of the study. A concise literature review of the study follows and will be elaborated on in subsequent chapters of the thesis. Included are the introduction as well are the study aims and objectives. The experimental design showing the broad protocol layout forms part of this introduction. Statistical analysis, brief methodology and expected results will append this section.

1.1 Thesis approach and layout:

The thesis is presented in an article format with key data prepared as published articles in scientific journals. Relevant materials not included in the articles are presented in the annexes section. Manuscripts that have been presented for publication will be presented in the format of the particular journal. There are generally five chapters in all excluding the preliminary pages. In the preliminary pages are the title page, the preface, the acknowledgements, the dedication, the table

of contents, list of figures and tables, followed by acronyms and the abstract coming in last. Chapter 1 is this introduction, chapter 2 is made up of three parts as follows:

- Chapter 2 Part I: Study comprises of an article published in the BioMed Central Complementary and Alternative Medicine. Mavondo et al. BMC Complementary and Alternative Medicine (2016) 16:357 DOI 10.1186/s12906-016-1338-z
- Chapter 2 Part II: This an article that has been published by the BioMed Central. Malaria Journal. Mavondo et al. Malar J (2016) 15:226 DOI 10.1186/s12936-016-1278-6
- Chapter 2 Part III: Article published by the Asia Pacific Journal of Traditional Medicine Mavondo et al, Asian Pacific Journal of Tropical Medicine (2016) 9(12): 1172–1180 http://dx.doi.org/10.1016/j.apjtm.2016.10.008
- Chapter 3: An article that has been published by the African Journal of Traditional, Complementary and Alternative Medicine (AJTCAM). Mavondo Afr J Tradit Complement Altern Med. (2016) 13(5):91-101 Doi:10.21010/ajtcam.v13n5.13
- Chapter 4: Full manuscript submitted for publication in African Journal of Traditional, Complementary and Alternative Medicine (AJTCAM). Manuscript Number:AJTCAM-D-16-00528
- Chapter 5: The synthesis and summary of the whole study is presented here together with conclusion and recommendations.
- Findings in not included in the articles will also be included in the addendum section.

The manuscripts that have been presented to journals for review and publication will be included and will be part of the chapter although not running in line with the pagination system. Also included in between chapters will be bridging statements indicating how the chapters flow into one another, an adjunct of sorts to the readability of the thesis. It should be noted at this stage that the study was a multidiscipline approach encompassing human physiology, malariology,

phytomedicinal antimalarial compounds, haematology and chemical pathology showing the extent of malaria pathophysiology.

1.2 Statement of the problem:

Notably, the human malaria disease is preventable and treatable. How and why we have not controlled the disease, let alone eradicate it, defies logic. The five *Plasmodium* species (*P. falciparum, P. vivax, P. malariae, P. ovale, P. knowelsi*) that infect humans are permanently superimposed between the human secondary host and the *Anopheles* mosquito (primary host and vector) irking out an indelible evolutionary trajectory in human history [1-3]. An estimated 207 million infections and 655 000 malaria deaths were recorded in 2012 constituting and overall mortality reduction of 45% and 51% in all age groups and children ≤ 5 years of age, respectively[4]. Current malaria treatment appears to be either "anti-parasitic" (clears parasitaemia) or "anti-infection" (prevents infection) with a few showing immunoregulatory effects are emerging. A limited number of antimalarial drugs are "anti-disease" (ameliorate malaria pathophysiology) and also the advent of multidrug resistance besetting the main line drugs [5] makes new antimalarial drugs discovery imperative [6]. Just as humans do when malaria infected, mosquitos depend on plant material for their survival when accosted by the *Plasmodium* parasite [7, 8] showing the central role phytomedicinal compounds may play in the malaria matrix mix. Phytochemicals hold a possible panacea to the malaria scourge having coevolved with the human being, the mosquito and the *Plasmodium* parasite [9-11]. The anti-parasitic drugs quinine and artemisinin are plant products [12-14]. Anecdotal evidence exist on the successful use of ethnobotanical preparations in malaria treatment [15,16]. In more recent times, a veritable example of phytochemical antimalarial is curcumin, a natural polyphenol, which has been reported to modulate cerebral malaria (CM) as well as suppress parasitaemia [17, 18].

Asiatic acid (AA) with well-known, varied biological [19-21] and medicinal properties [22, 23] in diseases that have similar presentation to malaria is here reported as an antimalarial agent.

Having observed that AA has antioxidant, pro-oxidant, [1] anti-inflammatory properties amongst others and modulates haemodynamic and metabolic alterations [24], we hypothesized that AA may be both an antimalarial and malarial pathophysiology elixir. Therefore, the aim of this study was to evaluate the influence of murine malaria infection with *P. berghei* and AA administration by three different delivery modes in young (90-120 g) Sprague Dawley male rats looking closely at parasitaemia suppression, glucose homeostasis, renal electrolyte handling and renal function.

1.3 Literature review

1.3.1 Malaria pathophysiology and Asiatic acid possible interventions:

Patient morbidity and mortality, key outcomes of malaria disease, are not the prerogative of the parasite *per se* [25] but parasite infection, parasitic effects and host physiological responses to the two constitutes malaria disease with the non-parasitic events being the major cause of mortality rather than the parasitic burden [17].

Pathophysiology of malaria is manifested by periodicity-exhibited paroxysms with phenotypic presentations of: i) innate immunity modulated inflammation [26], ii) severe malaria haemolytic anaemia [27, 28], iii) acute renal failure [29, 30], iv) anorexia leading to cachexia [31], v) cerebral malaria [CM], vi) severe hypoglycaemia [32-34], vii) hypoxia associated oxidative stress [6-7,

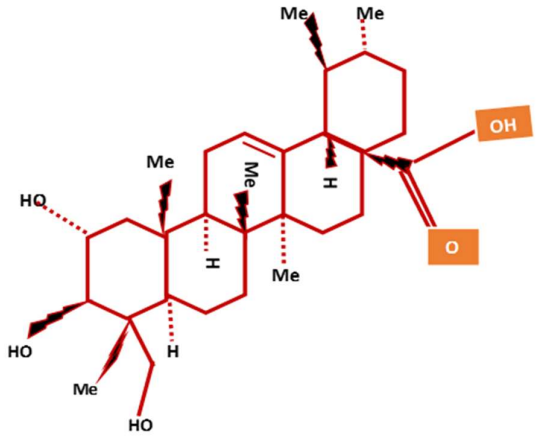

Figure 1: Chemical structure of Asiatic acid. Formula $C_{30}H_{48}C_5$. MW: 488.69912 (g/mole). Redox characteristics: Hydrogen bond donor (HBD) 7.1; Hydrogen bond acceptor (HBA) 4.176. [1]

10, 33-35]. Different people may manifest different syndromes depending on the affected organs, constitutively expressed proteins, receptors and ligands resulting in differential disease presentations and outcomes. There exists a high probability of end-stage multi-organ failure development in untreated non-immune cases. Anti-infection, anti-parasitic, anti-transmission (without a holistic anti-disease approach) may result in treatment failure as host related pathophysiology, the cause of disease fatalities, may remain unresolved. The challenge is finding a cure with anti-disease properties because even after successful treatment cytoadherence may persist with hypoxia and tissue damage [33-36].

One of the aetiology of malaria pathophysiology is the elicitation of glycosylphosphatidylinositol (GPI) with subsequent aberrant immune excitation and inflammatory response where tumour necrotic factor-α (TNF-α) is pivotally involved [37-39]. The inhibition of TNF-α by AA was reported [22] by Huang et al (2011) but its interaction with GPI in malaria remains to be investigated. Whether AA will interact with GPI directly or through inhibition of its downstream effectors is an interesting avenue that may be pursued. The effect of immunological and inflammatory activation amplifies oxidative stress in malaria with subsequent aberrant signalling processes coming into play. Interestingly, AA as other phytochemicals in the plant kingdom, may have coevolved with both the human host and the *Plasmodium* parasite. The phytochemical has been reported to have anti-oxidant capacity, anti-inflammatory and pro-oxidant capacities [6], facets that may combat both the parasitic and the pathophysiology of malaria. It is instructive to mention that the pro-oxidant effects of AA are being discussed here for the very first time in terms of their involvement in inflammatory conditions and malaria, specifically. The antioxidant capacity to attenuate oxidative stress has been the focus all along. However, AA by its capacity to donate hydrogen bond is by definition an oxidative agent (Figure 1) [1]. Generation of oxidative stress (OS) may affect ion channel transport systems as well Na^+/K^+ ATPase pump [40-42] with subsequent cellular functional changes ranging from RBC's reduced deformability leading to haemolysis, Na^+ wasting in the renal tubules, derangement of glucose homeostasis and general metabolic deficits. Vascular bed attenuation through nitric oxide (NO) uncontrolled

synthesis results in increased vascular adhesiveness and permeability with fluid extravasation and hypovolaemia [43], depletion of NO by free haemoglobin that may lead to various conditions including vasoconstriction, impaired peripheral perfusion, hypoxia, pulmonary oedema, renal dysfunction, increased cerebral and intracranial pressure, congestive heart failure, shock, coma and death [44].While AA has been shown to modulate inflammation in other conditions, its influence on the parasite, immunological response and malarial inflammatory processes is not known. Also, equally opaque to scientific probing is how AA will influence hypoglycaemia induced by the parasite as the phytochemical's anti-hyperglycaemic properties are known. The shades of grey in scientific knowledge also extends to AA's influence in malaria on renal electrolyte handling and renal function [45], claims of the diuretic effect of *Centella asiatic* notwithstanding [46]. *Centella asiatica* (parent plant for AA) has been used for various medicinal purposes including as a nerve tonic, anxiolytic agent, improving learning and memory [47] as well as in research on neuroprotection [48], but in the light of malaria which causes cerebral malaria the influence of AA on the latter needs to be explored.

The route of drug administration has an effect on the bioavailability, pharmacodynamics and pharmacokinetics of the drug [49]. AA concentration in beagle dog (12 kg) plasma (termed low plasma AA concentration) was (C_{max} 0.74 μg/mL) at 2.7h (T_{max}) after a single oral *Centella asiatica* extract dose with $T_{1/2}$ at 4.29 hours and another residual peak appearing 8 hours later [47, 50] which portrayed a phytochemical with anything but poor bioavailability. However, these findings make it imperative to determine further what this entails with reference to parasitaemia, glucose homeostasis, renal function alterations in rats administered with oral AA or as a once-off topical application in murine malaria. There are no reports that show how AA influences malaria outcomes when administered post-infection (chemotherapeutic) or pre-infection (chemoprophylaxis). The delayed secondary maximum concentration of AA at 8 hours observed by *Zheng et al* (2010) [47] may indicate slow release and long-acting effects of the amphiphilic AA necessary for chemoprophylaxis drug development deserving further exploration. Possessing both antioxidant and pro-oxidant properties, AA is both a hydrogen bond donor (HBD 7.1) and a

hydrogen bond acceptor (HBA 4.176) [1] [Figure 1], making it possible to have pleiotropic influences in malaria where aberrant ROS signalling, pro-inflammatory and anti-inflammatory immunomodulation are key determinants of the malaise seen in the disease process. The influence of AA on food and water intake in malaria or any other disease remains to be explored with the hormonal effect of insulin and glucagon equally unexplored when the phytochemical is administered. There is of necessity a need to investigate the possible interactions in malaria infection and concurrent administration of AA.

1.4 Study concept:

From the biological and medicinal knowledge of AA in other diseases similar to malaria and the missing links between the phytochemical AA and its influence in malaria, we embarked on the search to determine how infection with *P. berghei* will be influenced by the administration of AA in distinct and chronological time-separated studies. The influence of differing study designs and settings, differing chemical matrix, different doses of administration, using varied routes of administration on selected physiological and metabolic parameters were conducted in young (6 weeks weighing 90-120 g) male Sprague Dawley rats. The different chemical matrices included administration of an organic-aqueous suspension per oral and pectin hydrogel-AA matrix transdermal (the patch) delivery. The different settings included infecting the animals and administering AA after 7 days (chemotherapeutic) and administering the AA 48 hours prior to infection (chemoprophylaxis). The different doses were 5, 10, 20 mg/kg AA prepared as an aqueous suspension or as a patch. The differing routes of administration were per oral gavage needle transgastric delivery of aqueous-organic suspension of AA and transdermal drug delivery (TDDS) of AA (AA-amidated hydrogel matrix patch).

1.5 Significance of the Study:

Malaria elimination drive by the Global Health Group and WHO initiatives has been scaled up recently. However, antimalarial multidrug resistance evolution, high morbidity and mortality from malaria in pregnant women and children under five years of age are still at unsustainable and unacceptable rates. Therefore, it becomes important to continuously seek possible ways of combating malaria. The study seeks to increase the body of knowledge on malaria pathophysiology and malaria disease management using triterpene compounds which have been reported to have possible anti-disease properties. Drug delivery in malaria management is mainly by the oral route, intramuscular or intravascular injection. These methods are most likely to cause patient non-compliance due to the bitter taste of the drugs or pain associated with injectable, respectively. Oral AA administration has been shown to result in low bioavailability but here we show the possibility of phytochemical dissolution in organic-aqueous environment and its pharmacodynamics in the management of malaria, which is a novel approach to malaria treatment. Equally important and novel is the TDDS application which is an AA administration method invented in our laboratory with a possible high patient compliance and commercial impact factor in the future when fully developed. The reduction in the AA dose, dosage frequency and anti-disease paradigms that are expected to spin off from this study are envisaged as futuristic interventions in the fight against malaria. Prophylaxis anti-malaria effects of oral AA, which may be extended to TDDS, are novel findings that hold a lot of potential in drug discovery and malaria treatment. Moreover, the study lands itself in the path of the South African government's National Development Plan (NDP) Vision 2030 which advocates for the leverage of science and technology to solve some of the biggest challenges in heath, as it seeks to break new frontiers in fighting malaria disease. Finally, AA interaction with the malaria parasite in an animal model has the likelihood of providing information on the pathophysiology of malaria such as hypoglycaemia, acute renal injury and electrolyte derangements, which is difficult to obtain, if not impossible to study effectively in a human being for ethical reasons.

1.6 Study Objectives:

Overarching aim of this study was to investigate the influence of *Plasmodium berghei*-infection and Asiatic acid administration per oral (po) and by TDDS in young (6 weeks old weighing 90-120 g) male Sprague Dawley rats (anti-parasitic). In the process, the most effective route of AA administration, shown by the lowest dose with the highest efficacy against malaria, was investigated. The anti-disease effect of AA on selected malaria pathophysiology parameters was also tested.

1.6.1 Study objectives:

1.6.1.2 Determined the influence of AA on parasitaemia when delivered by oral route in pre-infection and post-infection treated rats

1.6.1.3 Established the effects of transdermal drug delivery system administration of AA on parasitaemia

1.6.1.4 Determined the influence of AA on inflammation in malaria

1.6.1.5 Demonstrated the influence of AA on severe malaria anaemia

1.6.1.6 Measured the effect of AA on oxidative stress in malaria

1.6.1.7 Established the influence of AA on glucose homeostasis and renal function with electrolyte handling

1.7 Experimental design: Animals were randomly assigned to groups (n=6 per group) of non-infected non-treated, non-infected but treated, infected non-treated, infected treated with either asiatic acid (5, 10, 20 mg/kg) by either oral or transdermal drug delivery system, infected treated

with chloroquine (30 mg/kg) by either oral or transdermal drug delivery system with various metabolic function examined.

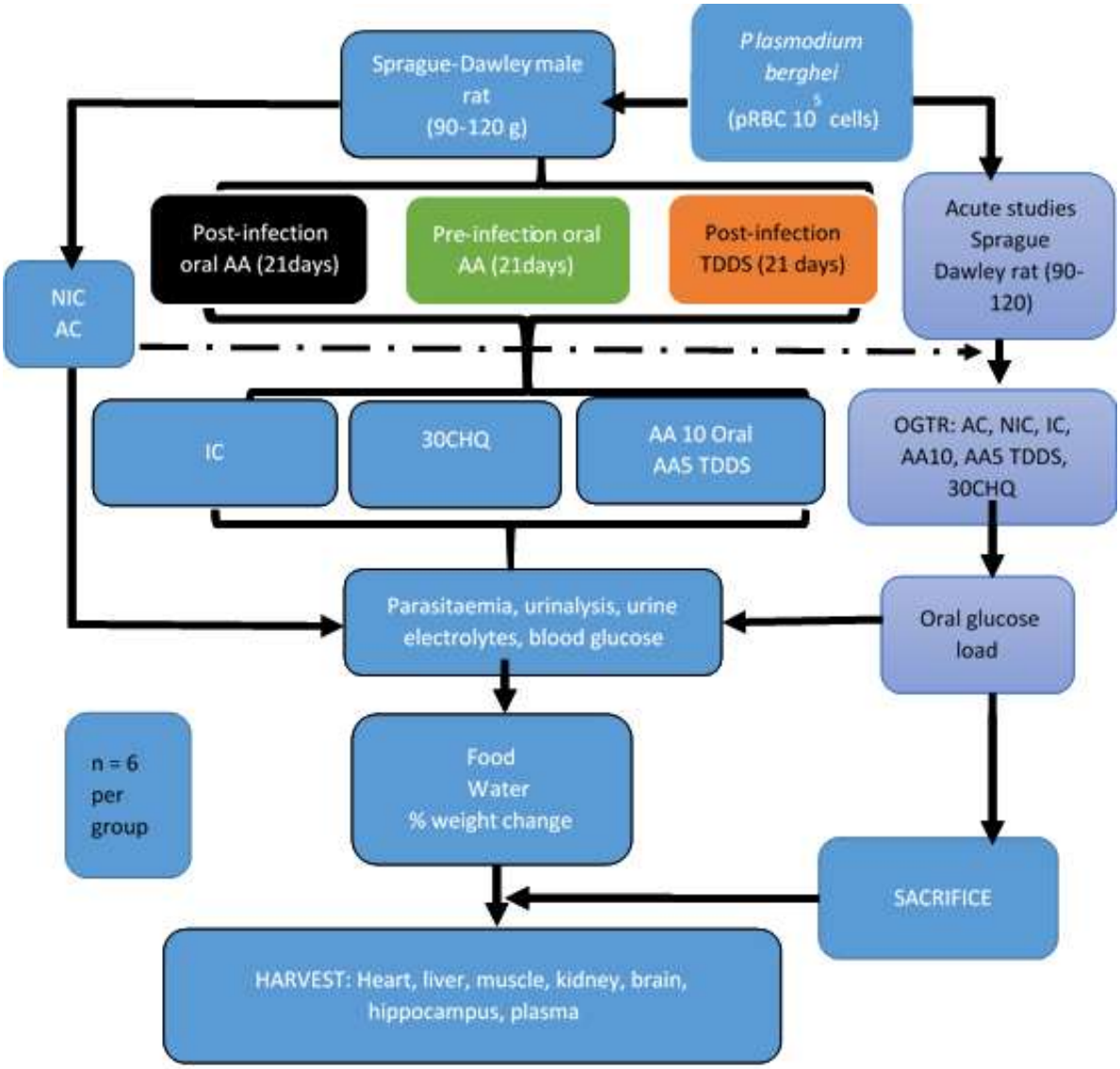

Figure 2: Research design flow diagram for different protocols used in study **Key:** AA5-Asiatic acid 5 mg/kg; AA10-Asiatic acid 10mg/kg; 20AA: 20 mg/kg Asiatic acid 30CHQ-chloroquine diphosphate 30 mg/kg; pRBC's-parasitized red blood cell; AA (21 days)-Asiatic acid sub-chronic

21 day study Asiatic acid; OGTR-oral glucose tolerance response. AC: absolute control; NIC: non-infected treated control; TDDS: transdermal drug delivery system.

1.8 Statistical analysis:

Data are presented as the means ± standard error of mean (SEM). Overall statistical comparisons between the control means and experimental groups (n= 6 per group) were performed with GraphPad Prism Software version 5.00, (GraphPad Prism Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer post hoc multiple comparison test. A value of $p < 0.05$ was considered significant.

1.9 Methodology briefs:

All materials used in the study were of analytical grade purchased from reputable suppliers. Four experimental animal studies were carried out: one acute study and three sub-chronic studies.

1.9.1 Acute studies: Animals were divided into none-infected absolute control (AC), none-infected treated (NIC), infected none-treated (IC), infected treated with either AA (AA5, 10, 20 mg/kg) or chloroquine (30 mg/kg) (30CHQ) which served as the positive control. The acute studies were conducted seven days after infection when parasitaemia was at patent or stable state malaria (15-20%). After a 16 hour of fasting, an oral glucose tolerance response was carried out.

1.9.2 Sub-chronic studies: Three consecutive 21-day sub-chronic studies were carried out over a period of time. AA concoctions were either aqueous-organic suspensions or AA-patch. The route of AA administration and the treatment time-points were either before or after infection with 10^5 pRBC's saline suspension. For oral treatment, AA was administered once daily for five days, CHQ twice daily for five days. TDDS application was a once-off three day application of both type of patches. Animals were sacrificed at days 0, 8, 12 and 21. All animals were monitoring for %parasitaemia, physicochemical, biophysical and biochemical changes. Pre-infection treatment oral administration involved administering different doses of AA or CHQ before infection. Post-infection treatment oral administration was after 7 days post infection. Post-infection treatment topical application of AA-amidated pectin hydrogel matrix patch involved preparing the animal by shaving off hair from the dorsal neck region, applying the patch and proceed as for their protocols.

1.10 References:

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Chapter 2:
Introduction of Chapter 2 Part I Article 1

The current thesis is presented in the "article format" as recognized by the College of Health Sciences, University of KwaZulu Natal. This chapter presents the findings for Part I, of a three part chapter, published in the BioMed Central Complementary and Alternative Medicine, a peer reviewed journal. Mavondo et al Mavondo et al. BMC Complementary and Alternative Medicine (2016) 16:357 DOI 10.1186/s12906-016-1338-z

Asiatic acid influences parasitaemia reduction and ameliorates malaria anaemia in *P. berghei* infected Sprague-Dawley male rats

The article begins with the title, contributing authors and their affiliations and contacts, corresponding author, followed by an abstract, key words, highlights and abbreviations. Thereafter comes the main body of the research including: Background, Materials and Methods, Results, Discussion and Conclusion. Figures, legends to figures, tables and legends to tables are included in the text. Acknowledgments, conflict of interests and references mark the end of the article.

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RESEARCH ARTICLE Open Access

Asiatic acid influences parasitaemia reduction and ameliorates malaria anaemia in *P. berghei* infected Sprague–Dawley male rats

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Abstract

Background: Current malaria treatment is either "anti-parasitic", "anti-infectivity" or both without addressing the pathophysiological derangement (anti-disease aspect) associated with the disease. Asiatic acid is a natural phytochemical with oxidant, antioxidant and anti-inflammatory properties whose effect on malarial and accompanying pathophysiology are yet to be investigated. Asiatic acid influence in *P. berghei*-infected Sprague Dawley rats on %parasitaemia and malarial anaemia were investigated.

Methods: *Plasmodium berghei*-infected rats (90–120 g) were orally administered with Asiatic acid (5, 10, 20 mg/kg) and 30 mg/kg chloroquine as a positive control. Changes in %parasitaemia and haematological parameters in Asiatic acid administered rats were monitored in a 21 day study and compared to controls.

Results: All animals developed stable parasitaemia (15-20 %) by day 7. Asiatic acid doses suppressed parasitaemia, normalised haematological measurements and influenced biophysical characteristics changes. Most positive changes were associated with intragastric administration of 10 mg/kg Asiatic acid dose. Peak %parasitaemia in Asiatic acid administration occurred at days 12 with a shorter time course compared to day 9 for chloroquine (30 mg/kg) treatment with a longer time course.

Conclusions: Oral Asiatic acid administration influenced %parasitaemia suppression, ameliorated malarial anaemia and increased biophysical properties on infected animals. Asiatic acid may be a replacement alternative for chloroquine treatment with concomitant amelioration of malaria pathophysiology. Due to different action time courses, Asiatic acid and chloroquine may be possible candidates in combination therapy.

Keywords: Asiatic acid, Chloroquine, Malaria parasitaemia, *Plasmodium berghei*

Background

One or more of the five *Plasmodium* species known to infect human beings cause malaria accounting the death of over 600, 000 people annually, a majority of which are pregnant women and children less than 5 years of age [1]. The pathophysiology of malaria, which is the "malaria disease", include immunological aberrations, inflammation, haemolysis with severe malaria anaemia [SMA] [2], acute renal failure and general cachexia [3] and malaria cachexia leading to cardiac failure [4]. Pathophysiological manifestations during or after successful treatment of infection are major causes of high morbidity and mortality associated with malaria [5]. Untoward post treatment effects with artemisinins and chloroquine and drug resistance affect the mono therapeutic usage of these historical drugs [6]. Novel anti-inflammatory and immunoregulatory functions of artemisinin and its derivatives has been reported to inhibit nitric oxide (NO) and proinflammatory cytokines production by suppressing MRPK and NF-kβ in macrophages cell line RAW 264.7 [7]. However, the continued need of for antipyretic supportive paracetamol

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(acetaminophen) therapy prolongs parasite clearance time by decreasing induced tumour necrosis factor-α (TNF-α) and may worsen the disease with its inadequate "anti-disease effects" necessitating continuing search for more effective "anti-parasitic" or "anti-infection" or both.

Efforts to use adjunctive therapy in malaria, born out of the identification of malaria pathophysiology resolution was key to treatment of the disease, have not managed to reduce malaria morbidity and mortality. Dexamethasone (steroid), antibodies against TNF-α, phenobarbital (anti-convulsant) and iron chelation with desferrioxamine administration in malaria have not yielded expected outcomes. Therefore, there still exists an acute need for antimalarial drugs with anti-parasitic, anti-infectivity and anti-disease properties. Aberrant immune response and uncontrolled inflammatory process driven by increasing parasitaemia in malaria, formulate a significant part of a vicious cycle in a feed-forward mechanism leading to severe malaria anaemia, general cachexia, coma and death from the disease [8]. Hypothetically, drugs that may inhibit or reverse malaria pathophysiology or the disease components have a higher chance of controlling malaria even without parasite eradication through targeting growth essential host related factors such as severe malaria anaemia [SMA] an independent malaria mortality predictor in pregnant women and children. We hypothesized that triterpenes with anti-disease properties in other conditions similar to malaria, like inflammation in sepsis and hypoxia in anaemia may be able to eradicate the *Plasmodium* parasite as well as resolve the ensuing pathophysiology.

Triterpenes with pleiotropic functions, sufficient to be anti-disease as well as anti-parasitic have been reported. Betulinic acid [BA] (IC_{50} 19.6 and 25.9 μg/mL), ursolic acid [UA] (IC_{50} 36.5 and 28 μg/mL) and oleanolic acid [OA] (88.8 and 70.6 μg/mL) have been shown to have moderate activity in vitro against the chloroquine insensitive (K1) and chloroquine sensitive (T9–96) *Plasmodium falciparum* parasites [9]. Maslinic acid, a possible multi targeting antimalarial, effectively inhibited proteolytic processing of the merozoite surface protein (MSP1) complex, inhibited the metalloproteases and revealed (in silico studies) two targets while suggesting several putative new binding sites for the natural triterpene [10]. This multi-target phenomenon suppresses the parasitaemia and avoids the age old preoccupation with targeting single process of the parasite infective cycle (which is mutation prone) to involve host-related responses potentiating anti-disease and anti-resistance outcomes.

Asiatic acid (AA), an amphiphilic triterpene, with known antioxidant and pro-oxidant capacity [11], anti-inflammatory and antinociception activity in mice [12], calcium-release associated apoptosis induction [13] and a potent immunomudulator shares structural and bioactivity properties with OA, MA, UA and BA making it a possible antimalarial agent. Indeed, prophylaxis activity of AA has recently been suggested [14] together with its influences on glucose homeostasis in malaria [15].

Proper interventions may inhibit malarial pathology development which should be the aim of malaria management seeing that people living in endemic areas develop partial to total immunity against the parasite and asymptomatic parasitaemia is common [16]. Therefore, targeting the pathophysiology of malaria as well as the parasite may provide a new mechanism of combating malaria. It is noteworthy that the diseases and conditions AA is known to attenuate, inhibit or ameliorate formulate the bedrock of malaria disease and sequalae as alluded earlier [2, 3, 17–19]. However, there is little information on the anti-malarial, haematological, immunological impact of AA in malaria, facets that require exploration for possible AA pharmacotherapeutic uses. Malaria is driven by glycosylphosphatidylinositol (GPI) which elicits excessive macrophages activation leading to proinflammatory cytokine release, tissue damage, erythophagocytosis, erythropoiesis dysfunction and general cachexia, require an animal model to unravel the complex disease pathophysiology [20]. AA modulates immunity by selective induction of mitochondria-dependent apoptosis of activated lymphocytes in the prevention of murine fulminant hepatitis [21] a mechanism that may be extendable to malaria. Using membrane DNA array technique, a wound-healing derivative of AA [2-Oxo-3, 23-isopropylidene-asiatate (AS 2006A)] exerted anti-inflammatory effect through selective cytotoxicity to activated macrophage cell line (L-929) by upregulating expression of apoptosis-inducing genes caspase-8, c-myc, inducible nitric oxide synthase (iNOS), mdm2, NF-kβα, I-kβα, and NF-kβ p105 [22]. The finding may allude to AA also exerting anti-inflammatory effect by cytochrome c release, caspase 3 activation and poly(ADP-ribose) polymerase cleavage mechanism as did AS2006A which may require possibly elucidation in vivo experiments.

The effect of AA in alleviating haemodynamic and metabolic alterations in rats with metabolic syndrome through restoration of endothelium nitric oxide synthase (eNOS)/iNOS expression [23] has been reported. Similar AA influence in murine malaria may be anticipated where eNOS/iNOS ratio determines the bioavailability of NO necessary for proliferation and angiogenesis [24]. Derangements of this relationship may formulate malarial microvascular pathology.

Limitations inherent in in vitro studies and the inaccessibility of the human host further navigates research of this nature to an animal model with works by Thaane, T on maslinic acid (MA) [25] and by Mbatha B OA [26] in the murine malaria animal model suggesting amelioration of pathophysiological derangements in malaria. Furthermore, *Centella asiatica*, from which naturally occurring AA is

obtained, was used to treat malaria related fevers [27] although no specific mention of the phytochemical has been associated with malarial treatment. In vitro studies, whilst they may show the molecular interaction of AA with the parasite, they fall short where the pathophysiology of the disease are being explored as in this current study. In recognition of this, AA antimalarial and its malaria pathophysiology ameliorative effects were investigated in *P. berghei*- infected young (90–120 g) male Sprague Dawley rats as a novel effect of AA in malaria.

Methods

Materials

Drugs, chemical and accessories

AA (97 % purity), chloroquine diphosphate and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and reagents used were of analytical grade.

Animals

Male Sprague–Dawley (SD) rats (90–120 g) were obtained from the Biomedical Research Unit (BRU), of the University of KwaZulu-Natal where they were bred and housed for the entire experiment period. Animals were housed communally and individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) during experiments. The animals were kept under maintained laboratory conditions of constant temperature (22 ± 1 °C); $C0_2$ (<5000 ppm), humidity of 55 ± 5 % and illumination (12 h light/dark cycles) with access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water ad libitum. All animals were sacrificed by day 21 through exposure to lethal anaesthetic inhalation of isofor (Safeline Pharmaceuticals, Rooderport, South Africa) for 3 min via an anaesthetic gas chamber (100 mg/kg). All experiments and protocols used in this study were reviewed and approved by the animal ethics committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

Plasmodium parasite

Chloroquine-susceptible strain of *Plasmodium berghei* ANKA, murine malaria parasite was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). The parasite was sub-cultured, harvested and stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until use.

Experimental design

The study was conducted over 21 day in animal groups ($n = 6$ per group) as follows:

Non-infected treated control groups (NIC)
Infected non-treated control groups (IC)
Infected treated with CHQ 30 mg/kg groups (30CHQ)
Infected administered AA 5 mg/kg groups (5 mg)
Infected administered AA 10 mg/kg groups (10 mg)
Infected administered AA 20 mg/kg groups (20 mg)

Methods

Induction of parasitaemia

Chloroquine-susceptible strain of *P. berghei* ANKA (10^6 parasitized red blood cells [pRBC's] suspension in saline) was inoculated intraperitoneal (ip). Control animals received equivalent amount of saline. Day of inoculation was regarded as experiment day 1.

Oral Asiatic acid and chloroquine preparation

Asiatic acid was dissolved in DMSO (0.5 mL) and made up to volume with distilled water such that the final concentration was 5 mg/kg AA. Dosage was administered as multiples of the stock volume equivalent to the dose required. Chloroquine [CHQ] (30 mg/kg) was dissolved in distilled water. Both compounds were prepared fresh each day.

Monitoring of %parasitaemia

Appearance of parasites in the blood after ip inoculation takes 2–3 days [28]. Pre-patent period of 72 h post infection and a stable state parasitaemia at 15–20 % on day 7 without intervention confirmed the models conform to the known murine malaria course. These periods can be used as set points for establishing predictive validity of the experiments. %Parasitaemia was assessed and measured on day 3 and 7 in infected groups. Treatment was only commenced when the %parasitaemia had reached patent level or stable state SM.

Influence of Asiatic acid on biophysical changes

In animals individually housed in metabolic cages, intake of food, water and body weights of non-treated infected (IC), non- infected treated (NIC) and infected treated were monitored gravimetrically at 09 h00 every third day for experiment duration. The effects of AA on the measured parameters were compared to those of controls.

Malaria treatment

Rats were treated per oral (po) using a ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 ml syringe. Due to preliminary studies on AA posology and literature reports AA (5, 10, 20 mg/kg) [29] was administered on day 7–12 (5 days), once daily at 09 h00. CHQ (30 mg/kg) was administered twice daily (09 h00 and 16 h00) according to local laboratory developed posology [25, 26]. The CHQ dose of 30 mg/kg was selected although it was larger than the highest dose of AA (20 mg/kg) because it is treatment

dose lethal to *P. berghei* which is closest to AA doses in the study that has been used by other researchers [30].

Influence of AA administration on % parasitaemia

Peripheral (tail) blood smears were used to monitor %parasitaemia. The actual number of pRBC's relative to 2×10^4 RBC's was used to calculate parasitaemia [28]. A 15–20 % parasitaemia, confirmed by Giemsa staining under a microscope (Olympus Cooperation, Tokyo, Japan), was considered as stable state of severe malaria (SM). For post-infection AA or CHQ po administration % parasitaemia was monitored at 72 h (pre-patent period), every third day up to Day 7 [patent period] [31], every day during treatment period (5 days) and thereafter every other day post-treatment period until day 21.

Influence of AA administration on malaria anaemia

Changes in full blood count (FBC) were used to further confirm the influence of AA in reversing low blood volume experienced in malaria. Animals were sacrificed on days 0, 8, 12, 21 and blood was collected by cardiac puncture into EDTA tubes for full blood count analysis.

Statistical analysis

Unless otherwise stated, data was presented as mean ± standard error of the mean (SEM). Statistical comparisons were performed by one way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison post hoc test using GraphPad InStat Software (version 5, GraphPad Software, San Diego, California USA). A $p < 0.05$ considered statistically significant.

Results

Asiatic acid influence in malaria

Per-oral AA administration on %parasitaemia

Figure 1 shows %parasitaemia changes over time. AA (5, 10, 20 mg/kg) decreased %parasitaemia in comparison to IC ($*p < 0.05$) during treatment. AA (5, 10, 20 mg/kg) administration varied significantly vs 30CHQ on days 8–12 ($\#p < 0.05$). AA (10 mg/kg) decreased %parasitaemia significantly in comparison to 30CHQ ($\#p < 0.05$) post treatment period day 15. Peak parasitaemia had significantly different time points when AA (5, 10, 20 mg/kg) administered groups were compared to 30CHQ ($\#p < 0.05$) over the 21 day study.

AA effects on %parasitaemia-time area under the curves

%parasitaemia-time course, indicated by area under the curve (AUC), compared the cumulative effect of different AA doses on the parasite and malaria pathophysiology to those of IC ($AUC_{0\text{-}12days}$) and CHQ ($AUC_{0\text{-}21days}$) as shown in Fig. 2. Suppression efficiency and malaria pathology amelioration of different doses of AA (AA5, 10, 20 mg/kg $UC_{0\text{-}21days}$) was in the order of 10 mg/kg > 5 mg/kg > 20 mg/kg. AA 10 mg/kg administration lowered the $AUC_{0\text{-}21days}$ significantly compared to the IC $AUC_{0\text{-}12days}$ and 30CHQ treatment $AUC_{0\text{-}21days}$ (*, # $p < 0.05$, respectively). AA 20 mg/kg administration had an increased $AUC_{0\text{-}21days}$ compared to both the IC $AUC_{0\text{-}12days}$ and 30CHQ $AUC_{0\text{-}21days}$ (*, # $p < 0.05$, respectively). There was no significant difference in %parasitaemia-time course of AA5 ($AUC_{0\text{-}21days}$) when compared to CHQ ($AUC_{0\text{-}21days}$) and IC ($AUC_{0\text{-}21days}$).

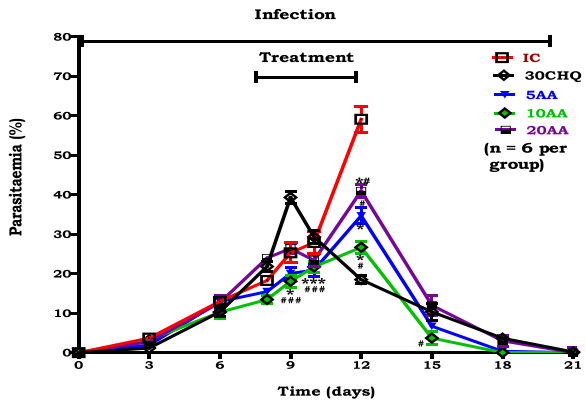

Fig. 1 Influence of oral AA on % parasitaemia compared to controls. IC is infected-non treated control. 30CHQ is chloroquine 30 mg/kg. Values are presented as means ± SEM, ($n = 6$ in each group). *, # $p < 0.05$ compared to IC, CHQ controls, respectively

Influence of AA on parasitaemia progression compared to controls

Table 1 showed %parasitaemia-time lines which revealed that AA doses influence varied according to the different doses when compared to controls. At day 7 AA20 had significantly lower %parasitaemia compared to CHQ

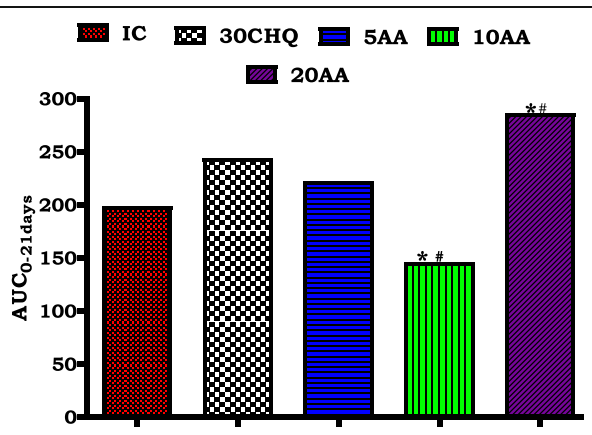

Fig. 2 Influence of AA administration on haemoglobin changes over time. NIC is non infected treated control; IC is infected non treated control; 30CHQ is chloroquine treated with 30 mg/kg. Values are presented as means ± SEM, ($n = 6$ in each group). α,*, # $p < 0.05$ compared to NIC, IC, CHQ controls

Table 1 Asiatic acid influence on parasitaemia % time lines compared to controls

Protocol	Dose	Infection rate	Pre-patent (days)	% parasitaemia day 3	% parasitaemia range on Day 7	% peak parasitaemia
Post-Infection per oral treatment	IC	100 %	2–3	3.70 ± 0.675	15.7 ± 3.264	69.3 ± 3.88
	30CHQ	100 %	2–3	1.167 ± 0.307	16.1 ± 1.325	43.3 ± 1.66
	5AA	100 %	2–3	1.928 ± 0.778	16.9 ± 2.774	41.7 ± 4.51*
	10AA	100 %	2–3	3.230 ± 2.209	21.3 ± 3.608*#	27.7 ± 1.96*#
	20AA	100 %	2–3	1.815 ± 1.016	8.4 ± 3.848*#	46.2 ± 2.94*

IC infected-non treated control, 30CHQ is chloroquine 30 mg/kg. Values are presented as means ± SEM. ($n = 6$ in each group)

(* $p < 0.05$) while other doses did not show significant differences to controls. At day 7 AA10 displayed a higher %parasitaemia compared to IC and CHQ (*, #$p < 0.05$, respectively). At peak % parasitaemia AA10 administration had a higher parasitaemia suppression effect compared to controls (*, #p <0.05.) AA5 and 20 had equally significant %parasitaemia suppression capacity at peak parasitaemia compared to CHQ (#$p < 0.05$). %parasitaemia progression was faster in AA10 administered animals. From day 3 to day 7 while it was slowest in AA20 administered animals.

Influence of Asiatic acid on eating and drinking habits as well as weight changes

Influence of AA on biophysical properties

Food and water intake plus weight changes reflected the general animal health among AA treated infected animals compared to controls. Tables 2, 3 and 4 showed the effects of AA on food and water intake, and %weight change. At day 12 and 21 food intake of AA administered animals increased in order of 5 mg/kg > 20 mg/kg > 10 mg/kg vs IC and 30CHQ (*$p < 0.05$ and #$p < 0.05$, respectively). Administration of 5 and 20 mg/kg AA significantly (*$p <$ 0.05) increased food intake by comparison to the IC on day 7–12 of treatment. AA administration (5, 10, 20 mg/kg) significantly increased water intake when compared to IC and 30CHQ group (*$p < 0.05$ and #$p < 0.05$, respectively) at patent/treatment and post treatment time points. Animals administered AA (5, 10, 20 mg/kg) had increased %weight change in comparison to IC and 30CHQ (*, #$p <$ 0.05, respectively) during treatment and post treatment periods. Overall the 10 mg/kg AA had the highest positive effect on biophysical changes by comparison to all controls (*, #$p < 0.05$). Generally, AA administration had a positive influence on the biophysical characteristics of the experimental animals compared to controls.

Influence of AA on anaemia development and resolution

Influence of AA administration on haemoglobin

Hb concentration estimated the degree of anaemia development and resolution compared to controls. Figure 3 AA (10 mg/kg) vs NIC, day 7–12 (α $p < 0.05$). AA (10 mg/kg) vs IC on days 7–12 (* $p < 0.05$). AA (10 mg/kg) vs 30CHQ on day 7–12 and post treatment (# p0.05). AA (5 mg/kg) vs NIC on days 7–12 (α $p < 0.05$). AA (5 mg/kg) vs IC on days 7–12 (* $p < 0.05$). AA (20 mg/kg) vs NIC on days 7–12 and post treatment (α $p < 0.05$). AA (20 mg/kg) vs IC on days 7–12 (* $p < 0.05$). AA (20 mg/kg) vs 30CHQ on days 7–12 and post treatment (# $p < 0.05$).

Influence of AA administration on haematocrit compared to controls

Haematocrit change over time was used as a surrogate marker for severe malaria anaemia development and resolution. Compared to the IC, AA10 administration

Table 2 Post-infection AA5, 10, 20 oral administration influence on food intake changes compared to controls

Parameter	Post-infection treatment	Pre-patent (D 3)	Patent/Treatment (D7–12)	Post-treatment (D13- 21)
Food intake (g/100 g)	NIC	12.3 ± 2.3	13 ± 9.0	13.4 ± 1.6
	IC	11.5 ± 1.9	6.7 ± 1.3	N/A
	30CHQ	12.3 ± 1.8	7.8 ± 2.0	8.6 ± 1.7
	5 AA	12.9 ± 1.3	9.8 ± 1.3*#	9.7 ± 1.5*#
	10 AA	12.3 ± 1.8	12.9 ± 2*#	13.2 ± 0.72#
	20 AA	12.7 ± 1.6	9.2 ± 1.6α*	10.3 ± 1.2#

NIC is non-infected treated control, IC is infected non-treated control and CHQ is chloroquine control (30 mg/kg). Values are presented as means ± SEM, ($n = 6$ in each group). α,*,#$p < 0.05$ by comparison with NIC, IC, 30CHQ groups, respectively. *NIC* non infected treated control, *IC* infected control, *CHQ* chloroquine, *AA* asiatic acid

Table 3 Post-infection AA5, 10, 20 oral administration influence on water intake changes compared to controls

Parameter	Post-infection treatment	Pre-patent (D 3)	Patent/Treatment (D7–12)	Post-treatment (D13- 21)
Water intake (mL/100 g)	NIC	14.3 ± 1.0	15.5 ± 1.3	15.2 ± 0.9
	IC	15.5 ± 1.3	8.4 ± 1.7	N/A
	CHQ	14.2 ± 1.2	10.5 ± 1.7	12 ± 1.2
	5AA	15 ± 3	12.7 ± 1*#	14.8 ± 1.9#
	10AA	15.8 ± 0.8	14.2 ± 0.9*#	15.1 ± 1.1#
	20AA	15.2 ± 1	11.6 ± 1.7α*	12.9 ± 1.6

NIC is non-infected treated control, IC is infected non-treated control and CHQ is chloroquine control (30 mg/kg). Values are presented as means ± SEM, ($n = 6$ in each group). α,*,#$p < 0.05$ by comparison with NIC, IC, 30CHQ groups, respectively. *NIC* non infected treated control, *IC* infected control, *CHQ* chloroquine, *AA* asiatic acid

Table 4 Post-infection AA5, 10, 20 oral administration influence on % weight change changes compared to controls

Parameter	Post-infection treatment	Pre-patent (D 3)	Patent/Treatment (D7–12)	Post-treatment (D13- 21)
% body weight change	NIC	8 ± 1	14.4 ± 6	18.9 ± 6
	IC	10.1 ± 5	4 ± 22.3	N/A
	CHQ	8.8 ± 1.5	9.4 ± 6	12.2 ± 0.5
	5AA	9.3 ± 2.2	14.2 ± 1.0*#	15.5 ± 1.9#
	10AA	8 ± 1	13.7 ± 1*	17.3 ± 0.8#
	20AA	9.5 ± 1	11.5 ± 1.9*	13.1 ± 0.2

NIC is non-infected treated control, IC is infected non-treated control and CHQ is chloroquine control (30 mg/kg). Values are presented as means ± SEM, ($n = 6$ in each group). *, #$p < 0.05$ by comparison with IC and 30CHQ groups, respectively. *NIC* non infected treated control, *IC* infected control, *CHQ* chloroquine, *AA* asiatic acid

was shown to influence a higher haematocrit at patent/ treatment periods (*$p < 0.05$). AA5, 10, 20 administration had generally lower haematocrit during patent/treatment and post treatment periods compared to NIC (α $p < 0.05$). AA10 administration influenced higher haematocrit compared to CHQ (# $p < 0.05$). AA10 administration proved to be the most efficacious of the three doses against malaria anaemia development.

Influence of AA administration on red blood cell mass compared of controls

Red blood cell mass was used as a critical indicator of anaemia development and resolution Compared to the IC, AA10 administration was shown to influence a higher red cell count at patent/ treatment periods (* $p < 0.05$). AA10 administration influenced higher red cell count compared to CHQ (# $p < 0.05$). Compared to NIC patent/treatment and post treatment period AA administration had lower red cell count. AA10 administration proved to be the most potent of the three doses in protecting red cell count.

Discussion

There is paucity of information with regard to AA treatment of malaria although anti-malaria anecdotal evidence has been forwarded for *Centella asiatica* [CA] from which AA is obtained. *P. berghei* has a similar pathophysiology with the *P. falciparum,* the most virulent human malaria parasite warranting its use as a murine malaria model. Studies in our laboratory have indicated, also proven in earlier research [32], that *P. berghei* causes severe malaria (SM) in younger animals which may proceed to cerebral malaria (CM).

The *P. berghei*-SD rat malaria model conformed to a high predictive validity at days 3 and 7 (Table 2) as expected. Infection effectuation by an ip inoculation of 10^6 parasitized red blood cells (pRBC's) saline suspension invariably resulted in SM. There was no significant difference in %parasitaemia observed amongst the groups during the pre-patent and patent periods. Decrease in %parasitaemia and changes in haematological parameters, therefore, could be attributable to the intervention.

We used young, 6 weeks old SD rats (90–120 g) which displayed SM, severe malaria anaemia (SMA) and in some cerebral malaria (CM) to demonstrate AA influence on murine malaria, SMA development and its resolution. Stable state SM was determined as 15–20 % parasitaemia at day 7 and >20 % at day 12 (Fig. 1) and SMA as an Hb <7.0 g/dL, Hct <15 % and red cell count <4 × 10^6/μL (Figs. 2, 3, 4).

In untreated animals peak parasitaemia was able to reach 69.3 % (Table 2) and Hb was 4.5 g/dL (Fig. 2) by day 12 when the animals were sacrificed to avert further pain and suffering. Suppression of parasitaemia to undetectable levels and resolution of haematological parameters to normal by administration of AA10mg/kg by day 21 was demonstrated. Once daily administration of AA10 corrected malaria manifestations compared to the IC and 30CHQ (twice daily treatment).

Partially pharmacodynamics of AA in influencing malaria was shown by comparing the efficacy of the different AA doses during the 21 day studies. Normally, a drug at higher concentrations is expected to exert a higher and more profound an effect and to be less effective at lower concentrations. While we did not determine the minimum inhibitory concentration for AA in this study, at highest (AA20) and lowest (AA5) doses, AA po administration may have been permissive to parasite growth through currently unknown mechanisms by displaying higher peak %parasitaemia comparable to CHQ although significantly

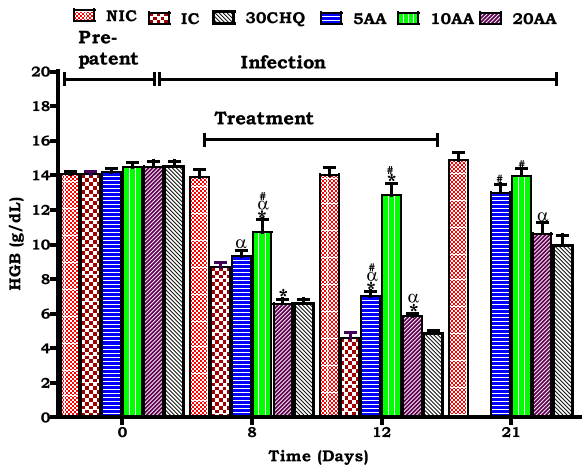

Fig. 3 Influence of AA administration on haematocrit changes over time. NIC is non infected treated control; IC is infected non treated control; 30CHQ is chloroquine treated with 30 mg/kg. Values are presented as means ± SEM, ($n = 6$ in each group). α,*, #$p < 0.05$ compared to NIC, IC, CHQ controls

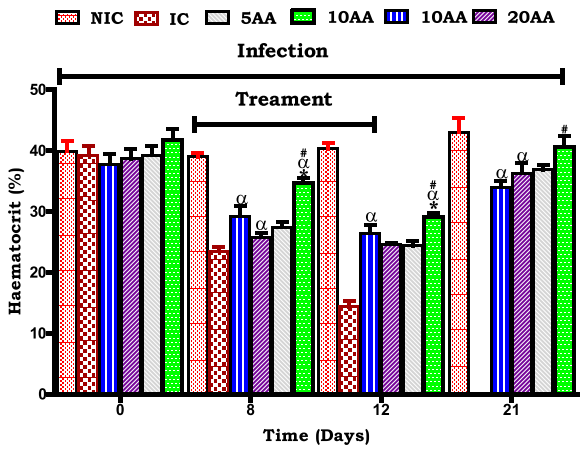

Fig. 4 Influence of AA administration on red blood cell count compared to controls. NIC is non infected treated control; IC is infected non treated control; 30CHQ is chloroquine treated with 30 mg/kg. Values are presented as means ± SEM, ($n = 6$ in each group). α,*, #$p < 0.05$ compared to NIC, IC, CHQ controls

lower than the IC (Table 2). AA5, AA20 and 30CHQ permitted %parasitaemia to increase by 1.47, 4.5 and 1.69 times, respectively, from day 7 to peak %parasitaemia at day 21 compared to AA10 which increased by 0.3. It may be poignant to note that AA20 had the highest effect on parasitaemia suppression during early days of AA administration, thereafter %parasitaemia dramatically increased subsequently culminating in the highest %parasitaemia-time course (Figs. 1, 5 and Table 2). Administration of AA5 may not have reached an optimum drug concentration to be lethal to the parasite during the treatment period while the AA20 dose may have been too high becoming protective to the parasite's survival over time. Administration of

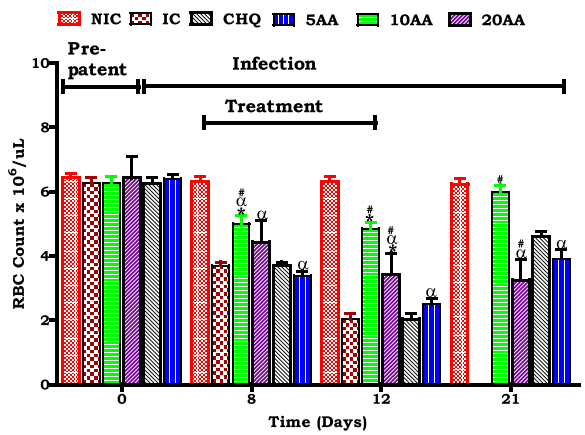

Fig. 5 $AUC_{0\text{-}21Days}$ of %parasitaemia for post-infection AA oral administration. IC is infected-non treated control. 30CHQ is chloroquine 30 mg/kg. Values are presented as means ± SEM, ($n = 6$ in each group). *, # $p < 0.05$ compared to IC, CHQ controls, respectively

AA10 provided the optimum dose as the suppression of %parasitaemia was evidenced by a higher patent/treatment period %parasitaemia without dramatic increase towards peak %parasitaemia.

The %parasitaemia-time under the curve (AUC) displayed the cumulative effect of the different AA doses collated over 21 days for AA, CHQ and over 12 days for the IC. Although the %parasitaemia-time course for IC (IC $AUC_{0\text{-}12days}$) was shorten to 12 days, compared to 21 days for the treated animals, still the latter had a comparatively higher AUC showing that AA intervention resulted in lowering of AA10 $AUC_{0\text{-}21days}$ as observed. Indeed, AA10 administration resulted in a lower $AUC_{0\text{-}21days}$ showing a more potent parasitic suppression effect of AA at this dose.

The observed continual %parasitaemia decline beyond AA administration at day 12 implies possible residual activity of AA with three possible effects: an accumulative AA concentration with increased direct parasite metabolism disruption or increased malarial pathophysiology resolution hostile to parasite survival or immune system activation. A combination of these and other phenomena is possible as well. However, this dynamic milieu, which may be explainable through recognition of the molecular characteristics of AA, was only attained by AA10 administration.

The phytochemical AA possesses both antioxidant (hydrogen bond acceptor 4.172) and pro-oxidant (hydrogen bond donor 7.1) capacities [11] which may concurrently function under physiological conditions and may eventually increase during subsequent dosing with possible chemoprophylaxis and chemotherapeutic effects. Hypothetically, the ratio of the oxidant and antioxidant capacity may dictate which facet of AA predominates. To the parasite, an oxidative interaction with AA will be detrimental to its survival while an antioxidant may be beneficial. There is a possibility that the optimum redox equilibrium of AA with a parasite killing potential was obtainable only at AA10 dose while it was diminished at AA20 and AA5 doses with resultant parasite proliferation seen by day 12.

In malaria, inflammatory response is usually exaggerated. The host's innate immune response to contain parasitaemia invariably involves the production and release of Th1 and Th2 cytokines, chemokines, growth factors, inflammatory effectors and mediators which throw the regulatory process off balance resulting in anaemia and other malaria pathology. AA has anti-inflammatory activity which could have resolved this pathophysiology, weakening malaria virulence and suppressing parasitaemia when AA10 was administered. Furthermore, the immunomodulatory effect of AA10 may also have curtailed aberrant immune reactivity that is common in malaria by selectively suppressing activated lymphocytes [21] and macrophages proliferation with possible enhancement

erythroid lineage propagation. An intricate balance in the host's eradication of the parasite exists during malaria proliferation through the production of inducible tumour necrosis factor (TNF), oxygen free radicals and other inflammatory mediators [33]. This could have implied that the anti-inflammatory and immunomodulatory effects of AA20 could have prolonged parasitaemia through over enhancement of these characteristics.

These the parasite suppression effects or lack off may also have been facilitated by a dose dependent AA absorptivity in the small intestines such that higher plasma concentrations were achieved through higher AA dose administration. Indeed, AA has been predicated to have a good intestinal absorption, mild Caco-2 cell permeability and a strong plasma protein binding [34] and low excretion. The strong protein binding of AA may have caused possible cumulative concentrations of the compound in plasma reaching a certain threshold that overwhelmed parasite defences with rapid parasitaemia decline. Moreover, a high affinity for proteins and low excretion rate may mean sustained, slow and constant release allowing AA to reach targets at optimum concentrations influenced by protein plasma concentrations which may be dependent on food and water intake.

Together with increased suppression of parasitaemia, administration of AA10 dose was associated with improved food and water intake and preserved weight gain compared to both 30CHQ dose and IC (Table 1). By preserving food and water intake at previous levels prior to infection, AA10 administration rebuffed onset of the sickness behaviour associated with malaria infection seeing that a significantly higher %parasitaemia was reported at day 7 (Table 2) showing infection patency. Malaria treatment with current drugs like CHQ does not counter parasite induced satiate but might actually exacerbate it. The decreased food and water intake and subsequently low weight gain in animals treated with CHQ could have been due a combined effect of both the parasite and the drug.

Chloroquine, like quinine, is known to have bitter taste which has been shown to reduce appetite [35]. The intestinal lining has receptors for both sweet and bitter substances with the latter associated with influencing decreased food intake through the gut-brain axis [36]. Nausea and vomiting are some side effects of CHQ which may increase aversion to food and water intake with worsening malarial pathophysiology, depletion of energy stores and decreasing foraging capacity. In this study, AA10 administration maintained food and water intake possibly due to its being tasteless and its antimalarial activity.

Furthermore, the hypoglycaemic effects of AA have been reported in streptozotocin-induced diabetes mellitus where the phytochemical increased activities of glycolytic enzymes while inhibiting gluconeogenesis and glycogenolysis [37]. Such an influence occurring in a normoglyaemic situation may invariably upregulate the energy mobilization processes to keep pace with the increased glucose utilization and demand of malaria. With food and water available ad libitum, an increased appetite as induced by AA10, physiologically inclines the animals to increase food and water intake. Therefore, it is plausible to assume that in malaria, a disease that influences induction of hypoglycaemia, AA10 had a causal relationship with weight gain and malaria complications alleviation.

One of the malaria sequalae that was observed to be averted is severe malaria anaemia (SMA). In malaria, SMA has multifaceted aetiologies ranging from parasitized red blood cells (pRBC's) rapture, pRBC's and non-parasitized red blood cells (npRBC's) phagocytosis [31], insufficient erythropoiesis, ineffective haematopoiesis and reduced erythropoietin production. While the haematological indices of haemoglobin (Hb) concentration, red blood cell count (RBC's) and haematocrit (Hct) were depressed with increasing %parasitaemia in the IC and CHQ controls, AA10 administration preserved these parameters and ameliorated SMA. SMA has been shown to persist even when parasitaemia has been resolved driven by an aberrant immune system and hemozoin-induced oxidative stress [38]. Therefore, current finding of AA10 parasitaemia suppression may not have been the only effect retarding and correcting SMA. There is possibility that more factors (including inflammatory mediators- impaired erythropoiesis) influencing SMA development may have been inhibited by AA10 per se together with preserved food and water intake.

Destruction of pRBC's occurs when the shyzoints mature and merozoites rapture cell membranes. Accompanying pRBC's destruction is the lysis of npRBC's at a ratio of 8.5 RBC's for each pRBC's haemolysed [39]. Anaemia develops as the RBC mass is reduced rapidly without concurrent replacement. Endogenous secretion of erythropoietin (EPO) may be overwhelmed by the high demand for erythropoiesis stimulation that is required to meet the rapid development of anaemia during the patent period. However, it was also observed that increasing EPO was not able to alleviate SMA associated with high parasitaemia showing that erythroid precursor response may also be inhibited by blood stage parasites resulting in low reticulocytosis [40]. Erythroid progenitor suppression in malaria has been ascribed to the increased free haemoglobin and hemozoin containing monocytes in the bone marrow and a shift of the transferrin receptor expression from erythroid to non-erythroid cell to increase immunological response to blood-stage parasite [41].

Alleviation of SMA involves the generation of reticulocytes, a process which requires the proliferation, differentiation and maturation of erythrocyte precursors in the bone marrow and other haematopoietic tissues. The

maintenance of RBC's count in this study may have implied that there was sufficient reticulocytosis or there was minimum RBC's destruction in AA10 administered animals compared to controls. However, the decrease in Hb and Hct at patent in IC and CHQ groups shows RBC's destruction as one of the key initiators of SMA which was minimized by AA10 administration.

The mechanism by which npRBC's are destroyed in malaria involves increased erythrocytic oxidative stress and parasite antigens which cause RBC's membrane to be less deformable and more fragile with shortened RBC's life spans. These cells are trapped during splenic sequestration and destroyed through phagocytosis. AA has known antioxidant, anti-inflammatory and immunomodulatory properties which could have protected cell membranes from oxidative damage and rigidity, reduced erythrophagocytosis and inhibited parasite proliferation. Masilinic acid (MA) a phytochemical similar in structure and polypharmacology with AA was shown to have multi-targeted inhibitory properties against malaria with possible blockade of parasite maturation from early ring to schizont stages [42]. Betulinic and ursolic acids also share carbon skeleton with MA and AA. Analogues of these two triterpenes have been shown to be antiplasmodial through disruption of parasite calcium homeostasis [43]. There is a possibility that AA may also possess the same inhibitory properties which could have limited RBC's haemolysis and preserved haematological indices.

Fully functional murine haemoglobinised RBC's take 7 days to be formed from an erythroid progenitor. Haematological indices in AA10 administered animals reflected the same time span for their recovery to near pre-infection levels showing an effectuation of a multifactorial remedy to SMA by AA10. The pleiotropic biological effect of AA may have influenced host control of the parasite through modulation of the immune system erythropoiesis suppressive effect resulting in the haematological indices correction towards normal in AA10 administered animals compared to controls. The involvement of cytokines and inflammation mediators, such as haeme-parasite derived hemozoin, influence the differentiation and maturation of erythroid cells. Increased levels of tumour necrosis factor (TNF), interleukin 6 (IL6), interleukin 1β (IL1β) and decreased levels of interleukin 12 have been associated with SMA [44]. The persistence of SMA beyond parasitaemia eradication is orchestrated and sustained by an immunological sequalae which upregulates hepcidin synthesis and modulation of iron metabolism [45]. Therefore, resolution of SMA (normalised haematological indices) indirectly indicated the abrogation of the immunological and inflammatory processes by AA10, which properties the triterpene is known to possess. However, the exact mechanism by which AA10 modulated SMA development and resolution requires further exploration.

Conclusion

Data demonstrating that AA positively influences food and water intake, %weight gain, % parasitaemia and SMA in *P. berghei*-infected SD rats has been presented. AA may have both anti-parasitic and anti-disease activities suppressing the parasite while ameliorating infection-induced pathology. AA10 administration showed a superior efficacy in preservation of food and water intake, parasitaemia suppression as well as resolution of SMA.

Abbreviations

AA: Asiatic acid; CA: *Centella* asiatica; CHQ: Chloroquine; DMSO: Dimethyl sulphoxide; GPI: Glycosylphosphatidylinositol; IC: Infected non treated control; ig: Intragastric; Ip: Intraperitoneal; NIC: Non infected treated control; po: Per oral; SMA: Severe malaria anaemia

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Availability of data and material

Supporting data to graphs and table can be accessed at: https://mynotebook.labarchives.com/Mavondo; This article is part of a large study whose common theme is AA influence on malaria parasitaemia and effects on pathophysiology parameters. Different experiments with distinct designs and settings were conducted in chronologically time-separations intervals. Certain parameters, therefore, (like parasitaemia and biophysical characteristics) may appear in other articles in print but depicting and based on different data sets.

Authors' contributions

MGA: research concept and design, collection and/or assembly of data, data analysis and interpretation, writing, critical revision of the manuscript and accountability towards work submitted. MBN: critical revision of the manuscript and accountability towards work submitted. MMV: critical revision of the manuscript and accountability towards work submitted. MCT (Deceased): research concept and design, initial data analysis and interpretation, research proposal critical revision and editing.

Competing interests

The authors declare that they have no competing interests in this work.

Consent for publication

All authors gave consent for this work to be published it in current form.

Ethics approval and consent to participate

All experiments and protocols used in this study were reviewed and approved by the Animal Ethics Committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

Ethical considerations

All experiments and protocols used in this study were reviewed and approved by the Animal Ethics Committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

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Chapter 2:

Introduction of Chapter 2 (Article 2) and Bridging gap to Chapter 2 (Article 1)

This chapter presents the findings on the Part II (Article 2), of a three part chapter, which has been published in the BioMed Central Malaria Journal, a peer reviewed journal. Mavondo *et al. Malar J (2016) 15:226* DOI 10.1186/s12936-016-1278-6. This article goes by the title:

Pre-infection administration of Asiatic acid retards parasitaemia induction in *Plasmodium berghei* murine malaria infected Sprague-Dawley rats

The article begins with the title, contributing authors and their affiliations and contacts, corresponding author, followed by an abstract, key words. Thereafter comes the main body of the research including: Background, Materials and Methods, Results, Discussion and Conclusion. Figures, legends to figures, tables and legends to tables are included in the text in the original document. The declaration section includes abbreviations, authors' contributions, acknowledgments and conflict of interests.

Bridging the gap between Chapter 2 (Article 1) and Chapter 2 (Article 2)

The results in Chapter 2 (Article 1) indicated (for the very first time) that AA10 suppressed parasitaemia, preserved food and water intake with concomitant %weight gain and reversed the severe malaria anaemia. However, the suppression started at the end of the treatment period on day 12, three days later than the time taken by 30CHQ to reach peak parasitaemia. The sudden drop in parasitaemia and the impressive efficacy compared to CHQ, overall, made us to hypothesize that AA may possibly have a long acting mode of action. This characteristic has been described by Zheng et al (2009) [reference in Article 2] when a single dose of *Centella asiatica* displayed two plasma AA peaks (C_{max}) in plasma concentrations which were four hours apart (4.7 hours and 8 hours) [reference in Article 2]. As a follow up to our result, we determined the effect of AA when administered prior to infection induction by giving AA10 48 hours before an intraperitoneal inoculation of *P. berghei* RBC's in saline. We report in Chapter 2 (Article 2) the pre-infection efficacy of AA in increasing the pre-patent period and retarding patent malaria in SD young male rats (90-120 g).

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Pre-infection administration of asiatic acid retards parasitaemia induction in *Plasmodium berghei* murine malaria infected Sprague-Dawley rats

Greanious Alfred Mavondo*, Blessing Nkazimulo Mkhwananzi and Musa Vuyisile Mabandla

Abstract

Background: Malaria prevention has remained a critical area in the absence of efficacious vaccines against malaria. Drugs currently used as chemotherapeutics are also used in chemoprophylaxis increasing possible drug resistance. Asiatic acid is a natural phytochemical with oxidant, antioxidant and anti-inflammatory properties with emerging anti-malarial potential. The influence of asiatic acid administration prior to *Plasmodium berghei* infection of Sprague-Dawley rats on parasitaemia induction is here reported.

Methods: Sprague-Dawley rats (90–120 g) were administered with asiatic acid (10 mg/kg) 48 h before intraperitoneal infection with *P. berghei*. Parasitaemia induction and progression, food and water intake as well as weight were compared to 30 mg/kg chloroquine-treated and infected control rats during sub-chronic studies (21 days).

Results: Asiatic acid pre-infection administration preserved food and water intake as well as increase in percentage weight gain of infected animals. In pre-infection treated animals, the pre-patent period was extended to day 6 from 72 h. Asiatic acid suppressed parasitaemia while oral chloroquine (30 mg/kg) did not influence malaria induction.

Conclusions: Per-oral, pre-infection, asiatic acid administration influenced parasitaemia patency and parasitaemia progression, food, water, and weight gain percentage. This may suggest possible chemoprophylaxis effects of asiatic acid in malaria.

Keywords: Asiatic acid, Malaria parasitaemia, *Plasmodium berghei*, Prophylaxis treatment

Background

Chemoprophylaxis can either be causal prophylaxis (absolutely prevents patent malaria development by eradicating liver stage parasites) or parasitaemia and malaria symptom suppression, referred to as suppressive or clinical prophylaxis, where blood stage parasites are destroyed from circulation [1]. Drugs used as prophylaxis need to be long acting or have longer half-lives as frequent dosing may lead to non-compliance [2, 3]. The drug also needs to be palatable and tolerable, facets absent in most prophylaxis drugs with phytochemical origins [4, 5]. In Uganda, an infusion of *Artemisia annua* consumed once weekly reduced risk of *Plasmodium falciparum* infection episodes due to as yet an unidentified constituent [6] with a longer half-life than artemisinin. However, its only drawback is the bitterness [7]. Fascinating results have also started to emerge where triterpenes oleanolic acid (OA) [8] and maslinic acid (MA) [9] have shown amelioration of metabolic dysfunction in malaria. These triterpenes have been reported to have anti-inflammatory activities as well [10, 11]. These findings suggest that other triterpenes may also have anti-malarial activity giving rise to this current investigation of asiatic acid (AA) as a potential anti-malarial. Asiatic acid is an intriguing molecule with antioxidant and pro-oxidant [12],

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anti-inflammatory [13] and immunomodulatory [14] activities. Chemically known as (4α)-2α, 3β, 23-trihydroxy-urs-12-en-28-oic acid, having redox reaction capability, amphiphilic with a hydrogen bond donor/acceptor ratio of 7.1/4.172 [15], AA has potential for anti-disease properties in malaria. Indeed, ongoing (unpublished) anti-malarial work with the triterpene AA has shown that the phytochemical has an abrupt or sudden parasite killing effect during the post-dosing period in infected Sprague-Dawley (SD) male rats. In those experiments high percentage parasitaemia seemed to just 'disappear' from subsequent peripheral slides without a predictable gradual decline seen with other anti-malarial drugs. With this in mind, the aim of the study was to establish whether the amphiphilic triterpenoid could have cumulative long-acting pharmacodynamics potentially useful for malaria chemoprevention. Findings on the influence of pre-infection administration of AA on the retardation of malaria development in *Plasmodium berghei*-infected SD male rats by monitoring malaria infection, percentage parasitaemia, as well as food and water intake are here reported.

Methods

Drugs and chemicals

The initial AA (500 mg) of 97 % purity used in the preliminary studies was a kind donation from Prof Van Heerden (University of KwaZulu Natal). Further quantities of AA (97 % purity) were purchased together with Giemsa stain, dimethyl sulfoxide (DMSO), chloroquine diphosphate (CHQ) from Sigma-Aldrich (St. Louis, MI, USA). All other chemicals and reagents were of analytical grade.

Animals

Male SD rats weighing 90–120 g were obtained from the Biomedical Research Animal Unit (BRU) of the University of KwaZulu where they were bred and housed for the entire experiment period. The animals were kept under maintained laboratory conditions of constant temperature (22 ± 1 °C); Co_2 (<5000 ppm), humidity of 55 ± 5 % and illumination (12 h light/dark cycles). Food, standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water were supplied ad libitum. All animals were sacrificed by day 21 through exposure to halothane for 3 min via an anaesthetic gas chamber (100 mg/kg). All experiments and protocols were reviewed and approved by the animal ethics committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

Murine malaria model

Chloroquine-susceptible strain of *P. berghei* ANKA, was a kind donation from Prof Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). The parasite was sub-cultured in SD rats and harvested into Na_2EDTA whole blood. The blood was washed and stored in freeze media containing 30 % glycerol at −80 °C until used.

Experimental design

Animal groups (n = 6) were divided according to whether they were infected or received treatment. Animals treated with CHQ (30 mg/kg) served as the positive control. The groups were as follows:

Non-infected treated control (NIC)
Infected non-treated control (IC)
Infection groups treated with CHQ 30 mg/kg (30CHQ)
Infected groups treated with AA 10 mg/kg (10AA).

Monitoring of physicochemical properties

Six animals per group were housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) with food and water availed to them ad libitum. Food, water intake and weight gain were determined gravimetrically every other day at 09.00 h.

Pre-infection oral administration

AA (10 mg/kg) and CHQ (30 mg/kg) were administered on successive days (days 0–5). AA was administered once daily (09.00) according to the posology developed for triterpenes [11, 16–18] and what doses others have advocated for treatment of other conditions [12, 19, 20]. CHQ was administered twice daily (09.00 and 16.00) for the same duration as AA. CHQ dose is a standard regimen for malaria prophylaxis in combination with doxycycline or proguanil [1]. A ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) Ltd, Benrose, South Africa) attached to a 1-ml syringe was used intragastric (ig) to deliver AA and CHQ.

Induction of parasitaemia

Plasmodium berghei (10^5 parasitized red blood cells (pRBCs) suspension in saline) was inoculated intraperitoneal (ip) [21]. Control animals received equivalent amounts of saline. Animals were inoculated 48 h after AA or CHQ administration. Administration of AA and CHQ was continued up to day 5 giving a total of 5 days administration inclusive of the pre-infection period.

Evaluation of parasitaemia

Appearance of parasites in blood after ip inoculation takes 2 to 3 days [22]. Pre-patent period was expected at 72 h post-infection and a stable parasitaemia at 15–20 % on day 7 [23]. After inoculation, parasitaemia was monitored at 72 h (pre-patent period) and every third day during the patent period [24] thereafter, until day 21. A 15–20 %

parasitaemia was considered as stable state severe malaria (SM) capable of inducing severe malaria anaemia (SMA). Stable state malaria was expected at day 7.

Influence of AA on percentage parasitaemia

Giemsa staining: peripheral blood obtained through a tail prick was made into thin blood smears and stained with Giemsa stain for monitoring of percentage parasitaemia by examination under a light microscope (Olympus Cooperation, Tokyo, Japan). The actual number of pRBCs relative to 2×10^4 RBCs was used to calculate parasitaemia [22].

Full blood count: To further explore the influence of AA on malaria and its co-morbidities of inflammation and SMA, white cell count (WBC) and haemoglobin estimations were made from blood obtained through cardiac puncture at days 0, 3, 9, 12, and 21 after administration of lethal anaesthesia with halothane. All IC animals were sacrificed by day 12 on ethical grounds to reduce pain and suffering using a humane method of halothane anaesthetic inhalation in gas chamber (100 mg/kg) and blood collected by cardiac puncture.

Statistical analysis

Unless otherwise stated, data were presented as mean plus standard error of the mean (M ± SEM). Statistical comparisons was performed by one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison post hoc test using Graph-pad Prism Software (version 5, GraphPad Software, San Diego, CA USA). $P < 0.05$ was considered statistically significant.

Results

Influence of AA on physicochemical properties

Table 1 shows the influence of AA administration on food and water as well as percentage weight gain. IC animals had significantly decreased water and food intake as well as body weight at day 12 compared to animals administered with AA (10 mg/kg) (*$p < 0.05$). CHQ treatment decreased food and water intake together with percentage weight gain when compared to AA (10 mg/kg) administration at relevant time points (**$p < 0.05$). Animals treated with 30CHQ had lower food and water intake as well as percentage weight gain when compared to NIC ($\gamma p < 0.05$).

Validation of parasitaemia

Table 2 shows the influence of AA (10 mg/kg) on prepatent period, percentage parasitaemia. AA (10 mg/kg) administration significantly influenced prolongation of the pre-patent period, parasitaemia inhibition by day 3 while reducing percentage parasitaemia at day 7 in comparison to the IC (*$p < 0.05$). AA (10 mg/kg), in comparison to 30CHQ (**$p < 0.05$) influenced prolongation of pre-patent period, parasitaemia inhibition at day 3 and reduction of percentage parasitaemia by day 7. In comparison to the IC, the positive control 30CHQ reduced percentage parasitaemia at day 3 (*$p < 0.05$). Animals administered AA (10 mg/kg) did not reach stable state malaria by day 7 in comparison to IC and 30CHQ (*, **$p < 0.05$, respectively). AA (10 mg/kg) had lower peak percentage parasitaemia compared to both the IC and 30CHQ controls (*, **$p < 0.05$, respectively). AA (10 mg/kg) peak period (day) was statistical different compared to that of 30CHQ (**$p < 0.05$).

Validation of asiatic acid influence on cellular morphology

As seen on Fig. 1, patent parasitaemia showed differential staining with Giemsa stain (×100 objective) where pRBCs showed as purple cells with or without parasites in them. By day 12 npRBCs in IC and CHQ groups were pail pick and reduced in number showing anaemia. There was minimum anisocytosis with slight polychromasia. Slides [C] and [D] from IC and CHQ-treated groups at days 12 and 9, respectively showed increased parasitaemia. Most cells were parasitized with visible parasite ring forms chromatin evident. Slides [E] was from AA (10 mg/kg)-administered group showing parasitaemia suppression at day 21. No pRBCs could be demonstrated in all AA-administered animals by day 21. Micrograph [E] from 30CHQ-treated animals showed that parasitaemia was still evident although significantly reduced compared to day 9.

Asiatic acid influence on percentage parasitaemia

AA administration and percentage parasitaemia

Figure 2 shows changes in percentage parasitaemia over time. AA (10 mg/kg) administration displayed significantly lower percentage parasitaemia compared to the IC (*$p < 0.05$) on days 3–12. Compared to 30CHQ, AA (10 mg/kg) had lower percentage parasitaemia (***$p < 0.05$) throughout the 21 days of the sub-chronic study. 30CHQ treatment lowered percentage parasitaemia significantly at day 12 in comparison to the IC (***$p < 0.05$).

AA administration and percentage parasitaemia-time area under the curve

Figure 3 shows the influence of AA (10 mg/kg) on percentage parasitaemia-time area under the curve ($AUC_{0-21days}$). AA (10 mg/kg) decreased the percentage parasitaemia-time curve significantly compared to the IC $AUC_{0-12days}$ (*$p < 0.05$). Compared to 30CHQ treatment, AA (10 mg/kg) administration reduced the $AUC_{0-21days}$ significantly (**$p < 0.05$) at the same time points. Compared to IC $AUC_{0-12days}$, 30CHQ treatment

Table 1 Influence of asiatic acid (10 mg/kg) on biophysical properties compared to controls

Parameter	Protocol name	Animal Groups	Pre-patent (D 3)	Patent/ Treatment (D7–12)	Post-treatment (D 21)
Food intake (g/100 g)	Pre-infection per oral AA administration	NIC	10 ± 3	11 ± 2	12 ± 1
		IC	9 ± 1	6 ± 2	N/A
		30 CHQ	10 ± 1	6 ± 1	8 ± 4
		AA (10 mg/kg)	10 ± 3	9 ± 2*, **	12 ± 1**
Water intake (mL/100 g/day)	Pre-infection per oral AA administration	NIC	15 ± 3	13 ± 1	16 ± 2
		IC	15 ± 2	7 ± 2	N/A
		CHQ	14 ± 1	10 ± 1	12 ± 2γ
		AA (10 mg/kg)	15 ± 3	14 ± 1*, **	15 ± 2**
% body weight change	Pre-infection per oral AA administration	NIC	8 ± 3	10 ± 2	15 ± 1
		IC	8 ± 2	−4 ± 2	N/A
		CHQ	8 ± 1	5 ± 1γ	6 ± 1γ
		AA (10 mg/kg)	8 ± 4	11 ± 2*, **	14 ± 1**

Changes on percentage body weight gain, food and water intake of *P. berghei*- infected treated and non-treated animals were monitored. Values are presented as mean ± SEM, (n = 6 per group)

NIC non infected treated control, *IC* infected non-treated control, 30*CHQ* chloroquine 30 mg/kg.

*, ** $p < 0.05$ by comparison to the IC, CHQ, respectively

Table 2 Percentage parasitaemia during different time points per different groups

Protocol	Groups	Pre-patent parasitaemia (days)	Parasitaemia on day 3 (%)	Parasitaemia on day 7 (%)	Parasitaemia at peak (%)	Peak period (day)
Pre-infection AA administration	IC	2–3	5.27 ± 1.17	15.72 ± 2.98	56.52 ± 3.20	12
	30CHQ	2–3	1.167 ± 0.31	16.08 ± 1.33	22.37 ± 4.36	9
	AA10 mg/kg	6*, **	0.00 ± 0.0*, **	0.13 ± 2.03*, **	7.51 ± , **	12**

Values are presented as mean ± SEM, (n = 6 per group)

IC infected non-treated control, 30*CHQ* chloroquine 30 mg/kg

*, ** $p < 0.05$ compared to IC, 30CHQ, respectively

reduced $AUC_{0-21days}$ significantly (***$p < 0.05$). Overall, the percentage parasitaemia-time IC $AUC_{0-12\ days}$ was significantly higher than either AA (10 mg/kg) or 30CHQ $AUC_{0-21\ days}$ (*, ***$p < 0.05$, respectively), regardless of the shorter time period.

AA influence on inflammation

Figure 4 shows the effect of AA (10 mg/kg) on WBC count over time. AA (10 mg/kg) administration lowered WBC count significantly compared to IC (*$p < 0.05$). Compared to 30CHQ, AA (10 mg/kg) decreased WBC significantly (**$p < 0.05$). At peak percentage parasitaemia AA (10 mg/kg) administered animal had a significantly higher WBC count compared to the NIC (α $p < 0.05$). Treatment with 30CHQ had higher WBC counts compared to the NIC (***$p < 0.05$) throughout the 21-day period.

Influence of AA on severe malaria anaemia

Figure 5 shows changes in haemoglobin (Hb) with administration of AA (10 mg/kg) over time. Administration of AA (10 mg/kg) had significantly higher Hb levels compared to the IC (**$p < 0.05$). Compared to 30CHQ, AA (10 mg/kg) had significantly higher Hb levels (***$p < 0.05$) throughout the 21-day study.

Discussion

Anecdotal information ascribes anti-malarial activity to *Centella asiatica* (CA) [25, 26] but there are no reports of chemoprophylaxis or chemotherapeutic effects of AA on malaria. There is a close resemblance in malaria pathophysiology between *P. berghei* and *P. falciparum*, the virulent species of human malaria parasites, warranting the former to be used as a safer analogue in experimental malaria. Studies have indicated, also proven by earlier

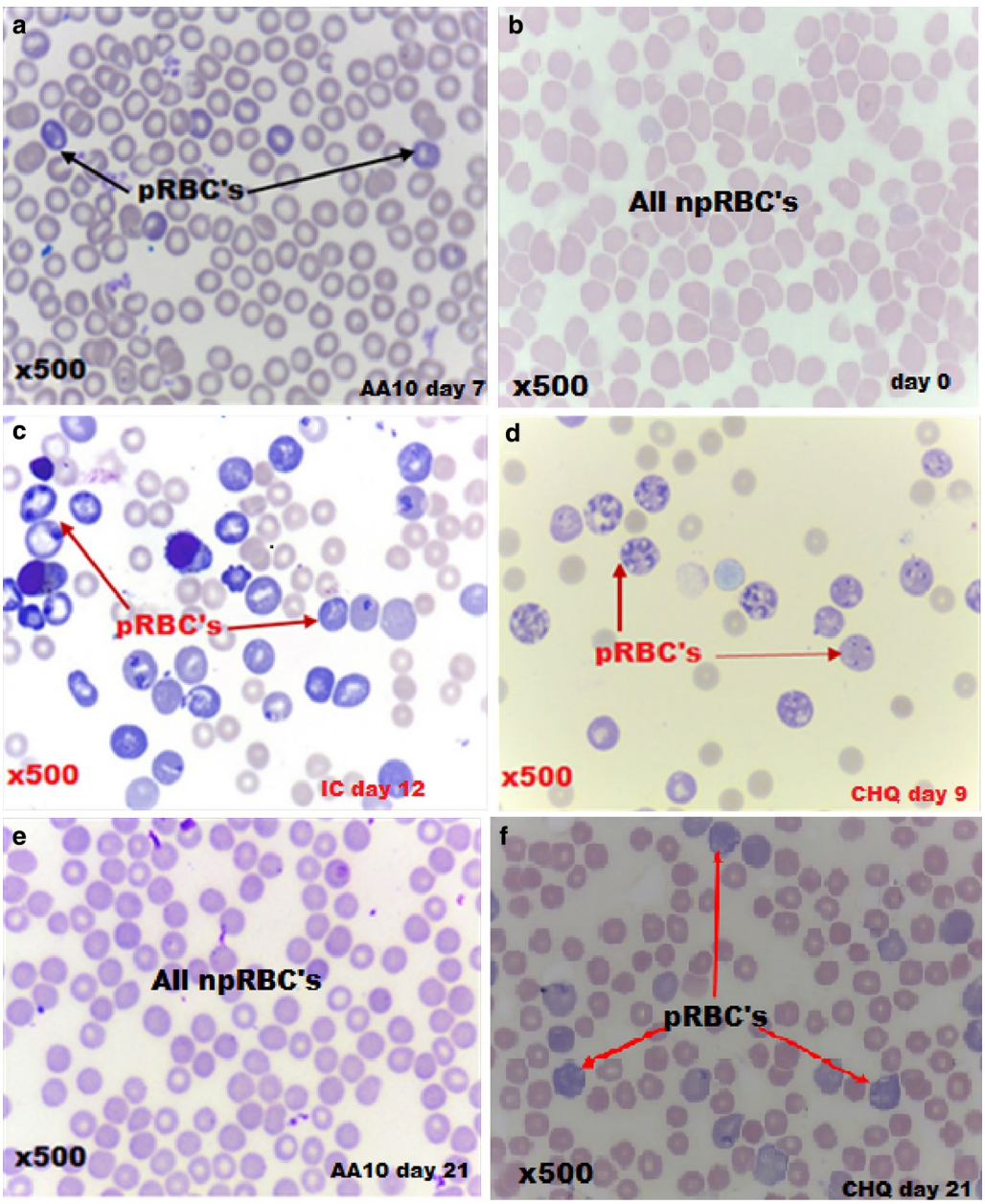

Fig. 1 Giemsa staining showing asiatic acid (10 mg/kg) influence on cellular morphology. Micrographs were before (day 0), during (day 7) and after malarial infection (day 21) and were compared to IC (day 12) and CHQ (days 9 and 21) controls. Slide are from: **a** AA 10 administered animals on day 7; **b** day 0; **c** IC day 12; **d** CHQ day 9; **e** AA 10 day 21; **f** CHQ day 21. Parasitized red blood cells (pRBCs) are indicated by *black arrows* in slide **a**, *red arrows* in slides **c**, **d** and **f**. Non-parasitized red blood cells (npRBCs) are shown in slides **b** and **e**. ×500 magnification used in all slides

research, that *P. berghei* causes SM proceeding to cerebral malaria (CM) in younger animals [23, 27–30]. Here, demonstrated is the novel influence of pre-infection po administration of AA on malaria in young, six weeks old SD rats (90–120 g) displaying hyperparasitaemia, SM and SMA.

Both murine malaria parasite (*P. berghei*) and SD rat malaria animal models conformed to a high predictive validity at days 3 and 7 in the IC and 30CHQ controls but not in the AA-administered group. Infection induction by an ip inoculation of 10^5 of pRBCs saline suspension invariably results in SM within 2 weeks, however this did not seem to happen in animals administered with AA (10 mg/kg) during the pre-infection period of 48 h. The pre-patent duration (the time it takes for parasites to

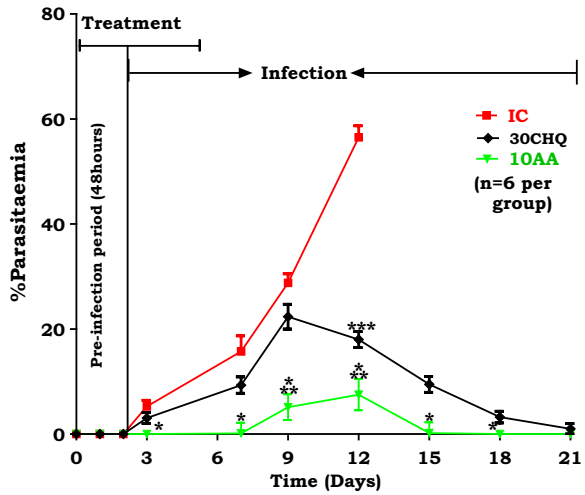

Fig. 2 Asiatic acid (10 mg/kg) influence on percentage parasitaemia compared to controls. Values are presented as mean ± SEM, (n = 6 per group). *, **p < 0.05 compared to IC and CHQ, respectively. ***p < 0.05 30CHQ compared to IC

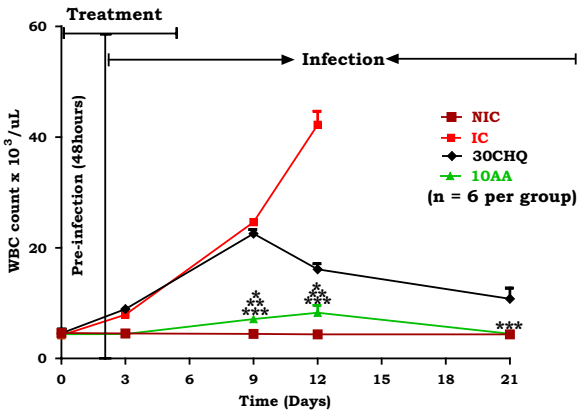

Fig. 4 Comparison of WBC count over time in AA10 administered animals with controls. Values are presented as mean ± SEM, (n = 6 per group). *NIC* non infected treated control, *IC* infected non-treated control, 30*CHQ* chloroquine 30 mg/kg. Values are presented as mean ± SEM, (n = 6 in each group). *, **, *** p < 0.05 by comparison with NIC, IC and 30CHQ-treated groups

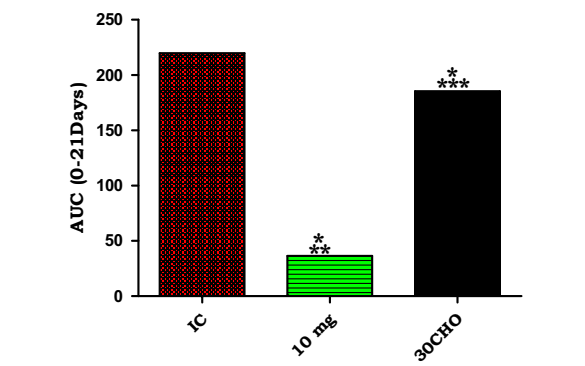

Fig. 3 Percentage parasitaemia-time AUC $_{(0-21\text{days})}$ for AA 10 mg/kg compared to controls. Controls included IC $AUC_{0-12\ days}$ and CHQ $AUC_{0-21\ days}$. *AA* asiatic acid, *IC* infected non-treated control, 30*CHQ* chloroquine 30 mg/kg. Values are presented as mean ± SEM, (n = 6 per group). *, **p < 0.05 compared to the IC and CHQ controls, respectively. ***p < 0.05 CHQ compared to IC

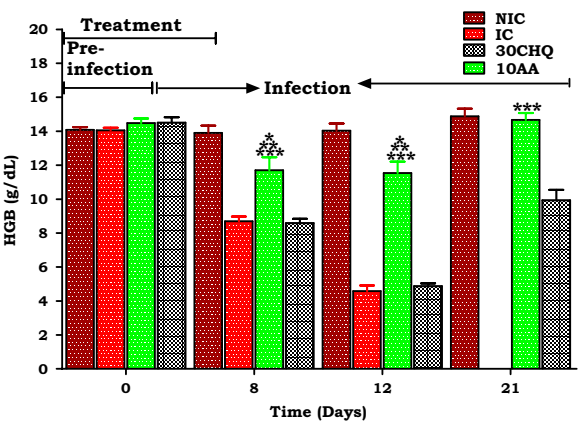

Fig. 5 Comparison of AA (10 mg/kg) administration on haemoglobin concentration with controls. Hb was estimated in *P. berghei*- infected AA10 treated SD rats with the NIC, IC and 30CHQ controls. *NIC* non infected treated control, *IC* infected non-treated control, 30*CHQ* chloroquine 30 mg/kg. Values are presented as mean ± SEM, (n = 6 in each group). *, **, *** p < 0.05 compared NIC, IC and CHQ, respectively

be detected in peripheral blood from the day of inoculation) is usually 72 h. This period was, however, prolonged by a further 3 days to a period beyond day 6 when AA (10 mg/kg) was administered 48 h before the animals were infected. The prevention of malaria could only be ascribed to the influence of AA as animal groups treated with CHQ (30 mg/kg) showed a malaria progression pattern as predicted [22]. Furthermore, subdued patent percentage parasitaemia, which failed to reach stable state malaria in AA (10 mg/kg) (Table 2)-administered groups, shows a possible cumulative concentration or effect of AA which continued to interact with the parasite well after administration of AA had ceased. This phenomenon was supported by observations of the percentage parasitaemia-time curve in this study (Fig. 2) which clearly showed AA10 administration causing a diminutive infection-time course compared to that of the IC and the CHQ controls. Notably, the IC percentage parasitaemia-time area under the curve covered only a maximum of 12 days, yet it was several times higher than that of AA10. Although others factors may be at play, the most probable cause of this difference may be attributable to

the influence of AA10 on parasitaemia development. The CHQ-positive control also showed a similar, albeit higher $AUC_{0-21\ days}$ than AA10, trend which effect may also be attributable to the influence of the drug on the percentage parasitaemia. The elongation of the bioavailability of AA in plasma, after oral administration, depends on a number of factors of which the amphiphilic nature of the triterpenoid is a major one [31, 32]. Indeed, AA percentage human intestinal absorption (% HIA) was predicted as 91.23 %, Caco-2 cell permeability as 20.97 nm sec^{-1} and plasma protein distribution as 96.45 %, suggesting well absorptivity, middle permeability and strong binding, respectively [33], which could account for possible AA accumulation in plasma relative to the albumin concentration [34–36]. Indeed, AA bioavailability and accumulation in circulation and tissues has been reported to increase with the duration of AA intake and with possible local and systemic protective effects [37]. This may explain how the parasitaemia was suppressed even when high erythrocytic-phase parasite inoculum (0.6–0.7 mL pRBC suspension), several-fold higher than human infection dose (50–100 µL), was used to establish malaria. An efficient chemoprophylaxis is expected to inhibit establishment of patent malaria. While this did not happen, the later clearance of parasitaemia may indicate that AA has suppressive or clinical prophylaxis and will require certain levels to be reached for efficacy to be achieved.

In the experiment by Yin et al. (2012) the recovery of intact AA from tissues and plasma after dietary intake which reached peak plasma concentrations quickly (0.5 h) after oral intake [37], the rapid metabolic rate of AA in rat liver microsomes and primary hepatocytes ($t_{1/2}$ = 9.493 min) and accompanying low AA bioavailability (16.25 % or 394.2 ng/mL) [38] further indicate that the phytochemical needs time to reach certain lethal levels against malaria. In the current study there was no indication of low bioavailability as the phytochemical managed to retard parasitaemia patency. Oral absorption of AA occurs throughout the small intestines with the highest absorption occurring in the jejunum [38]. Absorption is characterized by two peaks from a single dose inter-spaced by 8 hours, which could be attributable to the enterohepatic circulation [39] and avid binding by albumin [40].

Animals administered with AA (10 mg/kg AA) pre-infection besides suppressing parasitaemia, also preserved food and water intake as well as increased weight gain (Table 1). This may mean that po administration of AA is optimum at 10 mg/kg. Unlike some bitter tasting anti-malarials used for prophylaxis, AA is tasteless having most likely no effect on the brain-gut axis that senses the bitterness, inducing satiety, reduced food and water intake in treated animals [41–43]. Indeed, animals that received CHQ, which is bitter, posted reduced food and water intake as well as weight loss.

Infected animals that were not treated (IC) showed critically low food and water intake as well as negative weight gain. Prolonged reduced food intake results in increased breakdown of stored fat and proteins, production of keto acids with concurrent acidosis and increased oxidative stress (OS). These conditions weaken the animal, promoting parasitaemia, aspects which may have led to the spectacular percentage parasitaemia differences between the AA (10 mg/kg) and the IC. These same effects of hyperparasitaemia were also evident in the animals treated with 30CHQ showing lack of prophylaxis of the drug at this concentration. Furthermore, AA has been reported to have an anti-hyperglycaemic effect through attenuation of glycolytic enzymes and inhibition of glycogen phosphorylase [20]. Asiatic acid was administered in normoglycaemic animals. Consequently, AA inhibition of gluconeogenesis whilst increasing glucose oxidation (upregulation of glycolysis), resulted in energy deficits that could only be satisfied by exogenous sources. Animals administered with AA 10 mg/kg necessarily had to increase food and water intake, which resulted in an increase percentage weight gain, to avert hypoglycaemia. In other words, while the innate immune system combats the infection [44], continued food intake is paramount to alleviate parasitic effects [45] making AA's ability to increase feeding crucial in malaria [46]. Micronutrient malnutrition has been linked to malaria anaemia pathogenesis [47] and the three (malaria, malnutrition, anaemia) are the common face of childhood disease in many parts of the developing world [48].

To corroborate the reduced food intake was the retardation in RBC mass reduction, as shown by the SMA in thin blood smears (Fig. 1) as well as Hb measurements (Fig. 5), in animals administered with AA 10 mg/kg in comparison to the IC on day 12. There is a contrast between the RBC morphology on day 7 when compared to day 21 for the AA 10 mg/kg administered animals that reflects the slight slump in Hb observed on the earlier time period. Compared to the NIC this change in Hb in AA 10 mg/kg shows that no chemoprophylaxis agent may be 100 % effective all the time [1]. However, the low Hb observed in the IC demonstrates SMA caused by npRBC destruction [49], dyserythropoesis and/or ineffective erythropoiesis [50] and the general cachexia of the inflammatory disease [51] in the absence of effective chemoprophylaxis (Fig. 1c). Driving the hypochromic morphology observed with both IC and CHQ-treated animals (Fig. 1c, d) is a synchronous release of parasite pyrogens such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) that are also associated with anaemia, various pathologies and death from malaria

[52, 53]. While haemolysis results in reduced RBC mass, the more devastating effect is the release of merozoites which will infect more RBCs, inflammatory chemokines that upregulate leucocytosis, free Hb that rapidly inactivates nitric oxide with concomitant endothelium insults that follow which leads to vasoconstriction and damage to critical organs [54, 55]. Compared to the CHQ-treated and the IC groups, AA administration did avert anaemia showing also that npRBC haemolysis was inhibited and with it, deleterious inflammatory mediators were also suppressed. With malaria-induced anaemia being one of the major contributors to the 43 % anaemia prevalence in children between the age of six and 59 months [56], its inhibition by AA administration from developing in young rats, provides possible leads into preventive malarial disease management.

Elevated leucocytosis, a surrogate marker of inflammation, was observed in the IC and the CHQ-treated animals but not in the NIC or AA (10 mg/kg) administered animals (Fig. 4). In malaria, inflammation is initiated by the release of glycosylphosphatidylinositol (GPI) during or at the end of merogony or RBC death when the pRBCs rapture [51]. The parasite pleiotropic influence-exerting GPI induces high levels of cytokines (TNF-α, IL-1β, IL-6) release from macrophages, causing the pyrexia and cachexia of malaria when it substitutes for the host GPI-based signal transduction in regulating protein kinase C, calcium levels, cell adhesion and nitric oxide (NO) synthesis [57, 58]. Macrophages and polyclonal lymphocyte activation, which was observed as leucocytosis in the IC and CHQ treated animals (Fig. 4) but not in AA-administered animals, may be a reflection of the aberrant GPI hyperactivity and excitation of both pro-inflammatory and anti-inflammatory responses [59] involving the nuclear factor-Kβ (NF-Kβ) [60] in the malaria syndrome. With all this in perspective, it will be safe to infer that the prophylaxis administration of AA (10 mg/kg) did inhibit WBC count increase resulting from reduction in parasite infectivity, merogony abatement and subsequent insufficient GPI release as compared to the IC and CHQ-treated animals.

The founding principles of malaria lie in the successful activation of the immune system, incitement of the inflammatory cascade, abrogation of the haematopoietic function, systemic and endothelial changes with end organ failure, invariably initiated and orchestrated by an obligate intracellular protozoa [61, 62]. Therefore, it stands to reason that chemoprophylaxis approaches of malaria may of necessity focus on the prevention of these abnormalities from developing. While it might be impossible to have 100 % chemoprevention in malaria, infringement on the development of post-infection pathophysiology is crucial in keeping in check overt malaria disease occurrence [63]. Administered before infection or at the onset of the infection, AA10 may be able to avert the development of SM and the accompanying pathophysiology.

Conclusions

Presented here is data that demonstrate positive AA influence on food and water intake as well as percentage weight gain. Animals administered with AA (10 mg/kg) averted inflammation and severe malaria anaemia development. The anti-parasitic and anti-disease activities of AA in suppressing the parasite while inhibiting infection-induced pathology was evident. Administration of AA (10 mg/kg) showed a suppressive or clinical chemoprophylaxis better than chloroquine at 30 mg/kg, suggesting that AA may be used successfully in the prevention of malaria infection.

Abbreviations

AA: asciatic acid; ANOVA: analysis of variance; CA: *Centella asiatica*; CHQ: chloroquine; CM: cerebral malaria; DMSO: dimethyl sulfoxide; GPI: glycosylphosphatidylinositol; Hb: haemoglobin; HIA: human intestinal absorption; IC: infected non-treated control; ig: intragastric; ip: intraperitoneal; MA: maslinic acid; OA: oleanolic acid; OS: oxidative stress; po: per-oral; pRBC: parasitized red blood cell; SM: severe malaria; SMA: severe malaria anaemia; WBC: white blood cell.

Authors' contributions

MGA: research concept and design, collection and/or assembly of data, data analysis and interpretation, writing and critical revision of the manuscript. MBN: critical revision of the manuscript. MMV: critical revision of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Chapter 2:

Introduction to Chapter 2 (Article 3) and Bridging between Chapter 2 (Article 2) and Chapter 2 (Article 3)

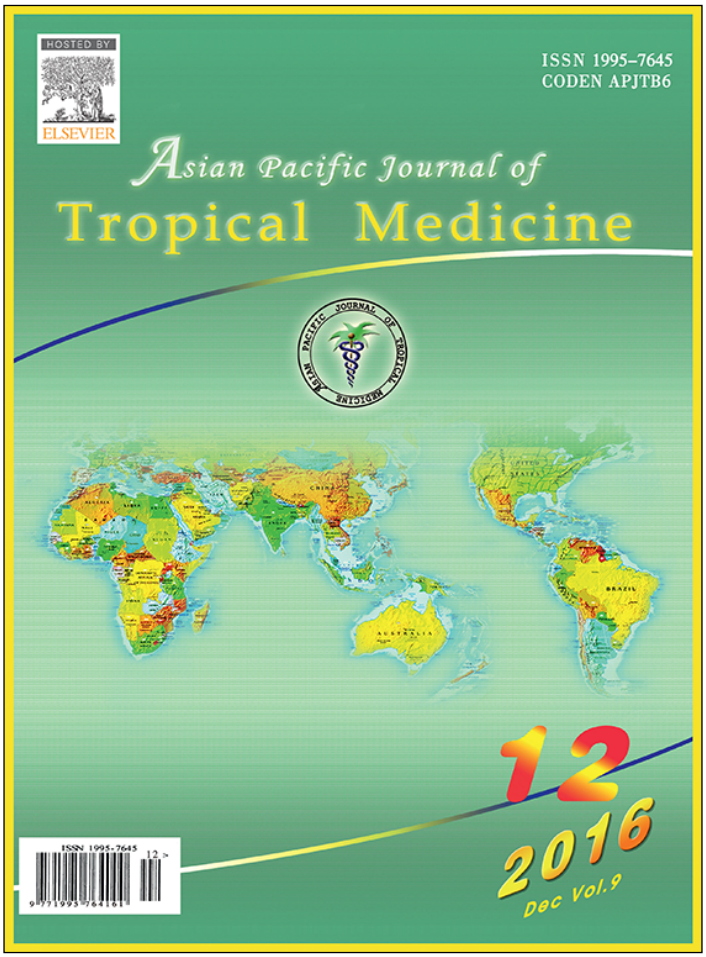
This chapter presents the findings on the Part III Article 3, of a three part chapter. The article has been accepted for publication and is in printing with the Asian Pacific Journal of Tropical Medicine (Ms. Ref. No.: APJTM-D-16-00801) and follows the format and instructions to the authors by the journal and goes by the title:

Asiatic acid-pectin hydrogel matrix patch transdermal delivery system influences parasitaemia suppression and inflammation reduction in *P. berghei* murine malaria infected Sprague-Dawley rats

The article begins with the title, contributing authors and their affiliations and contacts, corresponding author, followed by an abstract, key words, highlights and abbreviations. Thereafter comes the main body of the research including: Background, Materials and Methods, Results, Discussion and Conclusion. Figures, legends to figures, tables and legends to tables are included in the text. Acknowledgments, conflict of interests and references mark the end of the article.

Bridging gap between Chapter 2 (Article 2) and Chapter 2 (Article 3)

The novel prophylaxis effect of AA displayed in Chapter 2 Part II Article 2 buoyed us into experimenting with dose reduction techniques. An equally novel AA-amidated pectin hydrogel matrix patch was mooted as a means of decreasing the effect of AA low dissolution factor in aqueous medium. Transdermal drug delivery system-AA (TDDS-AA) patch aimed to reduce administered AA, the duration of administration and the frequency of dosing. These aspects enhance the novel antimalarial capacity of the triterpene as has been described here. Chapter 2 Part III (Article 3) describes a novel transdermal delivery system (TDDS), the AA-patch, for AA delivery through the epidermal layers of the skin. This is the first time that TDDS-AA is being described in malaria or any other condition. The reader is reminded that this innovative way of antimalarial delivery is a continuing effort in the search for new drugs delivery to combat malaria.



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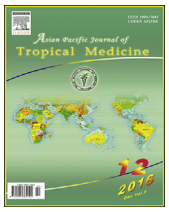

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Asiatic acid-pectin hydrogel matrix patch transdermal delivery system influences parasitaemia suppression and inflammation reduction in *P. berghei* murine malaria infected Sprague–Dawley rats

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ABSTRACT

Objective: To report the influence of transdermal delivery of asiatic acid (AA) in *Plasmodium berghei*-infected Sprague Dawley rats on physicochemical changes, %parasitaemia and associated pathophysiology.

Methods: A topical once-off AA (5, 10, and 20 mg/kg)- or chloroquine (CHQ)-pectin patch was applied on the shaven dorsal neck region of *Plasmodium berghei*-infected Sprague Dawley rats (90–120 g) on day 7 after infection. Eating and drinking habits, weight changes, malaria effects and %parasitaemia were compared among animal groups over 21 d.

Results: AA-pectin patch application preserved food and water intake together with % weight gain. All animals developed stable parasitaemia (15–20%) by day 7. AA doses suppressed parasitaemia significantly. AA 5 mg/kg patch was most effective. AA and CHQ displayed bimodal time-spaced peaks. CHQ patch had a longer time course to clear parasitaemia.

Conclusions: AA influences bio-physicochemical changes and parasitaemia suppression in dose dependent manner. In comparison by dose administered, AA has much better efficacy than CHQ. AA may be a useful antimalarial. AA and CHQ displays bimodal peaks suggesting possible synergism if used in combination therapy.

1. Introduction

Malaria still "rules the roost" in terms of the number of deaths it causes in children under five years and pregnant women, literally killing the unborn child [1,2]. The morbidities and mortality are caused by the parasite but much more by the pathophysiology of malaria or inadequate treatment, drug resistance, and drug induced toxicities [3]. Convoluted treatment regimens of malaria and frequency of dosing contribute significantly to malaria management failure [4]. Oral drug delivery is the major route by which most medication (74%) is administered as well as the standard method by which efficacy of a therapeutic agent is determined although the efficiency of this system is inadequate [5]. Novel methods of drug delivery, therefore become necessary to improve drug bioavailability and therapeutic efficacy [6]. Transdermal drug delivery system (TDDS) (the patch) has emerged as one of the novel means by which antimalarial drug may be delivered through the skin for systemic effects after overcoming the morphological, biophysical, and physicochemical characteristic of the skin barrier [7]. Advantages of TDDS include non-invasiveness, reduced dosing frequency, reduction of first pass hepatic metabolism, therapeutic enhancement and maintenance of steady state of drug concentrations in plasma [8–10]. Studies are ongoing on optimization of TDDS which has been patented for insulin and chloroquine (CHQ) delivery in diabetes mellitus and malaria, respectively [11,12]. The use of TDDS in malaria treatment with CHQ in a murine malaria model of *Plasmodium berghei* (*P. berghei*) was suggested to improve efficacy and ameliorate

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[3] Posthumously.

malaria pathophysiology [13]. Experimentation with maslinic acid (MA) and oleanolic acid (OA) TDDS has been embarked on (unpublished data). Furthermore, an oral aqueous-organic suspension of asiatic acid (AA, 10 mg/kg) has recently been shown to retard parasitaemia proliferation and a failure for %parasitaemia reach patent levels in a 21 d sub-chronic study [14] although the mode of action was not explored. AA is a triterpene with known anti-inflammatory and antinociceptive [15], anti-hyperglycaemia [16], immunoregulatory [17], haemodynamic modulation [18], inhibition of aberrant cell proliferation [19], anti-oxidant and pro-oxidant [20,21], and lipid peroxidation ameliorative properties [22].

The general principle of TDDS which makes it attractive in malaria treatment is the avoidance of single-pass hepatic metabolism and possible degradation that allows drugs to be delivered into the circulation directly, the smaller doses used and possible increased drug bioavailability in plasma. Buoyed by this knowledge, formulation (with some modifications) of an AA-pectin hydrogel matrix patch for the delivery of the amphiphilic triterpene via the skin was envisaged as an improved method for increased efficacy of the triterpene shown to be an effective suppressor of parasitaemia. The TDDS's sustained and controlled release of curative molecules was hypothesized as possible with AA due to its relatively moderate solubility in aqueous environment and its amphiphilic nature. Consequently, in a bid to increase AA bioavailability, reduce dosing frequency with concurrent diminution of the delivered dose, we purposed to investigate the effects of TDDS delivered AA in *P. berghei*-infected young (90–120 mg/kg) male Sprague Dawley (SD) rats. Here we present our findings on the influence of AA-pectin hydrogel matrix patch on food and water intake as well as %weight gain, its effects on malaria pathophysiology and parasitaemia reduction in a sub-chronic (21 d) study.

2. Materials and methods

2.1. Materials

2.1.1. Drugs, chemical and accessories

Different sources and suppliers were used for the procurement of drugs, chemicals and accessories but AA (>97% purity), Giemsa stain, dimethyl sulphoxide (DMSO), CHQ diphosphate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and reagents were of analytical grade.

2.1.2. Animals

Male SD rats (90–120 g) were obtained from Biomedical Research Unit (BRU) at University of KwaZulu-Natal where they were bred and housed for the entire experiment period. The animals were kept under maintained laboratory conditions of constant temperature [(22 ± 1) °C]; CO_2 (<5 000 ppm), humidity of (55 ± 5)% and illumination (12 h light/dark cycles). The animals had full access to food, standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water *ad libitum*. Animals were sacrificed by isofor (100 mg/kg) inhalation anaesthesia (Safeline Pharmaceuticals, Rooderport, South Africa) in a gas chamber by day 12 (infected none treated) and by day 21 (non-infected and infected treated animals). All experiments and protocols used in this study were reviewed and approved by the animal ethics committee of the University of KwaZulu-Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

2.1.3. Plasmodium murine malaria model

CHQ-susceptible strain of *P. berghei* ANKA, murine malaria parasite was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). The parasite was sub-cultured, harvested, and stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until use.

2.1.4. Experimental protocol design

Animals were inoculated at day 0, treated at day 7 and monitored up to day 12 for infected but non-treated control group (IC group) and day 21 for the non-infected control group (NIC group) and AA groups including AA-pectin patch applied groups at 5, 10, and 20 mg/kg doses. Animals groups sacrificed on day 0, 3, 9, 12, and 21. The protocol design was shown in Figure 1.

2.2. Methods

2.2.1. Induction of parasitaemia

P. berghei [10^5 parasitized red blood cells (pRBC's) suspension in saline] was used as an intraperitoneal (ip) inoculum [23]. Control animals received equivalent amount of saline.

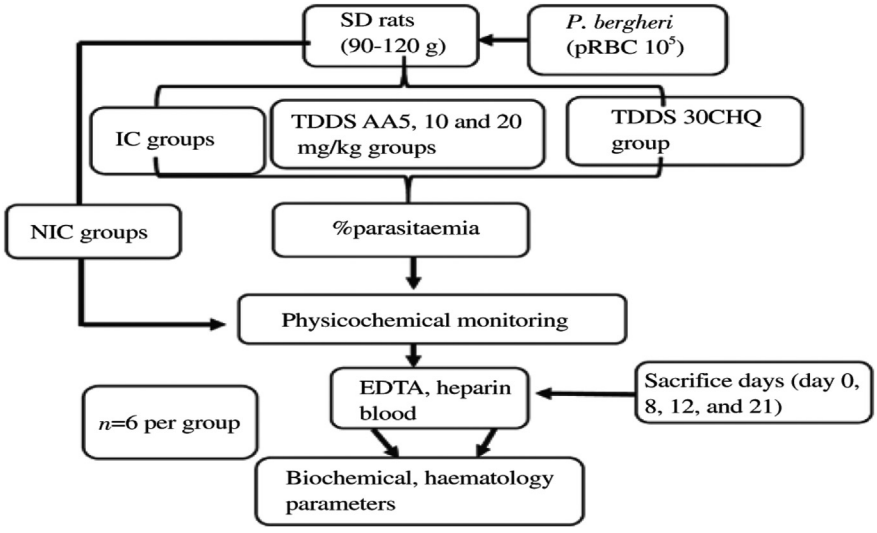


Figure 1. Flow diagram for the experimental protocol and design.
30CHQ was the 30 mg/kg CHQ TDDS treated group.

2.2.2. Parasitaemia monitoring

Peripheral blood was obtained by tail prick method and thin smears of rat blood were used in monitoring parasitaemia. Malaria monitoring consisted of Giemsa staining of the thin smear and examination under a microscope (Olympus Cooperation, Tokyo, Japan). The actual number of pRBC's relative to 2×10^4 RBC's was used to calculate %parasitaemia [24]. Change in % parasitaemia was monitored at 72 h (pre-patent period), every third day up to day 7 (patent period) [25], every day during treatment period for five days and thereafter every other day post-treatment period until day 21.

2.2.3. Evaluation of parasitaemia

Appearance of parasites in the blood after ip inoculation takes 2–3 d [24]. Two set points were used in the study to determine the performance of the malaria model. These were time-point at which malaria parasites were first detected in peripheral blood (pre-patent) which was 72 h post-infection and a parasitaemia of 15–20% on day 7, without intervention [26], signifying a stable state of severe malaria (SM).

2.2.4. Preparation of pectin patches for transdermal delivery system

Amidated pectin hydrogel matrix patches were prepared using a previously described protocol by Musabayane *et al.* [7], with slight modifications. Low methoxyl amidated pectin with a degree of esterification (DE) of 23% and an amidation of 24% was used for the preparation of the AA and CHQ patches. In separate beakers, water (110 mL) was used to dissolve either 30 mg/kg CHQ or AA (5, 10, and 20 mg/kg) with pectin (4.4 g) and agitated at 37 °C in a water bath at a speed of 38× G using an electric motor mixing rotor (Heidolph instruments GmbH & Co. KG, Schwabach, Germany) for 15 min. DMSO, vitamin E, and eucalyptus oil were added to the mixture, sequentially with continuous mixing. The mixture (11 mL) was pipetted into petri dishes and frozen at −81 °C for 18 h, following which a 2% $CaCl_2$ solution was added at room temperature (±25 °C) for matrix cross-linking. The patches were stored at 4 °C until use.

2.2.5. Application of the patch

Three discs [(4 ± 1) mm^2] were punched out from the different AA concentration (5, 10, and 20 mg/kg) patches or CHQ (30 mg/kg) and applied once-off onto the shaved dorsal region of the animal from day 7 to day 10 (three days). The jacket holding the patch in place was made from clinically sterile adhesive fabric plaster (Mediplast, Neomedic, Rickmansworth, and Herefordshire, UK) which caused no discomfort to the animals. The dorsal neck region was selected because it was the least accessible to the animal grooming habits and avoided removal of the patch by the animal. The TDDS aimed at reducing phytochemical amount delivered to the animal, dosing frequency, treatment duration and general animal discomfiture. A theoretical total AA yield of patch was estimated at 1 μg/per disc for 5 mg/kg AA dose and 3 μg was administered, therefore, per animal once-off to provide five day treatment. Other doses were multiples of this calculation.

2.2.6. Influence of AA-pectin patch application on % parasitaemia

A comparison was made between the different doses of topical AA (5, 10, and 20 mg/kg)-pectin patch, 30CHQ-pectin patch and the IC on changes of %parasitaemia over the 21 d period.

2.2.7. Haematological analysis

All animals were sacrificed by exposing to isofor (100 mg/kg) inhalation anaesthesia (Safeline Pharmaceuticals, Roodeport, South Africa) on day 0, 9, 12 and at the end of the study at day 21. Blood samples were collected by cardiac puncture into Na_2EDTA tubes for whole blood analysis. Liver, kidney, muscle and heart were removed, snap frozen in liquid nitrogen and stored together with the plasma in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80 °C until use in other studies.

2.2.8. Influence of AA-pectin patch on inflammation

Whole blood was analysed in a haematology four part white cell count (WBC) differential analyser (Coulter-Bachmann). WBC levels were used to estimate inflammatory response to malaria in AA-pectin patch applied animals, the NIC, IC, and CHQ-patch treated groups over time (21 d).

2.2.9. Influence of AA on inflammation in malaria C-reactive protein (CRP)

Inflammation is the bed rock to malaria pathophysiology. To indicate the influence AA (10 mg/kg) on inflammation, CRP was estimated using the Elabscience Rat hs-CRP (High Sensitive C-Reactive Protein) ELISA kit Catlog No: E-EL-R0506 (Elabscience Biotechnology Co. Ltd ELISA, Wuhan, P.R.C.) according to the manufacturer's instruction.

2.2.10. Influence of AA on severe malaria anaemia (SMA)

Anaemia presents as a low red blood cell mass relative to plasma. Haematocrit (Hct) is a compartmentalization of cellular components of whole blood expressed as a percentage such that the lower the percentage, the more severe the anaemia. Hct, therefore, as a surrogate marker for SMA was compared amongst the groups of topical AA (5, 10, and 20 mg/kg)-pectin hydrogel matrix patch applied, 30CHQ-pectin matrix patch treated, the NIC and the IC groups.

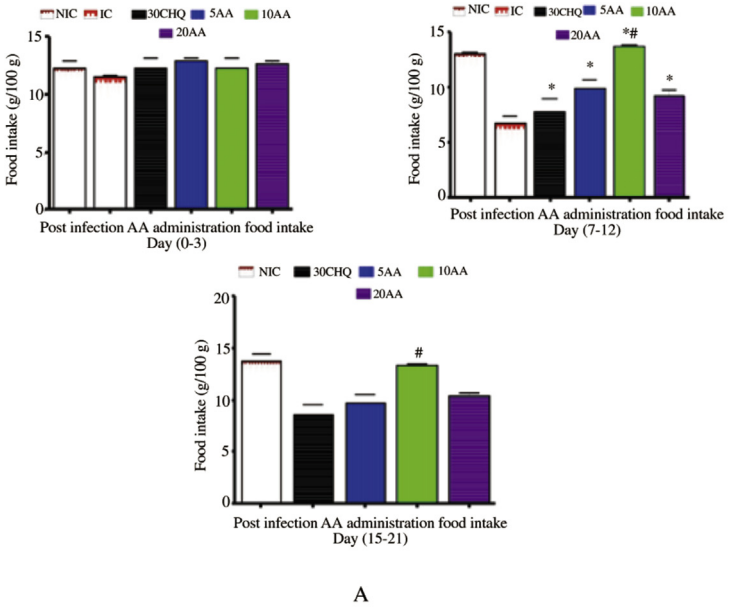
2.3. Statistical analysis

Unless otherwise stated, data was presented as standard error of the mean (SEM). Statistical comparisons performed by one way analysis of variances (ANOVA), followed by Tukey–Kramer multiple comparison ad hoc test using Graph-pad InStat Software (version 5, GraphPad Software, San Diego, California USA). A $P < 0.05$ was considered statistically significant difference.

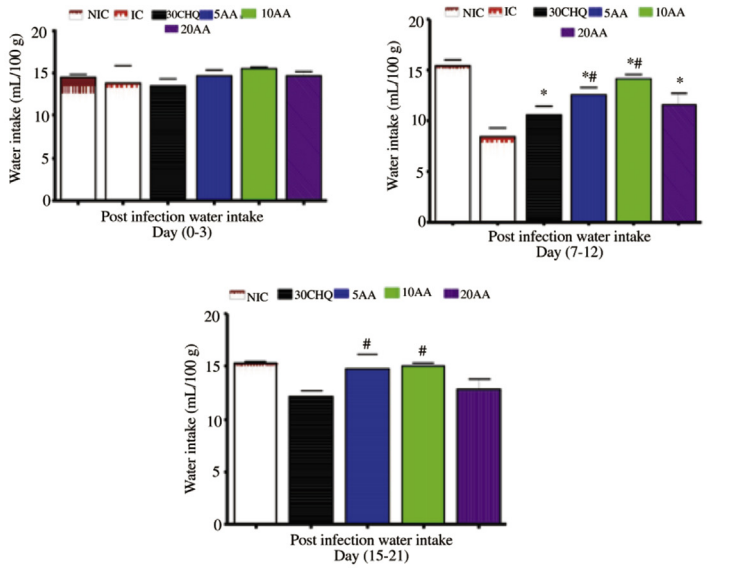
3. Results

3.1. Influence of AA on bio-physicochemical properties

Results of animals applied a once-off topical AA (5, 10, and 20 mg/kg)-pectin hydrogel matrix patch on changes of eating, drinking habits and %weight gain during the 21 d sub-chronic study were shown in Figure 2A, B and C. AA-pectin patch significantly preserved food and water intake together with increased %weight gain compared to IC and 30CHQ patch



A



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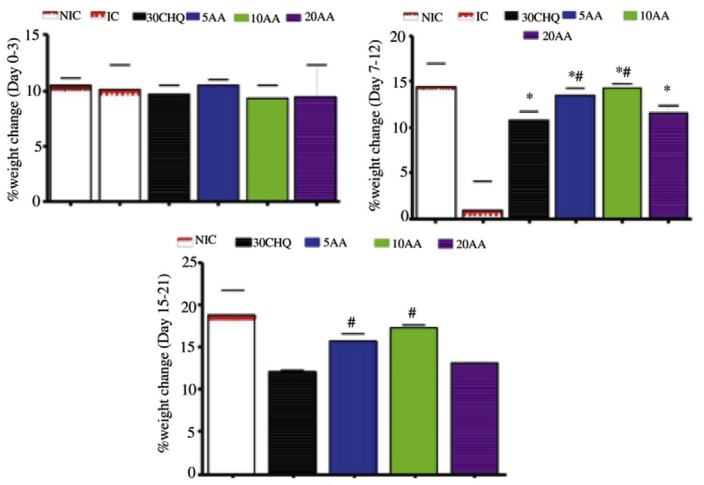

C

Figure 2. Comparison of AA influence on food intake, water take and %weight change over 21 d period (n = 6).
IC was infected but non-treated control group; NIC was non-infected control group; 30CHQ was the 30 mg/kg CHQ TDDS treated groups; 5AA, 10AA, and 20AA represented AA-pectin patch applied groups at 5, 10, and 20 mg/kg doses, respectively. A: food take; B: water take; C: %weight. $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to 30CHQ patch.

($P < 0.05$, respectively) during the treatment period. Compared to 30CHQ patch, IC significantly decreased food and water intake as well as %weight gain ($P < 0.05$) by day 12. Compared to the NIC, 30CHQ patch had significantly decreased food and water intake as well as %weight gain ($P < 0.05$) by day 21.

3.2. Validation of parasitaemia

Comparison of the various time points against the animal groups' %parasitaemia was shown in Table 1. AA (5, 10, and 20 mg/kg)-pectin patch application had significantly higher % parasitaemia at day 7 compared to the 30CHQ-pectin patch treatment ($P < 0.05$). Compared to the IC, AA (5, 10, and 20 mg/kg)-pectin patch had significantly lower %parasitaemia at peak period ($P < 0.05$). Time to peak %parasitaemia was significantly longer in 5AA, 10AA, and 20AA-pectin patch application than 30CHQ-pectin patch treatment ($P < 0.05$). AA (5 mg/kg)-pectin patch had a significantly lower peak %parasitaemia compared to 30CHQ-pectin patch ($P < 0.05$). Compared to the IC, 30CHQ patch had a significantly lower peak %parasitaemia ($P < 0.05$). Parasitaemia suppression continued until parasites were non-detectable by microscopy at day 18 for 5AA administered animals but persisted in 30CHQ treated animals.

3.3. AA-pectin patch application influence on % parasitaemia

A comparison of the influence of AA-pectin patch with controls was given in Figure 3. AA (5, 10, and 20 mg/kg)-pectin patch significantly reduced parasitaemia compared to IC ($P < 0.05$) at all relevant time points. In comparison to the 30CHQ-pectin patch treatment, AA (5 mg/kg)-pectin patch significantly lowered parasitaemia ($P < 0.05$) at all pertinent time points. Compared IC, 30CHQ patch had significantly lower %parasitaemia ($P < 0.05$) at day 10 and 12. AA and CHQ administration displayed significantly different bimodal peak parasitaemia time points.

3.4. AA-pectin patch application and %parasitaemia-time area under the curve (AUC)

Area under the curve for %parasitaemia and time [$AUC_{(0–21\ d)}$] showed the time course of AA administration relative to controls. A comparison of the time-course influence of AA on malaria was made in Figure 4. AA (5 mg/kg)-pectin patch displayed significantly lower $AUC_{(0–21\ d)}$ compared to the $ICAUC_{(0–12\ d)}$ ($P < 0.05$). Compared to the 30CHQ, AA (5, 10 mg/kg)-pectin patch had significantly lower AUC($P < 0.05$). Despite a shorter time period, $ICAUC_{(0–12\ d)}$ was equal to 10AA $AUC_{(0–21\ d)}$.

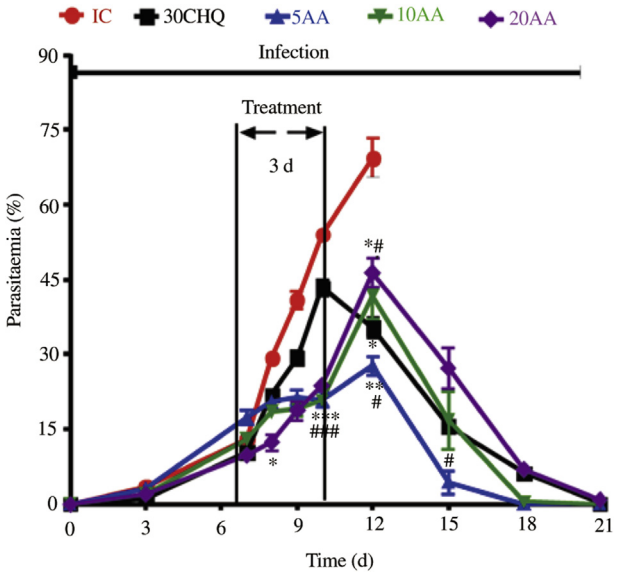

Figure 3. TDDS AA influence on %parasitaemia compared to controls ($n = 6$).
*$P < 0.05$, compared to the IC; #$P < 0.05$, compared to CHQ control.

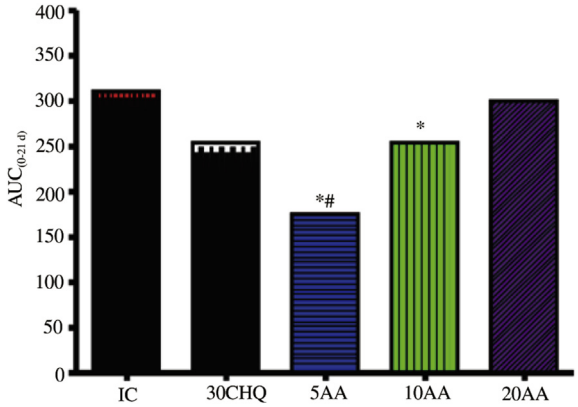

Figure 4. Area under the %parasitaemia-time curve [$AUC_{(0–21\ d)}$] for % parasitaemia in the pre-infection AA transdermal delivery system AA administration protocol ($n = 6$).
*$P < 0.05$, compared to the IC; #$P < 0.05$, compared 30CHQ control.

3.5. AA-pectin patch influence on inflammation

WBC count showed the presence or absence of immune response and/or inflammation. The effect of AA-pectin patch application on WBC counts over the 21 d study was compared to control in Table 2. At relevant time points AA (5, 10 mg/kg)-pectin patch significantly reduced WBC count compared to IC ($P < 0.05$). AA (5 mg/kg)-pectin patch reduced WBC count significantly in comparison to 30CHQ ($P < 0.05$) at all relevant time points. AA (10, 20 mg/kg)-pectin patch decreased WBC

Table 1
Comparison of %parasitaemia at different time points among animal groups ($n = 6$).

Protocol	Dose	Pre-patent parasitaemia (h)	%parasitaemia day 3	%parasitaemia day 7	Parasitaemia at peak (%)	Peak period (d)	Parasitaemia suppression (d)
Post-infection	IC	72	3.5 ± 0.6	13.0 ± 3.3	73.3 ± 6.7	N/A	N/A
TDDS	30CHQ	72	2.3 ± 0.3	10.3 ± 1.1	45.7 ± 1.8*	9	21>
administration	5AA	72	3.9 ± 2.2	21.3 ± 3.7#	24.5 ± 2.6*#	12#	<18
	10AA	72	1.9 ± 0.8	18.4 ± 3.7#	43.6 ± 4.5*	12#	18
	20AA	72	2.6 ± 2.4	12.4 ± 4.6	47.5 ± 3.4*	12#	18>

*$P < 0.05$, compared to IC; #$P < 0.05$, compared to 30CHQ. N/A means this criteria is not applicable; 18 means suppression of parasitaemia was attained on day 18; <18 and 18> means parasitaemia suppression was obtained before and after day 18, respectively; 21> means parasitaemia suppression was not attained at day 21.

Table 2
Comparison ofWBC count at pre-patent, patent, treatment, and post-treatment periods amongst the different animal groups (n = 6).

Protocol	Dose	Pre-patent period WBC	Patent period WBC	Treatment period WBC	Post-treatment period WBC
Post-infection TDDS treatment	NIC	4.6 ± 0.3	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.2
	IC	4.5 ± 0.3	14.6 ± 0.5	48.2 ± 3.3	N/A
	30CHQ	4.8 ± 2.8	17.6 ± 3.5$^{\alpha}$	36.1 ± 4.9$^{\alpha}$	10.8 ± 1.9$^{\alpha}$
	5AA	4.7 ± 0.8	7.1 ± 1.9$^{*\#}$	6.8 ± 0.1$^{*\#}$	4.5 ± 0.3$^{\#}$
	10AA	4.7 ± 0.1	10.7 ± 1.6$^{*\#}$	25.8 ± 2.5$^{*\#}$	3.4 ± 3.1$^{\#}$
	20AA	4.3 ± 0.1	14.9 ± 3.2$^{\alpha}$	34.9 ± 4.3$^{*\#}$	2.5 ± 0.2$^{\#\alpha}$

$^{\alpha}P < 0.05$, compared to NIC; $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$ compared to 30CHQ.

count significantly compared to 30CHQ ($P < 0.05$). Compared to the IC, 30CHQ patch had significantly higher WBC count ($P < 0.05$) at all relevant times.

3.6. Plasma CRP

CRP depicted the presence or absence of inflammatory response in malaria. The influence of 10AA on CRP concentration compared to controls in Figure 5. The 10AA administration reduced CRP concentrations significantly compared to the IC and CHQ treated animals ($P < 0.05$). There was no relative change in NIC compared to either the NIC or the CHQ.

3.7. AA-pectin patch influence on SMA

Haemtocrit was a positive indicator of anaemia presence. The influence of AA (5 mg/kg)-pectin patch on Hct over the duration of the study was made in Figure 6. Compared to the IC, AA (5 mg/kg)-pectin patch significantly preserved Hct ($P < 0.05$) during treatment and post-treatment periods. AA (5 mg/kg) preserved Hct compared to 30CHQ ($P < 0.05$) at all relevant time points. The 30CHQ patch had significantly lower Hct compared to the NIC ($P < 0.05$) (Figure 6).

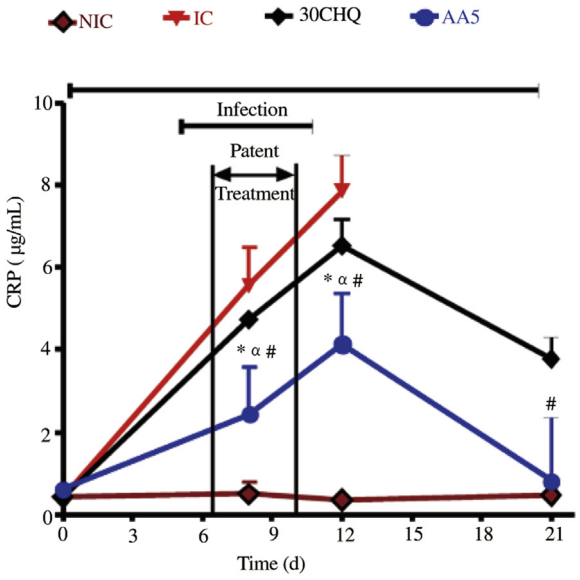

Figure 5. Changes of CRP concentration in 10AA administered animals compared to controls.
αP < 0.05, compared to the NIC; $^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to CHQ.

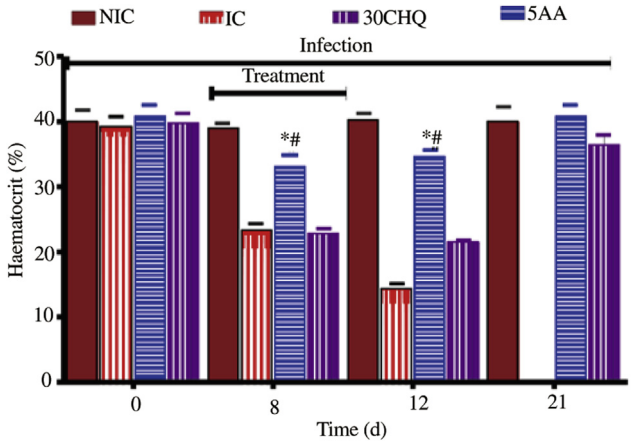

Figure 6. Comparison of AA-pectin patch application influence on Hct amongst the different animal groups (n = 6).
$^{*}P < 0.05$, compared to IC; $^{\#}P < 0.05$, compared to 30CHQ.

4. Discussion

Natural AA has relatively moderate dissolution factor in aqueous media that subsequently results in low bioavailability when administered by the oral route [27]. Pursuant to the quest of increasing the bioavailability of AA, a novel method of delivering the phytochemical in *P. berghei*-infected SD male rats was developed in this study.

Using young, 6 weeks old SD rats (90–120 g) which displayed SM, SMA, and in some CM [26,28] topical application of AA (5, 10, 20 mg/kg)-pectin hydrogel matrix patch suppressed parasitaemia to undetectable levels by day 21 post-infection and preserved the biophysical properties of the animals in the face of SM infection. Preservation of eating and drinking habits by TDDS AA administration may have had profound effect on the pathophysiology in general. This phenomenon is corroborated by findings that pre-infection AA 10 mg/kg oral administration retarded patent parasitaemia development with *P. berghei* infection [14]. In a similar report, post-infection AA (10 mg/kg) influenced glucose metabolism favourably [29]. The %parasitaemia showed bimodal peaks with 30CHQ and AA-pectin patches showing peaks at day 10 and 12, respectively. AA5-pectin patch displayed a significantly lower %parasitaemia-time area under the curve [$AUC_{(0-21\ d)}$]. Of note is that even though the IC animals were sacrificed by day 12 the % parasitaemia-time course was markedly higher to that of 5AA and 10AA $AUC_{(0-21\ d)}$, but equal to 20AA $AUC_{(0-21\ d)}$ showing that the administration of AA may have influenced this phenomenon. Indeed, this was also apparent with CHQ treatment

which posted comparable $AUC_{(0–21\ d)}$ to 10AA which was significantly higher that the 5AA $AUC_{(0–21\ d)}$. Compared to the 30CHQ patch, the drug load and efficacy ratio was favourable towards 5AA, equal to 10AA, and lower for 20AA when the phytochemical was administered by TDDS. The disparity observed needs further clarity through more investigations with special emphasis on the molecular dynamics and interactions of key proteins in the malaria parasite to AA.

Infection effectuation by an ip inoculation of 10^5 pRBC's saline suspension resulted in SM indicated by decreased Hct in the IC. There was a clear distinction of the influence of AA-pectin patch on SM when comparing to changes from day 7–21. In malaria, anaemia changes precede the actual overt malaria with low blood count indices and increased plasma volume from increased fibrinogen synthesis by the liver to counterbalance low whole blood viscosity by increasing plasma viscosity resulting in little to insignificant change of whole blood viscosity in SMA with characteristic hypovolaemia [30]. This is in agreement with observations that the rapture of pRBC's upon merozoite release play minor role in the early induction of anaemia which is contributed mainly by the loss of npRBC's [31] and impaired erythropoiesis [32]. Dyserythropoiesis on its own, however, may likely contribute little in SM aetiology [33] but will be a critical and predominant feature in chronic carriage of the parasite [34] where the haemodynamics studies revealed an initial hypovolaemia followed by hypervolaemia and normovolaemia [30]. Parasitaemia clearance does not automatically normalise Hct which may continue to fall [35] suggesting that other factors may be at play in the orchestration of malaria anaemia with increased destruction of RBC's and decreased synthesis. The production of hemozoin, a haemoglobin biocrystallization product, has also been implicated in immunological responses and bone marrow dyserythropoiesis [36] and cellularity manipulation towards myeloid lineage cell proliferation resulting in the erythroid lineage cell hypoplasia with consequent reduction in some haematological indices [37,38]. The observed Hct reduction, followed by subsequent resolution of SMA through AA intervention, is usually a chronic malaria processes in human beings.

Stretching the imagination somewhat, the 7th day when patent malaria was observed may actually be a chronic infection of 186.9 d [39], if the rat age to human conversion theorem is anything to go by [40]. Therefore, the anaemia displayed by Hct may have been a reflection of chronic anaemia of malaria which was controlled by the application of the AA-pectin patch. Furthermore, the reduced food intake, which may have been contributed by malaria anorexia and bitterness of CHQ [41], could have caused the reduction in Hct in IC and CHQ-pectin patch treated animals, respectively. While the TDDS of CHQ could have bypassed the gut where the bitter substance-sensing receptors lie, the entero-hepatic circulation may bring the drug into contact with the receptors several times over, with possibly the same effect. Consequently, animals treated with CHQ may tend to have appetite suppression which may be worsened by reduced drug inficacies. This understanding may counteracts earlier assertions that TDDS CHQ delivery camouflages the bitterness of the drug that could reduce compliance with treatment [7]. This may highlight the need to punctuate treatment with parenteral feeding when TDDS CHQ is used in malaria taking into consideration as well the hypoglycaemic effect of CHQ [42,43].

Administration of AA-pectin patch showed that the lower dose of AA (5 mg/kg) reduced %parasitaemia as well as inflammation and reduced leucocytosis to a significantly greater extent than the other two doses and 30CHQ. While there are possibly many reasons for this phenomenon, two speculative explanations are in order. First, the loading dose of AA in the pectin, although lower, may allow for a faster release of the phytochemical, accumulating relatively earlier in plasma than higher doses. However, diffusion kinetics will rather instruct that higher solute concentration have a higher capacity of delivery than the lower ones. Second, the higher doses carrying patches may result in the delivery of higher AA concentration to the extent that the anti-oxidant and pro-oxidant capacity of AA may favour parasite proliferation than its eradication. On the other hand lower patch with a lower dose may just deliver an optimum amount of AA to overwhelm parasite defences. Indeed, inflammatory processes in malaria are pivotal in the marshalling malaria pathogenesis. Inflammatory cytokines [macrophage migration inhibitory factor (MIF), tumour necrosis factor-α (TNF-α), IFN-γ, IL-12 may contribute to protection or pathology depending on production site, production timing, produced levels together with the presence or absence of immunoregulatory, *e.g.* cytokine IL-10 [25,44]. Unregulated expression of iNOS may result in vascular permeability, aberrant reactive oxygen species (ROS) production, peroxynitrite ($ONOO^-$) synthesis, sodium wasting. These lead to hypovolaemia and generalised tissue damage with multi organ failure, which are common in malaria. Stress is a common phenomenon in malaria which upregulates cortisol synthesis driven most likely by βHSD-1 with increases in gluconeogenesis and increase in glucose concentration [45] that increases oxidative stress (OS). Anorexia-induced cachexia invariably increases lipid breakdown and hyperlipidaemia upregulating HMG CoA reductase. Taken together, the anti-inflammatory, immunomodulatory, anti-oxidant, anti-hyperlipidaemic, and pro-oxidant properties if AA may have formulated the combined mechanism by which the phytochemical suppressed parasitaemia and ameliorated malaria pathophysiology.

The concept of inflammation in malaria was well articulated by the influence of AA (5, 10, and 20 mg/kg)-pectin patch application where both %parasitaemia, WBC count, and CRP were proportionally and progressively linked at corresponding time points. A fall in %parasitaemia was associated with a concomitant fall in WBC count and indirectly showing a decrease in inflammatory response. This may be plausible if inflammation may be regarded as a result of parasite antigen presentation to the innate immune system with subsequent production of inflammatory mediators that drive the cycle of more parasite production leading to more inflammation. The concentration of CRP throughout sub-chronic study indicated acute inflammation at day 12 that coincided with peak %parasitaemia, declining critically showing abrogation of inflammation or inflammatory insult by 5AA-pectin patch. Therefore, intervention that either infringes upon either the parasite or the inflammatory cascade or both may invariable cause a decline of the other by mutual association. In like manner, AA-pectin patch may have influenced both or either as we have observed a decline in %parasitaemia that has a reciprocal dose dependent with the least dose being the most efficacious of the three doses. However, the question of which parameter is affected first and then influence the other is fundamental in unravelling the effect of AA in malaria, or whether these are parallel processes.

The anti-inflammatory effect of AA has been reported in animal models and cell lines [46]. The 5AA-pectin hydrogel patch in decreasing WBC count and CRP was either influenced by a decrease in parasitaemia through abrogation of inflammation or retraction of inflammation from the disease milieu could have led to a decrease in parasitaemia. Similar phenomenon with AA has been shown where it induced activated T cell apoptosis to control fulminant hepatitis [17].

The pharmacodynamics of AA5 may be that the patch slow release maintained plasma AA concentration exerting both oxidative and the anti-oxidant effects concurrently. Hypothetically, the ratio of the oxidant and anti-oxidant capacity may dictate which facet of AA predominates. At medium (10 mg/kg) and higher (20 mg/kg) doses, TDDS may deliver higher levels of AA which may be toxic and exacerbate disease. This may account for the lower dose (5 mg/kg) being more effective compared to the medium and higher doses. Furthermore, WBC count was lowered in a dose dependent manner with the 20AA-pectin patch displaying the least count corresponding to a higher %parasitaemia-time $AUC_{(0–21\ d)}$ and lower %parasitaemia post-treatment decline. Moreover, AA has been observed to induce cytotoxic apoptosis and cell arrest in human breast cancer through the activation of extracellular signalling kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) [47], which may be the process by which lowed WBC count were observed with 20AA-pectin patch. However, the exact anti-disease mechanism of 5AA-pectin patch needs further research.

While the formation of ROS, nitric oxide (NO), and $ONOO^-$ may abate and reduce inflammatory response, host immune system necessary for phagocytosis of pRBC's, may be compromised through WBC depletion with lowered antimalarial efficacy. This may, however, not explain how the lower dose of AA (5 mg/kg) had a higher antimalarial efficacy in TDDS when the higher doses did not, contrary to our anticipation had lower efficacy. With oral AA administration it was observed in recent studies that the medium dose (10AA) was most efficacious in retardation of parasitaemia progression and control of malaria pathophysiology [14].

Bimodal peaks in %parasitaemia were conspicuous in this study. Treatment with 30CHQ patch had a steep rise in parasitaemia peaking at day 10 and gradually descending. On the other hand, AA-pectin patch displayed a reciprocal packing order with %parasitaemia precipitously decreasing in animals to which 5AA-pectin patch was applied. The shorter application time of three days for both AA and CHQ, compared to the five day treatment of twice daily dosing with CHQ and once daily with AA in po administration, portrayed a possible superior efficacy of TDDS in malaria. The bimodal peaks may show that the two compounds have different pharmacodynamics which could work well as double or triple combination therapy to avert drug resistance and prolong the useful life span of novel antimalarials [2].

In conclusion, our data demonstrates AA influence on % parasitaemia in murine malaria of SD rats. AA may have both anti-parasitic and anti-malarial disease activities supressing the parasite while ameliorating infection-induced pathology. Administration of AA post-infection suppressed parasitaemia by TDDS with the AA5-amidated pectin hydrogel matrix patch showing the most efficacy in anti-inflammatory response suppression, SMA amelioration, and general physicochemical properties outcomes. This may suggest that the 5AA-pectin patch may have both anti-parasitic and anti-disease facets necessary for a wholesome approach to malaria treatment seeing that no other supportive treatment was given to these animals besides patch application. The display of two distinct peak times in %parasitaemia progression may be attributable to different modes of action of the two compounds at the experiments concentrations and dosing regimens. Preservation of food and water intake, as well as increase in %weight gain in 5AA-pectin patch applied animals may indicate that the animals' appetite was not affected by sickness behaviour seen to be common in inflammatory and cachectic conditions like malaria.

Conflict of interest statement

We declare that we have no conflict of interest.

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Chapter 3:
Introduction to Chapter 3 (Article 4)

This chapter presents the findings presented in Article 4 which has been published in the Africa Journal of Traditional Complementary and Alternative Medicine (AJTCAM) a peer reviewed journal. Mavondo Afr J Tradit Complement Altern Med. (2016) 13(5):91-101 Doi:10.21010/ajtcam.v13n5.13. The article goes by the title:

Asiatic acid influences glucose homeostasis in *P. berghei* murine malaria infected Sprague-Dawley rats.

The article begins with the title, contributing authors and their affiliations and contacts, corresponding author, followed by an abstract, key words, highlights and abbreviations. Thereafter comes the main body of the research including: Background, Materials and Methods, Results, Discussion, Conclusion and References. Figures, legends to figures, tables and legends to tables are included in the text. Declarations are included, acknowledgments, conflict of interests and references mark the end of the article.

Bridging the gap between Chapter 2 (Articles 1-3) and Chapter 3 (Article 4):

Chapter 2 did show, in three different ways, that AA administration (oral pre-infection, oral post-infection, post-infection transdermal drug delivery system (TDDS) supressed parasitaemia efficiently at AA10mg/kg po and AA5mg/kg by TDDS. However, the ameliorative effect of AA on hypoglycaemia, which is common in malaria, has not been demonstrated as an anti-disease potential of the phytochemical. What has been reported in glucose homeostasis is that the phytochemical reduces glucose concentration in streptozotocin-induced diabetes mellitus by potentiating glycolytic enzymes and inhibiting gluconeogenesis and also reduces insulin resistance in rats with metabolic syndrome from high fat diet. The balancing tack between the unknown AA hypoglycaemic effect and the proven anti-hyperglycaemia makes an interesting dimension to track. Therefore, we saw it important to investigate the influence of AA on glucose homeostasis in malaria. Chapter 3 (Article 4) shows the results of AA influence on glucose metabolism in malaria.

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ASIATIC ACID INFLUENCES GLUCOSE HOMEOSTASIS IN *P. BERGHEI* MURINE MALARIA INFECTED SPRAGUE-DAWLEY RATS

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Abstract

Background: Glucose homeostasis derangement is a common pathophysiology of malaria whose aetiology is still controversial. The *Plasmodium* parasite, immunological and inflammatory responses, as well as chemotherapeutics currently used cause hypoglycaemia in malaria. Anti-parasitic and anti-disease drugs are required to combat malaria while ameliorating the pathophysiology of the infection. Asiatic acid has anti-hyperglycaemic, antioxidant, pro-oxidant properties useful in glucose homeostasis but its influence in malaria is yet to be reported. Here we present findings on the influence of asiatic acid on glucose metabolism in vivo using *P. berghei*-infected Sprague Dawley rats.
Materials and Methods: Acute as well as sub-chronic studies were carried out in vivo where physicochemical properties and glucose homeostasis were monitored after administration of asiatic acid (10mg/kg) in both non-infected and infected animals. Glucose metabolism associated biochemical changes in malaria were also investigated.
Results: In acute studies, asiatic acid improved oral glucose response while in the sub-chronic state it maintained food and water intake and suppressed parasitaemia. Normoglycaemic control was maintained in infected animals through insulin suppression and increasing glucagon secretion, in both acute and chronic studies. Asiatic acid administration curtailed lactate concentration towards normal.
Conclusion: Per oral post-infection asiatic acid administration preserved drinking and eating habits, inhibited sickness behaviour while suppressing parasitaemia. Reciprocal relationship between insulin and glucagon concentrations was maintained influencing glucose homeostasis positively and inhibition of hyperlactaemia in malaria.

Key words: Asiatic acid, malaria, *Plasmodium berghei*, glucose homeostasis, anti-disease, anti-parasitic

Abbreviations: ip -intraperitoneal, po -per oral, ig -intragastric, AA-Asciatic acid, OGTT-oral glucose tolerance test, OS-oxidative stress, ROS-reactive oxygen species, NO-nitric oxide, $ONOO^-$ - peroxynitrite, BRU-Biomedical Research Unit, SD-Sprague Dawley,

Introduction

Malaria is an immunological disease displaying systematic inflammatory aspects with marked cachexia (Goldring, 2004, Schofield, 2007). Glucose homeostasis in malaria is critical in the more vulnerable groups of pregnant women and children <5 years of age with highest disease associated morbidity and mortality rates (White N.J. et al., 1983, English et al., 1998). Either group has less immune competency, heighten or constant demand for energy supply, and tend to experience slight physiological deficits in a more exaggerated way (Miller et al., 2013). Hypoglycaemia, cognitive impairment, severe malaria anaemia (SMA) non-respiratory acidosis and renal insufficiency are recognised facets of a post malarial treatment syndrome (Mackintosh et al., 2004). Aetiology of malaria hypoglycaemia is multi-faceted. Anti-parasitic treatment therefore may be inadequate to avert or ameliorate malaria-induced glucose homeostasis derangements. Glycolysis has also been observed to have more homeostatic effects beyond being involved in nucleic acid, lipid and amino acid biosynthesis in what is called the "Warburg effect" shown by increased glycolysis in the presence of oxygen (Warburg, 1930, Warburg, 1956; Pedersen, 2007). Glycolysis is involved in signal transduction regulating immunometabolic processes (Ho, 2016), histone acetyl-CoA acetylation control of early differentiation of embryonic stem cells (Moussaieff, 2015) and immune system function modulation through interferon-γ and IL-2 biosynthesis (Zhao, 2016). This makes glucose metabolism controlling agents in malaria have possible influence on the disease outcomes.

Asiatic acid (AA), is a phytotherapeutic with antioxidant, antihyperglycaemic, antihyperlipidaemic (Ramachandran & Saravanan, 2014) as well as emerging antimalarial properties (Mavondo, 2016). However, there is a lack of information on AA's influence on glucose homeostasis in malaria. Desirous to unravel further the metabolic effects of AA, we have investigated the influence of this phytomedicinal in murine malaria. Here we report on the influence of AA on glucose homeostatic changes in *Plasmodium berghei* malaria infection in young (90-120g) male Sprague Dawley rats.

Materials and Methods

Drugs, Chemical and Accessories

Asiatic acid (AA 97% purity), Giemsa stain, dimethyl sulphoxide (DMSO), chloroquine diphosphate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and reagents were of analytical grade.

Animals

Male Sprague-Dawley (SD) rats weighing 90-120 g were obtained from the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal where they were bred and housed for the entire experiment period. The animals were kept under maintained laboratory conditions of constant temperature (22±1 ˚C); $C0_2$ (<5000 ppm), humidity of 55±5% and illumination (12 h light/dark cycle). Food, standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water were supplied to the animals *ad libitum*. Animals were sacrificed at specific time point up to 12 days for the non-treated infected control (IC) and up to 21 days for the rest of the experimental animals. Lethal anaesthetic inhalation of isofor (Safeline Pharmaceuticals, Rooderport, South Africa) in a gas chamber (100mg/kg) was used to sacrifice the animals. All experiments and protocols used in this study were reviewed and approved by the animal ethics committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

Malaria Parasite

Chloroquine-susceptible strain of *P. berghei* ANKA, was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). *P. berghei* (10^5 parasitized red blood cells [pRBC's] suspension in saline) was inoculated intraperitoneal (ip) into stock animals which were sacrificed after 12 days and the infected blood was harvested, washed and stored at -80ºC in freezing media (30% glycerol in phosphate buffer) until used.

Experimental Design

The study was divided into two viz; an acute and a sub-chronic study (lasting 21 days)**.** Experimental animals were divided into 5 groups (n = 6 per group) as follows:

Non infected absolute control [3mL/kg H_2O] (AC)
Non-infected treated control [AC+AA 10mg/kg] (NIC)
Infected non-treated control (IC)
Infected treated with CHQ 30mg/kg (30CHQ)
Infected treated with AA 10mg/kg (10mg) (AA10)

Preliminary studies using 3 concentrations of AA (5, 10, 20mg/kg) indicated that the AA10 oral administration had the most antimalarial efficacy hence it is the dose of choice in this study.

Malaria Induction

P. berghei (10^5 parasitized red blood cells [pRBC's] suspension in saline) was inoculated ip and control animals received equivalent amount of saline.

Monitoring of Parasitaemia

Peripheral blood was collected using the tail prick method (Rangaraj et al., 2014). Parasitaemia % monitored after 72 hours (pre-patent period), every third day up to day 7 [patent period] (Changa & Stevenson, 2004), every day during treatment period of five days and thereafter every other day (post-treatment period) until day 21. A 15-20% parasitaemia on day 7, confirmed by May-Grünwald-Giemsa staining under a microscope (Olympus Cooperation, Tokyo, Japan), was considered as stable state malaria.

Asiatic Acid Influence on Biophysical Changes

Body weights, food and water intake were monitored gravimetrically in all animals at 09h00 every third day during the pre-treatment, treatment and post treatment periods. The effects of AA on these parameters were examined.

Asiatic Acid Administration in Acute Studies (Oral Glucose Tolerance Response)

With parasitaemia at 15-20% in infected animals, all animals were fasted for 16 hours and a 240-minute oral glucose (0.86g/kg) tolerance response (OGTR) was carried out to distinguish effects of AA and malaria on glucose homeostasis. The time-glucose concentration course was determined to map out the influence of AA on the rate of glucose disappearance from plasma. Animals were sacrificed after the experiments by exposing to isofor (100 mg/kg) inhalation anaesthesia [Safeline Pharmaceuticals, Rooderport, South Africa]. Blood samples were collected by cardiac puncture into pre-cooled lithium heparin tubes onto melting ice, immediately centrifuged for 15 minutes at 959 x G in a 4^0C centrifuge (Eppendorf International, Hamburg, Germany) and plasma separated. Plasma was stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80°C until AA influence on hormonal changes was assessed.

Asiatic Acid Influence on Insulin and Glucagon Concentrations Post Glucose Bolus Administration

Insulin concentration was determined quantitatively in plasma using the Mercodia Ultrasensitive Rat Insulin ELISA (Mercodia AB, Uppsala, Sweden) following the manufacturer's instructions. The lower and upper limits of detection were ≤3.38pmol/L and 783pmol/L, respectively. Plasma glucagon concentration was determined using Elabscience Rat GC (Glucagon) ELISA kit Catalog No: E-EL-R0425 (Elabscience Biotechnology Co. Ltd, WuHan, P.R.C.) as per the manufacturer's instructions. Same methods were used in the sub-chronic studies.

Sub-chronic Studies

Post-infection po AA administration's influence on glucose metabolism and murine-malaria in SD rats was carried out in animals housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa), with food and water availed *ad libitum*, at the Biomedical Resource Unit, University of KwaZulu Natal over a 3-week period. Chloroquine (CHQ) control (30mg/kg) was dissolved in distilled water and administered as for AA. AA was dissolved in DMSO (0.5mL) and diluted with distilled water to give a stock solution of 10mg/kg. AA (10mg/kg) was administered once daily po (at 09h00) for five days. CHQ (30mg/kg) was administered twice daily (09h00 hours and 17h00 hours) according to posology developed in our laboratory. A ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 ml syringe was used. Animals were sacrificed at day 8, 12 and 21. The IC animals were sacrificed at day 12 for ethical reasons.

Plasma and Tissue Sample Harvesting

Influence of AA on glucose homeostasis, hormonal, immunology and inflammatory changes as well as other biochemical parameters were determined in either plasma or organ tissues. Animal sacrifice and plasma collection was as described in the acute studies (5.2.4). The liver, kidney, muscle and heart were removed, snap frozen in liquid nitrogen and stored together with the plasma in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at −80°C until use.

Influence of Asiatic Acid on Glucose Utilization in Sub-Chronic Conditions

The effects of AA on blood glucose homeostasis in malaria were evaluated by measuring blood glucose every other day during pre-patent, daily during patent/treatment and every three days post treatment periods in all the animal groups using blood glucose testing strips (Lifescan, Zug, Switzerland).

Influence of Asiatic Acid on Tissue Glycogen Storage

Influence of AA10 on glycogen storage capacity in liver, muscle and kidney were compared amongst AA treated infected animals and controls according to the method by Seifter (Seifter *et al.*, 1949), with some modifications.

Influence of Asiatic Acid on Plasma Lactate Concentration

The influence of futile glucose homeostasis was indicated by levels of lactate in plasma which were estimated using Cobas^{R} Accutrend Plus using Accutrend BM-Lactate strips (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analysis

Data are presented as the means ± standard error of mean (SEM). Overall statistical comparisons between the control means and experimental groups were performed with GraphPad Prism Software version 5.00, (GraphPad Prism Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer post hoc multiple comparison test. A value of $p < 0.05$ was considered significant.

Results

Parasitaemia Monitoring

Compared to IC and CHQ, AA10 administration had significantly lower %parasitaemia (★, ≠ $p<0.05$. Table 1) at relevant time points. Compared to both IC and CHQ, AA (10mg/kg) had a lower peak %parasitaemia (★, ≠ $p<0.05$. Table 1) AA10 suppressed parasitaemia to undetectable levels compared to CHQ (ψ $p<0.05$. Table 1) Compared to IC, CHQ had lower %parasitaemia during patent/ treatment period (ψ $p<0.05$. Table 1)

Table 1: Comparison of %parasitaemia changes over time (21 days). NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are presented as means ± SEM, (n=6 per group). ★, ≠ $p<0.05$ compared to IC and CHQ, respectively. ψ $p<0.05$ CHQ compared to IC

Protocol	Experimental Groups	%Parasitaemia per time point					
		Pre-patent	Patent/ treatment period			Post-treatment	
			Day 7	Day 9	Day 12	Day 15	Day 21
Post-infection AA administration	IC	3.7±0.21	13.68 ±0.98	33.43 ±1.07	56.52 ±3.20	N/A	N/A
	30CHQ	2.7±0.41	18.68 ±1.89ψ	42.08 ±1.325 ψ	37.37 ±4.36ψ	23.38 ±1.32	1.14 ±1.21
	AA10	3.2±0.90	13.47 ±0.95★≠	21.13 ±2.031★≠	26.67 ±2.91★≠	3.71 ±0.12≠	0.00 ±0.00≠

Food and Water Intake

A10 administration significantly preserved food and water intake as compared to the IC, and CHQ (★, ≠ p<0.05, respectively. Figure 1) Compared to the AC and NIC, CHQ treated animals had significantly lower food and water intake (**ψ**, **γ** p<0.05, respectively). IC had significantly lower intake compared to AC and NIC (**δ**, **χ** p<0.05, respectively. Figure 1) Compared to the NIC, AC had higher food and water intake (**β** p<0.05. Figure 1)

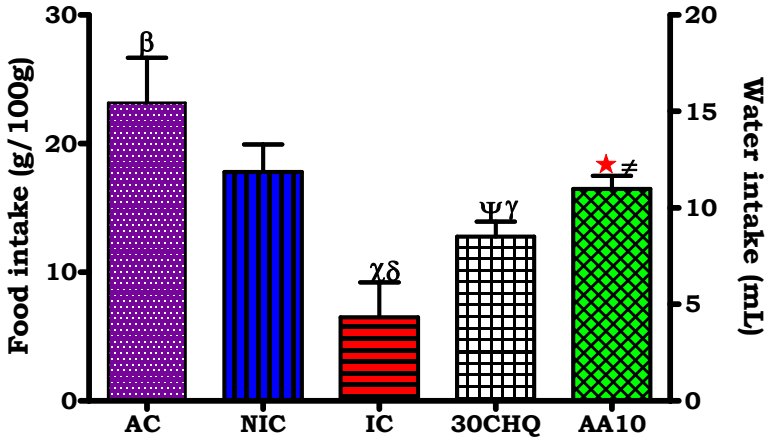

Figure 1: Comparison of food and water intake as influenced by AA10. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are represented as means ± SEM. α,★, ≠ p<0.05 compared to the NIC, IC and CHQ, respectively. **ψ** p<0.05 CHQ compared to IC. **γ** p<0.05 CHQ compared to the NIC. **χ** p<0.05 IC compared to NIC. **δ** p<0.05 IC compared to AC. **β** p<0.05 AC compared to NIC.

Influence of AA on Glucose under Acute Conditions (OGTR)

OGTR as influenced by AA10 administration was compared to the NIC, IC and 30 CHQ groups in Figure 2. AA10 administration significantly improved OGTR compared to IC and CHQ (★, ≠ p<0.05, respectively. Figure 2) At the 15 minute time point, AA10 ablated glucose spike compared to both IC and CHQ (★, ≠ p<0.05, respectively. Figure 2) Compared to NIC, the IC and CHQ groups had significantly poor OGTR (γ, ψ p<0.05, respectively).

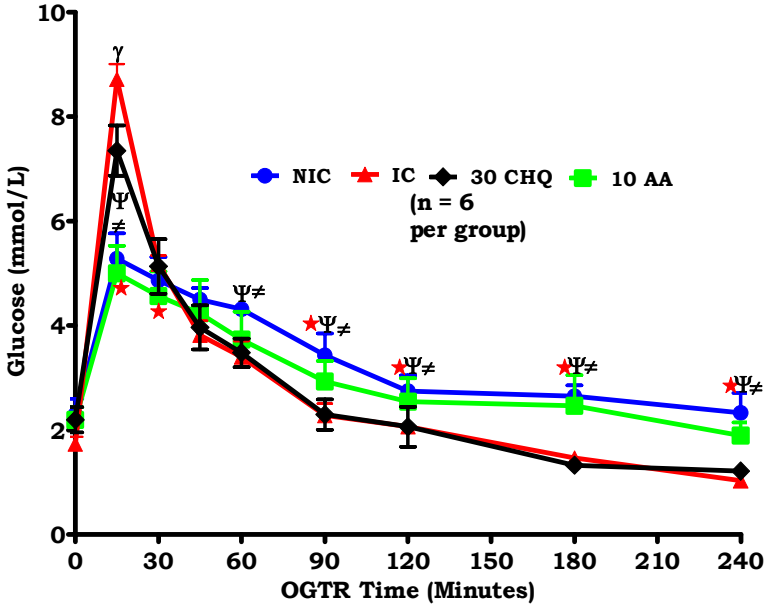

Figure 2: Oral glucose test response curve for AA10 compared to controls. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are presented as means ± SEM, (n=6 per group). ★, ≠ p<0.05 compared to IC and CHQ, respectively. **ψ** p<0.05 CHQ compared to IC. **γ** p<0.05 IC compared to NIC.

Asiatic Acid Influence on Insulin and Glucagon Concentrations Post Bolus Administration

Insulin and glucagon concentration changes in OGTR as influenced by AA10 administration was compared to controls in Table 2. AA10 significantly increased glucagon and lowered insulin compared to IC and CHQ (★, ≠ p<0.05, respectively). NIC had significantly lower insulin and higher glucagon compared to IC and CHQ (γ, χ p<0.05, respectively).

Table 2: Influence of AA (10mg/kg) on acute insulin and glucagon secretion compared to controls. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are presented as means ± SEM, (n=6 per group). ★, ≠ p<0.05 compared to IC and CHQ, respectively. γ, χ p<0.05 NIC compared to CHQ and IC.

Hormone	Parameter	Concertation after 20 hour fast
Insulin (pmol/L)	NIC	8.12±10.76
	IC	225.38±5.72 χ
	30 CHQ	258.53±11.78γ
	AA10	8.93±3.87★≠
Glucagon (pmol/L)	NIC	628±32.66
	IC	98±6.98χ
	30CHQ	76±3.87γ
	AA10	498.76±7.12★≠

Influence of AA on Glucose Utilization in Sub-Chronic Conditions

Administration of AA10 significantly preserved blood glucose compared to IC and CHQ (★, p<0.05, respectively. Figure 3) at days 7-12 and CHQ (≠ p<0.05 Figure 3) at days 7-18). IC displayed lower glucose compared to NIC (χ p<0.05 Figure 3). CHQ administration lowered glucose levels compared to NIC (γ p<0.05 Figure 3) but increased it compared to IC (ψ p<0.05 Figure 3) at days 7-12. CHQ treatment glucose lowering continued post treatment compared to NIC at day 18

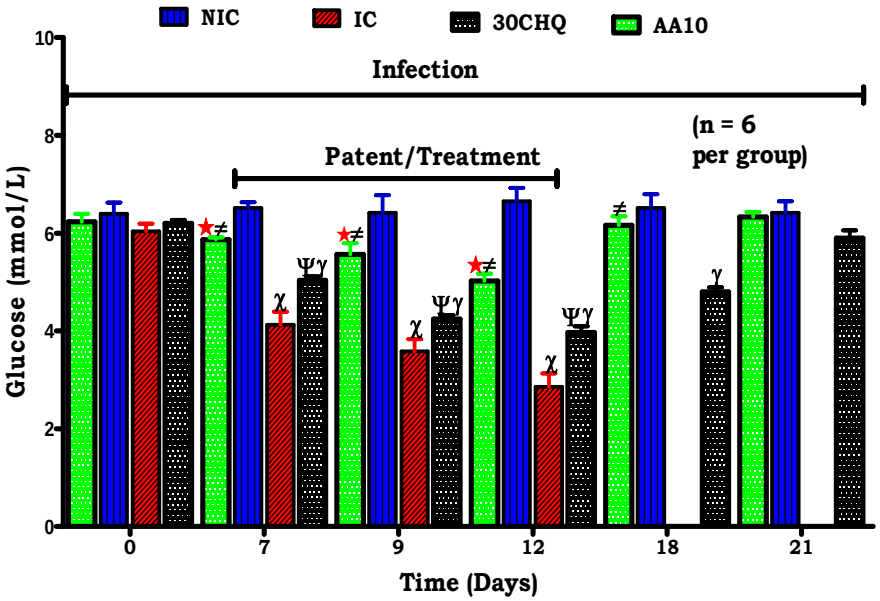

Figure 3: A comparison of the influence of AA on blood glucose concentration in sub-chronic studies over time. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are represented as means ± SEM. α,★, ≠ p<0.05 compared to the NIC, IC and CHQ, respectively. ψ p<0.05 CHQ compared to IC. γ p<0.05 CHQ compared to the NIC. χ p<0.05 IC compared to NIC.

Influence of AA Administration on Liver Glycogen Storage

Glycogen concentration, as influenced by AA10, was compared amongst animal groups in Figure 6. Administration of AA significantly increased glycogen in liver as compared to the IC, and CHQ (★, ≠ p<0.05, respectively). Compared to the AC and NIC, CHQ treated animals had lower glycogen levels (ψ, γ p<o.o5, respectively). IC had significantly lower glycogen compared to AC and NIC (δ, χ p<0.05, respectively).

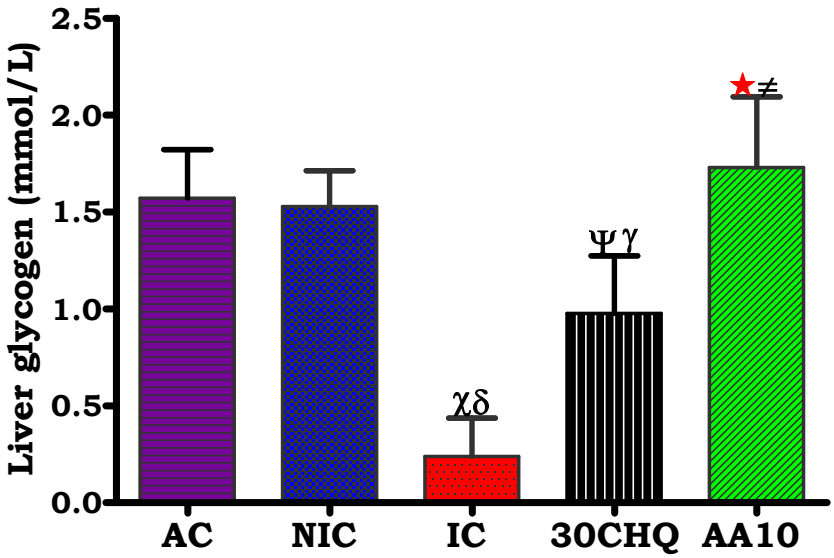

Figure 4: A comparison of liver glycogen concentration as influenced by AA 10mg/kg administration. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Values are represented as means ± SEM. α,★, ≠ p<0.05 compared to the NIC, IC and CHQ, respectively. ψ p<0.05 CHQ compared to IC. γ p<0.05 CHQ compared to the NIC. χ p<0.05 IC compared to NIC. δ p<0.05 IC compared to AC.

Influence of AA Administration on Muscle Glycogen Storage

Glycogen concentration, as influenced by AA10, was compared to controls in figure 5. Administration of AA10 significantly increased glycogen when compared to the IC, and CHQ (★, ≠ p<0.05, respectively). Compared to the AC and NIC, CHQ treated animals had lower glycogen levels (ψ, γ p<0.05, respectively). IC had significantly lower glycogen compared to AC and NIC (δ, χ p<0.05, respectively).

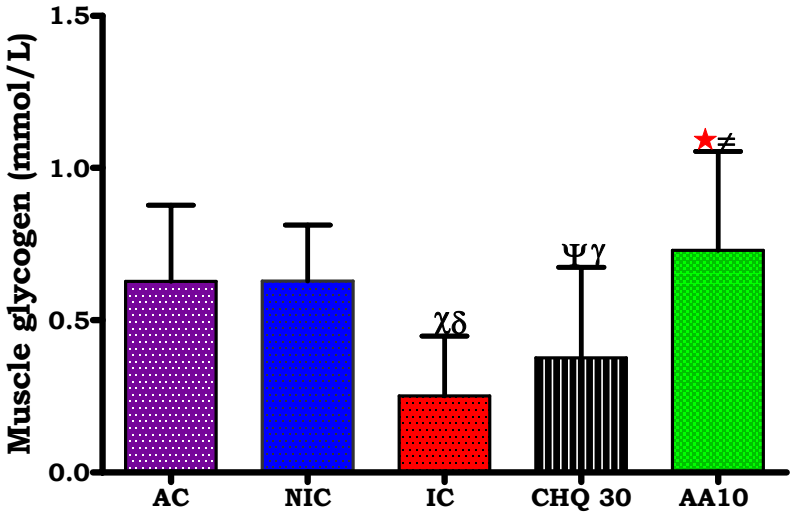

Figure 5: AA (10mg/kg) influence on muscle glycogen compared to other animal groups. NIC-non infected treated control, IC-infected control; CHQ-chloroquine treated infected control. Values are represented as means ± SEM. α,★, ≠ p<0.05 compared to the NIC, IC and CHQ, respectively. ψ p<0.05 CHQ compared to IC. γ p<0.05 CHQ compared to the NIC. χ p<0.05 IC compared to NIC. δ p<0.05 IC compared to AC.

Plasma Lactate Production

A comparison of the influence AA10 had on lactate production to controls was made in Figure 6. AA10 administration significantly lowered blood lactate concentration compared to the IC and 30CHQ (★, ≠ p<0.05, respectively). IC had higher blood lactate concentrations than NIC and CHQ (χ, ψ p<0.05, respectively). CHQ displayed higher blood lactate concentrations than NIC (γ p<0.05).

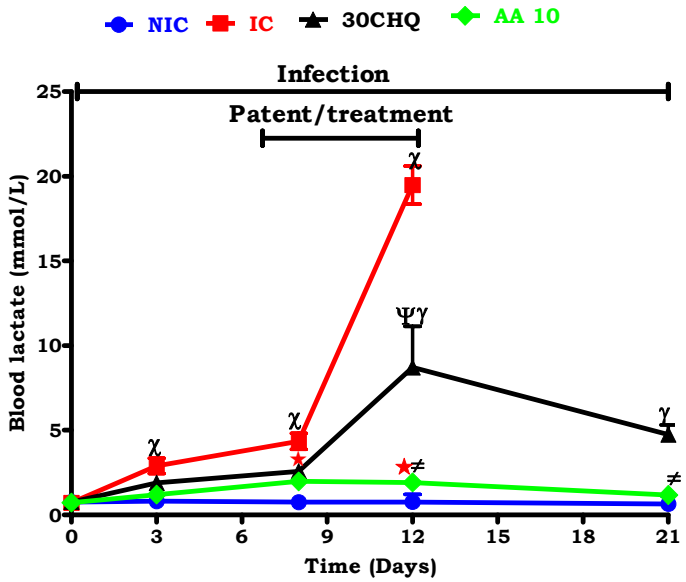

Figure 6: Influence of AA10 administration on lactate concentration changes compared to controls. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Concentrations are presented as means ±SEM (n = per group). ★, ≠ $p<0.05$ compared to the IC and CHQ, respectively. ψ $p<0.05$ CHQ compared to IC. γ $p<0.05$ CHQ compared to the NIC. χ $p<0.05$ IC compared to NIC.

Plasma Insulin Levels

Relationships of AA10 administration to insulin secretion were compared to various animals groups in Figure 7, displaying significantly lowered values compared to AC, NIC, IC and CHQ (**μ**, **α**,★, ≠ $p<0.05$, respectively). AC had significantly higher basal insulin levels compared to NIC (**β** $p<0.05$), although below reference interval (RR). NIC insulin was significantly lower than IC (χ $p<0.05$). Compared to the NIC and IC, CHQ treatment had significantly higher (γ $p<0.05$) and lower (ψ $p<0.05$) values, respectively.

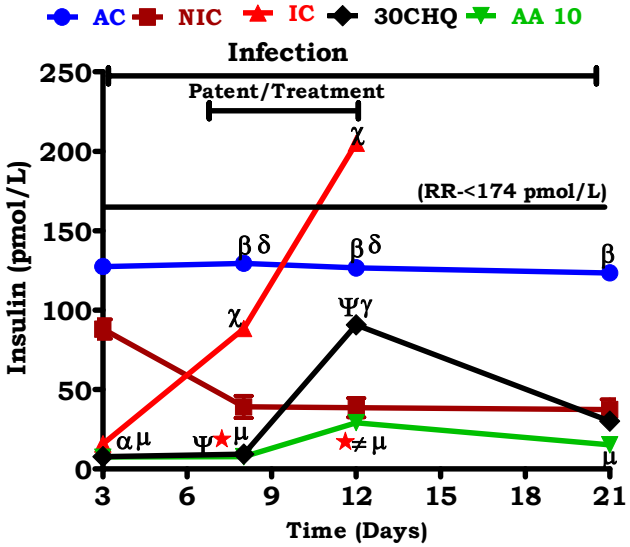

Figure 7: AA10 influence on insulin levels in comparison to the AC (absolute control), NIC, IC and 30 CHQ. NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Concentrations are presented as means ±SEM (n = per group). **μ**, **α**,★, ≠ $p<0.05$ compared to the AC, NIC, IC and CHQ, respectively. ψ $p<0.05$ CHQ compared to IC. γ $p<0.05$ CHQ compared to the NIC. χ $p<0.05$ NIC compared to IC. **β** $p<0.05$ NIC compared to AC. **δ** $p<0.05$ IC compared to AC.

Glucagon Concentrations

Relationships of changes of glucagon concentrations over time as influenced by AA10 were compared to controls in Figure

8. AA10 administration had significantly higher concentrations compared to AC, NIC, IC and CHQ treatment (**μ**, **α**,★, ≠ p<0.05, respectively). Glucagon concentrations were higher in animals treated with CHQ compared to NIC and IC groups (**γ**, **ψ** p<0.05, respectively). IC group glucagon concentrations were significantly higher than NIC (**χ** p<0.05).

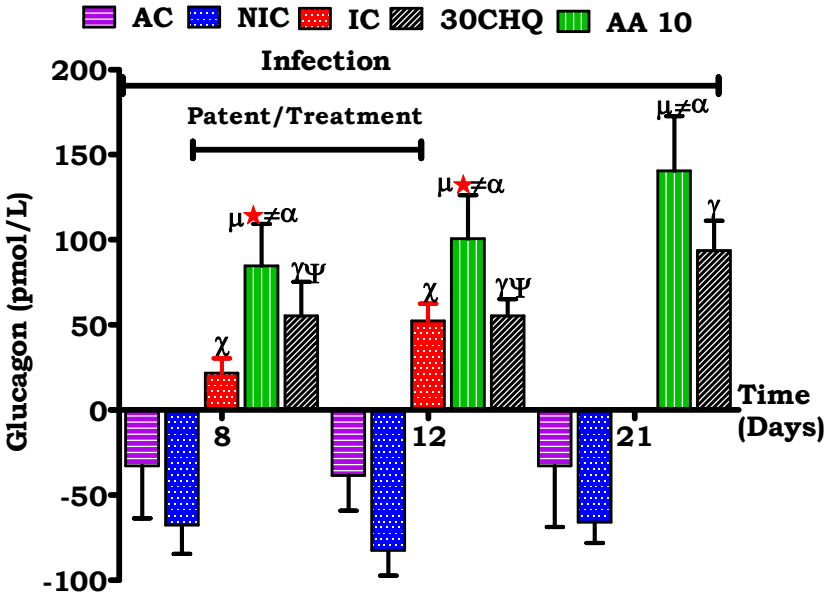

Figure 8: Influence of AA10 on glucagon (GLN) concentration changes over time in malaria. AC-absolute control, NIC-non infected treated control, IC- infected control; CHQ-chloroquine treated infected control. Concentrations are presented as means ±SEM (n = per group). **μ**, **α**,★, ≠ p<0.05 compared to the AC, NIC, IC and CHQ, respectively. **ψ** p<0.05 CHQ compared to IC. **γ** p<0.05 CHQ compared to the NIC. **χ** p<0.05 NIC compared to IC.

Discussion

Glucose metabolic disturbance is an intricate balance between supply, transportation, utilization or storage which the malaria parasite exploit successfully to systematically weaken the body defence systems while optimising its own survival (Olszewski et al., 2009), with poor prognosis in children under five years (English et al., 1998, Agbenya et al., 2000) and in pregnant women (Croft, 2000). Hypoglycaemia has a prevalence of approximately 10% in both adults and paediatric patients (White N.J. et al., 1983) and up to 25% in children (Osier et al., 2003). There are a number of systems that deplete plasma glucose in malaria which can be targets for AA intervention. We report here the influence of AA10 on glucose utilization in an acute study and over a period of 21 days in *P. berghei*-infected Sprague Dawley rats. All animal groups which were inoculated with *P. berghei* murine malaria parasite developed severe malaria (SM) by Day 7 (patent malaria period) at which period treatment was effectuated. Parasitaemia percentages were extreme in non-treated animals with a perceptible difference in suppression of the parasite in the AA10 orally administered groups which may be attributable to the influence of AA. Moreover, AA10 surpassed the efficacy of 30CHQ oral treatment (Table 1) although a single dose of AA10 was administered compared to the double dose of CHQ, daily.

Concomitant with % parasitaemia changes was a significant disparity in food and water intake between AA10 administered animals and IC groups. Animals that were administered with oral AA10 displayed a positive preservation of food and water intake at all relevant time periods during the study which correlated well with AC and contrasted with the IC and 30CHQ treated groups (Figure 1). There seems to be a causal relationship between parasite infection and food and water intake together with %weight gain (results not shown) seeing that, compared to the AA10, the IC group posted more averse outcomes in the experiments.

Malaria, like other chronic diseases, is associated with reduced food and water intake in what is termed the sickness behaviour (Hart, 1988).The natural response to infection or injury is for the animal to down-regulate normal activities (including eating and drinking), reorganize its resources to overcome the infection and re-establish homeostatic functions (Clark et al., 2008). However, when the insult perpetuates, the associated somnolence becomes a liability to the animal with energy depletion and anorexia leading to cachexia (Clark & Vissel, 2014). The resulting malnutrition and inanition accelerated a wasting diathesis as we have observed in the IC group despite *ad libitum* provision of food and water. The loss of appetite in sickness behaviour is driven by tumour necrosis factor-α (TNF-α). TNF-α secretion is an intrinsic mechanism to create a hostile environment for the pathogen (Grimble, 1992) which becomes aberrant in malaria due to unregulated immune response. However, AA10 administration preserved the social habit of eating and drinking showing possible inhibition of TNF-α and inflammation or the nuclear factor-kβ (NF-Kβ) suppression which directs inflammatory responses (Huang et al., 2011).

In the acute study, AA10 improved oral glucose tolerance response [OGTR] (Figure 2). Administration of AA ablated the rise in glucose at the 15 minute time point while the there was a sharp rise in glucose in both the IC and CHQ treated groups. This could be the result of the inflammatory cytokines (TNF-α)-induced insulin resistance that occurs in diabetes mellitus (Fernµndez-Real & Ricart, 1999) as well as in malaria (Acquah et al., 2014). The anti-inflammatory effect of AA has been reported as well as its capacity to potentiate insulin (Ramachandran & Saravanan, 2013, Ramachandran & Saravanan, 2014) resulting in a faster uptake of glucose without inducing hypoglycaemia. Insulin levels were observed to be lower in AA10 administered animals as well as in the NIC as compared to either the IC or the 30CHQ treated groups showing the suppressive effect of low glucose levels (Table 2).

Terminal glucagon concentration, on the other hand, was elevated in the AA10 group as well as the NIC showing how the animals did not experience severe hypoglycaemia even after a 20 hour fast. The same hormone levels were, however, suppressed in the IC and the 30CHQ treated animals during the acute studies. This scenario seemed to occur in acute circumstance of low glucose concentration during fasting than in the sub-chronic studies.

In the sub-chronic study there was an inflection in the glucose-time course which corresponded with the rise in parasitaemia in the AA10 administered animals. But there was a steep decline in glucose concentration showing a terminal hypoglycaemic trend that is typical of the malaria parasite effect on glucose homeostasis. 30CHQ treatment showed a contraction of glucose-time curve during the patent/treatment phase of the study. Chloroquine (Cansu & Korkmaz, 2008) and quinine (Elbadawi et al., 2011) have been shown to have hypoglycaemic effects due to their insulin memetic effect (English *et al.*, 1998) which persisted well after treatment had ceased and parasitaemia resolved (English *et al.*, 1998). We have also shown in our laboratory that CHQ had a glucose lowering effect in Sprague Dawley rats in malaria with a concomitant rise in insulin (Musabayane et al., 2010) which was ameliorated by transdermal delivery of CHQ (Murambiwa et al., 2013). The hyperinsulinemia of malaria was clearly enunciated by our finding in the IC group which displayed a steep rise in insulin (Figure 7) correlating well with the changes in %parasitaemia (Table 1). Indeed, this collateral rise in insulin predetermined suppression of glucagon (Figure 8) which invariably decreased plasma glucose in the group.

Malaria causes hyperinsulinemia as a result of increasing insulin resistance created by inflammation which disturbs glucose homeostasis. However, we observed correction of this trend when AA10 was administered. To maintain glucose concentration in malaria, glucagon was elevated in the AA10 administered animals in comparison with both the AC and NIC. The presence of AA and absence of malaria potentiated insulin activity such that a low concentration of glucose was maintained in the NIC compared to the AC during the fed state of the experiments. Indeed, AA has been reported to increase glycolysis enzymes in streptozotocin-induced diabetes while inhibiting gluconeogenesis and glycogenolysis (Ramachandran & Saravanan, 2013) facets that will have preserved food and water intake until the animal recovered from malaria. Rationally, in an increased in vivo glucose oxidation that is accompanied by either inaccessible stored energy sources (AA effect) or depleted stores (malaria effect), the animal will seek to replenish the energy deficit by foraging. The food and water intake which was preserved in our AA10 administered animals but not observed in the IC may be attributed to AA influencing these biophysical activities fostered by parasitaemia eradication. The antimalarial, anti-inflammatory, antioxidant and anti-hyperglycaemia influence of AA may have given the animals time to recover from toxic effects of parasitaemia and re-establish homeostasis.

Glucagon has a catabolic role on glycogen adjacent to the anabolic insulin action that tightly regulates blood glucose concentration (Schulman et al., 1957). We observed that liver glycogen stores were significantly higher in in the AA10, the NIC and the AC groups compared to either the IC and the CHQ controls (Figure 4). The glycogen levels correlated well with the glucagon activities in the relevant groups, being high where glycogen was low and being low where glycogen was high. This reciprocity was also evident in muscle glycogen concentrations showing that glucose homeostasis critically depended on the presence of AA10. However, it is now known that glucagon receptors are expressed in many tissues other than the liver showing extra influence of the hormone outside of glucose metabolism. One of these mechanisms is the involvement of glucagon in satiety initiation (Salter, 1960). We observed that animals in the well-fed disease-free groups (NIC, AC) had suppressed glucagon concentration and increased glycogen levels in either the liver or the muscles. The opposite was seen in the IC group where anorexia was apparent by reduced feeds and cachexic tendencies as shown by low muscle and liver glycogen concentrations.

Glucagon-induced hypophagia is regulated in the hypothalamus (Heppner et al., 2010) but only at low doses such that the high levels we saw in the acute studies (Table 1) and the sub-chronic studies may have initiated glucose mobilization regulating energy balance through thermogenesis and oxygen consumption (Davidson et al., 1957) by stimulation of brown adipose tissue (BAT) (Billington et al., 1991). In other normal conditions, this will have had a favourable effect on the animals' wellbeing, but in malaria the disease process was promoted instead. Low insulin concentration is necessary for the pharmacological effects of glucagon as the hyperinsulinemia we saw in the IC group would invariably blunt glucagon thermogenic effects (Calles-Escandon, 1994). Administration of AA resulted in increased glucose utilization and may, therefore, utilize the glucagon-induced glucose mobilization consequently quenching thermogenesis. Thermogenesis and febrile response are, on the other hand, cardinal features of malaria which could be orchestrated by either glucagon or malaria glycosylphosphatidylinositol (GPI)-induced TNF-α secretion and other cytokines by acting on the hypothalamus in severe malaria (Schofield & Hackett, 1993). GPI moieties have pleiotropic effects in malaria one of which is insulin memetic action which regulates glucose utilization by adipocytes and causing profound hypoglycaemia (Schofield & Hackett, 1993, Krishnegowda et al., 2005).

Overall, the administration of AA10 may have had an influence on the insulin-glucagon axis through amelioration of the debilitating effects of malaria by suppression parasitaemia and inhibiting the subsequent sequelae of malaria or may have abated malaria inflammatory effects of malaria and emasculating the disease in the process. Either way, AA10 administration positively influenced glucose homeostasis as compared to infected non-treated or CHQ-treated animals.

Glucose utilization in malaria readily becomes dysregulated early in the disease course driven by over expression of glucose transporter-1 (GLUT-1) in virtually all the cells, which depletes plasma glucose autonomous of insulin activity. The over expression of GLUT-1 in the muscle is instituted through the influence of GPI on TNF-α increased secretion and subsequent over expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) synthesis (Balon & Nadler, 1996, Bedard et al., 1997). While we did not investigate GPI interaction with AA or AA with GLUT-1 receptors, this may formulate gaps for future studies. Furthermore, the high concentrations of glycogen in the liver and the muscle may also point to an upregulations of GLUT 2 and GLUT 4 influenced by AA administration which may need to be investigated.

Plasma hyperlactataemia (Figure 6) observed in the IC group is evidence of increased energy demands of stress in the host or in the parasite or both and not necessarily hypoxia as lungs have been shown to produce increased amounts on lactate in disease (Opdam & Bellomo, 2000). Higher %parasitaemia was associated with higher levels of lactate production. Infection with malaria results in increased glucose production by approximately 50% (Davis et al., 1993) but also an expedited non-insulin mediated glucose disposal in glycolysis has been reported (Binh et al., 1997). This may mean possibly gluconeogenesis upregulation at the same time when glycolysis is increased. Therefore, the possibility that the intermediary molecule feeding both systems could be lactate is plausible. In sepsis, a condition that has many similarities with malaria infection, hyperlactaemia has been reported as an

independent predictor of mortality (Garcia-Alvarez et al., 2014). We have also observed that administration of AA10 preserved the concentration of lactate at lower concentration close to those of the NIC showing that possibly the phytochemical is also able to inhibit futile glycolysis either through its antioxidant or anti-inflammatory activity and also gluconeogenesis. The preservation of glycogen in the AA10 administered animals may have been as a result of lactate conversion to the glucose storage unit or the high glycogen stores may have inhibited hyperlactaemia development, both facets may be attributable to the influence of AA administration on glucose homeostasis.

Conclusion

We have shown the novelty of AA administration in malaria influencing both acute and sub-chronic glucose homeostasis, the preservation of food and water intake and modulating hormonal glucose handling. Insulin and glucagon preserved their reciprocal relationship which is usually dysregulated in malaria and other forms of malaria treatments showing the anti-disease aspect of AA. The preservation of glucose in malaria is a novel finding for a phytochemical that has be known to be anti-hyperglycaemic in diabetes mellitus.

The link between AA and glucose homeostasis may be in that the phytochemical potentiates the activity of glycolytic enzymes creating a demand for glucose. This energy source deficit cannot be satisfied by glycogenolysis which was suppressed as shown by liver and muscle stores leaving exogenous food source as the only alternative for energy metabolism, initiating foraging habits. Lactate metabolism was normalised showing efficient lactate utilization through oxidation or gluconeogenesis. The satiation drive by glucagon was suppressed in the treated animals requiring higher concentrations for the same effect. The findings suggest that AA may be an antimalarial which preserves glucose homeostasis and therefore, may be an anti-disease phytotherapeutic with both anti-parasitic and anti-infection properties.

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Conflict of Interests: The authors declare no conflict of in interest in this work.

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Chapter 4:
Introduction to Chapter 4 Article 5

This chapter presents the findings which is under review with the African Journal of Traditional Complementary and Alternative Medicine (AJTCAM), a peer reviewed journal. Manuscript Number: AJTCAM-D-16-00280 and title is:

Transdermal drug delivery of Asiatic acid influences renal function and electrolyte handling in *Plasmodium berghei*-infected Sprague-Dawley male rats

Instructions to authors from the journal are included in the annexes section. The manuscript begins with the title, contributing authors and their affiliations and contacts, corresponding author, followed by an abstract, key words, highlights and abbreviations. Thereafter comes the main body of the research including: Background, Materials and Methods, Results, Discussion and Conclusion. Figures, legends to figures, tables and legends to tables are included in the text. Acknowledgments, conflict of interests and references mark the end of the article.

Bridging the gap between Chapter 3 (Article 4) and Chapter 4 (Article 5)

The finding that AA5-pectin patch was most efficacious as an antimalarial was unexpected. Oral AA administration had indicated that the medium dose, AA10mg/kg, was the most effective and had hoped for the same trend to continue. Hypoglycaemia and renal dysfunction are malaria sequelae leading into coma and death connected through glycogen storage and gluconeogenesis. These share common aetiology or downstream effectors to malarial pathophysiology. In Chapter 3 (Article 4) preservation of glucose homeostasis was observed and we speculated whether the same anti-disease aspect could be demonstrated with renal function and renal electrolyte handling in malaria, hence the results presented in Chapter 4 (Article 5). Glucose is wholly absorbed in the proximal convoluted tubules, in a normal glucose homeostasis and renal function. This process depends mainly on GLUT 2, sodium-glucose transporters (SGLT) where glucose rides on the Na^+ electro-gradient potential for its transportation in the later and passively in the former. We hypothesized that the glucose homeostasis ameliorative effect of AA may also influence renal function and renal electrolyte handling. Chapter 4 (Article 5), therefore, reports on the influence of AA on renal function and renal electrolyte handling in malaria infected SD rat, an extension of the anti-hypoglycaemia, antioxidant and renoprotective effects AA.

Chapter 4 Article 5

Transdermal drug delivery of Asiatic acid influences renal function and electrolyte handling in *Plasmodium berghei*-infected Sprague-Dawley male rats

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1.0 Abstract:

Background: Higher prevalence of malaria related renal failure, current malaria drugs nephrotoxicity and drug resistance to malaria calls for continued research in anti-parasitic as well as anti-disease pharmaceuticals. Asiatic acid has antioxidant, pro-oxidant and diuretic properties. Here we report influence of asiatic acid-pectin hydrogel matrix patch application in *P. berghei*-infected Sprague Dawley rats on renal function and electrolyte handling.

Materials and Methods: Asiatic acid (5mg/kg)-pectin patch was applied on the dorsal neck region of the rat on day 7 post infection and monitored for parasitaemia, physicochemical changes. Urine, blood and plasma were collected for measuring various biochemical parameters.

Results: Asiatic acid-pectin patch application had significant influence on food and water intake as well as weight changes, urine electrolytes, glomerular filtration rate, inflammatory and antioxidant markers together with hormonal changes of aldosterone and vasopressin.

Conclusion: Application of the once-off asiatic acid (5mg/kg)-pectin patch influence renal function and renal electrolyte handling while ameliorating, biochemical and hormonal derangments induced by malaria.

1.2 Novelty of the work:

Here we show for the first time a) the efficacy of asiatic acid (AA) in suppressing murine malaria by way of administering the phytochemical using the amidated pectin hydrogel matrix patch transdermal drug delivery system, b) diminution of asiatic acid dose applied from 10mg/kg oral to 5mg/kg by transdermal, c) reduction of time once-off patch application from five days to three days, (d) attenuation of oxidative and hormonal derangements in malaria and e) the amelioration of renal function together with improvement in renal electrolyte handling. The results may be of use in patient care replacing the multiple approaches used in malaria management.

1.3 Key words: Asiatic acid, malaria, transdermal drug delivery, *Plasmodium berghei*, Sprague Dawley, renal failure.

1.4 Abbreviations: AA-asiatic acid, TDDS-transdermal drug delivery system, AVP-arginine vasopressin, ADH-antidiuretic hormone, po-per oral, ig-intragastric, ip-intraperitoneal; CA-*Centella asiatica*

2.0 Introduction:

When not properly managed, acute renal failure (ARF) may develop without warning in malaria. Malaria ARF (MARF) may develop into malaria chronic renal failure (MCRF) or the later might develop independently without an acute phase [Khan R *et al*, 2013]. In non-immune adults and children MARF is one of the leading causes of ARF in Africa South of the Sahara [Naicker S *et al*, 2008]. Mortality from MARF has been rated at 14-45% [Barsoum RS, 2000]. Aetiology of MARF depends on the immunological and inflammatory host responses [Pino P *et al*, 2003], microcirculation blockage by parasitized red blood cells [pRBC's] [Moxon CA *et al*, 2013, Hanson J *et al*, 2009], hypovolaemia from peripheral blood pooling and ischaemic acute tubular necrosis [ATN] [Naqvi R *et al*, 2003]. Treatment, therefore should be aimed at avoiding ARF development or reversing the renal pathophysiology where it has occurred.

Current malaria treatment regimens have a number of setbacks. Amongst these, use of 4-or 8-aminoquinolene drugs in malaria has been associated with increased sodium wasting and hyperkalaemia [Musabayane CT *et al*, 1996]. Artemisinins and their derivatives rapidly clear parasitaemia but create reactive oxygen species (ROS) leading to RBC's damage with post artemisinin treatment induced drug haemolysis (PADH) and ARF [Khan FY, 2009 , Plewes K *et al*, 2015, Rolling T *et al*, 2014]. Multi-drug resistance has emerged in antimalarial treatment introducing the most prominent obstacle in the fight against malaria which calls for continuing antimalarial research.

Phytochemicals open up an avenue worth exploring as anti-parasitaemia as well as malaria renal pathophysiology (anti-disease) alexia. Per oral (po) administration of asiatic acid (AA) has been demonstrated to have both anti-parasitic as well as anti-disease influence in malaria. This followed pre-infection administration of AA (chemoprophylaxis) and post-infection administration of the phytochemical in *Plasmodium berghei*-infected male Sprague Dawley rats [Mavondo GA *et al*, 2016]. Not only does this increase the number of potential antimalarial therapies, but it also introduces a malaria pathophysiology-directed approach for both malaria prevention and treatment.

Asiatic acid is both an antioxidant (hydrogen bond acceptor BDA) and a pro-oxidant (hydrogen-bond donor HBD) capable of redox reactions participation [Patel H *et al*, 2015] with a potential to eradicate the parasite while ameliorating untoward malaria disease sequelae. AA is pleiotropic in nature [Huang S-S *et al*, 2011, Yan S-L *et al*, 2014, Xu MF *et al*, 2012] with its diuretic effect [Pakdeechote P *et al*, 2014] and non-nephrotoxicity [Zhang J *et al*, 2013] makes it more amenable in the management of MARF. However, the influence of AA in the management of MARF has not been reported. But, it makes sense that therapeutics with anti-disease targets in malaria have a higher success chance in eradicating the parasite as well as forefending MARF development.

Renal dysfunction in malaria is commonly associated with electrolytes mismanagement by the nephron accompanied by arginine vasopressin (AVP) and aldosterone (ALD) aberrations before overt disease signs and symptoms are apparent [Musabayane CT *et al*, 1996, Musabayane CT *et al*, 2000] through increased oxidative stress from oxygen free radicals and $ONOO^-$ [Locatelli F *et al*, 2003, Forbes JM *et al*, 2008]. These formulate the renal disease aspects of malaria as contrasted to parasitic infection, which need to be managed concurrently with parasitaemia reduction strategies. Hypovolaemia of malaria, through red blood cell (RBC's) haemolysis and reduced water and food intake, increases both AVP and ALD. Therefore, investigation of MARF will of necessity involve demonstration of both AVP and ALD activities together with changes in electrolytes concentrations in both plasma and urine. The influence of AA on AVP and ALD in malaria or renal function is not yet reported. Also still a grey area is the influence of AA's antioxidant and anti-inflammatory capacities in malarial electrolyte handling. We hypothesise that the antioxidant capacity of AA may influence electrolyte transporters by reducing oxidative stress which is common in malaria infection and thus play a crucial anti-disease role in malaria pathology.

Studies have indicated that the mode of drug delivery, e.g. transdermal drug delivery system (TDDS), plays a crucial role in the ultimate efficacies of the drug to the extent of salvaging

antimalarial potency of chloroquine [Musabayane CT *et al*, 2003, Murambiwa P *et al*, 2013]. To further enhance the efficacy and reduce the dosage of AA in malaria management, an AA-amidated pectin hydrogel matrix patch, commonly referred to as "the patch" was mooted as a novel method of phytochemical drug delivery and antimalarial therapy. Therefore, the aim of this study was to investigate the influence of AA-TDDS on malaria, renal function and renal electrolyte handling in male Sprague Dawley rats, a novel approach in both AA administration and AA malaria treatment.

3.0 Materials and Methods:

3.1 Materials:

3.1.1 Drugs, chemical and accessories:

Asiatic acid (AA >97% purity), Giemsa stain, dimethyl sulphoxide (DMSO), chloroquine diphosphate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals and reagents used were obtained from reputable suppliers and were of analytical grade.

3.1.2 Animals: Male Sprague-Dawley (SD) rats weighing 90-120 g (6 weeks old) were obtained from Biomedical Research Unit (BRU), of the University of KwaZulu-Natal where they were bred and housed for the entire experiment period. The animals were kept under maintained laboratory conditions of constant temperature (22±1 ˚C); $C0_2$ (<5000 ppm), humidity of 55±5% and illumination (12 h light/dark cycles). The animals had full access to food, standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water *ad libitum*. Infected non-treated control (IC) animals were sacrificed by day 12 post infection. All other animal groups were sacrificed by day 21. Lethal inhalation anaesthesia with isoform (Safeline Pharmaceuticals, Rooderport, South Africa) for 3 minutes via a gas anaesthetic chamber (100 mg/kg) was used to sacrifice all the animals. All experiments and protocols used in this study were reviewed and approved by the animal ethics committee of the University of KwaZulu Natal (UKZN) with ethical clearance numbers 079/14/Animal and 013/15/Animal issued.

3.1.3 Malaria parasites: Chloroquine-susceptible strain of *Plasmodium berghei* ANKA, was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). *P. berghei* (10^5 parasitized red blood cells [pRBC's] suspension in saline) was inoculated intraperitoneal (ip) [Gumede B *et al*, 2003] into stock animals which were sacrificed after 12 days and the infected blood was harvested, washed and stored at -80^{o}C in freezing media (30% glycerol in phosphate buffer) until used.

3.1.4 Experimental design: The study was a 21 day protocol of animals groups (n=6 per group) administered either with AA-pectin matrix patch or chloroquine-pectin patch post-infection with *P. berghei*. The groups were as follows:

i) Non-infected treated control DMSO (NIC)

ii) Infected non-treated control (IC)

iii) Infection groups treated with CHQ 30mg/kg patch (30CHQ)

iv) Infected groups treated with AA 5mg/kg patch (AA5)

Preliminary findings have shown that AA 5mg/kg had the highest efficacy and it will be the only dose reported unless where it is necessary to stress a point.

3.2 Methods:

3.2.1 Induction of parasitaemia: Chloroquine-susceptible strain of *P. berghei* ANKA, was a kind donation from Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). *P. berghei* (10^5 parasitized red blood cells [pRBC's] suspension in saline) was inoculated ip [Gumede B *et al*, 2003]. A patch containing DMSO was applied to non-infected treated controls (NIC).

3.2.2 Influence of AA on sub-chronic studies: Experiments were conducted over a period of 21 days to establish the influence of AA on renal function and electrolyte handling in murine-malaria affected SD rats. Animals per group (n = 6 per group) were housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) with food and water availed *ad libitum*.

3.2.3 Preparation of AA-pectin and CHQ hydrogel patches: Amidated pectin hydrogel matrix patches were prepared using a previously described protocol by Musabayane *et al.,* (2003) with slight modifications [Musabayane CT *et al*, 2003]. Low methoxyl amidated pectin with a degree of esterification (DE) of 23% and an amidation of 24% was used for the preparation of the AA and CHQ patches. AA (5mg/kg) or CHQ (30mg/kg) were added to amidated low methoxyl pectin (4.4g) and dissolved in deionized water (110mL) in separate beakers. The concoctions were agitated at 37^0C in a water bath at a speed of 38 x G using an electric motor mixing rotor (Heidolph instruments GmbH & Co. KG, Schwabach, Germany) for 15 minutes**.** Subsequently, DMSO (2mL), 1.65mL eucalyptus oil (Barrs Pharmaceutical Industries, Cape Town, South Africa) and 1.65mL vitamin E (Pharmacare Ltd, Johannesburg, South Africa) were added to the mixtures and mixed for 1h 30 minutes. Aliquots (11 mL) were transferred to petri dishes with a known diameter and frozen at -4^{o}C for 18 hours following which 2% $CaCl_2$ solution was added on top of the frozen

pectin and left to stand at room temperature (±25º C) for 10 minutes to allow for cross-linking and formation of the matrix patch. The patches were then stored in a refrigerator at 4ºC until use.

4.2.4 Application of the patch: Three discs (4±1mm^2) were punched out from AA (5mg/kg) patch or CHQ (30mg/kg) and applied once-off onto the shaved dorsal region of the animal from Day 7 to Day 10 (three days). The jacket holding the patch in place was made from clinically sterile adhesive fabric plaster (Mediplast, Neomedic, Rickmansworth, and Herefordshire, UK) which caused no discomfort to the animals. The dorsal neck region was selected because it was the least accessible to the animals' grooming habits and avoided removal of the patch by the animal. The transdermal delivery (TTD) system aimed at reducing phytochemical amount delivered to the animal, dosing frequency, treatment duration and general animal discomfiture. A theoretical total AA yield of the patch was estimated at 1µg/per disc for 5mg/kg AA dose and 3µg was administered as three discs were applied per animal to provide equivalent of five day treatment.

3.2.5 AA5-pectin patch influence on parasitaemia monitoring: Parasitaemia % monitored 72 hours post inoculation (pre-patent period), every third day up to day 7 [patent period] [Changa K-H and Stevenson MM, 2004], every day during patent/treatment period of five days and thereafter every other day (post-treatment period) until day 21. A 15-20% parasitaemia on day 7, confirmed by Giemsa staining under a microscope (Olympus Cooperation, Tokyo, Japan) [Mavondo GA *et al*, 2016], was considered as stable state malaria where administration of AA and treatment with 30CHQ was commenced

3.2.6 AA5-pectin patch influence on physicochemical monitoring: Body weights, food and water intake were monitored gravimetrically using a balance Mettler balance PC 180-instruments (Protea Laboratory Services, Johannesburg, South Africa) in control and treated animals at 09h00 every 3rd day during the pre-treatment, treatment and post treatment periods.

3.2.7 Plasma and tissue sample harvesting: All animals were sacrificed by exposing to isofor (Safeline Pharmaceuticals, Rooderport, South Africa) for 3 minutes via a gas anaesthetic chamber (100 mg/kg). Blood samples were collected by cardiac puncture into pre-cooled lithium heparin tubes onto melting ice. Blood in heparin tubes was immediately centrifuged for 15 minutes at 959 x G in a 4^0C centrifuge (Eppendorf International, Hamburg, Germany) and plasma separated. The liver, kidney, muscle and heart were removed, snap frozen in liquid nitrogen and stored together with the plasma in a Bio Ultra freezer (Snijers Scientific, Tilburg,

Netherlands) at −80°C until use. Protein content of all organs was quantified using the Lowry method and samples standardized to one concentration (1 mg/mL).

3.2.8 AA5-pectin patch influence on urinalysis: Urine was collected over a 24 hour period in metabolic cages every third day during the pre-patent, patent/treatment and post treatment periods to investigate influence of AA on urine parameters. Gravimetric method was used to determine urine volume. Quantitative measurements of total urinary outputs and plasma concentrations of Na^+, K^+, Cl^-, urea and creatinine were performed using Beckman Coulter (Synchron CX3 Clinical Systems, Fullerton, California, USA) with reagent kits from Beckman Coulter (Dublin, Ireland). Creatinine concentrations used to calculate the glomerular filtration rate (GFR) using formulae: GFR = ([U_{creat}] x UV) / ([P_{creat}] x 1440 minutes) where U_{creat} is urine creatinine concentration, P_{creat} is plasma creatinine concentration and UV is 24 hour urine volume. Renal clearance (C) and fractional excretions (FE) were calculated with the standard formulae C = U x V/P and FE = C/GFR where U is the urinary concentration, V is the urine flow rate and P is plasma concentration as used by Salman et al [Salman IM *et al*, 2010].

3.2.9 AA5-pectin patch influence on haemolysis: To corroborate both haematological aberrations in malaria and hypoxia-induced tissue damage, plasma enzymatic activity of lactate dehydrogenase (LDH) was estimated. Haemolysis extent, renal tissue damage or renal disease as well hyperlactaemia affect renal function. The enzymes is raised in these conditions to underscore its increased release from tissues in necrosis or increased synthesis. LDH was determined in plasma using the Architect c8000 Abbott Diagnostic Clinical Chemistry Analyser (Abbott Laboratories, Illinois, USA).

3.2.10 AA5-pectin patch influence aldosterone (ALD) and arginine vasopressin (AVP): Levels of ALD and AVP are affected in malaria and were estimated to show the influence of AA administration on renal electrolyte handling. Malaria increases levels of both hormones through sodium wasting and volume depletion for ALD and AVP, respectively. Blood samples collected from treated and untreated groups of SD rats were used in ELISA methods hormonal estimates.

3.2.11 ALD estimations: An Elabscience ALD (Aldosterone) ELISA kit Catalog No, E-EL-0070 (Elabscience Biotechnology Co. Ltd., WuHan, P.R.C.) was used to measure the hormone concentration following manufacturer's instructions. The detection limit was 15.63-1000pg/mL with a sensitivity of 9.38pg/mL.

3.2.12: AVP estimations: An Elabscience Rat ADH (Antidiuretic Hormone) ELISA kit Catalog No, E-EL-0552 (Elabscience Biotechnology Co. Ltd., WuHan, P.R.C.) was used to

measure the hormone concentration following manufacturer's instructions. The detection limit was 31.25-2000pg/mL with a sensitivity of 18.75pg/mL.

3.2.13 Influence of AA on oxidative stress (OS) in malaria: OS infringes upon normal electrolytes handling in the proximal and distal convoluted tubules. The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and levels of malonyl aldehyde (MDA) were measured in kidney tissues to estimate influence of AA on OS.

3.2.14 SOD activity estimation: Tissue SOD activities of kidney homogenates from NIC, AA treated and IC SD rats were assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format using a commercially available from abcam Superoxide Dismutase Activity Assay kit (colorimetric) catalogue no. ab65354 (abcam, Canada) according the manufacturer's instructions.

3.2.15 GPx activity estimation: Activity of GPx was determined in AA-pectin patch applied and control animals' kidney tissues using abcam Glutathione Peroxidase Activity Assay kit (colorimetric) catalogue no. ab102530 (abcam, Canada) according the manufacturer's instructions.

3.2.16 MDA concentration estimation: MDA levels in kidney tissues from NIC, IC and infected-AA treated animals were estimated using a previously described method [Kasapoglu M and Özben T, 2001] with some modifications.

3.3 Statistical analysis: Data are presented as the means ± standard error of mean (SEM). Overall statistical comparisons between the control means and experimental groups were performed with GraphPad InStat Software (version 5.00, GraphPad Prism software (San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer post hoc multiple comparison test. A value of $p < 0.05$ was considered significant.

4.0 Results:

4.1 Influence of AA5-pectin patch on %parasitaemia: The degree of %parasitaemia decrease indicated the efficacy treatment on malaria. A comparisons of %parasitaemia during the sub-chronic study was made between AA5-pectin applied animals and controls in Table 1. AA5-TDDS administration had lower %parasitaemia at days 7-12 compared to IC and CHQ treatment (*, # $p<0.05$, respectively) and at day 21 compared to CHQ treatment.

4.2 Influence of AA5-pectin patch on physicochemical changes: Eating and drinking habits indicated the bio-physiochemical status of both infected and non-infected animals.

Comparison of food and water intake together with %weight gain was made between AA5-pectin and controls as shown in Table 2. Physiochemical properties were significantly preserved by AA5-pectin patch compared to the IC, and CHQ (***, #** $p<0.05$, respectively).

4.3 Influence of AA5-pectin patch on Glomerular filtration rate [GFR]: Changes of GFR overtime reflected the status of renal function in the NIC, IC and treated animals in Figure 1. Once-off AA-pectin patch influence on GFR compared to NIC, IC and 30CHQ. AA5-TDDS application preserved GFR compared to IC and 30-patch treatment **(*, #** $p<0.05$, respectively) at pertinent time periods.

4.4 Influence of AA5-pectin patch on urine Na^+ output (mmol/L/day): Renal Na^+ preservation was shown by the amount of Na^+ in the urine is shown. Comparison of urine Na^+ output in AA5-pectin patch applied animals and controls is shown in Figure 2. AA5 patch significantly preserved Na^+ excretion compared to IC and 30CHQ patch (***, #** $p<0.05$, respectively) at all relevant times.

4.5 Influence of AA5-pectin patch on K^+ output (mmol/L/day): Renal handling of K^+ was shown by the urine K^+ activity. Urine K^+ output in AA5-pectin patch applied animals and controls was shown in Figure 3. AA5 patch significantly preserved K^+ excretion compared to IC and 30CHQ patch (***, #** $p<0.05$, respectively) at all relevant times.

4.6 Influence of AA5-pectin patch on urine Cl^- output (mmol/L/day):): Urine Cl^- output in AA5-pectin patch applied animals and controls was shown in Figure 5. AA5 patch reduced Cl^- excretion compared to IC and 30CHQ patch (***, #** $p<0.05$, respectively) at all relevant times.

4.7 Influence of AA5-pectin patch on absolute Na^+ excretion (µmol/mL/min): The influence of AA5-pectin patch on absolute Na^+ excretion and the controls was compared in Figure 6. Absolute Na^+ excretion was significantly lower in the AA5-pectin matrix patch administered infected animals compared to the IC and the 30CHQ-pectin matrix patch treated groups (***, #** $p<0.05$, respectively).

4.8 Influence of AA5-pectin patch on lactate dehydrogenase (LDH) activity: The degree of haemolysis was reflected by the activity of LDH in plasma. The influence of AA5-pectin hydrogel matrix patch on LDH activity was compared in Table 3. Application of the AA5-pectin patch decreased LDH activity significantly on day 12 as compared to either IC or 30CHQ patch (***, #** $p<0.05$, respectively).

4.9 Influence of AA5-pectin patch on aldosterone: Hormonal renal modulation of electrolyte handling was indicated by concentrations of ALD. A comparison of the influence of

AA-pectin patch on ALD with controls was shown in Figure 7. Application of AA5-pectin patch significantly reduced ALD (*, # $p<0.05$, compared to IC and CHQ, respectively).

4.10 Influence of AA5-pectin patch on Arginine vasopressin (AVP) secretion: Dehydration status and water balance was reflected by AVP concentrations in plasma. The influence of AA-pectin patch application is compared to controls in Figure 8. Once-off AA-pectin patch application suppressed AVP significantly compared to both the IC and CHQ patch (*, # $p<0.05$, respectively).

4.11 Influence of AA5-pectin patch on superoxide dismutase (SOD): Oxidative status of the animals administered AA, CHQ and IC groups was indicated by SOD concentration in tissues of the kidney. The influence of AA5-pectin patch application on SOD activity is compared to control animals in Table 4 (A). Compared to IC and CHQ, AA5-pectin patch administration did significantly increase enzyme activity (***, #** $p<0.05$) in kidney.

4.12 Influence of AA5-pectin patch on glutathione peroxidase (GPx): Oxidative status of the animals administered AA, CHQ and IC groups was indicated by GPx concentration in tissues of the kidney.AA5-pectin patch influence on GPx was compared to control animal groups in Table 4 (B).

4.13 Influence of AA5-pectin patch on malonyldialdehyde (MDA): The level of lipid peroxidation, a marker of oxidative stress in malaria, was reflected by amount of MDA in the kidney tissues. The levels of MDA in AA-pectin patch applied animals was compared to controls in Table 4 (C). AA5-pectin patch significantly reduced levels of MDA in kidney significantly compared to IC (***,** $p<0.05$).

Table 1: % Parasitaemia comparisons between AA5-TDDS application and controls. Values are presented as means ±SEM (n = 6 per group). *, # $p<0.05$ compared to the IC and 30CHQ patch, respectively.

Protocol	Parameter	Sub-chronic Study Time-Course		
Post- infection Asiatic acid Transdermal Drug Delivery		Pre-patent (D3) %Parasitaemia	Patent/Treatment (D7-10) %Parasitaemia	Post-treatment (D21) %Parasitaemia
	IC	3.5± 0.2	13.00-69.33±8.6	N/A
	30CHQ	2.17±0.3	10.33-43.33±11.5	0.3±0.23
	AA5-TDDS	3.23±0.4	17.42-27.68±5.7*#	0.00#

Table 2: AA5-TDDS influence on %weight changes, food and water intake. Compared to controls values are represented as means ± SEM. ***, #** $p<0.05$ compared to the NIC, IC and CHQ, respectively.

Parameter	Protocol		Pre-patent (D 3)	Patent/ Treatment (D7-10)	Post-treatment (D 21)
A. Food intake (g/100g)	Post-infection AA TDD treated	NIC	11 ± 1	10 ± 2	12 ± 2
		IC	11 ± 1	6±4	N/A
		30CHQ patch	11 ± 2	9 ± 1	8 ± 1
		AA5-TDDS	11 ± 1	10 ± 2*#	12 ± 2#
B. Water intake (mL/100g)	Post-infection AA TDD treated	NIC	15 ± 1	13 ± 2	15 ± 2
		IC	14 ± 1	7± 2	N/A
		30CHQ patch	15 ± 1	13 ± 1	14 ± 3
		AA5-TDDS	15 ± 1	15± 2*#	15 ± 2#
C. %body weight change	Post-infection AA TDD treated	NIC	8±1	12±1	14±1
		IC	8±4	-4±2	N/A
		30CHQ patch	8±1	10±1	12±1
		AA5-TDDS	8±1	11+1*#	15±1#

Table 3: Influence of AA5-TDDS on lactate dehydrogenase activity compared to controls. Results presented as mean ± SEM (n = 6 in each group). *, # p<0.05 compared to IC and 30CHQ-pectin patch, respectively.

Parameter	Transdermal Delivery Post Treatment Day 12
	LDH (U/L)
NIC	551.00±42.29
IC	2453.00±235.20
CHQ 30mg/kg Patch	1063.00±51.85
AA 5mg/kg Patch	598.00±46.59*#

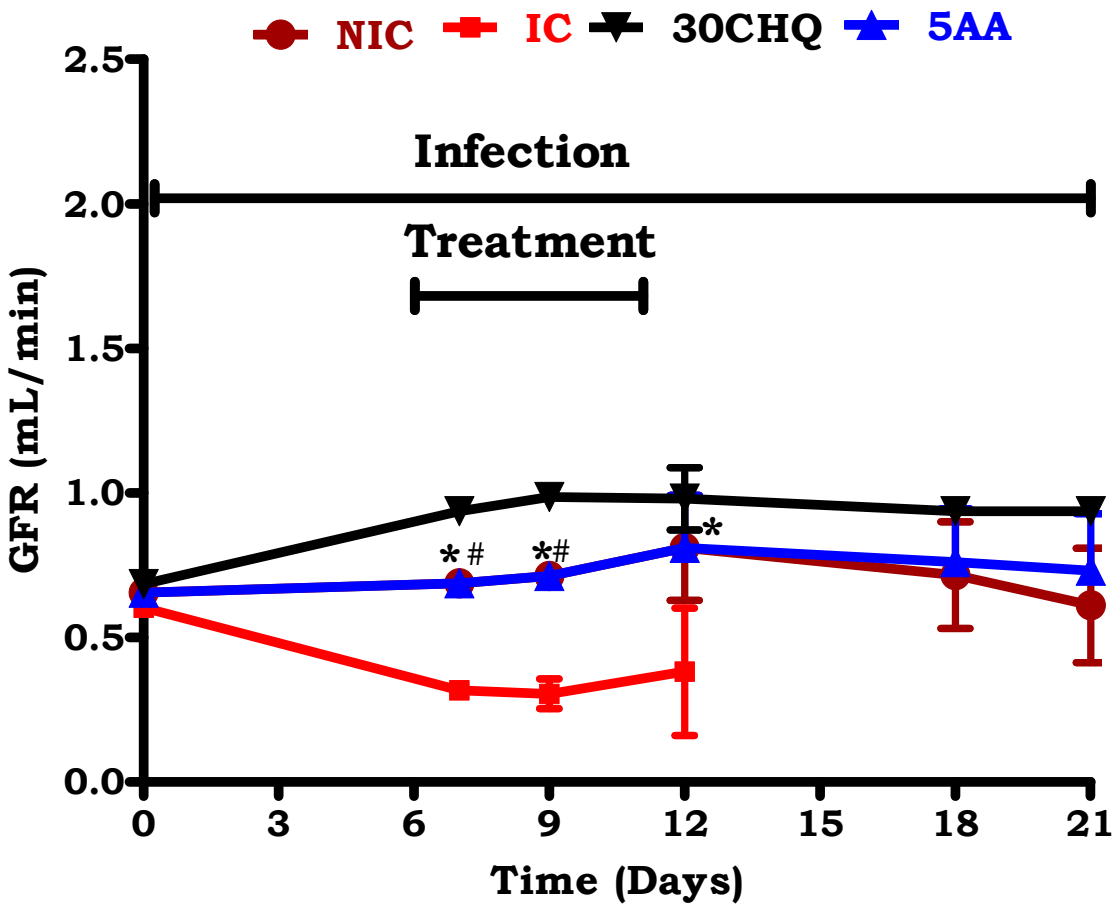

Figure 1: Comparison of GFR changes in AA5-TDDS and controls. Values are presented as means and vertical bars indicate SEM (n = 6 per group). *, # p<0.05 compared to IC, NIC, 30CHQ pectin matrix patch, respectively.

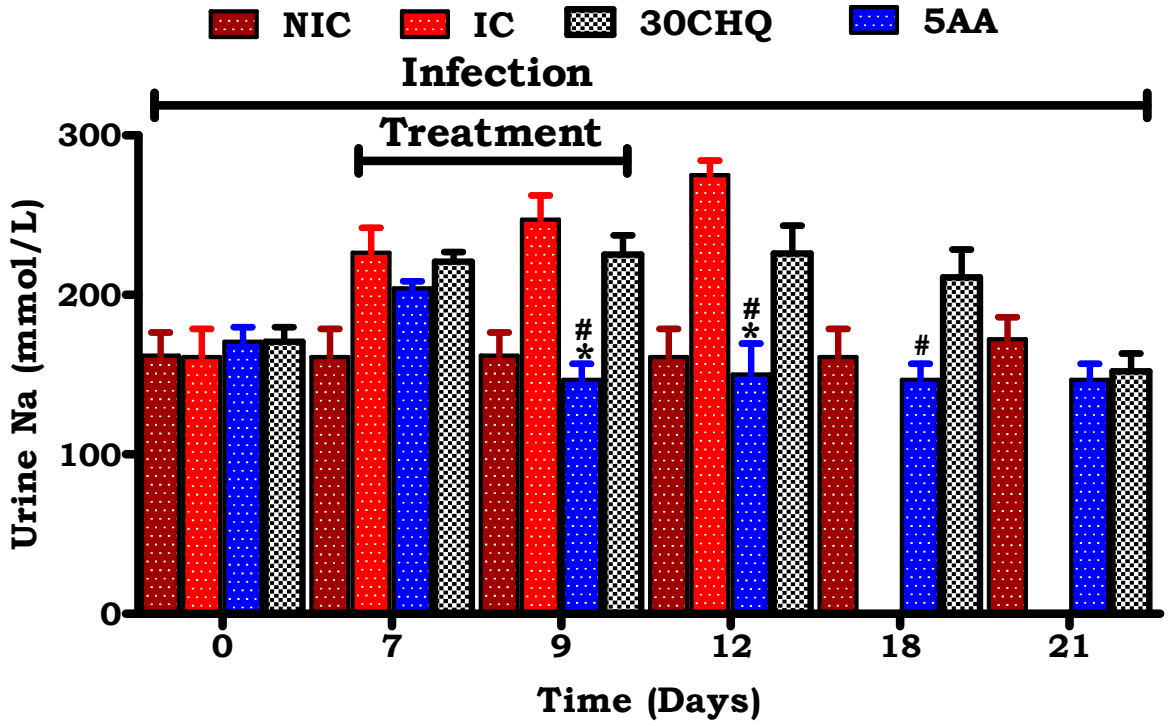

Figure 2: Urine Na^+ of AA5-TDDS applied animals compared to controls. Results presented as mean ± SEM (n = 6 in each group). *, # $p<0.05$ compared to IC, 30CHQ-pectin patch, respectively.

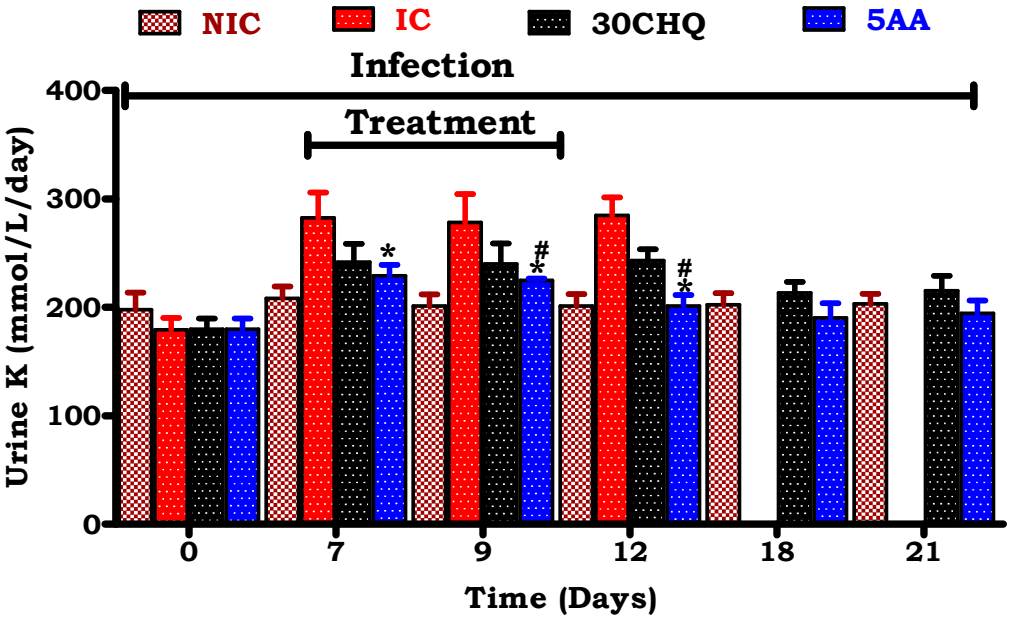

Figure 3: Urine K^+ output for AA5-TDDS compared to controls. Values are presented as

means and vertical bars indicate SEM (n = 6 per group). *, # P<0.05 compared to IC and 30CHQ, respectively.

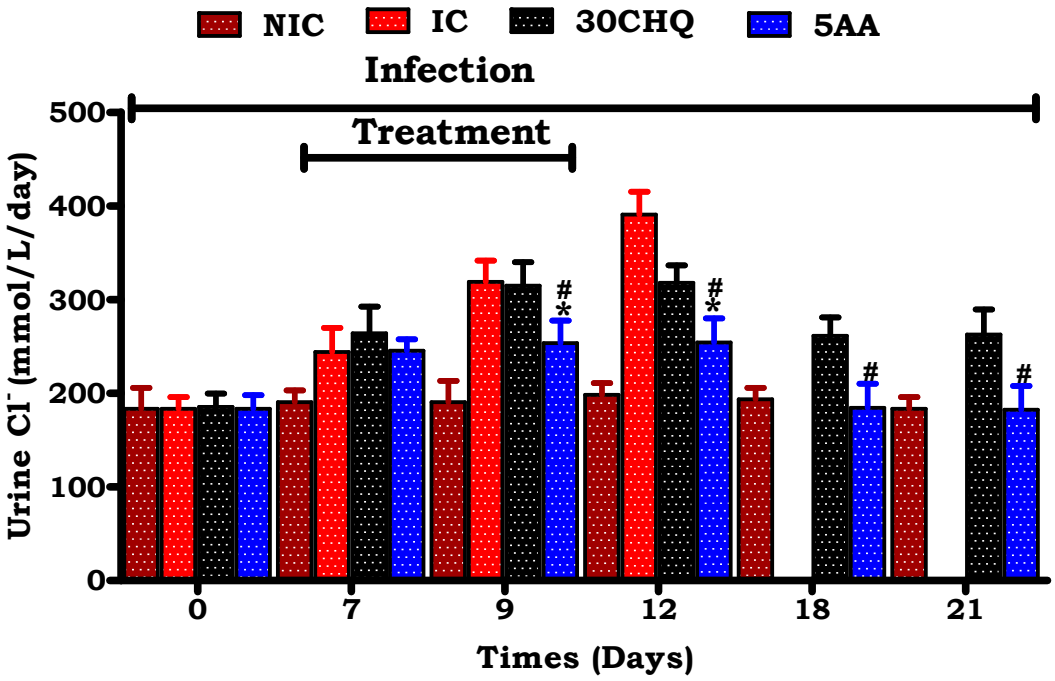

Figure 4: Urine Cl^- output of AA5-TDDS compared to controls. Results presented as mean ± SEM (n = 6 in each group). *, # p<0.05 compared to IC, 30CHQ-pectin patch, respectively.

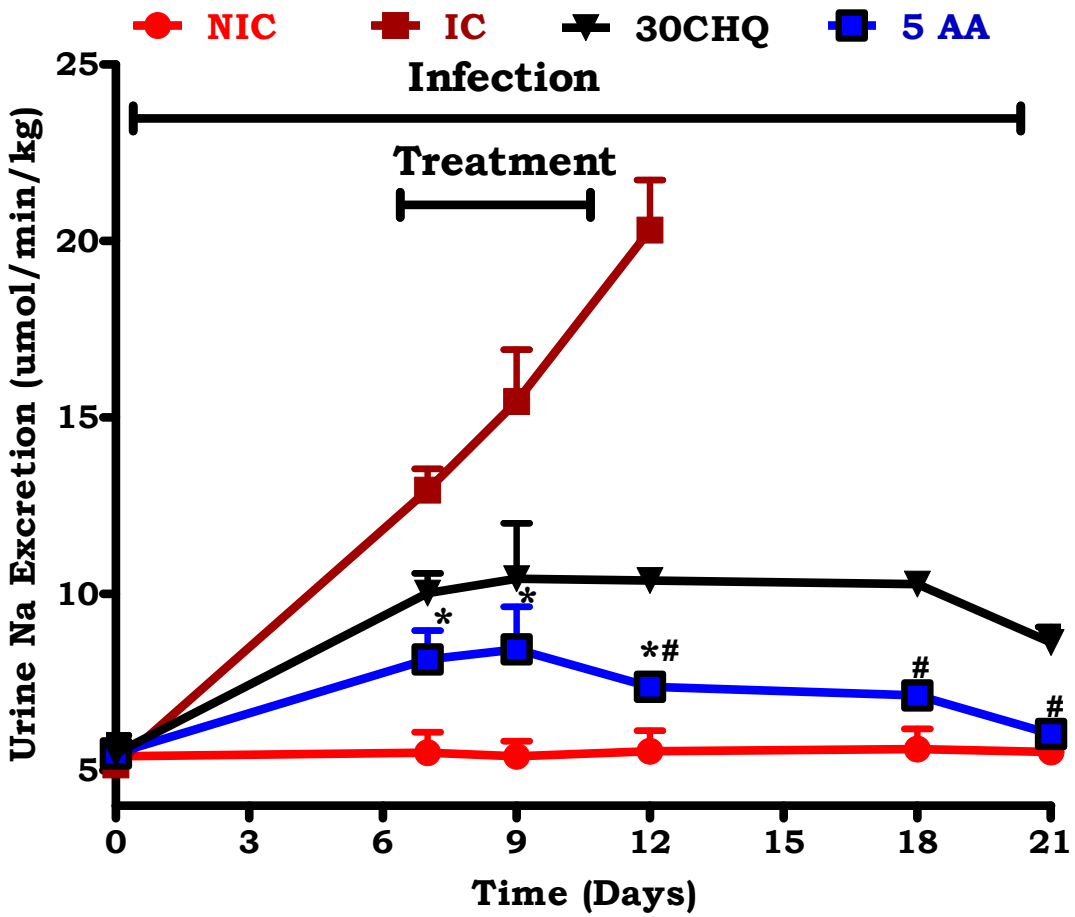

Figure 5: AA5-TDDS influence on absolute Na^+ excretion compared to controls. Results presented as mean ± SEM (n = 6 in each group). *, # $p<0.05$ compared to IC, 30CHQ-pectin patch, respectively.

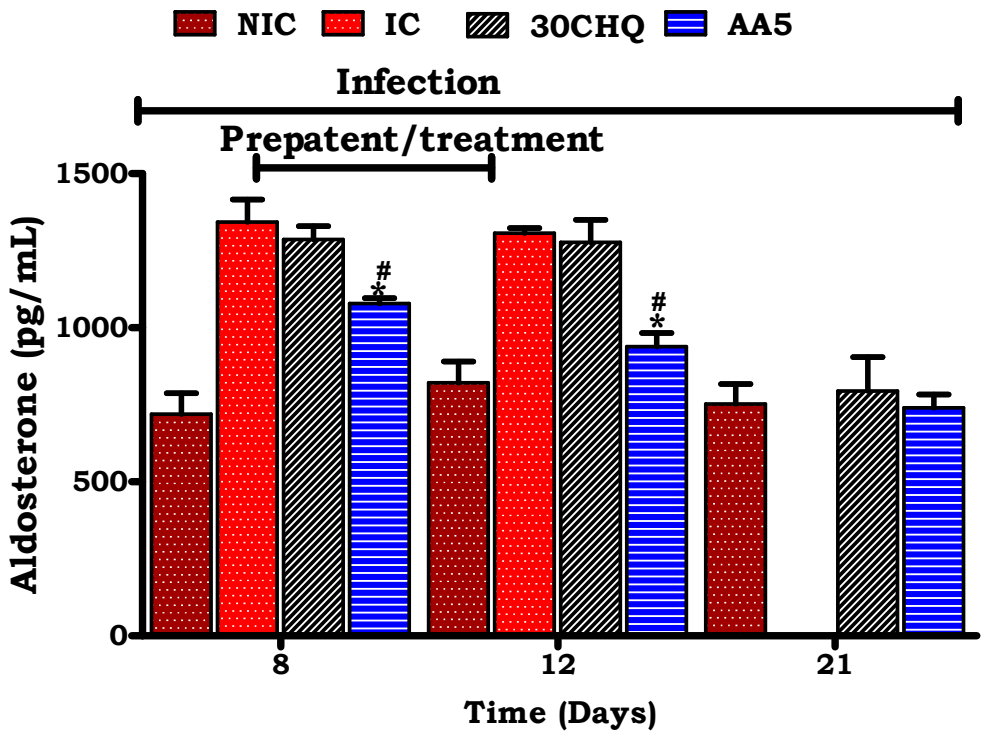

Figure 6: A comparison of AA5-TDDS influence on ALD against controls. Results presented as mean ± SEM (n = 6 per group). *, # $p<0.05$ compared to NIC, IC and CHQ, respectively.

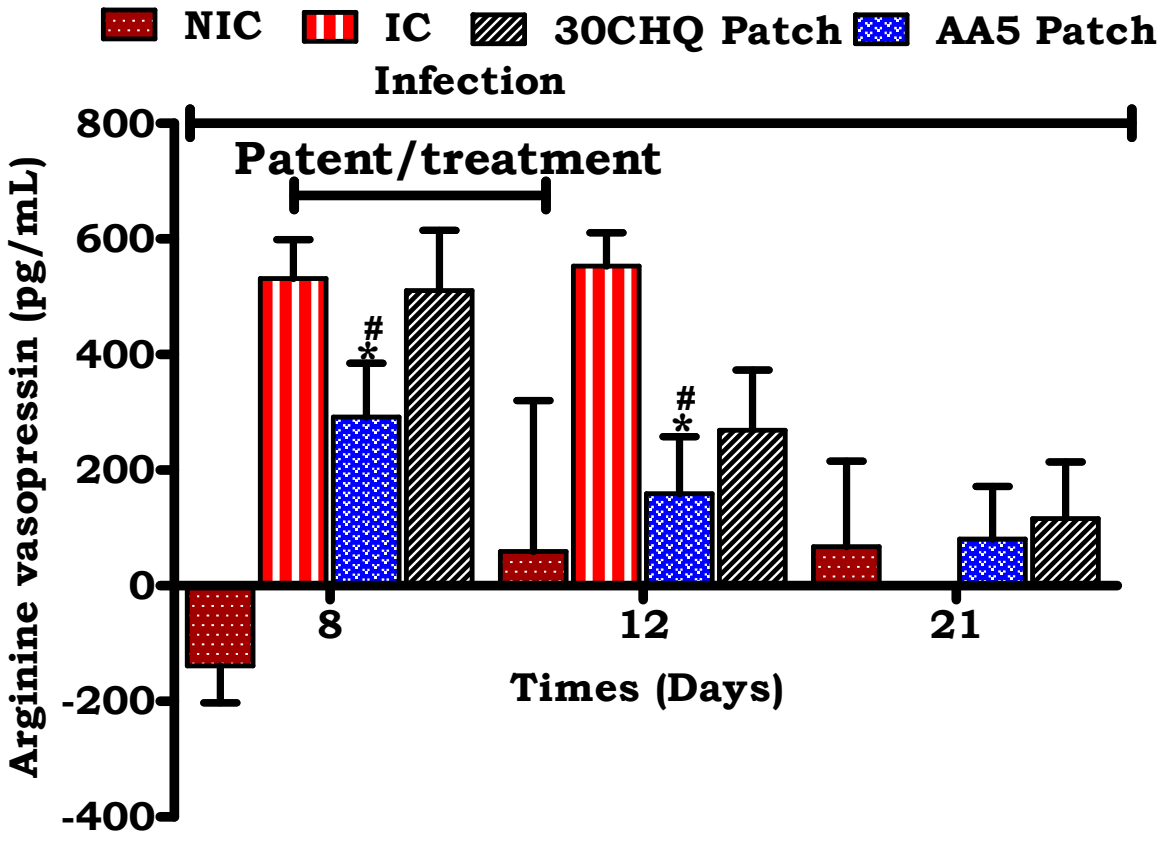

Figure 7: Influence of AA5-TDDS application compared with controls. Results presented as mean ± SEM (n = 6 per group). *, # $p<0.05$ compared to NIC, IC and CHQ, respectively.

Table 4: AA5-pectin patch effects on antioxidant status compared to controls. Superoxide dismutase (SOD), GPx and malonyldialdehyde (MDA) were determined. Values are presented as means ±SEM (n = per group). *, # $p<0.05$ compared to the NIC, IC and CHQ, respectively.

Measured parameter	Experimental group	Tissue source/organ
		Kidney
(A) SOD activity (mU/mL)	NIC	26.34±13.6
	IC	2.58±2.9
	CHQ 30mg/kg	12.11±3.7
	AA5-TDDS mg/kg	23.34±4.1*#
(B) GPx Activity (mU/mL)	NIC	56.67±2.9
	IC	8.23±2.6
	CHQ 30m/kg	44.87±2.5
	AA5-TDDS mg/kg	55.67±4.7*#
(C) MDA (nmol/g protein)	NIC	18.98±3.5
	IC	49.78±5.8
	CHQ 30mg/kg	22.76±6.8
	AA5-TDDS mg/kg	20.12±7.7*

5.0 Discussion:

The systemic pathophysiology of malaria acute renal failure (MARF) requires a wholesome approach bed-rocked on antimalarial agents with multiple anti-disease properties, divergent from parasite load-reduction approaches [Miller LH *et al*, 2013]. We, therefore, report here for the very first time the influence of transdermal drug delivery system (TDDS) of asiatic acid (AA) on renal function and electrolyte handling in *P. berghei*-infected SD male rats, an approach qualifying to be regarded as an anti-disease paradigm.

The combination of amphiphilic AA and TDDS provide a robust framework for combating malaria providing a revolutionary way in which malaria is managed. AA-hydrogel matrix application provides a once-off treatment for malaria, in its own way, a drastic divergence from the known convoluted regimens of current antimalarial drugs in both dosage, frequency and administration route. The theoretic concentration delivered into the systemic circulation was

several amplitudes lower than either oral or TDDS chloroquine at 3µg/kg (6.14nmol/L) giving credence to the efficacy of AA5-TDDS malaria suppression. In our laboratory, malaria posology is designed for a five day twice daily treatment course but AA-TDDS reduced treatment to a once-off-three-day treatment protocol. This prototype reduced animal manipulation frequency, stress and conferred improved food and water intake as well as %weight gain. Continued feeds during infection is tacit indication of the animal overcoming the sickness behaviour which characterised by food and water intake aversion, anorexia, cachexia and death. Post-infection application of the patch preserved the animal wellbeing in general with profound effect of renal function and electrolyte handling. Indeed, malaria and malnutrition from inadequate food intake co-exist as factors contributing to high mortality in children [Kateera F *et al*, 2015]. An anorexic exposition in the infected non-treated (IC) animals (absent from AA-TDDS treated animals) was typified by a negative %weight gain by day 12 necessitating their sacrifice. The preserved bio-physicochemical characteristic of animals on AA5-pectin patch resulted in the improved glomerular filtration rate (GFR) compared to either 30CHQ patch or the IC. The influence of AA on renal function is only limited to a report of *Centella asiatica* having diuretic effect cited by Singh et al (2010) from an untraceable source [Singh S *et al*, 2010], making this current report the first to show improved GFR from AA5-TDDS administration. Moreover, the anti-parasitic efficacy of AA has been reported recently in pre- and post-infection po AA administration animal experiment [Mavondo GA *et al*, 2016, Mavondo GA, Mkhwanazi, B.N., Mabandla, M.V., Musabayane, C.T., 2016]. Treatment with CHQ patch resulted in a higher GFR during the treatment period as has been reported that CHQ inhibits arginine vasopressin (antidiuretic hormone-ADH) which will invariably increase urine output and GFR [Musabayane CT *et al*, 1993]. Observations have also been made that po CHQ has more pronounced effects on GFR than TDDS [Musabayane CT *et al*, 2003]. Furthermore, Patel *et al* (2015), using silico structure-based virtual screening approach have shown AA to be non-nephrotoxicity [Patel H *et al*, 2015], findings may corroborate AA-TDDS's renoprotective effect in malaria.

The influence of AA-TDDS application on urine Na^+ output was significantly lower compared to the IC and 30CHQ patch treatment. However there was an increase in urine Na^+ on day 7, at the initiation of treatment, which was reflected by a non-significant inflection in the GFR when compared to the NIC during the pertinent time point which we could not explain based on these results. We, however, would want to speculate as an interpretation, that the observed urine Na^+ output rise could have been a parasite induced effect as the trend was as for the IC urine Na^+ output and GFR. The initial diffusion, for lack of knowledge on the AA transportation mechanism through the dermis at present, into the systemic circulation may have coincided with an increase in parasitaemia causing a fall in GFR and subsequently a rise in urine Na^+ output. A

cursory inspection of the %parasitaemia will show that indeed, there was a higher transition in parasitaemia between pre-patent (day 3) and the patent/treatment (day 7) periods in the order of 14.19% >9.5%>8.16% for AA5, IC and 30CHQ, respectively. It may also be instructive to mention that a single urine Na^+ output is more an indication of the concentration of the urine and its volume, in the absence of the urine osmolality and may not be reflective of incipient kidneys disease. Our observation were that the higher the urine Na^+ the less urine volume excreted and the more concentrated the urine was. The homeostasis of urine outflow by convention, depends on a variety of factors to include water intake. We were able to demonstrate that the AA-pectin patch applied animals did preserve their water intake for the duration of the study. However, a constantly higher and rising urine Na^+ may have indicated kidney disease as we observed with the IC and 30CHQ patch groups.

Indeed, malaria induces increased Na^+ excretion through the induction of nitric oxide (NO) [Guzman NJ *et al*, 1995, Roczniak A and Burns KD, 1996]. Inducible nitric oxide synthesis (iNOS) is upregulated in malaria generating excessive NO which inhibits Na^+/K^+ ATPase pump responsible for the active reabsorption of the ion in the proximal convoluted tubules (PCT) and other ells. AA has been suggested to inhibit iNOS, cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), interleukin-1β (IL1-β) and tumour necrosis factor-α (TNF-α) expression by downregulating nuclear factor-kappa beta (NF-kβ) via 1kβ kinase and mitogen-activated protein kinase [Huang S-S *et al*, 2011], which are upregulated in malaria. There is a higher probability that the preservation of Na^+ excretion within expected range, when the IC had significantly higher excretion, could also be through protection of the ATPase from oxidative inhibition by AA-TDDS administration. On the other hand, 30CHQ patch high Na^+ urine excretion during patent/treatment period and beyond was expected. Other researchers have reported that CHQ increased renal Na^+ excretion by inhibiting arginine vasopressin (AVP) or antidiuretic hormone (ADH) [Musabayane CT *et al*, 1996, Musabayane CT *et al*, 2000]. Synergistic strategies between the parasite and CHQ in Na^+ depletion, may be involved in the hyponatraemia of malaria and in post-treatment [van Wolfswinkel ME *et al*, 2010].

The absolute urine Na^+ excretion was observed to be continuously rising in the IC showing a sodium wasting process as the transporters were inhibited. We cannot currently explain why the parasite causes Na^+ depletion, weakening its own survival chances with the demise of the host. We speculate that an excessive Na^+ diuresis is not an intended outcome by the parasite. A sequelae from host over-responsiveness to infection through immune reactivity, oxidative stress and inflammation might also be at play. These aspects were resolved by the application of AA5-amidated pectin patch in our experiments as compared to IC and 30CHQ controls. This may

suggest that application of AA-TDDS may have an anti-disease effect on renal Na^+ handling through restoration of the Na^+/K^+ ATPase pump or preservation of other mechanism affected by malaria infection.

The urine K^+ output was elevated in the untreated as well as the 30CHQ patch treated animal groups when compared to the AA5-pectin patch treated animals. This was possibly a reflection of the hyperkalaemia occurring as result of the ATPase pump failure to Na^+ maintenance within the extracellular compartment as well as increased haemolysis. The ATPase pump does not only affect renal electrolyte handling but most cell membranes including red blood cells (RBC's).

The wider ramification of inflammation driven NO excess tends to affect RBC's metabolism and membrane integrity and deformability which results in RBC's (both parasitized-pRBC's and non-parasitized-npRBC's) to be removed by the spleen [Maitland K and Marsh K, 2004] with resultant severe malaria anaemia (SMA) [Ghosh K and Ghosh K, 2007]. We did not observe a rise in urine K^+ output in the AA5-pectin patch applied animals as compared to the IC and the 30CHQ patch treated animals indicating a reduction or absence of haemolysis which may be traced back to the anti-inflammatory effect of AA on RBC membrane integrity. The antioxidant capacity of AA could also have quenched ROS generation while the pro-oxidant capacity may have eradicated the parasite to preserve the kidney function.

To corroborate the renal electrolyte preservation of AA5-pectin patch was the estimation of urine Cl^- output. Urine Cl^- output was elevated in the IC as well as the 30CHQ patch treated animals, but remained significantly correlated to the NIC values except during treatment. Noteworthy is the fact that, application of the AA5-pectin patch was commenced when the %parasitaemia had already reached patent and stable state and the reversal of the electrolyte dysregulation observed for Cl^- but not observed in the IC urine Cl^- output indicated the influence of AA on renal electrolyte handling. An increase in urine Cl^- output, besides depicting a concentrated urine excretion, may also be an indicator of acid base balance disturbances. In non-respiratory acidosis there is a depletion of HCO_3^- and an increase in H^+ which necessitates conversation of the latter in the PCT. To maintain an electrolyte neutrality, Cl^- shifts from the tubular cells into the lumen in exchange and preservation of HCO_3^-. The hydronium ion is also excreted in exchange for K^+ or Na^+ but due the ATPase pump failure in malaria this may not happen and as a result non-respiratory acidosis escalates worsening the compromised renal function.

Besides impairment of Na^+/K^+ pump by increased oxidative stress, ATP rundown seen in malaria through Poly(ADP-ribose) polymerase activation by $ONOO^-$ in malaria may also contribute to pump failure with the same results. Besides inhibition of Na^+/K^+ ATPase pump,

NO also inhibits endothelial Na^+ channels (ENaC). Other electrolyte transporters such as the Na^+/H^+ exchanger that facilitate proton excretion and HCO_3^- reabsorption depend on the Na^+ electrochemical gradient created by the ATPase. Inhibition of the pump may, therefore, result in acidosis [Clark IA and Cowden WB, 2003]. However, AA has been reported to alleviate haemodynamic and metabolic aberrations in rats with metabolic syndrome via the equilibration of endothelial nitric oxide synthase (eNOS) and induced nitric oxide synthase (iNOS) expression together with oxidative stress and inflammation reduction [Pakdeechote P *et al*, 2014]. These reports are in support of our findings that AA5-pectin patch application suppressed parasitaemia as well as the abolition of other malaria pathophysiology.

Due to the inhibition of ENaC, aldosterone is unable to effect Na^+ retention resulting in salt-losing nephropathy characterised by dehydration and non-respiratory acidosis and a concomitant rise in the hormone's plasma concentration. When aldosterone activity is elevated, in rising natriuresis, a condition termed pseudohypoaldosteronism type 1 results in congenital conditions characterised by ENaC failure [Amin N *et al*, 2013]. We observed the same scenario during the initiation of AA-TDDS application on day 8. Significantly higher activity of aldosterone corresponded to raised urine Na^+ output and a downward transitory inflection in the GFR which could indicate hypovolaemia from reduced food and water intake, increased RBC's destruction or both. This may also imply that before therapeutic dose of AA was reached, there might have been inhibition of the ENaC resulting in hyperaldosteronism. Reactivation of the channel, through possible quenching of oxidative stress or oxygen reactive species (ROS) or both might have normalized Na^+ excretion. In stark contrast to AA-pectin patch effect was the IC and 30CHQ patch treatment showing raised aldosterone and Na^+ concentrations, indications of possible excessive ATPase pump inhibition.

Arginine vasopressin (AVP) activity for AA5-pectin patch applied animals, during the day 8 time-point, was higher than for other time-points triggered by possible hypovolaemia and increasing osmolality. However, the activity was comparatively lower than both the IC and 30CHQ patch groups. Indeed, chloroquine has been shown to influence increased Na^+ urine excretion [Musabayane CT *et al*, 1996] through inhibition of cyclic AMP (cAMP) or via generation of NO [Ahmed MH and Osman MM, 2007]. Both mechanism cause an increase in plasma vasopressin as we observed with 30CHQ patch treatment. The resultant diuresis, natriuresis and increased GRF are hall marks of chloroquine treatment which we were able to show in 30CHQ patch treatment but were corrected with AA5-TDDS application. In contrast to this finding on CHQ, the phytochemical AA (as an extract of *C. asiatica*) has been reported to

increase cAMP content in tissues [Tholon I *et al*, 2002] which could also be the mechanism AA uses in preservation of renal electrolyte handling.

To probe further the influence of AA5-TDDS on oxidative stress, we measured the activity of antioxidant enzymes and the level of peroxidation in the kidney. Significantly higher superoxide dismutase activity was observed in the animals on AA5-pectin patch. Also significantly raised was glutathione peroxidase as compared to both the IC and the 30CHQ patch treated animals. On the other hand animals applied the AA5-TDDS patch had lower MDA compared to the 30CHQ patch and the IC groups. Others workers have also described similar findings with oral administration of AA at higher doses, than reported here, in non-malaria conditions [Huang S-S *et al*, 2011]. The antioxidant role of AA5-pectin patch in the preservation of renal electrolyte handling may have been through reactivation of the ATPase pump as well as hormonal influence of water metabolism showing possible anti-disease effects of AA on kidney function. Further work, however, may need be done to explore the possible interaction of AA with electrolyte channels and transporters, the interaction of AA with malaria toxins that elicit inflammatory reactions and the general metabolic effects of the amphiphilic nature of AA in malaria.

6.0 Conclusion:

All of the above taken together, we have been able to demonstrate that AA5-amidated pectin hydrogel matrix patch application reduced the dosing frequency, dosage and the duration of treatment. Also shown was the ability of AA5-pectin patch application to preserve food and water intake as well as %weight gain and suppress parasitaemia. We also showed that AA5-TDDS ameliorated the disease aspect of malaria through increasing antioxidant capacity, reduction in renal lipid peroxidation, preservation of renal function and electrolyte homeostasis together with maintenance of renal hormonal function. We also observed that pseudohypoaldosteroism was a reversible condition in malaria with proper treatment as we saw a slight increase in urine Na^+ output which was attenuated by AA5-TDDS application. We speculate that the antioxidant capacity of AA5-TDDS through quenching of NO, ROS, $ONOO^-$ seem to influence renal function and electrolyte handling through preservation of the Na^+/K^+ ATPase pump and ENaC.

7.0 Declarations:

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7.3 Conflict of interests: The authors declare no conflict of in interest in this work.

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Chapter 5

Synthesis, Conclusions and Recommendations

5.1 Synthesis and discussion:

The link between murine malaria development, prevention and management by way of Asiatic acid (AA) administration, orally and transdermal, was elucidated as novel findings in this study. Demonstration of %parasitaemia on Giemsa stained glass slides was the cardinal point used to show *Plasmodium berghei* patent infection at day 7, peak parasitaemia at day 12 for AA administration or day 9 for chloroquine treatment and parasitaemia suppression during studies termination. Successful intervention was proven by a reduction in %parasitaemia with efficacy of the treatment agent administered reached when parasitaemia was not detectable anymore in the peripheral circulation. By three different protocols, the efficacy of AA in suppressing malaria was presented mainly: i) retardation of patent parasitaemia development with subsequent diminution of the cumulative parasite load in per oral chemoprophylaxis investigations, ii) suppression of very high parasitic burdens by per oral post-infection administration of AA and iii) through post-infection chemotherapeutic suppression of parasitaemia by way of AA-transdermal drug delivery system (AA-TDDS) administration. The three chronologically-spaced experiments yielded the same results although with varied specific outcomes indicating a common basic mechanism of action for AA in combating malaria. In the pre-infection AA administration, the phytochemical may have interfered with parasite patent development through direct interaction with the parasite exposing parasite antigens to the innate immunity with subsequent mounting of competent immunological response to the infection. While we were unable to demonstrate the oxidative capacity of AA in this study, maslinic acid (MA), a similar compound to AA, is known to interfere with the cellular membranes of the schyzoint stages of malaria parasite, through possible pro-oxidant mechanism and AA may have similar action on the parasite thereby inhibiting mature merozoite formation. Schyzoint maturation is a critical step in the propagation of patent

parasitaemia which when disrupted may retard malaria parasite cycle. Per oral AA administration may have necessitated a cumulative lethal dose build up in plasma for an oxidative anti-parasitic action to be attained during post-infection administration of AA. This drug build up in plasma may not have been required in pre-infection administration as parasite introduction occurred when such drug levels had been reached already. The transmission of the phytochemical directly into the intravascular compartment through AA-TDDS may have circumvented the hepatic single pass with possible faster plasma build-up of the triterpene lethal dose thereby requiring shorter time course and lower dose to clear the parasite. By these three distinct AA administration processes, three different %parasitaemia-time trajectories (curves) could be traced which invariably terminated in parasitaemia suppression before or at the day 21 time points intoning inalienable efficacy of the triterpene against *P. berghei*-induced murine malaria.

The anti-inflammatory activity of AA may also have had a common influence on parasitaemia development in either chemoprophylaxis or chemotherapeutic, by either po or TDDS delivery routes, which forefended macrophages recruitment necessary for providing parasite passage during infection initiation. By this process parasite infectivity may have been educed. By reducing inflammation, AA could also have removed the foothold of malaria pathogenesis quilling existing and incipient pathophysiology. Without the disease aspects of malaria, parasite infection may have been emasculated and semi-immune status, seen in people living in malaria endemic areas entrenched with subsequent parasite eradication. These anti-disease aspects of AA are unassailable novel findings that may be useful in the combat of malaria.

We have reported that, besides parasitic proliferation inhibition, AA ameliorated severe malaria anaemia (SMA) as well as inflammation when administered per oral and by transdermal drug delivery system (TDDS) of which the former pathophysiology and the latter drug delivery method are novel findings in the fight against malaria. The mechanism of malaria anaemia and aetiology was explained elsewhere with inflammation being closely linked to its development such that

amelioration of inflammatory pathology by AA, which was reported by others, may be subsequently lead to anaemia resolution.

Transdermal drug delivery of AA resulted in a reduced dose having the same efficacy with the higher dose po, a finding that is of utmost important redirecting malaria management to non-invasive and most likely patient convenience and compliance. The rats to which we applied the patch seemed to tolerate the patches as they did not display signs of discomfort showing the safety aspect of the delivery method and the delivered phytochemical. Patient non-compliance to treatment is one major method by which treatment failure may be experienced and drug resistance may be acquired through suboptimal drug delivery. The use of AA-TDDS also showed that po administration of AA is an inefficient or inadequate method of delivery of the phytochemical as a much lower dose of AA-TDDS was able to suppress parasitaemia to the same extent as the po dose. The convenience of the patch application in malaria, in that it is an "apply and walk away" prototype suggests a higher potential of use in malaria chemoprophylaxis where it may benefit both children under the age of 5 years and pregnant women. Children under the age of 5 years usually require care-givers close attention during po disease management which care could be extremely difficult to effect when current malaria is not the compelling factor for drug administration. Pregnant women and visitors to endemic areas may also benefit from AA-TDDS chemoprophylaxis or chemotherapeutics. Therefore, AA-TDDS becomes an area with promise for long-term malaria prevention and treatment especially in patients who find it cumbersome to adhere to treatment or prophylactic regimens.

We observed that untreated parasitaemia led to pRBC's and npRBC's haemolysis (elevated lactate dehydrogenase) which reduced cell volume (low RBC count), increased plasma volume relative red cell volume and increased rheological abnormalities (low GFR), released GPI and activated innate immune response with inflammation commandeering TNF-α secretion (elevated WBC count, CRP). Hemozoin release may have induced SMA [1] (Giemsa micrographs) through erythropoiesis suppression (low haemoglobin, haematocrit). However, per oral pre-and post-

infection AA administrations circumvented as well as reversed these pathophysiology of malaria showing that AA may be a potential antimalarial with anti-disease effects. The pathophysiology seen in malaria is usually the cause of death from malaria than the parasite itself and proscribing these developments in malaria, has a higher potential of containing malaria. Asymptomatic malaria, with undetectable levels of parasitaemia, is premised to hold sway based on the immune system containing the pathophysiology of malaria. Therefore, AA administration may play a prophylaxis role as a supplement in food or directly to suppress parasitaemia. On the more practical side *Centella asiatic* is a natural plant growing wild in many malaria endemic areas which can be domesticated easily and included in the diet as a green salad to provide adequate levels of AA and other phytochemicals for the combating of malaria.

There was a strong display in preservation of eating and drinking habits as well as %weight gain in animals administered with AA either before or after *P. berghei* infection, by oral route or by TDDS. Infected non-treated controls showed a picture conforming to the sickness behaviour induced anorexia [2, 3]. This anti-disease effect of AA had a roller coaster effect on the downstream effectors of disease, concomitantly influencing the aspects of inflammation (significantly decreased CRP) and immune dysregulation (significantly reduced WBC count) [4] which are bedrocks of malaria. Appetite suppression and satiation are an intrinsic mechanism built within the animal for self-preservation during disease-induced stress. AA's effect on food intake may have rested on the potentiation of insulin of glucose homeostasis as does other phytochemicals [5]. Efficient utilization of glucose, which was observed, averted hypoglycaemia or hyperlactaemia of malaria. Lactate invariably increases from anaerobic glycolysis facilitated by and generated from increased GLUT-I expression in muscle and lung tissue in malaria [6,7]. Lactate may be converted to energy in the tricarboxylic acid cycle or converted to glucose through the Cori cycle in the liver [7]. But due to the increased amount produced in malaria hypoglycaemia [8], the reclamation of lactate through pyruvate may be overwhelmed. AA administration induced clearance of lactate through either efficient utilization as energy or conversion to glycogen as the

glycogen stores were high in AA administered animals and lactate was low. Insulin remained low during the 21 day study as well as in the acute studies showing some protective effect on either its function or secretion which was not observed in infected non-treated groups [9-10]. The disparity may be attributable to AA effects on malaria. In acute studies improved oral glucose tolerance response may have indicated patent GLUT 4 transporters in muscle and adipose tissues a fact that was corroborated by the demonstration of increased muscle glycogen stores. On the other hand glucagon increased significantly showing that higher concentrations might have been as a result of gluconeogenesis enzymes inhibition by AA which caused a "resistance" to its activity. In hyperglycaemic rats AA was shown by others to increase glycolytic enzyme function while inhibiting gluconeogenesis an effect that may result in energy deficits in normoglycaemic cases resulting in food and water intake as the only source of energy. Glucagon in turn may have increased appetite promoting increased animal foraging, forestalling anorexia and preserving food and water intake as well as %weight gain. Glucagon was suppressed while insulin was elevated in infected non-treated control groups with opposite effects being observed. This may imply that the antihyperglycaemic effect of AA seen in streptozotocin-induced diabetes mellitus (DM) [11] may be extended to malaria management too although with the opposite effect of glucose preservation than depletion. This ameliorative role of AA may therefore not be regarded as antihyperglycaemic effect but a "glucose modulation function" since it lowers high glucose concentrations in DM and rescues hypoglycaemia in malaria. The situational influence of AA on glucose homeostasis is a novel finding attributable most likely to the pleiotropic effects of AA which intones the possibility of an evolutional biological control function of phytochemicals. This may also imply a profound effect on malaria in diabetic patients. Indeed, it has since been reported that animals that have a co-existence of DM and malaria tended to be higher transmitters of the infection to the mosquito [12] and that DM type 2 predisposes patients to *P. falciparum* infection by 46% [13]. AA5-pectin patch application modulated inflammation (significantly decreased plasma CRP at day 21) together with decreasing oxidative stress (raised liver, kidney, muscle SOD and GPx and low MDA) [14]. Ultimately, renal function and renal electrolyte

handling was preserved. Preservation of electrolyte homeostasis is an intricate balance between sodium reabsorption in the proximal convoluted tubule and facilitated retention at the distal convoluted tubules. However, in malaria sodium wasting is experienced in the face of increased aldosterone (ALD) a condition referred to a pseudohyperaldosteronism orchestrated by reduction in the functional salt channels necessary for the movement of the ion between the ultrafiltrate and the tubular cells. Increased oxidative stress of malaria through increased oxygen free radical, $ONOO^-$, and other species may affect the sodium/glucose transporters (SGLUT) in the proximal convoluted resulting in reduced sodium reabsorption and increased load reaching the distal convoluted tubules where the capacity to reabsorb sodium is overwhelmed and the electrolyte lost in urine with subsequent volume depletion. Hypovolaemia in malaria is worsened by reduced food and water intake, glucagon-induced satiation, anorexia which will induce arginine vasopressin (AVP) secretion. While we observed increases of both AVP and ALD, decreased glomerular filtration rate and increased urinary sodium in the infected non-treated animals, AA-TDDS administration ameliorated these untoward effects of malaria.

As indicated earlier, AA may have anti-disease pleiotropic functions like most molecules which co-evolved to foster ecological equilibrium [14].

5.2 Conclusion:

AA is an effective antimalarial by oral or transdermal drug delivery in pre-and post *P. berghei* infection making it possible a chemoprophylaxis and chemotherapeutic in malaria. Transdermal drug delivery system reduce the effective dose used in malaria, frequency of dosing, treatment duration and easier drug intake compliance. AA abrogated parasitaemia by all doses when administered pre-infection po, post-infection po per oral and po TDDS. The AA 10mg/kg dose had the most efficacy per oral while the AA 5mg/kg dose was the most efficacious anti-parasitic by transdermal route. AA improved oral glucose tolerance response under acute conditions through moderation of insulin and glucagon functions. AA had renal-protective effect in malaria and may have reversed pseudohyperaldosteronism, restored Na^+ loss and water balance through

equilibrating of aldosterone and arginine vasopressin activities. AA has antimalarial, antioxidant, glucose homeostasis modulatory, and renoprotective capacity in malaria.

5.3 Recommendations:

Elucidate in silico structure identification and binding of AA to gycosylphosphatidylinositol toxin and effects on cAMP generation

Investigate the interaction of AA with proteins involved in the DNA machinery

Create a malaria-diabetes mellitus model and investigate the effect of AA in this condition

5.4. Strengths and Limitations of the study:

5.4.1 Strengths:

The strength to the study have been discussed in the findings that have been reported in the various chapters mainly:

- Parasitaemia suppression of AA by both oral and TDDS in a murine malaria model

- Amelioration of severe malaria anaemia and influencing malaria induced inflammation of AA

- Influence on glucose homeostasis in malaria of AA by oral administration

- Moderation on acute kidney injury and renal electrolyte handling among

5.4.2 Weaknesses:

Although it was not the scope of this study certain aspect could have been included in the research protocol to strengthen and corroborate current finings, were it not for limitations of funding, mainly:

- The current findings were carried out in and animal model which may not translate human efficacy directly

- The study was carried out as a 21 day study with parasitaemia suppression as the end point where as a longer period may have possibly a different outcomes

- More advanced molecular studies such as demonstration of GLUT and Na^+ transporters mRNA reduction or increase may have awarded the research more results to answer some of the unanswered question

- Estimation of more robust acute kidney injury biomarkers such as Kidney Injury Molecule 1, Neutrophil Gelatinase Associated Lipocalin, Interleukin 18, N-Acetyl-Glucosaminidase, Asymmetric Dimethyl Arginine, and Liver Type Fatty Acid Binding Protein could have indicated the most affected areas for renal system.

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ANNEXES LIST

Pictorial depiction of AA-patch preparation process, products, application, treatment course and outcomes. AA journeys from materials [A] to AA-hydrogel pectin patch in petri dish [B], shaved animal dorsal neck region [C] (day 7), past malaria parasite infection [C] (day 7-10), to animal healing, health and wellness [E] (day 21) and to used up, shrivelled AA-pectin patch discs ready for the dust bin [F1, F2].

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

7 April 2014

Reference: 079/14/Animal

Mr G Mavondo
Discipline of Physiology
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Mavondo

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Evaluation of efficacy of selected medicinal plant extracts on malaria parasites in *Plasmodium berghei*-infected Sprague-Dawley rats: effects on blood glucose and renal electrolyte handling."

Yours sincerely

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
Head of School – Prof. W Daniels
BRU – Dr S Singh

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 December 2014

Reference: 013/15/Animal

Mr G Mavondo
Discipline of Physiology
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Mavondo

RENEWAL: Ethical Approval of Research Projects on Animals

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Yours sincerely

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

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//Posthumously

Introduction

Glucose homeostasis derangement is a common pathophysiology of malaria whose aetiology is still controversial. The Plasmodium parasite, immunological and inflammatory responses, as well as chemotherapeutics currently used cause hypoglycaemia in malaria. Anti-parasitic and anti-disease drugs are required to combat malaria while ameliorating the pathophysiology of the infection. Asiatic acid has antihyperglycaemic, antioxidant, pro-oxidant properties useful in glucose homeostasis but its influence in malaria is yet to be reported. Here we present findings on the influence of asiatic acid on glucose metabolism in vitro together with in P. berghei-infected Sprague Dawley rats.

Methods

Acute as well sub-chronic studies were carried out in vivo where physicochemical properties and glucose homeostasis were monitored, after administration of asiatic acid (10mg/kg) in both non-infected and infected animals. Glucose metabolism associated biochemical changes in malaria were also investigated.

Results

In acute studies, asiatic acid improved oral glucose response while in the sub-chronic state it maintained food and water intake as well as %weight gain, suppressed parasitaemia and maintained normoglycaemic control in infected animals.

Conclusions

In vitro asiatic acid did not display significant effect on glucose homeostasis. Per oral post-infection asiatic acid administration influenced physicochemical parameters, parasitaemia, glucose metabolism and associated biochemical processes.

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