*IN VITRO*ANTIMALARIALSUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* ISOLATES FROM PATIENTSATTENDING A HEALTH FACILITY IN WUSASA, ZARIA, NIGERIA

BY

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DECLARATION

I declare that the work in the dissertation titled "*In vitro* antimalarial susceptibility of *Plasmodium falciparum*isolates from patients attending a Health facility in Wusasa, Zaria, Nigeria'' has been performed by me in the Department of Pharmaceutics and Pharmaceutical Microbiology under the supervision of Professor J.O. Ehinmidu and Prof (Mrs) A.R. Oyi. The information derived from the literature has been fully acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another degree or diploma at any University.

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation titled "*In vitro*antimalarial susceptibility of *Plasmodium falciparum* isolates from patients attending a Health facility in Wusasa, Zaria, Nigeria" meets the regulations governing the award of Master of Science (Pharmaceutical Microbiology) of Ahmadu Bello University and it is approved for itscontribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to Almighty Allah (SWT) for giving me the health and strength to carry out this research work.

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ABSTRACT

The continous emergence and spread of resistant strains of *Plasmodium falciparum*isolates to single and combined antimalarial drug has resulted to increase in mortality rate associated with the disease, especially in the African Continent as a whole and Nigeria in particular where the disease continues to be endemic. *In vitro*antimalarial susceptibility of *Plasmodiumfalciparum*isolates from patients attending a Health facility in Wusasa, Zaria, Nigeria was carried out using WHO standard Mark III Micro- Test System. A retrospective study determined the incidence of malaria and pattern of antimalarial prescription at the Health Facility. A total of 953 people, comprising of 448 males (47%) and 505 females (53%) were reported to be infected with malaria from 2012 through 2014 in a Health facility in Wusasa, Zaria. Of this total number of reported cases, 265 (28%) were children between the ages of 0-5 years, 503(53%) were between the ages of 6-39 years and 185 (19%) were aged 40 and above. The incidence of malaria at the Health Facility, overthe three year period (2012-2014) was calculated to be 2.8%. Chloroquine, Quinine, Fansider/ Falcidine(Sulphadoxine+Pyrimethamine),Artemether, Artesunate, Coartem (Artemether+Lumefantrine), ASAQ (Artemether+Piperaquine), were the antimalarials that were routinely prescribed at a Health facility in Wusasa, Zaria from 2012 through 2014. Chloroquine was the most prescribed in year 2012(35.1%) and 2013(35.9%), While Coartem was the most prescribed in 2014(31.7%). A total of 120 blood samples of patients with *Plasmodium falciparum* malaria wereused in this study. However, only 30 samples were successfully evaluated, the remaining were not used due to patients admitting to taking antimalarial drugs 14 days prior to presentation, low parasite density, Failure of schizont to mature satisfactorily in the control well and contamination. Out of the total of 30 isolates of *Plasmodium falciparum* used in this study, comprising of 17 females and 13 males,8 were between the ages of 0-5, 14 were between the ages of 6-39 years and 8 were 40 and above. Most of the isolates (90%)were resistant to Quinine and Chloroquine, with median IC_{50} of 7.8µg/ml and 5.3µg/ml respectively. There was however 100% *invitro*sensitivity to Artemeter and Artemeter - lumefantrine combination. The crude extract of *Psidium guajava* and *Carica papaya*showed good antiplasmodial activity against *Plasmodium falciparum* isolates in the study conducted, with Median IC_{50} values of 9.2µg/ml and 5.4µg/ml respectively.Ten (10) of the resistant *Plasmodium falciparum* isolates were assayed to determine the prevalence of two of the established antimalarial drug resistance markers: the Chloroquine resistance transporter gene (*pfcrt*)and the multi-drug resistance transporter 1gene (*pfmdr1*). All 10 (100%) of the *in vitro*resistant isolates harboured the *pfcrt* chloroquine resistance gene, while 8(80%) harboured the *pfmdr1*gene. Theresult from this study shows that high level of chloroquine resistance still remains in this region and there is an alarming emergence of resistance to Quinine. Artemether and artemisin based combinations still remain very potent in treating malaria based on the *in vitro* results from this study. *Psidium guajava* and *Carica papaya* leaf extracts showed promising activity against resistant *Plasmodium falciparum* isolates in this study, making them plants of choice to be considered for the development of newer antimalarials.

TABLE OF CONTENT

TITLE **PAGE**

Title Page- - - - - - - - - - - i

Declaration- - - - - - - - - - - ii

Certification - - - - - - - - - - - iii

Dedication- - - - - - - - - - -iv

Acknowledgement-- - - - - - - - - - v

Abstract- - - - - - - - - - -vi

Table of Content- - - - - - - - - - vii

List of Tables - - - - - - - - - - xii

List of Figures- - - - - - - - - - xiii

List of Appendices- - - - - - - - - - xiv

CHAPTER ONE

1.0 Introduction- - -- - - - - - - 1

1.1 Background Information- - - - - - - - - - 5

1.2 Statement of Problem- - - - - - - - - - 6

1.3 Justification- - - - - - - - - - 6

1.4 Null Hypothesis- - - - - - - - - - 6

1.5 Alternate Hypothesis- - - - - - - - - - 6

1.6 Aims and Objectives- - - - - - - - - - 7

1.6.1 Aims- - - - - - - - - - 7

1.6.2 Objectives- - - - - - - - - - 7

CHAPTER TWO

2.0 Literature Review- - - - - - - - - - 8

2.1 Historical Outline- - - - - - - - - - 8

2.2 Malaria Parasite- - - - - - - - - - 9

2.3 Malaria Disease- - - - - - - - - - 11

2.3.1 Epidemiology- - - - - - - - - - 11

2.3.2 Etiological Agent- - - - - - - - - - 12

2.3.3 Life Cycle- - - - - - - - - - 12

2.3.4 Pathophysiology- - - - - - - - -- - 14

2.3.5 Symptoms- - - - - - - - - - 16

2.3.6 Diagnosis- - - - - - - - - - 17

2.4 Antimalarial Drugs and Mechanisms of Action- - - - - 19

2.4.1 Quinine- - - - - - - - - - - 19

2.4.1.1 Mechanism of Action of Quinine- - - - - - - 19

2.4.2 Chloroquine- - - - - - - - - - 20

2.4.2.1 Mechanism of Action of Chloroquine- - - - - - 20

2.4.3 Amodiaquine- - - - - - - - - - 21

2.4.4 Pyrimethamine- - - - - - - - - - 21

2.4.4.1 Mechanism of Action of Pyrimethamine- - - - - - 22

2.4.5 Proguanil- - - - - - - - - - 22

2.4.5.1 Mechanism of Action of Proguanil- - - - - - - 22

2.4.6 Sulfonamide- - - - - - - - - - 23

2.4.6.1 Mechanism of Action of Sulonamide- - - - - - 23

2.4.7 Mefloquine- - - - - - - - - - 24

2.4.7.1 Mechanism of action of Mefloquine- - - - - - - 24

2.4.8 Primaquine- - - - - - - - - - 25

2.4.8.1 Mechanism of action of Primaquine -- - - - - - 25

2.4.9 Artemisinin and Derivatives- - - - - - - 25

2.4.9.1 Mechanism of action of artemisinin- - - - - - - 26

2.4.10 Halofantrine- - - - - - - - - - 27

2.4.10.1 Mechanismof Action of Halofantrine- - - - - - 27

2.4.11 Doxycyline- - - - - - - - - - 28

2.4.11.1 Mechanism of Action of Doxycyline- - - - - - 28

2.4.12 Clindamycin- - - - - - - - - - 29

2.5 Traditional Antimalarial Herbs- - - - - - - - 29

2.6 Antimalarial Drug Resistance- - - - - - - - 33

2.6.1 Types of Drug resistance - - - - - - - - 34

2.6.2 Mechanism of antimalarial resistance- - - - - - - 34

2.6.2.1 Mechanism of Chloroquine resistance- - - - - - 35

2.6.2.2 Mechanism of Antifolate resistance- - - - - - - 36

2.6.2.3 Mechanism of Atovaquone resistance- - - - - - 36

2.6.2.4 Mechanism of Artemisin resistance- - - - - - - 36

2.7 Spread of Drug resistance- - - - - - - - 37

2.8 *In vitro* cultivation- - - - - - - - - 38

CHAPTER THREE

3.1 Materials - - - - - - - - - - 41

3.1.1Study Area - - - - - - - - -41

3.1.2 Plant material - - - - - - - - - 41

3.1.3 Antimalarial drugs - - - - - - - - - 41

3.1.4 Reagents and Chemicals - - - - - - - - - - - 41

3.1.5 Equipments - - - - - - - - - - - - - - - - - - 41

3.1.6 Glass wares - 41

3.1.7 Culture Media- - - - - - - - - - - - - - - - - - 42

3.1.7.1 Preparation of incomplete culture medium- - - - - - - - -42

3.1.7.2 Preparation of complete culture medium- - - - - - - - - 42

3.2 Methods - 42

3.2.1 Retrospective Study- - - - - - - - - 42

3.2.2 Identification of plant material- - - - - - - - - - - - - 43

3.2.3 PlantExtraction- - - - - - - - - 43

3.2.4 Preparation of Plant extract- - - - - - - - 43

3.2.5 Ethical Consideration- - - - - - - - - 43

3.2.6 Sample collection- - - - - - - - - 44

3.2.7 Patients and study protocol- - - - - - - - -44

3.2.8 Processing of samples - - - - - - - - - - - - - - - - - - 44

3.2.9 Microscopic Examination - - - - - - - - - - - - - - - - - - 44

3.2.10 Performance of the *in vitro* micro test- - - - - - -45

3.2.11 Examination of the post culture blood slide- - - - - - -46

3.2.12Molecular Characterization of Chloroquine and quinine resistant genes- -47

3.2.12.1 DNA extraction- - - - - - - - - -47

3.2.12.2 PCR for the detection of pfcrt gene - - - - - - -47

3.2.12.4 Nested PCR for pfcrt mutation-specific detection - - - -50

CHAPTER FOUR

4.0 Results- - - - - - - - - - -52

4.1 Incidence of Malaria (Retrospective Study)- - - - - - -52

4.2 Incidence of *Plasmodiumfalciparum* among age groups- - - -54

4.3 Pattern of Antimalaria prescription (Retrospective study)- - - -56

4.4 *In vitro* assay- - - - - - - - - -61

4.5 *In vitro* antimalarial drug sensitivity test- - - - - -61

4.5.1 Quinine- - - - - - - - - - -62

4.5.2 Chloroquine- - - - - - - - - -62

4.5.3 Artemether- - - - - - - - - -63

4.5.4 Arthemether- Lumefantrine- - - - - - - -63

4.6*In vitro*Antimalarial activity of plant extract- - - - - -64

4.7Prevalence of Drug resistance molecular marker- - - - -73

CHAPTER FIVE

5.0 Discussion- - - - - - - - - -77

5.1Conclusion- - - - - - - - - -83

5.2 Recommendation- - - - - - - - - -84

References- - - - - - - - - - -85

LIST OF TABLES

TITLE **PAGE**

Table 3.1: Primers for Chloroquine and Quinine resistance genes- - - - 48

Table 3.2: Reaction Mixture for the detection of *Pfcrt* and *Pfmdr* genes- - - 49

Table 3.3: Thermal cycling conditions used for nested PCR- - - -51

Table 4.1: Monthly Distribution of reported malaria cases over a three year period in a Health facility in Wusasa, Zaria- - - - - -53

Table 4.2: Degree of quinine inhibition of Schizont maturation (%) at different concentrations (µmol/l)- - - - - - - - 65

Table 4.3: Degree of chloroquine inhibition of Schizont maturation (%) at different concentrations (µmol/l)- - - - - - - - 66

Table 4.4: Degree of arthemether inhibition of Schizont maturation (%) at different concentrations (µmol/l)- - - - - - - - 67

Table 4.5: Degree of artemether-lumefantrine combination inhibition of Schizont maturation (%) at different concentrations (µmol/l)- - - - 68

Table 4.6: Degree of*P. Guajava* leaf extract inhibition of Schizont maturation (%) at different concentrations of (mg/ml)- - - - - - - 69

Table 4.7: Degree of *C. papaya*inhibition of Schizont maturation (%) at different concentrations (mg/ml)- - - - - - - - 70

Table 4.8: Concentration of test antimalarial agent that give 50% inhibition of *Plasmodium falciparum* isolates (IC_{50}) from patients in a Health facility in Wusasa, Zaria- - - - - - - - - 71

Table 4.9: Resistant *Plasmoidium falciparum*profile against test antimalarial agent in a Health facility in Wusasa, Zaria Nigeria.- - - - - 72

LIST OF FIGURES

TITLEPAGE

Fig 2.1: Life cycle of malaria parasite- - - - - - - 13

Fig 2.2: Chemical Structure of Quinine- - - - - - - 19

Fig 2.3: Chemical Structure of Chloroquine- - - - - - - 20

Fig 2.4: Chemical Structure of Amodiaquine- - - - - - - 21

Fig 2.5: Chemical Structure of Pyrimethamine- - - - - - - 21

Fig 2.6: Chemical Structure of Proguanil- - - - - - - - 21

Fig 2.7: Chemical Structure of Sulfonamide- - - - - - - - 23

Fig 2.8: Chemical Structure of Mefloquine- - - - - - - - 25

Fig 2.9: Chemical Structure of Primaquine- - - - - - - - 25

Fig 4.1: Age Distribution of patients diagnosed with malaria at a Health facility in Wusasa- - - - - - - - - - 55

Fig 4.2: Pattern of Antimalarial prescription at a Health facility in Wusasa in 2012-58

Fig 4.3: Pattern of Antimalarial prescription at a Health facility in Wusasa in 2013- 59

Fig 4.4: Pattern of Antimalarial prescription at a Health facility in Wusasa in 2014- 60

LIST OF APPENDICES

TITLE **PAGE**

APPENDIX I: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolates for Chloroquine- - - - - - 93

APPENDIX II: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolates for quinine- - - - - - - 94

APPENDIX III: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolates for Artemether- - - - - - 95

APPENDIX IV: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolates for Artemether-lumefantrine combination- - - 96

APPENDIX V: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolates for aqueous extract of *P. guajava* leaf- - - 97

APPENDIX VI: Schizont count (per 200 parasites) of *Plasmodium falciparum* isolatesfor aqueous extract of *C. papaya* leaf- - - -98

APPENDIX VII: Concentration of test antimalarial agent that gives 90% inhibition of *Plasmodium falciparum* isolates (IC_{50}) from patientsin a Health facility inWusasa, Zaria- - - 99

APPERNDIX VIII: Concentration of test antimalarial agent that gives 99% Inhibition of *Plasmodium falciparum* isolates (IC_{50}) from patient Health facility in Wusasa, Zaria- - - - 100

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Malaria, one of the oldest diseases known to mankind is a mosquitoe-borne infectious disease of humans and other animals caused by protists (a type of microorganism) of the genus *plasmodium.*

Human malaria likely originated in Africa and has coevolved along with its hosts, mosquitoes and non-human primates(Poiner, 2005). The first evidence of malaria parasites was found in mosquitoes preserved in amber from the Palaeogene period that are approximately 30 million years old (Poiner, 2005). For over 2500, years the idea that malaria fevers were caused by miasmas rising from swamps persisted and it is widely held that the word malaria comes from the Italian word 'malaria' meaning spoiled air although this has been disputed. Malaria may have been a human pathogen for the entire history of the species. Humans may have originally caught *Plasmodium falciparum (P.falciparum)*from gorillas (Liu *et al*., 2010). About 10,000 years ago malaria started having a major impact on human survival which coincides with the start of agriculture. References to the unique periodic fevers of malaria are found throughout recorded history. Malaria was once common in most of Europe and North America, where it is now for all purposes non-existent(Liu *et al*., 2010).Throughout history the most critical factors in the spread or eradication of disease have been human behavior (shifting population centers, changing farming methods and the like) and living standards.

Precise statistics of the disease do not exist because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. Poverty has been and remains a reason for the disease to remain while it has undergone a decline in other location. Climate change is likely to affect future trends in malaria transmission, but the severity and geographic distribution of such effects are currently uncertain, though attracting increasing scientific attention.

Malaria affects 3.3 billion people, or half of the world's population, in 106 countries and territories. WHO estimated that 216 million cases of malaria occurred in 2010, 81% in the African region. WHO estimates that there were 655,000 malaria deaths in 2010, 91% in the African Region, and 86% were children under 5 years of age. Malaria is the 3rd leading cause of death for children under five years worldwide, after pneumonia and diarrheal disease (World malaria report, 2012). Using a different set of predictive models to estimate mortality, a 2012 study determined the number of documented and undocumented deaths in 2012 to be 1.24million (Murray, *et al*., 2012). The majority of cases (65%) occurred in children under 15 years old (Murray, *et al.,* 2012). There are about 10,000 malaria cases per year in Western Europe, and 1300-1500 in the United States. Thirty countries in Sub-Saharan Africa account for 90% of global malaria deaths. Nigeria, Democratic Republic of Congo (DRC), Ethiopia, and Uganda account for nearly 50% of the global malaria deaths. Malaria is the 2nd leading cause of death from infectious diseases in Africa, after HIV/AIDS. Almost 1 out of 5 deaths of children under 5 in Africa is due to malaria. About 125 million pregnant women are at risk of infection each year, in Sub-Saharan Africa. Maternal malaria is associated with up to 200,000 estimated infant deaths yearly (Hartman, *et al*., 2010). According to the World malaria report 2016, there were 212 million new cases of malaria worldwide in 2015 (range 148-304 million). The African region accounted for most global cases

(90%)(World malaria report, 2016).In 2015, there were an estimated 429000 malaria deaths (range 235000-639000) Worldwide. Most of these deaths occurred in the African region (92%).In 2015, malaria killed an estimated 303000 under-fives globally including 292000 in the African region(World malaria report, 2016).Malaria is a major public health problem in Nigeria where it accounts for more cases and deaths than any other country in the world. Malaria is a risk for 97% of Nigeria's population. The remaining 3% of the population live in the malaria free highlands. There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria. This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. Malaria contributes to an estimated 11% of maternal mortality. Malaria accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria. Malaria has the greatest prevalence, close to 50%, in children age 6-59 months in the South West, North Central, and North West regions. Malaria has the least prevalence, 27.6 percent, in children age 6 to 59 months in the South East region (World malaria report,2010). Eradication of malaria is one of the cardinal points of the sustainable developmental goals (SDG 3) which seeks to combat HIV/AIDS, malaria and other diseases.

The first effective treatment for malaria came from the bark of cinchona tree, which contains quinine. Quinine was the predominant malarial medication until the 1920s, when other medications began to be developed. In the 1940s, chloroquine replaced quinine as the treatment of both uncomplicated and severe malaria until resistance supervened, first in the Southeast Asia and South America in the 1950s and then globally in the 1980s.Other drugs were developed for the treatment of malaria such as pyrimethamine, proguanil, sulphonamides, mefloquine, primaquine. Over the years, due to the continuous emergence and spread of resistance to these drugs, Artemisinins,

discovered by Chinese scientist, Tu you in the 1970s from the plant *Artemisia annua*,became the recommended treatment for *P. falciparum* malaria. It is administered in combination with other antimalarials (known as artemisinin-combination therapy, or ACT), which reduces the ability of the parasite to develop resistance to any single drug component (Kokwaro, 2009). These additional antimalarials include amodiaquine, lumefantrine, mefloquine or sulfadoxine/pyrimethamine . Another recommended combination is dihydroartemisinin and piperaquine (Keating, 2012; Manyando *et al*., 2011). ACT is about 90% effective when used to treat uncomplicated malaria (Howitt *et al.,* 2012). In the 2000, malaria with partial resistance to artemisins emerged in Southeast Asia (Obrien *et al*., 2011).

Several medicinal plants have also been used locally to treat malaria infections, some of which include *Azadirachta indica*, *Cissus rotundifolia*, *Echium rauwalfii*, *Boswellia elongate* (Merlin, 2004; Clarkson *et al*., 2004; Alshawsh *et al*., 2007). The use of these local herbs for the treatment of malaria has helped to reduce mortality and morbidity rates especially in rural areas of developing countries worldwide where antimalarial drugs are not readily available or cannot be afforded (Olashinde, 2014). One way to prevent drug resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agent (Azas *et al*., 2002).

The efficacy of antimalarial drugs depends primarily on their ability to kill malaria parasites by interrupting their essential life functions, leading to inhibition of multiplication and allowing the immune system to remove damaged parasites completely from the circulation. This efficacy varies according to the susceptibility of each parasite clone within a natural, often genetically heterogeneous, population of malaria parasites, and is generally referred to as drug sensitivity. With spreading resistance of *P. falciparum* to available treatments in the past five decades, drug

sensitivity has become an issue of utmost importance for the development of therapeutic guidelines and policies (Herald *et al*., 2003).

Resistance to antimalarial drugs can be assessed *in vivo* and also *invitro* by parasite susceptibility assays or by the use of molecular techniques including Polymerase Chain Reaction (PCR) methods to detect genetic markers .The *in vitro* micro-test provides information on the quantitative drug response of *P. falciparum* irrespective of the patient's immune status. (WHO, 2001) It is an epidemiological tool for assessing baseline sensitivity and for monitoring the drug response of *P. falciparum* over time and place and therefore can provide background information for the development and evaluation of drug policies. Changes in parasite drug sensitivity *in vitro* can be an indicator of future therapeutic failure. Unlike the *in vivo* tests, the results of an *in vitro* test are not disturbed by ongoing malaria transmission. The *invitro* test may be carried out with several drugs simultaneously and is independent of the patient's clinical condition. (WHO, 2001)

PCR is the most important new scientific technology to come along in the last hundred years (Beck,1999;De monbrison *et al*., 2003). This technique is more accurate than microscopy and its value lies in its sensitivity with the ability to detect ≤5 parasites /µl of blood with 100% sensitivity and equal specificity. PCR methods are particularly useful for studies on strain variation, mutation and studies of parasite genes involved in drug resistance. Molecular studies using various markers can provide the advance information on the emergence of drug resistance pattern in the field and such can be used to design malaria control strategies(Beck, 1999; De monbrison *et al*., 2003).

1.2 Statement of Problem

The eradication of malaria is far from reaching its completion due to the continuous emergence and spread of antimalarial drug resistant pathogens, and this has significantly affected the wellbeing of humans across social and economic levels and health budgets. The eventual failure of drugs in the face of parasite evolution is now accepted as inevitable by the World Health Organization (WHO) and others (American Academy of Microbiology, 2009). A key component of the Global Malaria Action Plan is an explicit plan for a discovery pipeline to deliver replacement drugs continuously .According to a 2016 report by WHO, there were 212 million new cases of malaria worldwide in 2015 . Malaria is a risk for 97% of Nigeria's population. The remaining 3% of the population live in the malaria free highlands (World malaria report, 2010). There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria (World malaria report, 2010). This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. Malaria contributes to an estimated 11% of maternal mortality. Malaria accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria(World malaria report,2010).

1.3. JUSTIFICATION

- Emergence of resistant strains of malaria parasites to single and combined antimalarials has worsened the health and economic burden of malaria globally.
- Emergence of resistant strains of malaria parasites has made it imperative to verify the efficacy of commonly used antimalarial drugs to treat malaria in Nigeria.
- There is a need to test the efficacy of locally used herbal antimalarials so as to pave the way for possible discovery of novel antimalarial drugs.

1.4. NULL HYPOTHESIS

P. falciparum isolates are not sensitive to all single and combined antimalarials drugs used as chemotherapeutic agents and *Carica papaya (C. papaya) and Psidium guajava (P.guajava)*extracts have no antimalarial activity.

1.5 ALTERNATE HYPOTHESIS

P. falciparum isolates are sensitive to all single and combined antimalarial drug commonly used as chemotherapeutic agents and *C. papaya* and *P.guajava* leafextracts haveantimalarial activity.

1.6 AIMS AND OBJECTIVES

1.6.1. Aim of the study

The study was designed to determine the sensitivity of *P. falciparum*isolates to some commonly prescribed antimalarials and to the ethanolic leaf extract of *C. papaya and P. guajava* in a Health facility in Wusasa Zaria, Kaduna state, Northern Nigeria, using WHO standard procedures.

1.6.2. Objectives

1. To carry out a retrospective study in the Health facility in Wusasa, to determine the incidence of malaria, and pattern of antimalarial drugs prescription over a three year period (Jan 2012- Dec 2014).
2. To isolate andidentify *P. falciparum*from patients attending the Health facility, Wusasa.
3. To determine the susceptibility pattern of the isolated *P.falciparum* to some single and combined antimalarials drugs commonly prescribed in this locality and to the ethanolic extracts of *C. papaya* and *P.guajava* leaves
4. To determine parasites genetic factors responsible for antimalarial ineffectiveness in Wusasa, Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORICAL OUTLINE

Malaria is an ancient disease and references to what was almost certainly malaria occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC. Such historical records must be regarded with caution but moving into later centuries we are beginning to step onto firmer ground. The early Greeks, including Homer in about 850 BC, Empedocles of Agrigentum in about 550 BC and Hippocrates in about 400 BC, were well aware of the characteristic poor health, malarial fevers and enlarged spleens seen in people living in marshy places. For over 2500 years the idea that malaria fevers were caused by miasmas rising from swamps persisted and it is widely held that the word malaria comes from the Italian "malaria" meaning spoiled air although this has been disputed(Russell, 1965).With the discovery of bacteria by Antoni van Leeuwenhoek in 1676, and the incrimination of microorganisms as causes of infectious diseases and the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879, the search for the cause of malaria intensified. Scientific studies only became possible after the discovery of the parasites themselves by Charles Louis Alphonse Laveran in 1880 and the incrimination of mosquitoes as the vectors, first for avian malaria by Ronald Ross in 1897 and then for human malaria by the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900. Excellent histories of this disease include those by Celli (Celli,1933), Stephens (Stephens and blackwater, 1937), Scott, Russell (Russell,1965).

2.2MALARIA PARASITE

The storyof malariabegins with the discovery of the stages in the blood. The discoveries of Pasteur and Koch had precipitated a search for a bacterial cause for many diseases including malaria. By 1879 the miasma theory was going out of favour and the two theories vying for contention were whether the microorganisms responsible were transmitted (Celli, 1933) by air and inhalation or by water and ingestion. The leading theory was that proposed by the Italian Corrado Tommasi-Crudeli and the German, Theodor Albrecht Edwin Klebs, an eminent microbiologist who had been the first person to see the bacteria responsible for typhoid and diphtheria. Tommasi-Crudeli and Klebs claimed that they had isolated from the waters of the Pontine Marshes, where malaria was prevalent, a bacterium, *Bacillus malariae*, which when isolated in culture and injected into rabbits caused febrile infections accompanied by enlarged spleens reminiscent of malaria. It was against this background that Charles Louis Alphonse Laveran, an unknown French army officer working in Algeria, challenged the perceived wisdom and began in his own words 'to follow the pigment'. Beginning with the known fact that the spleens of malaria patients contained pigment he began to look for pigment in the fresh unstained blood of patients and observed it first in leucocytes and then in or on red blood cells. Looking more carefully, he observed several different forms of erythrocytic organism including crescents, spherical motionless bodies with pigment, spherical moving bodies with pigment and bodies that extruded flagella-like structures all of which he thought were on the outside of the red cells. These observations are particularly interesting because Laveran not only used fresh blood but also a dry objective with a maximum magnification of×400 diameters. He also suggested a course of events that began with clear spots that grew, acquired pigment and filled the corpuscle which then burst coinciding with the fevers associated with malaria. Laveran

meticulously examined the blood of 200 patients and in 148 observed the crescentic bodies in all cases of malaria but never in those without malaria. He also noted that quinine removed these stages from the blood. Laveran quickly realised that he had found a parasitic protozoan which he called *Oscillaria malariae*. He presented his findings to the French Academy of Medical Sciences in December 1880 (Laveran, 1884) but failed to persuade any of the eminent microbiologists, zoologists or malariologists of the day that he was seeing anything other than disintegrating red blood cells. Nevertheless he persevered and by 1884 had convinced the leading Italian malariologists including Bignami, Golgi and Marchiafava that malaria was caused by a protozoan and not a bacterium (Laveran,1884). His biggest triumph came in the same year when he also convinced the more cynical microbiologists Louis Pasteur, Charles Edouard Chamberland and Pierre Paul Émile Roux. Robert Koch, one of the most influential microbiologists of his time, however, remained sceptical until 1887. Nevertheless in some quarters the miasma theory persisted and as late as 1895 the American R. C. Newton, a supporter of Tommasi-Crudeli, wrote that 'Aerial and aquatic transportation of malaria has been proved' (Newton, 1895). Laveran was awarded the Nobel Prize for Medicine in 1907.

By this time it had also become clear that the paroxsms characteristic of malaria coincided with the bursting of infected red blood cells and the release of the products of multiplication something that Laveran, who had also realised that in the case of malignant tertian malaria the brain was involved, had proposed (Laveran, 1893). Thus by 1890 it was known that malaria was caused by a protozoan parasite that invaded and multiplied in red blood cells and, after a lot of confusion, that there were three species with specific periodicities and other characteristics responsible for benign tertian (*Haemamoeba vivax*), malignant tertian (*Laverania malariae*) and quartan

(*Haemamoeba malariae*) malaria now respectively *Plasmodium vivax*, *P. falciparum* and *P. malariae*. In 1918, John Stephens, working in West Africa, discovered a fourth species which resembled *P. vivax* which was described as *P.ovale* (Laveran, 1893).

2.3MALARIA DISEASE

2.3.1Epidemiology

The geographic distribution of malaria within large regions is complex, and malaria-afflicted and malaria-free areas are often found close to each other (Greenwood and Mutabingwa, 2002). Malaria is prevalent in tropical and subtropical regions because of rainfall, consistent high temperatures and high humidity, along with stagnant waters in which mosquito larvae readily mature, providing them with the environment they need for continuous breeding (Jamieson*et al*., 2006). In drier areas, outbreaks of malaria have been predicted with reasonable accuracy by mapping rainfall (Abeku,2007).Malaria is more common in rural areas than in cities. For example, several cities in the Greater Mekong subregion of Southeast Asia are essentially malaria-free, but the disease is prevalent in many rural regions, including along international borders and forest fringes (Cui *et al*., 2012) In contrast, malaria in Africa is present in both rural and urban areas, though the risk is lower in the larger cities.(Machault *et al*., 2011)

Malaria is presently endemic in a broad band around the equator, in areas of the Americas, many parts of Asia, and much of Africa; in Sub-Saharan Africa, 85–90% of malaria fatalities occur.(Layne,2006)

The World Health Organization (WHO) estimates that in 2010 there were 219 million cases of malaria resulting in 660,000 deaths. (World malaria report,2012), equivalent to roughly 2000 deaths every day.(Nadjmn and Behrens, 2012). Using a different set of predictive models to estimate mortality, a 2012 study determined the number of

documented and undocumented deaths in 2012 to be 1.24million. (Murray *et al*.,2012). The majority of cases (65%) occur in children under 15 years old.(Murray*et al.*, 2012). About 125 million pregnant women are at risk of infection each year, in sub-SaharanAfrica; maternal malaria is associated with up to 200,000 estimated infant deaths yearly. (Hartman*et al*., 2010). There are about 10,000 malaria cases per year in Western Europe, and 1300-1500 in the United States. (Taylor *et al*., 2012). In Sub-Saharan Africa, 85-90% of malaria fatalities occur. World Health Organization (WHO) in 2016 reported that there were 212 million new cases of malaria worldwide in 2015, 90% of these cases occurring in the African region. In 2015, there were an estimated 429000 malaria deaths. Most of these deaths (92%) occurred in the African region (World malaria report, 2016). In 2015, malaria killed an estimated 303000 under-fives globally including 292000 in the African region (World malaria report, 2016) There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria.(World malaria report,2010).

2.3.2 Etiological Agent

The parasite responsible for the disease malaria is most commonly transmitted by an infected female Anopheles mosquitoe. There are about 460 species of the Anopheles mosquito, but only 68 transmit malaria. Malaria parasites belong to the genus Plasmodium. In humans, malaria is caused by *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium knowlesi* (Mueller *et al*., 2007).Among those infected , *P.falciparum* is the most common species identified (75%) followed by *P. vivax*(20%). Although *P.falciparum* traditionally accounts for the majority of deaths (Nadjem and Behrens, 2012), recent evidence suggests that *P.vivax* malaria is associated with potentially life- threatening conditions about as often as with a diagnosis of *P. falciparum* infection (Sarkar *et al*., 2009). *P. vivax* proportionally is

more common outside Africa. There have been documented human infections with several species of plasmodium from higher apes, however, except for *P.knowlesi*- a zoonotic specie that causes malaria in macaques (Collins, 2012) these are mostly of limited public health importance.(Collins and Barnwell,2009).

2.3.3 Life Cycle

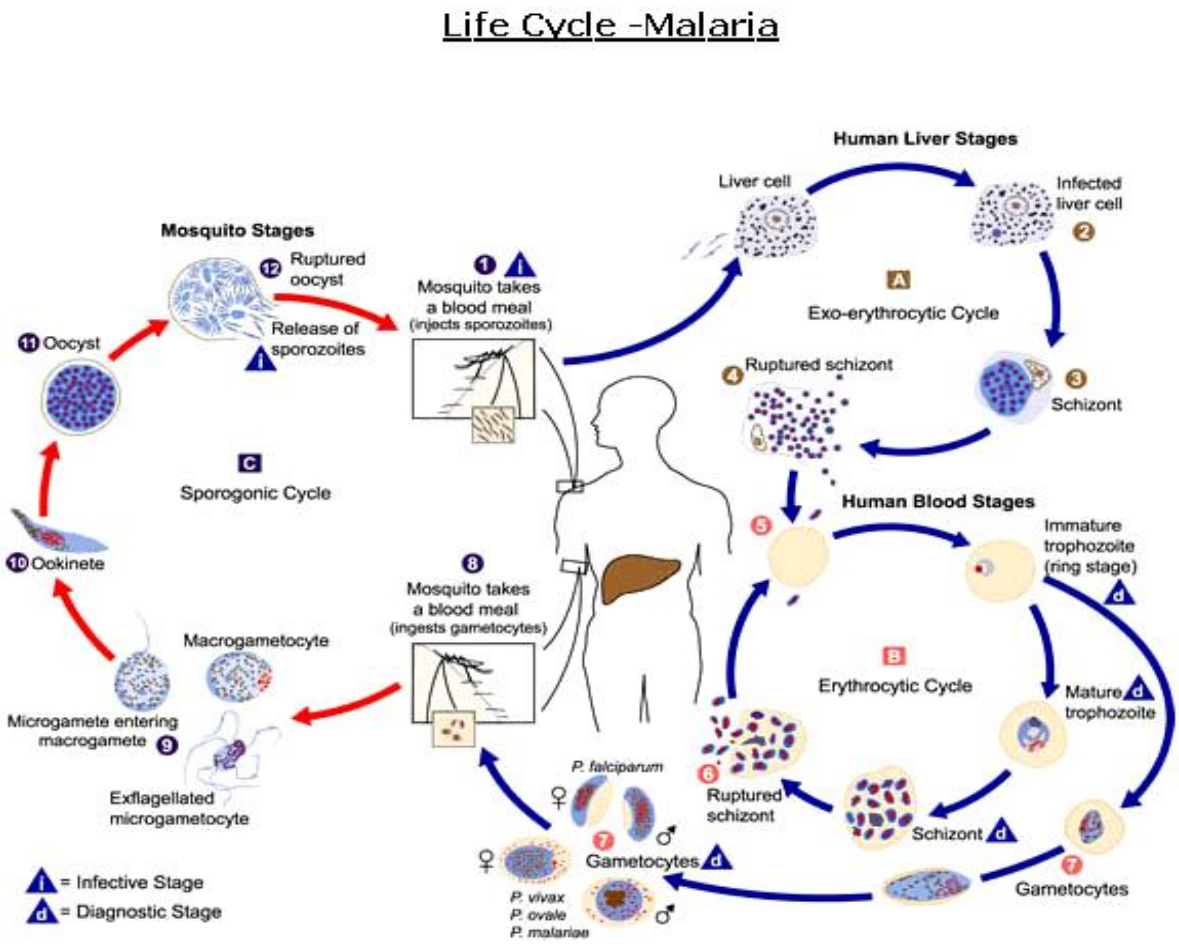


Fig. 2.1: Malaria parasite life cycle (www.cdc.gov/malaria)

In the life cycle of Plasmodium, a female Anopheles mosquitoe transmits a motile infective form (called the sporozoite) to a vertebrate host such as a human (the secondary host), thus acting as a transmission vector. A sporozoite travels through the blood vessels to liver cells (hepatocytes), where it reproduces asexually (tissue schizogony), producing thousands of merozoites. These infect new red blood cells and initiate a series of asexual multiplication cycles (blood schizogony) that produce 8 to 24 new infective merozoites, at which point the cells burst and the infective cycle begins anew. (Schalagenhauf-lawlor, 2008).

Other merozoites develop into mature gametocytes, which are the precursors of male and female gametes. When a fertilized mosquitoe bites an infected person, gametocytes are taken up with the blood and mature in the mosquitoe gut. The male and female gametocytes fuse and form an ookinetea fertilized, motile zygote. Ookinetes develop into new sporozoites that migrate to the insect's salivary glands, ready to infect a new vertebrate host. The sporozoites are injected into the skin, in the saliva, when the mosquitoe takes a subsequent blood meal (Cowman *et al*. 2012).

Only female mosquitoes feed on blood, male mosquitoes feed on plants nectar, and do not transmit the disease. The females of the Anopheles genus of mosquitoe prefer to feed at night. They usually start searching for a meal at dusk, and will continue throughout the night until taking a meal. (Arrow *et al*., 2004). Malaria parasites can also be transmitted by blood transfusions, although this is rare (Owusu-ofori *et al*., 2010).

2.3.4Pathophysiology

Malaria infection develops via two phases,one that involves the liver (exoerythrocytic phase), and one that involves red blood cells, or erythrocytes (erythrocytic phase). When an infectedmosquitoe pierces a person's skin to take a blood meal, sporozoites in

the mosquitoe's saliva enter the bloodstream and migrate to the liver where they infect hepatocytes, multiplying asexually and asymptomatically for a period of 8-30 days (Bledsoe,2005)

After a potential dormant period in the liver, these organisms differentiate to yield thousands of merozoites, which following rupture of their host cells, escape into the blood and infect red blood cells to begin the erthrocytic stage of the cycle (Bledsoe, 2005). The parasite escapes from the liver undetected by wrapping itself in the cell membrane of the infected liver cell (Vaughan *et al*., 2008)

Within the red blood cells, the parasites multiply further, again asexually, periodically breaking out of their host cells to invade fresh red blood cells. Several such amplication cycles occur. Thus, classical descriptions of waves of fever arise from simultaneous waves of merozoites escaping and infecting red blood cells (Bledsoe, 2005).

Some *P. vivax* sporozoites do not immediately develop into exoerthrocytic phase merozoites, but instead produce hypnozoites that remain dormant for periods ranging from several months to several years. After a period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for long incubation and late relapses in P. vivax infections(White, 2011), although their existence in *P.ovale* is uncertain.

The parasite is relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating infected blood cells are destroyed in the spleen.To avoid this fate, the *P. falciparum* parasites displays adhesive proteins on the surfaceof the infected blood cells, causing the blood cells to stick to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation and the spleen(Tilley *et al*., 2011). The blockage of the

microvasculature causes symptoms such as in placental malaria(Mens *et al*., 2012). Sequestered red blood cells can breech the blood- brain barrier and cause cerebral malaria (Renia *et al*., 2012).

2.3.5 Symptoms

The signs and symptoms of malaria typically begin 8-25 days following infection (Fairhurst and Wellems, 2010) however, symptoms may occur later in those who have taken antimalarial medications as prophylaxis (Nadjem and Behrens, 2012). Initial manifestations of the disease- common to all malaria species are similar to flu-like symptoms (Bartoloni and Zammarchi, 2012) and can resemble other conditions such as sepsis, gastroenteritis and viral diseases (Nadjem and Behrens, 2012). The presentation may include headache, fever, and shivering, joint pain, vomiting hemolytic anaemia, jaundice, hemoglobin in the urine, retinal damage and convulsion.

The classic symptomof malaria is paroxysm- a cyclical occurrence of sudden coldness followed by shivering and then fever and sweating, occurring every two days in P. vivax and P.ovale infections and every three days (quartan fever) for *P. malariae*. *P. falciparum* infection can cause recurrent fever every 36–48 hours or a less pronounced and almost continuous fever (Ferri,2009).

Severe malaria is usually caused by *P. falciparum* (often referred to as falciparum malaria). Symptoms of falciparum malaria arise 9–30 days after infection (Bartoloni and Zammarchi, 2012). Individuals with cerebral malaria frequently exhibit neurological smptoms, including abnormal posturing, nystagmus, conjugate gaze palsy (failure of the eyes to turn together in the same direction), opisthotonus, seizures, or coma (Bartoloni and Zammarchi,2012).

There are several serious complications of malaria. Among these is the development of respiratory distress, which occurs in up to 25% of adults and 40% of children with severe *P. falciparum* malaria. Possible causes include respiratory compensation of metabolic acidosis, non-cardiogenicpulmonary oedema, concomitant pneumonia, and severe anaemia. Acute respiratory distress syndrome (ARDS) may develop in 5–25% in adults and up to 29% of pregnant women but it is rare in young children (Taylor *et al*., 2012). Coinfection of HIV with malaria increases mortality (Korenromp, 2005). Renal failure is a feature of blackwater fever, where hemoglobin from lysed red blood cells leaks into the urine (Bartoloni and Zammarchi,2012).

Infection with *P. falciparum* may result in cerebral malaria, a form of severe malaria that involves encephalopathy. It is associated with retinal whitening, which may be a useful clinical sign in distinguishing malaria from other causes of fever(Beare *et al.,* 2011). Splenomegaly, severe headache, hepatomegaly (enlarged liver), hypoglycemia, and hemoglobinuria with renal failuremay occur (Bartoloni and Zammarchi,2012).

Malaria in pregnant women is an important cause of stillbirths, infant mortality and low birth weight (Hartman et al., 2010)particularly in *P. falciparum* infection, but also with *P. vivax* (Rijken *et al*., 2012).

2.3.6 Diagnosis

Malaria is usually confirmed by the microscopic examination of blood films or by antigen-based rapid diagnostic tests (RDT) (Abba *et al*., 2011).Microscopy is the most commonly used method to detect the malarial parasite-about 165 million blood films were examined for malaria in 2010 (Wilson, 2012). Despite its widespread usage, diagnosis by microscopy suffers from two main drawbacks: many settings (especially

rural) are not equipped to perform the test, and the accuracy of the results depends on both the skill of the person examining the blood film and the levels of the parasite in the blood. The sensitivity of blood films ranges from 75–90% in optimum conditions, to as low as 50%. Commercially available RDTs are often more accurate than blood films at predicting the presence of malaria parasites, but they are widely variable in diagnostic sensitivity and specificity depending on manufacturer, and are unable to tell how many parasites are present (Wilson, 2012)

In regions where laboratory tests are readily available, malaria should be suspected, and tested for, in any unwell patient who has been in an area where malaria is endemic. In areas that cannot afford laboratory diagnostic tests, it has become routine to use only a history of subjective fever as the indication to treat for malaria-a presumptive approach exemplified by the common teaching "fever equals malaria unless proven otherwise". A drawback of this practice is overdiagnosis of malaria and mismanagement of non-malarial fever, which wastes limited resources, erodes confidence in the health care system, and contributes to drug resistance(Perkins, 2008). Although polymerase chain reaction-based tests have been developed, these are not widely implemented in malaria-endemic regions as of 2012, due to their complexity(Nadjem and Behrens, 2012).

2.4 ANTIMALARIA DRUGS AND MECHANISMOF ACTION

2.4.1Quinine(6-Methoxyquinolin-4-Yl)(2s,4s,8r)-8-Vinylquinuclidin-2-Yl) Methanol)

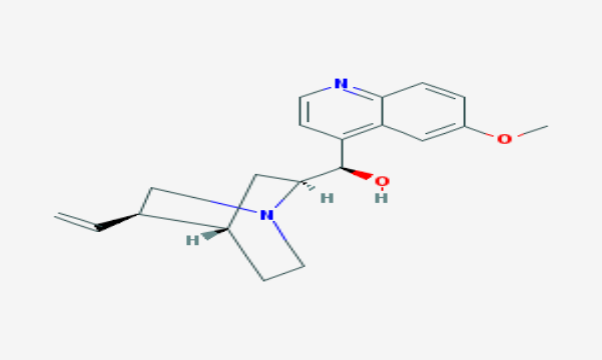


Fig 2.2: Chemical structure of quinine (Sandhya *et al*., 2010)

Cinchona contains a mixture of more than 20 structurally related alkaloids, the most important of which are quinine and quinidine. Both compounds contain a quinoline group attached through a secondary alcohol linkage to a quinuclidine. They differ only in the steric configuration at two of the three asymmetrical centers: the carbon bearing the secondary alcohol group and at the quinuclidine junction. Although quinine and quinidine have been synthesized, the procedures are complex; hence they are still obtained from natural sources. Quinidine is both somewhat more potent as an antimalarial and more toxic than quinine (Karle *et al*., 1992).

2.4.1.1Mechanism of action

As an alkaloid it is accumulated in the food vacuoles of plasmodium species, especially *P. falciparum*. It acts by inhibiting hemozoin biocrystallization, thus facilitating an aggregation of cytotoxic heme.

2.4.2 Chloroquine (Rs)-N-(7-Chloroquinolin-4-Yl)-N, N-Diethyl-Pentane-1, 4-Diamine

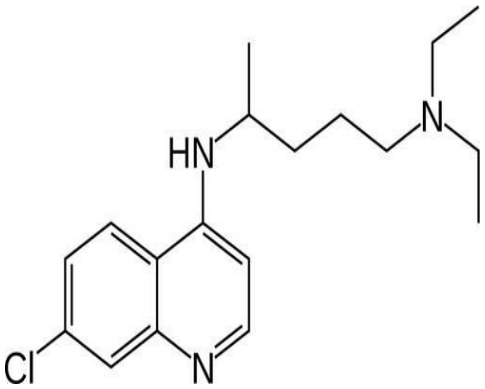


Fig 2.3:Chemical structure of chloroquine (Sandhya *et al*., 2010)

Chloroquine is a 4-aminoquinolone compound. A chlorine atom attached to position 7 of the quinolone ring confers the greatest antimalarial activity in both avian and human malaria.

2.4.2.1 Mechanism of action

It has a complicated and still unclear mechanism of action. It is believed to reach high concentrations in the vacuoles of the parasite, which, due to its alkaline nature, raises the internal pH. It controls the conversion of toxic heme to hemozoin by inhibiting the biocrystallization of hemozoin, thus poisoning the parasite through excess levels of toxicity. Other potential mechanisms through which it may act include interfering with the biosynthesis of parasitic nucleic acids and the formation of a chloroquine-haem or chloroquine-DNA complex. The most significant level of activity found is against all forms of the schizonts (with the obvious exception of chloroquine-resistant *P. falciparum* and *P. vivax* strains) and the gametocytes of *P. vivax, P. malariae, P. ovale* as well as the immature gametocytes of *P. falciparum*. Chloroquine also has a

significant anti-pyretic and anti-inflammatory effect when used to treat *P. vivax* infections, and thus it may still remain useful even when resistance is more widespread.

2.4.3 Amodiaquine (4-[(7-Chloroquinolin-4-Yl) Amino]-2-[(Diethylamino) Methylphenol

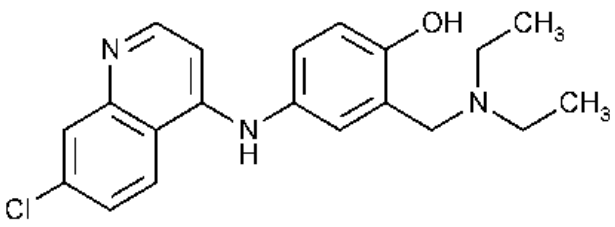


Fig 2.4: Chemical structure of Amodiaquine (Sandhya *et al*., 2010)

Amodiaquine is a 4-aminoquinolone anti-malarial drug similar in structure and mechanism of action to chloroquine. Amodiaquine has tended to be administered in areas of chloroquine resistance. Amodiaquine is now available in a combined formulation with artesunate (ASAQ) and is among the artemisinin-combination therapies recommended by the World Health Organisation. Combination with sulfadoxine-pyrimethamine is no longer recommended (WHO guidelines 2010).

2.4.4 Pyrimethamine (5-(4-Chlorophenyl)-6-Ethyl-2, 4-Pyrimethamine)



Fig 2.5: chemical structure Pyrimethamine (Sandhya *et al*., 2010)

Pyrimethamine is used in the treatment of uncomplicated malaria. It is particularly useful in cases of chloroquine-resistant *P. falciparum* strains when combined with sulfadoxine.

2.4.4.1 Mechanism of action

It acts by inhibiting dihydrofolate reductase in the parasite thus preventing the biosynthesis of purines and pyrimidines, thereby halting the processes of DNA replication, cell division and reproduction. It acts primarily on the schizonts during the erythrocytic phase, and nowadays is only used in concert with a sulfonamide.

2.4.5 Proguanil (1-(4-Chlorophenyl) -2- (N-Propan-2-Ylcarbamimidoyl) Guanidine

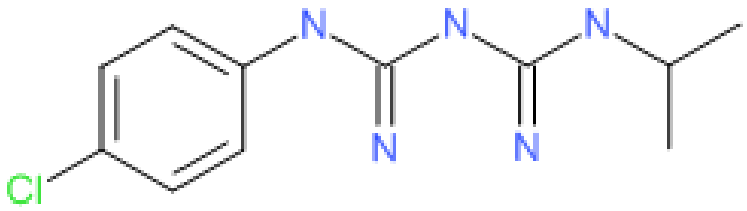


Fig 2.6:Chemical structure of proguanil (Sandhya *et al*., 2010)

Proguanil (chloroguanide) is a biguanide; a synthetic derivative of pyrimidine. It was developed in 1945 by a British Antimalarial research group.

2.4.5.1Mechanism of action

It has many mechanisms of action but primarily is mediated through conversion to the active metabolite cycloguanil. This inhibits the malarial dihydrofolate reductase enzyme. Its most prominent effect is on the primary tissue stages of *P. falciparum*, *P. vivax* and *P. ovale*. It has no known effect against hypnozoites therefore is not used in the prevention of relapse. It has a weak blood schizonticidal activity and is not

recommended for therapy of acute infection. However it is useful when combined with atovaquone or chloroquine (in areas where there is no chloroquine resistance). The proguanil- chloroquine combination does not provide effective protection against resistant strains of *P. falciparum*.

2.4.6 Sulfonamides (4-Aminobenzenesulfonamide)

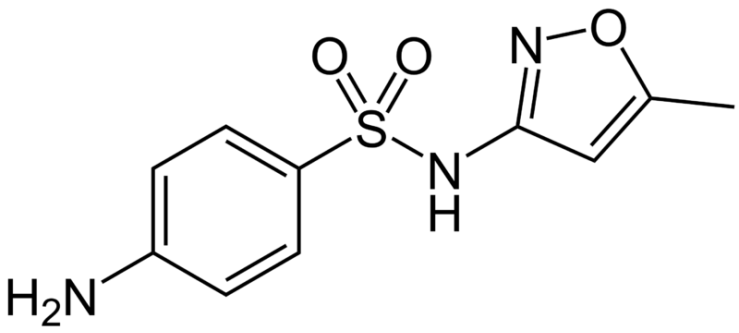


Fig 2.7:Chemical structure of sulfonamides (Sandhya *et al*., 2010)

2.4.6.1 Mechanism of action

Sulfadoxine and sulfamethoxypyridazine are specific inhibitors of the enzyme dihydropteroate synthetase in the tetrahydrofolate synthesis pathway of malaria parasites. They are structural analogues of *p*-aminobenzoic acid (PABA) and compete with PABA to block its conversion to dihydrofolic acid. Sulfonamides act on the schizont stages of the erythrocytic (asexual) cycle. When administered alone sulfonamides are not efficacious in treating malaria but co-administration with the antifolate pyrimethamine, most commonly as fixed-dose sulfadoxine-pyrimethamine (Fansidar), produces synergistic effects sufficient to cure sensitive strains of malaria.

2.4.7 Mefloquine (2, 8- Bis (Trifluoromethyl) Quinolin-4-Yl]-(2-Piperidyl) Methanol

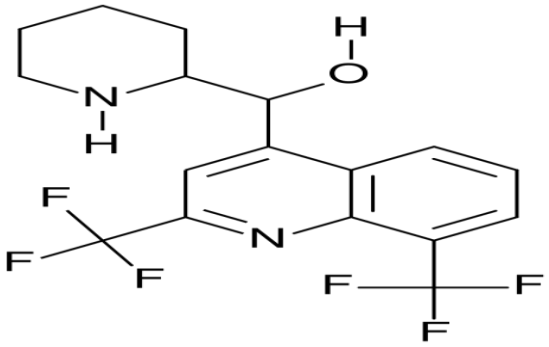


Fig 2.8:Chemical structure of mefloquine (Sandhya *et al*., 2010)

Mefloquine was developed during the Vietnam War and is chemically related to quinine. It was developed to protect American troops against multi-drug resistant *P. falciparum*.

2.4.7.1 Mechanism of action

It is a very potent blood schizonticide with a long half-life. It is thought to act by forming toxic heme complexes that damage parasitic food vacuoles. It is now used solely for the prevention of resistant strains of *P. falciparum* despite being effective against *P. vivax, P. ovale* and *P. marlariae*. Mefloquine is effective in prophylaxis and for acute therapy. It is now strictly used for resistant strains (and is usually combined with Artesunate). Chloroquine/proguanil or sulfa drug-pyrimethamine combinations should be used in all other *Plasmodia* infection.

2.4.8 Primaquine ((Rs)-N-(6-Methoxyquinolin-8-Yl) Pentane-1, 4-Diamine)

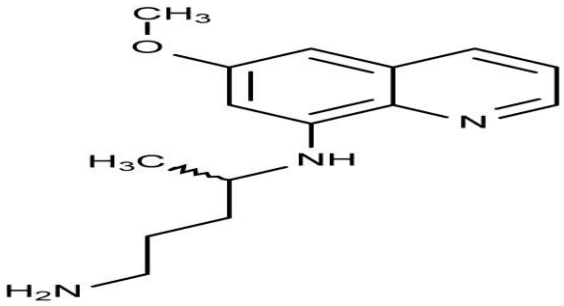


Fig 2.9:Chemical structure of primaquine (Sandhya *et al*., 2010)

Primaquine is a highly active 8-aminoquinolone that is used in treating all types of malaria infection. It is most effective against gametocytes but also acts on hypnozoites, blood schizonticytes and the dormant plasmodia in *P. vivax* and *P. ovale*. It is the only known drug to cure both relapsing malaria infections and acute cases.

2.4.8.1 Mechanism of action

The mechanism of action is not fully understood but it is thought to block oxidative metabolism in Plasmodia.

2.4.9 Artemisinin and Derivatives

Artemisinin(OCTAHYDRO-3,6,9-TRIMETHYL-312-EPOXY-2H-PYRANOL[4,3-]-1,2-BENZODIOXEPIN-10)-ONE)

Artemisinin is a Chinese herb (qinghaosu) that has been used in the treatment of fevers for over 1,000 years, (Rectal artemisinins) thus predating the use of Quinine in the western world. It is derived from the plant *Artemisia annua*, with the first documentation as a successful therapeutic agent in the treatment of malaria in 340 AD by Ge Hong in his book *Zhou Hou Bei Ji Fang* (*A Handbook of Prescriptions for Emergencies*). Ge Hong extracted the artemesinin using a simple macerate and this method is still in use today. The active compound was isolated first in 1971 and named

artemsinin. It is a sesquiterpene lactone with a chemically rare peroxide bridge linkage. It is this that is thought to be responsible for the majority of its anti-malarial action, although the target within the parasite remains controversial. At present it is strictly controlled under WHO guidelines as it has proven to be effective against all forms of multi-drug resistant *P. falciparum*, thus every care is taken to ensure compliance and adherence together with other behaviors associated with the development of resistance. It is also only given in combination with other anti-malarials.

2.4.9.1 Mechanism of action

Artemisinin is a sesquiterpene lactone that contains a labile peroxide bridge (David, 2001). The mechanism of artemisinin action involves two steps (Meshnick, 2001). First, heme iron within produce a carbon-centered radical that alkylates and damages macromolecules in the parasites, likely including the orthology of sarco/endoplasmic reticulum ca2+-ATpase. Artemisinin and its detivatives exhibit anti-parasitic activity *in vitro* against other protozoa, including *leishmania major*and *Taxoplasma gondii*, and have been used alone or in combination in patient with schistosomiasis (Utzinger *et al*, 2003).

Three semi synthetic derivative with improved potency and bioavailability have since largely replaced the use of artemisinin. They are easier to use than the parent compound and are converted rapidly once in the body to the active compound dihydroartemesinin.

Artemether is a methyl ether derivative of dihydroartemesinin. It is similar to artemesinin in mode of action but demonstrates a reduced ability as a hypnozoiticidal compound, instead acting more significantly to decrease gametocyte carriage. Similar restrictions are in place, as with artemesinin, to prevent the development of resistance,

therefore it is only used in combination therapy for severe acute cases of drug-resistant *P. falciparum*.

Artesunate is a hemisuccinate derivative of the active metabolite dihydroartemisin. Currently it is the most frequently used of all the artemesinin-type drugs. Its only effect is mediated through a reduction in the gametocyte transmission. It is used in combination therapy and is effective in cases of uncomplicated *P. falciparum*.

Dihydroartemisinin is the active metabolite to which artemesinin is reduced. It is the most effective artemesinin compound and the least stable. It has a strong blood schizonticidal action and reduces gametocyte transmission. It is used for therapeutic treatment of cases of resistant and uncomplicated *P. falciparum*.

Arteether is an ethyl ether derivative of dihydroartemisinin. It is used in combination therapy for cases of uncomplicated resistant *P. falciparum*.

2.4.9.10 Halofantrine (3-Dibutylamino-1-[1,3-Dichloro-6-(Trifluoromethyl) Phenanthren-9-Yl]-Propan-1-Ol)

Halofantrine is a relatively new drug developed by the Walter Reed Army Institute of Research in the 1960s. It is a phenanthrene methanol, chemically related to Quinine and acts as a blood schizonticide effective against all plasmodium parasites.

2.4.10.1 Mechanism of action

Its mechanism of action is similar to other anti-malarials. Cytotoxic complexes are formed with ferritoporphyrin XI that cause plasmodial membrane damage. Despite being effective against drug resistant parasites, halofantrine is not commonly used in the treatment (prophylactic or therapeutic) of malaria due to its high cost. It has very

variable bioavailability and has been shown to have potentially high levels of cardiotoxicity. It is still a useful drug and can be used in patients that are known to be free of heart disease and are suffering from severe and resistant forms of acute malaria. A popular drug based on halofantrine is Halfan. The level of governmental control and the prescription-only basis on which it can be used contributes to the cost, thus halofantrine is not frequently used.

2.4.11 Doxycycline (4-(Dimethylamineamino)-3, 5, 10, 12-Pentahydroxy-6-Methyl-1,11-Dioxo-Octahydrotetracene-2-Carboxamide)

Probably one of the more prevalent antimalarial drugs prescribed, due to its relative effectiveness and cheapness, doxycycline is a tetracycline compound derived from oxy tetracycline. The tetracyclines were one of the earliest groups of antibiotics to be developed and are still used widely in many types of infection.

2.4.11.1 Mechanism of action

It is a bacteriostatic agent that acts to inhibit the process of protein synthesis by binding to the 30S ribosomal subunit thus preventing the 50s and 30s units from bonding. Doxycycline is used primarily for chemoprophylaxis in areas where chloroquine resistance exists. It can also be used in combination with quinine to treat resistant cases of *P. falciparum* but has a very slow action in acute malaria, and should not be used as mono therapy.

2.4.12 Clindamycin (Methyl 7-Chloro-6, 7, 8 –Trideoxy-6-{[(4r)-1-Methyl-4-Propyl-L-Proly] Amino-1-Thio-L-Threo-D-Galacto-Octopyranoside)

Clindamycin is a derivative of lincomycin, with a slow action against blood schizonticides. It is only used in combination with quinine in the treatment of acute cases of resistant *P. falciparum* infections and not as a prophylactic. Being more expensive and toxic than the other antibiotic alternatives, it is used only in cases where the Tetracyclines are contraindicated (for example in children).

2.5TRADITIONAL ANTIMALARIAHERBS

The first effective treatment for malaria came from the bark of cinchona tree, which contains quinine. Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine (Hoareau and Dasilva, 1999) and for a long time, natural products were the sources of medication (Boyrdy *et al*., 2008). A WHO 2002 report showed that 80% of African population used traditional medicine for primary health care (WHO, 2002). In Asia and Africa as a whole and in Nigeria particularly, several medicinal plants have been used locally to treat malaria infection. Some of which include, *Cymbopogon giganteus, Morinda lucida, Dendrosicyos socotrana, Boswellia elongate etc.*

The world's poorest are the most affected and many treat themselves with traditional herbal medicine, because they are often more available and affordable, and sometimes are perceived as more effective than conventional antimalarial drugs (Merlin).In Nigeria, the reduction in the cost of malaria treatment through the introduction of cheap antimalarials lead to a decrease in the use of herbal plants in malarial treatment , however due to continous emergence and spread of multi resistant strains of *Plasmodium sp*, there seems to be an increase in the use of the almost forgotten herbal

plants. Due to the successes recorded by the use of Quinine derived from cinchona tree, and Artemisinin derived from artemisua annua as chemotherapeutic agents for malaria treatment, the study of plants to develop new antimalaria drugs is important as the emergence and spread of multi resistant strains of *Plasmodium spp*, continous to be on the increase.

C. papaya Linnaeus, (pawpaw), belongs to the family of Caricaceae. Papaya is not a tree but an herbaceous succulent plant that possessesself-supporting stems (Dick Gross, 2003). Papaya is a large perennial herb with a rapid growth rate. The plants are usually short-lived, but can produce fruit for more than 20years. The papaya has a rather complicated means of reproduction. The plants are male, hermaphrodite, or female (Bruce and Peter, 2008).The male trees are uncommon, but sometimes occur when homeowners collect their own seeds. Hermaphrodite trees (flowers with male and female parts) are the commercial standard, producing a pear shaped fruit. These plants are self-pollinated(Jari, 2009).

*C. papaya*leaves(pawpaw leaves) is a widely used herb in tropical countries. Believed to have some therapeutic effects, pawpaw leaves has been used for centuries in West Africa, South America and India and had also become popular in Europe and US. In Nigeria, it has been used for some time by herbal medicine practitioners and locals in addition with other herbal plants in the treatment of malaria. It is commonly referred to as Ibe kpe in Yoruba, Gwanda in Hausa, Acharaehi in Igbo.*C. papaya* leaves contain rich source of mineral elements like Ca, Mg, Na, K, Fe and Mn and phytochemicals such as alkaloids, saponnins and cardiac glycosides. The presence of this minerals and phytochemicals in *C. papaya* leaves indicates the usefulness of the leaves in treatment of diseases (Atta, 1999). It has been suggested to be effective as a tumour- destroying agent (Walter last, 2008), treatment of gastroenteritis, ulcer and impotence (Elizabeth,

1994). Fresh, green pawpaw leaf is used as an antiseptic, whilst the brown, dried pawpaw leaf is used as a tonic and blood purifier (Atta, 1999).

Chewing the seeds of ripe pawpaw fruit also helps to clear nasal congestion, (Elizabeth, 1994). The green unripe pawpaw has a therapeutic value due to its antiseptic quality. It cleans the intestines from bacteria, more so that (only a healthy intestine is able to absorb vitamin and minerals, especially vitamin B12). The tea, prepared with the green papaya leaf, promotes digestion and aids in thetreatment of ailments such as chronic indigestion, overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart (Mantok, 2005).

The presence of alkaloids in the leaves shows that these plants can be used as effective anti-malaria (Robinson, 1985).

In a study carried out in Tamil Nadu, India to determine the antimalarial activity of the ethanol leaf extract of *C. papaya*, blood stages of CQ-sensitive and CQ resistant strains against *P. falciparum*as target species. Plant extracts showed moderate to good antiparasitic effects. These four concentrations (25, 50,100 and 150 µg/mL) of ethanol leaf extracts exhibited promising inhibitory activity against the CQ sensitive strain with (IC50) values 40.75%, 36.54%, 25.30%, and 18.0% and in CQ resistant 50.23%, 32.50%, 21.45%, and 23.12% against *P. falciparum* (Kovandum *et al.*, 2012).

P. guajava (Guava) belongs to the family Myrtaceae It is a low evergreen tree or shrub 6 to 25 feet high, with wide-spreading branches and square, downy twigs, is a native of tropical America. It is a common vegetation cover by roads and in waste places in Hawaii. Guava is a tropical and semitropical plant. It is well known in the islands for its edible fruit. It is common in the backyards. The branches are crooked, bringing opposite leaves. The flowers are white, in curved petals, 2 or 3 in the leaf axils; they are fragrant, with four to six petals and yellow anthers. The fruit is small, 3 to 6 cm long, pear-

shaped, reddish-yellow when ripe. Guava leaves is a widely used herb in Nigeria, commonly referred to asgwaabaa (Hausa); woba (Efik); ugwoba (Igbo); guafa (Yoruba).

The leaves contain essential oil with the main components being α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene and curcumene. The essential oil from the leaves has been shown to contain, nerolidiol, β-sitosterol, ursolic, crategolic, and guayavolic acids have also been identified (Iwu, 1993)

The leaves extract show *in-vitro* antibacterial activity against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Shigella dysenteria* (Iwu, 1993). Another paper showed the effectiveness of the leaf extract against *Staphylococcus aureus* (Gnan and Demello, 1999). The leaves are rich in tannin, and have antiseptic properties (Hernandez, 1980). The leaves are used as an ingredient in the preparation of fever "teas". They are also used as part of the pot herb used in steam treatment for malaria. Indeed, the main ethno-therapeutic use in Africa is said to be for malaria. *P. guajava* stem-bark extract contained anthraquinones, flavonoids, seccoirridoids and terpenoids and was found to be effective for the treatment and/or prophylaxis of malaria in KwaZulu-Natal province of South Africa. The *in vitro* anti-plasmodial assay was carried out using a chloroquine-sensitive strain of malarial parasite (Nundkumar *et al*., 2002).

2.6ANTIMALARIA DRUG RESISTANCE

Anti-malariadrug resistance has been defined as: "the ability of a parasite to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject. The drug in question must gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action." In most instances this refers to a parasite that remainsafter an observed treatment. Thus excluding all cases where anti-malaria prophylaxis has failed. In order for a case to be defined as resistant, the patient under question must have received a known and observed anti-malarial therapy whilst the blood drug and metabolite concentrations are monitored concurrently. (White, 2004)

Antimalarial drug resistance develops when spontaneously occurring parasite mutants with reduced susceptibility are selected and are then transmitted. Drugs for which a single point mutation confers a marked reduction in susceptibility are particularly vulnerable. Low clearance and a shallow concentration- effect increase the chance of selection.

The first type of resistance to be acknowledged was to chloroquine (White, 2004).Drug resistance has emerged as one of the greatest challenges facing malaria control today. It has been implicated in enhanced mortality from malaria in hyper and holo-endemic areas and in the development of new and expanding foci of falciparum malaria, but above all it has been identified as a factor in the economic constraints of malaria control. Over the past five decades, the drug resistance of *P. falciparum* has become an issue of utmost concern.

2.6.1 Types of drug resistance

Drug resistance in malaria management control may take one of several forms as described by black, (1946). In the first type of resistance, RI, clearance of asexual parasites from the blood occurs within 7 days after chloroquine treatment, but there is recrudescence within 28 days. These 28 days of observation may be too long as it possible for reinfection to occur and the wrong conclusions to be drawn concerning resistance. In the second type of resistance.RH, there is marked reduction in the asexual forms of the parasites but such parasites are never cleared completely from the blood following drug treatment .In RHI resistance, the level of parasitaemia remains high despite drug treatment. Intense malaria transmission and intense drug pressure have been considered important as factors in the development and spread of resistant strains(Verdrager,1986).

2.6.2 Mechanisms of antimalarial resistance

The genetic events that confer antimalarial drug resistance (while retaining parasite viability) are spontaneous and are thought to be independent of the drug used (White,1998). In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. Provided the mutations are not deleterious to the survival or reproduction of the parasites, drug pressure will remove susceptible parasites while resistant parasites survive.Single malaria isolates have been found to be made up of heterogeneous populations of parasites that can have widely varying drug response characteristics, from highly resistant to completely sensitive (Thaitthong,1983).

The biochemical mechanism of resistance has so far been well described in three major drugs used for malaria treatment. These are: chloroquine, antifolate combination drugs, and atovaquone (Holland, 2001).

2.6.2.1Mechanisms of Chloroquine Resistance

Though the mechanism of resistance of chloroquine has been extensively studied, it is not yet fully understood. Parasites resistant to chloroquine have been found to accumulate much less chloroquine in their digestive vacuoles than their sensitive counterparts. As the malaria parasites digests large amount of a toxic by- products are formed. The parasites polymerizes this by- product in its food vacuole, producing non-toxic hemozoin (malaria pigment) Chloroquine resistance have been clearly associated with multiple mutations in genes encoding for membrane transporters located in the digestive vacuole , namely the chloroquine resistance transporter *pfCRT* and the multi-drug Resistance transporter 1 *pfMDR1* (Wongarichanalai *et al.,* 2002). It is believed that resistance of *P.falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization (Foley and Tilley, 1997). The chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones. Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system. (Martin *et al*., 1987).It is unclear whether parasites resistance to other quinolones antimalarials (amodiaquine , mefloquine , halofantrine and quinine) occurs via similar mechanisms.(Foley and Tilley, 1997).

2.6.2.2Mechanisms of Antifolate Resistance

Analysis of resistance to the antifolate drugs, sulfadoxine and pyrimethamine has been considerably straight forward. Pyrimethamine targets the dihydrofolate reductase (*DHFR*) activity while sulfadoxine drugs inhibit the dihydropteroate synthetase (DHPS) activity.*DHPS* is used in the bio synthesis of key folate coenzymes whereas DHFR is essential to maintain a supply of fully reduced (tetra-hydro) forms of such cofactors for the provision of nucleotids for DNA synthesis and the metabolism of certain amino acids. A small number of point mutations in these two targets genes appear to be responsible for anti folates resistance (Hyde, 2007).

2.6.2.3 Mechanism of Atovaquone Resistance

Atovaquone acts through inhibition of electron transport at the cytochrome bc1 complex (Ittarat *et al.,*1994).Although resistance to atovaquone develops very rapidly when used alone, when combined with a second drug such as proguanil(the combination used is Malarone TM) or tetracycline, resistance develops more slowly . Resistance is conferred by single point mutations in the cytochrome-b gene (Holland, 2001).

2.6.2.4Mechanisms of Artemisinin Resistance

Several studies indicate that PfATP6, a transporter found on membraneous structures within the parasite cytoplasm, plays a key role in artemisinin sensitivity. Stable resistance to artemisinin and artesunate, a derivative of artemisinin has been induced in laboratory lines of a rodent parasite, suggesting that resistance to artemisinin derivatives may develops in future. Early signs of resistance to arteminisin were first reported in 2007-2008 and were reported on the Cambodia-Thailand border in 2009. It has since been reported in Myanmar and Vietnam.

2.7 SPREAD OF DRUG RESISTANCE

The spread of drug resistance cuts across aspects of economic, human behavior, pharmacokinetics and biology of vectors and parasites.

The biological influences are based on the parasites ability to survive the presence of an anti-malarial thus enabling the persistence of resistance and the potential for further transmission despite treatment. In normal circumstances any parasites that persist after treatment are destroyed by the host's immune system, therefore any factors that act to reduce the elimination of parasites could facilitate the development of resistance. This attempt to explain the poorer response associated with immuno-compromised individuals, pregnant women and young children.(White, 2011)

The use of anti-malarials developed from similar basic chemical compounds can increase the rate of resistance development, for example cross-resistance to chloroquine and amiodiaquine, two 4-aminoquinolones and mefloquine conferring resistance to quinine and halofantrine. This phenomenon may reduce the usefulness of newly developed therapies prior to large-scale usage. (White, 2011)

The resistance to anti-malarials may be increased by a process found in some species of plasmodium, where a degree of phenotypic plasticity was exhibited, allowing the rapid development of resistance to a new drug, even if the drug has not been previously experienced.(White, 2011)

The pharmokinetics of the chosen anti-malarial are key; the decision of choosing a long half-life over a drug that is metabolised quickly is complex and still remains unclear. Drugs with shorter half-life require more frequent administration to maintain the correct plasma concentrations, therefore potentially presenting more problems if levels of

adherence and compliance are unreliable, but longer-lasting drugs can increase the development of resistance due to prolonged periods of low drug concentration.(White, 2011)

The pharmokinetics of anti-malarials is important when using combination therapy. Mismatched drug combinations, for example having an 'unprotected' period where one drug dominates can seriously increase the likelihood of selection for resistant parasites.

Ecologically there is a linkage between the level of transmission and the development of resistance, however at present this still remains unclear.(White, 2011)

The treatment regime prescribed can have a substantial influence on the development of resistance. This can involve the drug intake,combination and interactions as well as the drug's Pharmokinetic and dynamic properties (White ,2001).

2.8*IN VITRO* CULTIVATION

One of the most important breakthroughs in malaria research was the development of techniques that enabled scientists to grow the erythrocytic stages of malaria parasites in continuous culture pioneered by William Trager and J.B. Jensen (Jensen,1988) thus freeing investigators from the need to use animals for chemotherapeutic and biochemical studies. The importance of this discovery cannot be over emphasised. For the first time, scientists had access to unlimited quantities of human malaria parasites, particularly *P. falciparum*, thus reducing their dependence on laboratory animals and blood taken from humans. The ease with which the erythrocytic stages could be grown in bulk made it possible not only to test the effects of drugs directly but also to isolate and purify parasite components in order to identify biochemical pathways and molecules of potential use in the development of vaccines and chemotherapy. The

cultivation of sexual stages provided insights into the genetics of human malaria parasites and the development of drug resistance. The cultivation of liver stages, although more difficult to achieve, made it possible to develop and test drugs against these stages and provided vital information about the immune responses in the liver.

In a study carried out in Cambodia on the decreases in *In vitro* susceptibility of *P. falciparum isolates* to Artesunate, Mefloquine, chloroquine and quinine from 2001 to 2007, a total of 2,041 isolates were used. Among these 1588 were examined for their *in vitro* susceptibilities to four antimalarials (Artesunate, Mefloquine, chloroquine and quinine). The geometric mean of 50% inhibitory concentration($GMIC_{50}$) for isolates from patients who failed artesunate-mefloquine therapy were significantly higher than those for patients who were cured(p,<0.001) (Pharath *et al*.,2007).

In another research on the *In vitro* studies on the sensitivity pattern of *P. falciparum* to anti-malarial drugs and local herbal extract using the *in vitro* micro test (markiii) technique to determine IC_{50} the drugs. All the isolates tested were sensitive to quinine, mefloquine, and artesunate. Fifty-one (51%) of the isolates were resistant to chloroquine, 13% to amodiaquine and 5% to sulphodoxine /pyrimethamine (Olasehinde *et al*., 2014).

In another study carried out in Abuja to check the *invitro* susceptibility of *P. falciparum* to Chloroquine, Quinine, Dihydroartemisinin and Monodesethylamodiaquine. The standard WHO *in vitro* micro test methodology was used in the study. Of 18 isolates evaluated for susceptibility to chloroquine, 16 (88.9%) were resistant with median IC_{50} of 0.06 μmol/1 of blood (range 0.36 – 1.32 μmol/1). The other 2 (11.1%) were however susceptible with median IC_{50} of 0.27 μmol/1 (range 0.24 – 0.30 μmol/1) of blood. With quinine, 13 (72.2%) were susceptible with median IC_{50} of 1.8 μmol/1 of

BMM (range 1.4 – 2.3 μmo1/1). For dihydroartemisinin, an IC_{50} of 13.5nmol/1 of BMM (range 7.8 – 26 nmol/1 of BMM) was indicated from the isolates. Withmonodesethyl amodiaquinine, 10 (55.6%) were susceptible with median IC_{50} of 0.09 μmol/1 (range 0.08 – 0.10 μmol/1 of blood) (Peletiri, 2012)

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1STUDY AREA

This study was carried out in a health facility inWusasa, Zaria, a Local Government Area in Kaduna State, Northern, Nigeria. The 2006 census population was estimated as 408,198.The language of majority of people of Zaria is Hausa. The area experiences two seasons, the dry season (November to May) and the Wet Season (June to October).The annual temperature range is from 18.5^{o}c to 55.7^{o}c and the mean annual rainfall is about 1050mm. Malaria has been reported to be present throughout the year with a marked increase during the rainy season.

3.1.2 Plant material

Carica papaya and *Psidium guajava* leaves

3.1.3 Antimalarial drugs

Chloroquine, Quinine, Artemether, Artemether-lumefantrine combination.

3.1.4 Reagents and chemicals

RPMI 1640 media, methanol, Giemsa stain, rhesus ‘O’ positive blood, sodium bicarbonate, Dimethyl sulfoxide(DMSO).

3.1.5 Equipment

Incubator, Hot air oven,tissue culture plates 12×8, microscope, autoclave, Pasteur pipette, rapid diagnostic test strip (RDT), forceps, rack, aluminum foil, Glass writing pencil

3.1.6 Glass wares

EDTA bottles, sterile syringes, microscope slides, beaker, measuring cylinder, conical flasks.

3.1.7 Culture media

One packet of RPMI which contains 25mM of HEPES buffer was dissolved in 960ml of triple distilled water.Two (2gm) of glucose was added and dissolved. 40µg/ml of Gemtamycin Sulphate was added to prevent microbial contamination. The solution was further sterilized by passing through a Millipore Filter of 0.22µm porosity and stored at 4^{o}C as 96ml aliquots in sterilebottles.

3.1.7.1 Preparation of Incomplete Culture Medium(Washing medium)

Four(4 ml) of 5% Sodium Bicarbonate was added to 96ml of Stock RPMI 1640.

3.1.7.2Preparation of complete Culture Medium

To prepare 100ml of the medium.90 ml of RPMI 1640 solution Containing 5% Sodium Bicarbonate was mixed with 10ml of O positive human serum. The medium was then stored in the refrigerator.

3.2 METHODS

3.2.1 Retrospective study

A retrospective study was carried out to know the pattern of antimalarial prescription and age distribution of patients presenting with malaria in a health facility in Wusasa, Zaria.

All files of patients diagnosed with malaria between the years 2012 through 2014 were collected from the records office, after ethical approval to execute the research was granted

Data on age, sex and pattern of antimalarial drug prescription was recorded. The data was analyzed and the information obtained provided a guide as regards the choice of antimalarial that wasused in the prospective study and the age group of persons commonly affected with malaria in this locality.

3.2.2 Identification of plant material

The leaves of *C. papaya* and *P. guajava* collected from Zaria wereidentified and authenticated at the herbarium section of the Department of Biological Sciences Ahmadu Bello University, Zaria Kaduna State, Nigeria. Voucher specimen with numbers (3071 and 3253 respectively) weredeposited for future reference.

3.2.3 Plant Extraction

The ethanolic extract was obtained by macerating the leaf powder in 95% ethanol, in the proportion of 100grms of powder to 300mls of ethanol (Ivoke, 2005) for 72 hours. The mixture was heated and exhaustively extracted with ethanol at a temperature of 40^{o} c for about 6hrs. Water bath was used to evaporate the solvent at 40^{o}C. The extract was left overnight at room temperature for the remaining solvent to evaporate.

3.2.4 Preparation of plant extract

One millilitre of Dimethyl sulphoxide (DMSO) was added to 0.100mg of each extract to make 100µg/ml. Further dilutions were made to obtain a working concentration of 50, 25 12.5 and 6.25µg/ml for each extract.

3.2.5 Ethical consideration

Ethical approval was obtained from the ethical committee of the Health facility in Wusasa,zaria. Approvalletter attached in appendix.

3.2.6 Sample Collection

Blood samples werecollected from the Medical Laboratory of the health facility in Wusasa, Zaria from patients diagnosed with malaria. The collected blood samples were kept in EDTA bottles.

3.2.7Patients and study protocol

Blood samples were collected with the assistance of Medical Laboratory Scientist from the patients attending the health facility. The malaria patients who agreed with the research work after informed consent was obtained or in case of children from their parents were 120. They were aged between 1-70 years, male and female. It was only malaria patients with auxiliary temperature greater than 37.5^{o}C, mono infection with *P. falciparum* and parasitemia level ranging from 1000-80,000 asexual parasites per µl of blood that were used for the *in vitro* study (WHO, 2001). All patients that have taken antimalarial drugs within 2 weeks preceding the study or with any other symptoms or signs of non-malaria etiology were excluded from the study.

3.2.8Processing of samples

3.2.9Microscopic Examination

Thick and thin films stained with Giemsa were prepared for the microscopic examination of the malarial parasite. The thin films were fixed with methanol and all the films were stained with 3% Giemsa of P^{H}7.0 for 30 min as recommended by WHO (WHO, 2000). Blood films were examined microscopically using 100x (oil immersion) objectives as described by Cheesbrough(2000). The thick films were used to determine the parasite densities whilethin films were used to identify the parasite species and infective stages. Parasite density per microliter of blood (Parasitemia) was estimated from the thick film. This was carried out by multiplying the average number of parasites per high power field (100x objective) by 500.Ten to fifty fields (depending on

parasitaemia) were examined to determine the average number of trophozoites per high power field(HPF). Ten fields are sufficient when the parasite density is high (Greenwood and Armstrong,1991). Patients who have mono- infections with *P.falciparum* and asexual parasitaemias in excess of 1000 parasites, but less 80 000 parasites per µl blood, were considered suitable for the work (WHO, 2001).

3.2.10 Performance of the *in vitro* micro –test

The *in vitro* tests and drug susceptibility tests were carried out following standard culture techniques by Trager and Jensen(1976) and under strict aseptic conditions. The RPMI 1640 media was prepared from the powder following strict aseptic techniques.

Stock solutions of antimalarial drugs (Chloroquine, Quinine, Artemether and Artemether + Lumefantrine) and the extract of the leaves of *C. papaya* and *P. guajava* wereprepared. The solution was filtered with 0.22 membrane filter (millipore) aseptically. Furtherdilutions of each drug were made using distilled water to obtain a working solution of desired concentration. From the working solutions, seven two - fold dilutions of each drug was made and used to dose 96 well flat bottom micro culture plates and left to dry in an incubator at 37^{o}C.

Following centrifugation of infected blood samples at low speed (200 rpm) the plasma was discarded and the erythrocytes (packed cells) washed three times in culture medium (RPMI 1640 withL-glutamine, without sodium bicarbonate) at 2000 rpm for 5 minutes. Washed infected erythrocytes (100µl) was mixed aseptically with 900µl of complete culture medium (1:10) and 50µl of theblood-medium mixture (BMM) was transferred into each well of the pre-dosed plates, using Eppendorf pipette with a sterile tip. This was done in descending order, starting from well A (control) and ending at well H with the highest concentration of the test drug.

The plates were incubated at 37^{o}C for 24-30 hours using the candle jar method (5% CO_2) (Trager and Jensen, 1976). All the susceptibility assays for each drug was done in duplicate.

After incubation, the plates was allowed to stand for 30 minutes in a semi-vertical position (about 45 ^{o} inclined). The supernatant was then removed, erythrocytes re-suspended in the remaining fluid, and a thick blood film was made from each well. The blood smears were air-dried for 24 hours and stained with 3% Giemsa stain for 30 minutes.

3.2.11 Examination of the Post –Culture Blood Slide

The stained thick films were examined with the oil immersion objective(100 x) and the number of malaria parasitescalled schizonts were counted and compared with the control. The reading started with the control well. The basis of the count is that at the end of incubation schizont maturation in the control well should be ≥10% out of 200 asexual parasites. The counts read in each well were expressed as percentages of the control as shown below:

Z= Number of Schizonts per 200 parasites in the control well after incubation

Y= Number of Schizonts per 200 parasites in other wells dosed with drugs after incubation

X= % of Schizonts relative to control sample

$X\ (\%) = Y/Z \times 100$

The inhibition of parasite growth for each of the isolates used was calculated as follows

Percentage inhibition= 100-X

The individual percentage Schizont maturation inhibitions were fed directly into Microsoft Excel. Using linear regression lines their inhibitory concentrations IC_{50} (IC_{50}), 90 (IC_{90}) and 99 (IC_{99}) were determined. Drug resistant *P. falciparum* parasites

were identified as parasites with IC_{50} values greater than the peak Plasma concentration of the Antimalarial drugs.

3.2.12Molecular Characterization of Chloroquine and Quinine Resistant Genes

3.2.12.1DNA Extraction

Resistant *P. falciparum* DNA was extracted from patient blood. 3ml of blood sample was put in Eppendorf tube, 20ml of protenase k and 400mlof lysis buffer was addedand the solution was vortexted for 5 minutes. It was then incubated at 56^0c for 30 minutes, it was vortexed again for 5 minutes, the mixture was then transferred into spin column, after which it was purified using wash 1& 2 and elutin buffer.(Dneasy blood and tissue handbook, 2006).

3.2.12.2PCR for the detection of pfcrt gene

The oligonucleotides were designed from published sequences. For the amplification of the 1.6kb fragment of *pfcrt,* the reverse primer was 5′-CCGTTAATAATACATAAAGTCA-3′ and the forward primer was 5′-CTTTTAAAAATGGAAGGGTGT-3' (Table 3.1)(Dorsey *et al*., 2001). The primary PCR components, in a final volume of 25µl was 2.5mgcl_2, 1µl of Taq polymerase, 2µl of DNA template, 1µl of 10X buffer, 1µl of dNTP, 11.5µl of Nuclease free water(Table 3.1).

The cycling protocol was as follows: 94^oc for 5 min for initial denaturation, 40 cycles of 94^oc for 30s, 52^oc for 40s and 72^oc for 30s and a final extension of 72^oc for 5min (Table 3.3.

Table 3.1: Primer for chloroquine and quinine resistant genes

S/N	Primer Names	Oligonucliotide Sequence	Anti-malaria Drug	Base pair	References
1	Pfmdr – Forward	ATGGGTAAA GAGCAGAAAGA	Chloroquine and Quinine	76bp	Olasehinde *et al.,* 2014
	Pfmdr – Reverse	AACGCAAGTAATACATAAAGTCA			
2	Pfcrt-Reverse	CCGTTAATAATAAATACAGGC	Chloroquine putative transporter	1.6kbp	
	Pfcrt-Forward	CTTTTAAAAATGGAAGGGTGT			

3.2.12.3PCR Method

An aliquot of the below reagents were added together to make a 25µl reaction as in Table 3.1

Table 3.2: Reaction mixture for the Detection of *Pfcrt* and *Pfmdr* genes

Reagent	**Quantity(µl)**
Nuclease free water	11.5
10X Buffer	5
10uM dNTPs	1
Primer Forward	1
Primer Reverse	1
Taq polymerase (AmpliTaq Gold, USA)	1
DNA template	2
$MgCl_2$	2.5
Total	25

3.2.12.5Nested PCR for *Pfcrt* mutation-specific detection.

The PCR products from the amplification reactions were evaluated by electrophoresis on 2% agarose gels containing ethidium bromide. 10 µl of the nested PCR product reaction mixture were treated directly with 3U of the restriction enzyme *Apo* I for 6 to 16 hours at 50°C as recommended by the manufacturer (New England Biolabs, Beverly, MA). The enzyme *Apo* I recognize and cut the 76K codon and leaves the 76T codon found in chloroquine-resistant *P. falciparum*. The conditions used for thermal cycling conditions are contained in the Table 3.2 below:

Table 3.3:Thermal Cycling Conditions Used For Nested PCR

Step	Temperature ^{0}C	Time	Number of Cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30sec	40 cycles
Annealing	52	40 sec	1
Extension	72	45sec	1
Final extension	72	5mins	1
Final hold	4	Total time is 11mins, 55 sec.	

CHAPTER FOUR

4.0 RESULTS

4.1 INCIDENCE OF MALARIA (RETROSPECTIVESTUDY)

A total of 953 people comprising448 males (47%)and 505 females (53%) were reported to be infected with malaria from 2012 through 2014 in the Health Facilityin Wusasa, Nigeria.The number of reported malaria cases in 2012, 2013 and 2014 was 151, 449 and 353 respectively. The monthly distribution of reported cases is presented inTable 4.1. The number of reported cases increased significantly from 151 cases in 2011 to 449 cases in 2013 but reduced to 353 cases in 2014.

The total number of patients that presented to a Health facility inWusasa, Zaria with malariaor other illnesses from 2012 through 2014 was 35,035.The incidence of malaria over the three year period (2012-2014) was therefore calculated to be 2.8%

Table 4.1: Monthly Distribution Of Reported Malaria Cases Over A Three Year Period At A Health Facility In,Wusasa Zaria, Nigeria.

MONTH	2012	2013	2014
JANUARY	5	6	9
FEBUARY	3	7	5
MARCH	3	5	11
APRIL	5	15	6
MAY	15	23	15
JUNE	20	78	20
JULY	33	99	76
AUGUST	52	140	129
SEPTEMBER	3	45	55
OCTOBER	3	17	15
NOVEMBER	4	9	10
DECEMBER	5	5	2
TOTAL	**151**	**449**	**353**

4.2INCIDENCE OF *P. falciparum* INFECTION AMONG AGE GROUPS

The distribution of malaria among age groups 0-5, 6-39 and 40 and above from 2012 through 2014 is presented in the Fig 4.1 below.

In 2012, out of the 151 reported malaria cases, 28 were children between the ages of 0-5years, 100 were between the ages of 6-39 years and 23 were 40 and above. Of the total 449 reported malaria cases in 2013, 148 were children between the ages of 0-5 years, 202 were between the ages of 6-39 years and 99 were 40 and above. In 2014, 89 patients out of the total 353 reported cases were children between the ages of 0-5 years, 201 were between the ages of 6-39 and 63 patients were 40 and above.

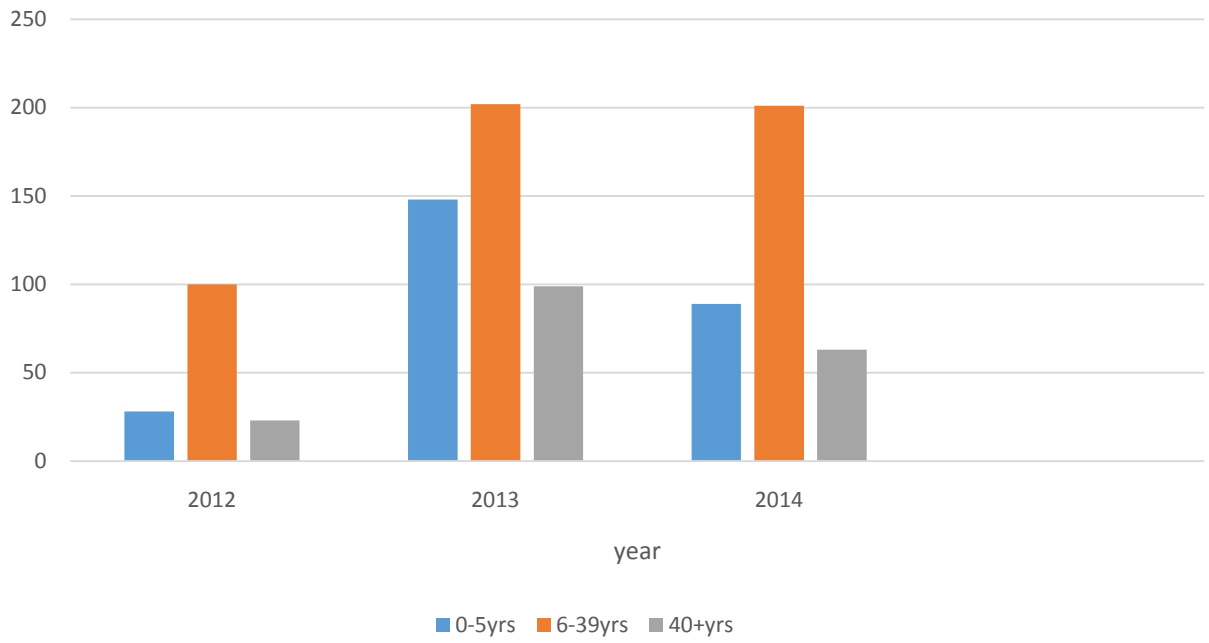


Fig 4.1: Age distribution of patients diagnosed with Malaria at a Health facility from 2012-2014.

4.3PATTERN OF ANTIMALARIAL DRUG PRESCRIPTION (RETROSPECTIVE STUDY)

Chloroquine, Quinine, Fancidar/Falcidine (Sulphadoxine+ Pyrimethamine), Artemether, Coartem (Artemether + Lumefantrine), ASAQ (Artemether + Piperaquine), and Artesunate were the antimalarial drugs that were routinely prescribed for the treatment of malaria at a Health facility inWusasa, Zaria Nigeria over the three year period. The summary of drug prescription pattern is presented inTable 4.2.

Chloroquine was the most highly prescribed antimalarial (35.1%) for malaria treatment in year 2012 and Artesunate the least prescribed,(3.9%) while Amodiaquine was not prescribed at all (Fig 4.2). In 2013, 35.9% of the total number of patients that had malaria were treated with chloroquine, making it the most prescribed drug for malaria treatment in that year, Amodiaquine was the least prescribed (1.4%) while coartem (Atresunate –lumefantrine) was not prescribed at all (Fig 4.3). In 2014Coartem was reported to beprescribed more frequently (31.7%), Artequine was the least presc ribed (0.8%) whileAmodiaquine wasnot prescribed at all, (Fig 4.4). These results show thatsingle antimalarial drug (chloroquine) was still very much in use in the study center in the years 2012 and 2013 as it was the most prescribed, for the treatment of malaria in the center,while in 2014 there was a shift to combination therapy, Coartem (Arthemether-lumefantrine), for malaria treatment, as it was the most prescribed in that year

.

Table 4.2: Summary of Antimalarial prescription pattern ina Health facility inWusasa Zaria, Kaduna State, Nigeria

ANTIMALARIAL	2012	2013	2014
A	1	45	18
B	12	97	70
C	53	161	65
D	20	100	72
E	6	12	12
F	13	8	0
G	14	20	3
H	0	6	0
I	32	0	112
J	0	0	1

Key:

A= Artesunate-Piperaquine, B=Arthemether, C=Chloroquine, D= Quinine, E= Artesunate, F= Falcidine, G= Fansider, H=Amodiaquine, I= Coartem, J=Artequine

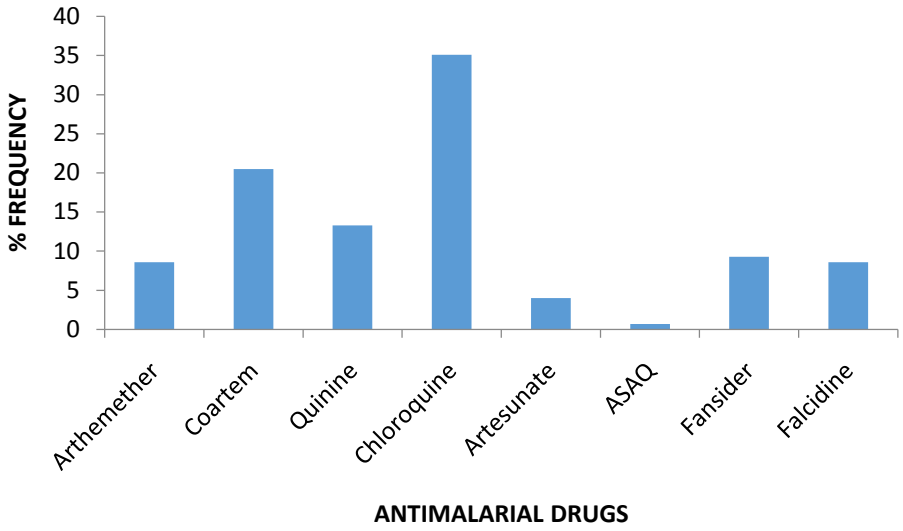


Fig 4.2: Pattern of antimalarial drug use in at theHealth facility in2012

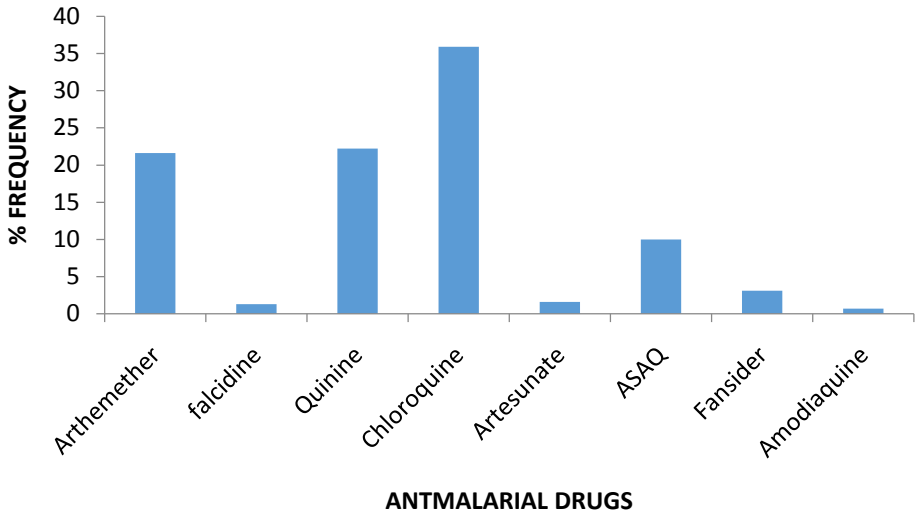


Fig 4.3: Pattern of Antimalarial Drug use at the Health facility in 2013

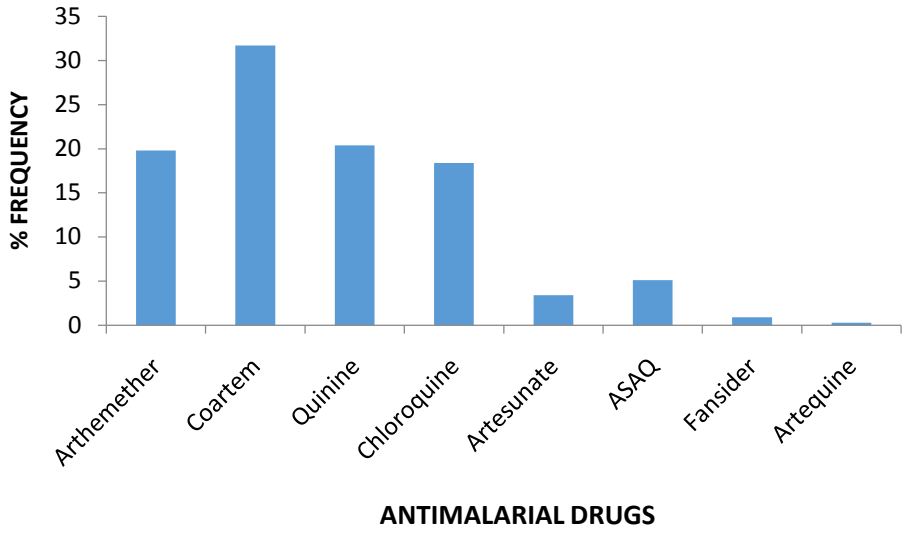


Fig 4.4: Pattern of antimalarial use at the Health facility in 2014

4.4*IN VITRO* ASSAY

A total of 120 malaria positive patients were used in this study, after obtaining informed consent. However, out of these 120 patients, 30 patients admitted to have taken one antimalarial or the other, either in the form of over the counter Fansider, or other herbal extract before presentation to the hospital, so were excluded from the study. 40 patients did nothave the required parasitaemia of (greater than or equal to 1000 but less than 80,000 parasites per µl of patients' blood), so were alsoexcluded from the study.

A total of 50 *P. falciparum* isolates were processed, but 30 isolates were successfully evaluated forthe in *vitro* susceptibility of the isolates to Chloroquine, Quinine, Artemether, combination of Artesunate- Lumefantrine, C. papaya, *P. guajava* extract. The remaining 20 were discarded due to contamination and failure of the schizont to mature satisfactorily (200 asexual ring forms) in the control well. Out of the 30 isolates evaluated, 17were from females(56.7%) and 13 were from males (43.3%), out of which 8 were from patients between the ages of 0-5 years, 14 were from patients between the ages of 6-39 years and 8 were from patients 40 and above.

4.5*IN VITRO*- ANTIMALARIAL SENSITIVITY TESTS

In vitro assay of the isolates to Chloroquine,Quinine, Artemether and Artemether + Lumefantrine(Coartem) and local herbal leaf extracts (*C. papaya*and *P. guajava*) were carried out according to WHO (2000) and the IC_{50}, IC_{90} and IC_{99} for each drug was determined by linear extrapolation using Microsoft excel 2007. The *in vitro*response of *P. falciparum* Isolates to varying concentrations of the test drug is expressed as the 50% inhibitory dose value IC_{50}. $IC_{50}$is the concentration of an antimalarial that inhibits 50% of schizont maturation as comparedto the development in drug-free control wells(Basco, 2007). The drug- resistant *P.falciparum* isolates were identified as isolates with IC_{50} values greater than the peak plasma level of the drug in question. The reported

peakplasma level of Chloroquine is 0.5µmol/l, Quinine: 2.2µmol/l, Arthemether: 332µmol/l, Lumefantrine: 6.3µmol/l , the peak plasma level of Artemether –lumfantrine combination (Coartem) is 332µmol/l considering the upper limit of 332µmol/l as the peak plasma break point of Artemether-lumefantrine combination.\

4.5.1 Quinine

The schizont maturation counts at different concentrations of quinine used arepresented in Appendix III. The degree of quinine inhibition of schizont maturation (%) at different concentration of quinine in µmol/l ispresented in Table 4.2.The concentration of quinine that gives 50% inhibition (IC_{50}) of *P. falciparum* isolates is presented in Table 4.8. The IC_{90} and IC_{99} values of quinine for the isolates are presented in Appendix VIII and IX.

Of the 30 *P. falciparum*isolates evaluated, 27(90%) were resistant to quinine (Table 4.9) with a median IC_{50} of 7.8µmol/l(range 2.6-26µmol/l), 3(10%) were however susceptible with a median IC_{50} of 2µmol/l(Table 4.9), based on comparison with the peak plasma concentration of quinine (2.2µmol/l).

4.5.2Chloroquine

The degree of chloroquine inhibition of schizont maturation (%) at different concentration of chloroquine in µmol/l is presented in table 4.3. The schizont maturation counts at different concentrations of chloroquine used arepresented in Appendix IV. The concentration of chloroquine that gives 50% inhibition (IC_{50}) of *P. falciparum*isolates is presented in Table 4.8. The IC_{90} and IC_{99} values of chloroquine for the isolates are presented in Appendix VIII and IX

Of the 30 *P. falciparum*isolates evaluated 27 (90%) were resistant to chloroquine (Table 4.9) with a median IC_{50} of 5.3µmol/l (range 1.1 - 24µmol/l). 3 (10%) were susceptible with a median IC_{50} 0f -1.1µmol/l (Table 4.9), based on comparison with peak plasma concentration of Chloroquine (0.5µmol/l).

4.5.3Artemether

The degree of Artemether inhibition of schizont maturation (%) at different concentration of artemether in µmol/l is presented in Table 4.4.The schizont maturation counts at different concentration of artemether used is presented in Appendix V. The concentration of artemether that gives 50% (IC_{50})inhibition of *P.falciparum* isolates is presented in Table 4.8. The IC_{90} and IC_{99}values of Artemether are presented in Appendix VIII and IX.

Of the 30 *P.falciparum* isolates evaluated, all 30 (100%) were susceptible to artemether with a median IC_{50} of 7.2µmol/l (range3.1-15µmol/l) (Table 4.9) with 0% resistance (Table 4.9) basedon comparison with peak plasma concentration of Artemether(332µmol/l).

4.5.4Artemether –Lumefantrine combination (Coartem)

The degree of inhibition of schizont maturation (%) of Artemether-lumefantrine combination is presented in Table 4.5. The schizont maturation counts at different concentrations of Artemether-Lumefantrine combination is presented in Appendix V.

All the *P. falciparum* isolates tested were fully susceptible (Table 4.9) to the commercially available arthemether and lumefantrine combination (coartem) with no schizont maturation recorded.

4.6*IN VITRO* ANTIMALARIAL ACTIVITY OF HERBAL EXTRACTS

The aqueous extract of paw paw and guava leaves showed good anti-plasmodial activity *in vitro* against *P. falciparum*. Paw paw leave extract had a median IC_{50} of 5.4µg/ml (range 2.3-20µg/ml), while Guava leaf extract had a median IC_{50} value of 9.2µg/ml (range 5.5-64µg/ml) (Table 4.8).

Table 4.2: Degree of Quinine inhibition of Schizont maturation (%) at different concentrations of Quinine(µmol/l)

Blood Sample	A Control	B 4	C 8	D 16	E 32	F 64	G 128	H 256
1	0.0	0.0	0.0	8.3	16.7	37.5	54.2	66.7
2	0.0	36.0	72.0	82.0	94.0	98.0	100	100.0
3	0.0	25.0	25.0	16.7	16.7	8.3.0	29.2	37.5
4	0.0	78.0	82.0	87.0	94.0	93.0	93.0	96.0
5	0.0	60.0	67.0	67.0	65.0	72.0	85.0	95.0
6	0.0	44.0	56.0	62.0	70.0	77.0	84.0	88.0
7	0.0	60.0	67.0	67.0	70.0	72.0	78.0	93.0
8	0.0	55.4	58.9	66.1	73.2	82.1	87.5	92.9
9	0.0	75.0	78.0	83.0	86.0	94.0	94.0	96.0
10	0.0	19.0	31.0	53.0	75.0	86.0	92.0	92.0
11	0.0	72.0	72.0	79.0	82.0	87.0	92.0	94.0
12	0.0	64.0	72.0	79.0	88.0	91.0	90.0	97.0
13	0.0	75.0	78.0	83.0	86.0	94.0	94.0	96.0
14	0.0	65.0	70.0	77.0	80.0	92.0	92.0	95.0
15	0.0	65.0	73.0	80.0	88.0	92.0	90.0	97.0
16	0.0	73.6	77.3	81.1	84.9	86.8	86.8	98.1
17	0.0	45.0	55.0	67.0	75.0	85.0	88.3	88.3
18	0.0	9.0	9.0	22.0	17.0	87.0	87.0	96.0
19	0.0	0.0	6.7	4.0	4.0	13.3	31.0	40.0
20	0.0	73.0	77.0	87.0	87.0	93.0	100.0	97.0
21	0.0	89.0	91.0	87.0	100.0	98.0	100.0	100.0
22	0.0	65.0	73.0	80.0	88.0	92.0	90.0	97.0
23	0.0	17.0	17.0	37.0	63.0	80.0	80.0	88.0
24	0.0	60.0	62.0	78.0	78.0	83.0	91.0	95.0
25	0.0	95.0	95.0	95.0	92.0	97.0	97.0	100.0
26	0.0	89.0	94.0	95.0	94.0	97.0	100.0	98.0
27	0.0	96.0	96.0	94.0	98.0	98.0	100.0	100.0
28	0.0	88.0	92.0	92.0	97.0	95.0	98.0	98.0
29	0.0	21.0	35.0	44.0	58.0	69.0	85.0	92.0
30	0.0	82.0	84.0	89.0	87.0	92.0	92.0	95.0

Key: A= control, B-H represent different concentrations of test drug

Table 4.3: Degree of Chloroquine inhibition of Schizont maturation (%) at different concentrations of Chloroquine (µmo/l)

Blood Sample	A Control	B 0.2	C 0.4	D 0.8	E 1.6	F 3.2	G 6.4	H 12.8
1	0.0	58.3	50.0	50.0	66.6	75.0	83.3	83.3
2	0.0	54.0	62.0	78.0	90.0	96.0	100.0	100.0
3	0.0	12.5	0.0	20.8	25.0	29.2	45.8	70.8
4	0.0	49.0	56.0	70.0	57.0	63.0	73.0	79.0
5	0.0	63.0	78.0	78.0	88.0	90.0	90.0	95.0
6	0.0	38.0	46.0	65.0	61.0	74.0	90.0	96.0
7	0.0	64.3	74.5	81.8	87.3	92.7	92.7	96.2
8	0.0	69.6	67.9	73.2	73.2	83.9	94.6	96.4
9	0.0	64.0	75.0	83.0	88.0	94.0	98.0	98.0
10	0.0	8.0	8.0	31.0	75.0	81.0	86.0	86.0
11	0.0	64.0	74.0	76.0	87.0	93.0	96.0	96.0
12	0.0	97.0	90.0	91.0	88.0	79.0	72.0	64.0
13	0.0	64.0	75.0	83.0	88.0	94.0	98.0	98.0
14	0.0	63.0	75.0	82.0	86.0	93.0	95.0	97.0
15	0.0	50.0	63.0	72.0	75.0	83.0	86.0	95.0
16	0.0	64.2	64.2	75.5	83.0	88.7	92.5	98.1
17	0.0	47.0	58.0	75.0	75.0	82.0	88.3	95.0
18	0.0	0.0	0.0	13.0	17.0	26.0	78.0	87,.0
19	0.0	0.0	0.0	0.0	4.0	46.7	46.7	55.6
20	0.0	3.0	30.0	27.0	43.0	57.0	87.0	87.0
21	0.0	4.0	4.0	11.0	18.0	13.0	40.0	33.0
22	0.0	50.0	63.0	72.0	75.0	83.0	86.0	95.0
23	0.0	51.0	60.0	63.0	77.0	83.0	88.0	88.0
24	0.0	31.0	38.0	49.0	52.0	71.0	83.0	90.0
25	0.0	25.0	47.0	67.0	82.0	80.0	87.0	87.0
26	0.0	41.0	49.0	55.0	61.0	66.0	75.0	85.0
27	0.0	6.0	12.0	8.0	25.0	22.0	31.0	33.0
28	0.0	47.0	45.0	62.0	71.0	75.0	79.0	83.0
29	0,.0	23.0	21.0	31.0	35.0	60.0	58.0	46.0
30	0.0	5.0	8.0	0.0	0.0	26.0	26.0	37.0

Key: A= control, B-H represent different concentrations of test drug

Table 4.4: Degree of Artemether inhibition of Schizont maturation (%) at different concentrations of Artemether (µmol/l)

Blood sample	Control A	B 2	C 4	D 8	E 16	F 32	G 64	H 128
1	0.0	50.0	50.0	50.0	66.7	58.3	75.0	83.3
2	0.0	54.0	68.0	78.0	86.0	100.0	100.0	100.0
3	0.0	12.5	25.0	29.2	37.5	45.8	58.3	83.3
4	0.0	82.0	88.0	85.0	91.0	94.0	96.0	99.0
5	0.0	62.0	65.0	73.0	80.0	93.0	98.0	100.0
6	0.0	77.0	81.0	91.0	95.0	98.0	99.0	100.0
7	0.0	65.5	72.7	76.4	87.3	92.7	98.2	0.0
8	0.0	62,5	67.9	73.2	80.4	92.9	96.4	0.0
9	0.0	80.0	82.0	83.0	89.0	97.0	100.0	100.0
10	0.0	17.0	17.0	39.0	92.0	97.0	100.0	100.0
11	0.0	72.0	65.0	78.0	83.0	93.0	97.0	97.0
12	0.0	67.0	76.0	84.0	90.0	97.0	100.0	100.0
13	0.0	69.0	75.0	78.0	87.0	92.0	96.0	100.0
14	0.0	68.0	73.0	75.0	77.0	85.0	97.0	100.0
15	0.0	63.0	72.0	83.0	88.0	95.0	100.0	100.0
16	0.0	67.9	71.7	77.4	86.8	92.5	96.2	0.0
17	0.0	80.0	85.0	87.0	95.0	98.0	100.0	100.0
18	0.0	43.0	43,0	52.0	60.0	78.0	96.0	96.0
19	0.0	4.0	9.0	11,1	48.9	86.6	88.9	93.0
20	0.0	73.0	77.0	87.0	90.0	100.0	100.0	100.0
21	0.0	40.0	53.0	64.0	98.0	98.0	100.0	100.0
22	0.0	63.0	72.0	83.0	88.0	95.0	100.0	100.0
23	0.0	66.0	74.0	71.0	83.0	88.0	88.0	97.0
24	0.0	75.0	73.0	82.0	87.0	88.0	95.0	100.0
25	0.0	75.0	82.0	87.0	92.0	100.0	100.0	100.0
26	0.0	85.0	85.0	89.0	95.0	100.0	98.0	98.0
27	0.0	98.0	98.0	98.0	96.0	100.0	100.0	100.0
28	0.0	79.0	79.0	82.0	90.0	99.0	100.0	96.0
29	0.0	10.0	10,0	17.0	58.0	90.0	85.0	94.0
30	0.0	84.0	89.0	95.0	92.0	97.0	97.0	95.0

Key: A= control, B-H represent different concentrations of test drug

Table 4.5: Degree of inhibition of Schizont maturation (%) of Artemether-lumefantrine combination

Blood Sample	Control	% inhibition
1	0.0	100.0
2	0.0	100.0
3	0.0	100.0
4	0.0	100.0
5	0.0	100.0
6	0.0	100.0
7	0.0	100.0
8	0.0	100.0
9	0.0	100.0
10	0.0	100.0
11	0.0	100.0
12	0.0	100.0
13	0.0	100.0
14	0.0	100.0
15	0.0	100.0
16	0.0	100.0
17	0.0	100.0
18	0.0	100.0
19	0.0	100.0
20	0.0	100.0
21	0.0	100.0
22	0.0	100.0
23	0.0	100.0
24	0.0	100.0
25	0.0	100.0
26	0.0	100.0
27	0.0	100.0
28	0.0	100.0
29	0.0	100.0
30	0.0	100.0

Key: A= control, B-E represent different concentration of the extract

Table 4.6: Degree of *P. Guajava* leaf extract inhibition of Schizont maturation (%) at different concentrations of the extract (µg/ml)

Blood Sample	Control A	B 6.25	C 12.5	D 25	E 50
1	0.0	70.8	87.5	95.8	100.0
2	0.0	94.0	98.0	100.0	100.0
3	0.0	79.2	95.8	100.0	100.0
4	0.0	98.0	98.0	100.0	100.0
5	0.0	93.0	95.0	98.0	100.0
6	0.0	94.0	97.0	100.0	100.0
7	0.0	85.5	89.1	92.7	100.0
8	0.0	82.1	94.6	98.2	100.0
9	0.0	92.0	96.0	99.0	100.0
10	0.0	75.0	81.0	94.0	94.0
11	0.0	81.0	88.0	95.0	100.0
12	0.0	79.0	89.0	96.0	99.0
13	0.0	90.0	94.0	99.0	100.0
14	0.0	88.0	95.0	98.0	100.0
15	0.0	90.6	94.3	100.0	100.0
16	0.0	90.6	94.3	100.0	100.0
17	0.0	95.0	98.0	100.0	100.0
18	0.0	87.0	96.0	100.0	100.0
19	0.0	100.0	100.0	98.0	100.0
20	0.0	87.0	97.0	100.0	100.0
21	0.0	91.0	98.0	98.0	96.0
22	0.0	78.0	88.0	92.0	98.0
23	0.0	77.0	86.0	97.0	100.0
24	0.0	83.0	88.0	95.0	99.0
25	0.0	57.0	70.0	100.0	100.0
26	0.0	95.0	98.0	100.0	100.0
27	0.0	94.0	98.0	100.0	100.0
28	0.0	90.0	92.0	98.0	98.0
29	0.0	92.0	100.0	96.0	98.0
30	0.0	100.0	100.0	97.0	92.0

Key: A= control, B-E represent different concentrations of the extract

Table 4.7: Degree of*C. papaya* leaf extract inhibition of Schizont maturation (%) at different concentrations of the extract (µg/ml)

Blood sample	A Control	B 6.25	B 12.5	C 25	D 50
1	0.0	25.0	54.2	66.7	79.2
2	0.0	90.0	94.0	94.0	100.0
3	0.0	83.3	87.5	87..5	100.0
4	0.0	70.0	74.0	88.0	98.0
5	0.0	67.0	78.0	87.0	98.0
6	0.0	79.0	94.0	99.0	100.0
7	0.0	64.3	64.3	87.3	96.2
8	0.0	80,4	83.9	87.5	98.2
9	0.0	94.0	96.0	98.0	99.0
10	0.0	47.0	56.0	81.0	81.0
11	0.0	26.0	58.0	74.0	78.0
12	0.0	48.0	62.0	72.0	88.0
13	0.0	92.0	96.0	98.0	98.0
14	0.0	92.0	95.0	97.0	98.0
15	0.0	62.0	77.0	83.0	93.0
16	0.0	81.1	84.9	98.1	100.0
17	0.0	88.0	95.0	100.0	100.0
18	0.0	70.0	78.0	100.0	100.0
19	0.0	71.0	76.0	80.0	88.9
20	0.0	47.0	57.0	77.0	87.0
21	0.0	38.0	53.0	80.0	84.0
22	0.0	62.0	77.0	83.0	93.0
23	0.0	77.0	80.0	88.0	97.0
26	0.0	86.0	92.0	95.0	95.0
27	0.0	78.0	76.0	94.0	94.0
28	0.0	77.0	84.0	92.0	92.0
29	0.0	67.0	69.0	77.0	85.0
30	0.0	66.0	71.0	76.0	87.0

Key: A= control, B-E represent different concentrations of the extract

Table 4.8: Concentration of Test Antimalarial Agents that Give 50% Inhibition of *Plasmodium Falciparum* Isolates (Ic50) Growth in a Health Facility In Wusasa, Nigeria.

Plasmodium falciparum	**Chloroquine PPL(0.5µmol/)**	**Quinine PPL(2.16µmol/l)**	**Arthemeter PPL(332µmol/l)**	***C.papaya* Leaves MLD (5000mg/kg)**	***P.guajava*MLD (50000mg/kg)**
1	24	2.0	5.5	2.9	6.6
2	8.1	7.6	8.3	11	17.7
3	2.0	2	3.1	10.4	9.0
4	7.8	13	15	5.8	64
5	6.4	5.9	7.1	5.8	22.1
6	4.9	6.4	12.4	5.6	17.7
7	10.8	9.7	3.9	3.9	7.5
8	8.3	9.6	8.7	9.1	10.2
9	8.6	13.7	9.6	20	13.5
10	3.2	4.1	5	2.7	5.5
11	7.0	9.6	8.7	3.0	5.9
12	5.5	6.8	7.0	3.8	8.9
13	6.9	7.9	7.2	17	11
14	6.6	10	9.8	17	9.3
15	5.3	3.3	7.1	5.7	7.5
16	5	13.7	8.9	6.5	12
17	4.8	4.6	8.3	5.2	11
18	2.7	2.6	8.4	2.7	8.8
19	-1.1	-0.04	3.9	3.2	8.1
20	2.3	5.5	8.6	3.2	8.9
21	1.1	13	4.2	2.3	10.2
22	5.3	6.7	4.8	3.6	5.8
23	5.3	3.3	7.1	5.7	7.5
24	5.0	6.8	9.1	6.2	-95
25	3.7	21	6.9	3.2	8.2
26	5.8	12	12.7	11.1	21
27	-1.8	26	5.1	3.8	9.3
28	3.9	11	6.9	6.5	13
29	1.3	5.7	4.1	6.5	35.1
30	-0.2	8	9.5	2.8	7.3

Key- PPL = Peak plasma level

MLD= Mean lethal dose

Table 4.9: Resistant *Plasmodium falciparum* Isolates Profiles Against Test Antimalrial Agent In A Health Facility InWusasa Zaria, Nigeria.

Drug	Antimalarial Peak plasma concentration(µmol/l)	% Resistant
Chloroquine	0.5	90
Quinine	2.16	90
Arthemether	332	0
Coartem	332 for arthemether and 6.3 for lumefantrine.	0

4.7 PREVALENCE OF DRUG RESISTANT MOLECULAR MARKERS

Resistant *P.falciparum* isolates (10) collected from the Health facility in Wusasa Zaria, Nigeria were assayed to determine the prevalence of established antimalarial drug resistant markers.Plate 4.1 shows the amplification of extracted DNA from the resistant*Plasmodium falciparum* isolates .The molecular markers assayed for in the study were *pfcrt* and *pfmdr*. Eighty percent (80%) of the isolates had pfmdr1resistance gene, which amplified around 1600 base pair (bp), while all (100%) had *pfcrt* chloroquine resistance gene, which amplified around 76bp. All the isolates that showed invitro resistance to chloroquine also harboured the genes coding for resistance to chloroquine (*pfcrt and pfmdr1*) however out of the 10 isolates that showed invitro resistance to quinine, 8(80%) of them harboured the *Pfmdr1* gene. Plates 4.1-4.2 shows gel electrophoresis results for the detection of *pfcrt* and *pfmdr1*genes respectively.

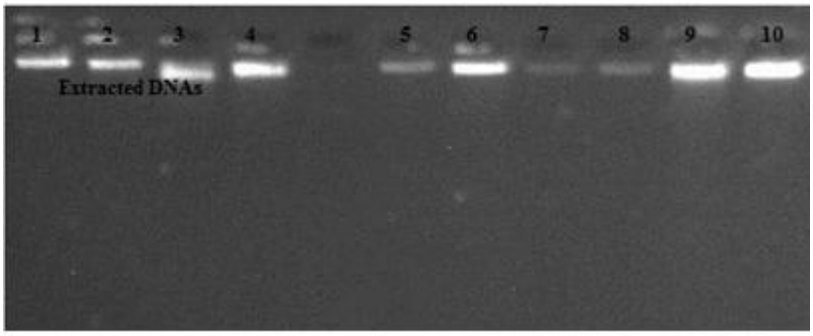


Plate 4.1: Electrophoresis to show Extracted DNA from blood samples of *P. falciparum* infested patients in Zaria, Nigeria

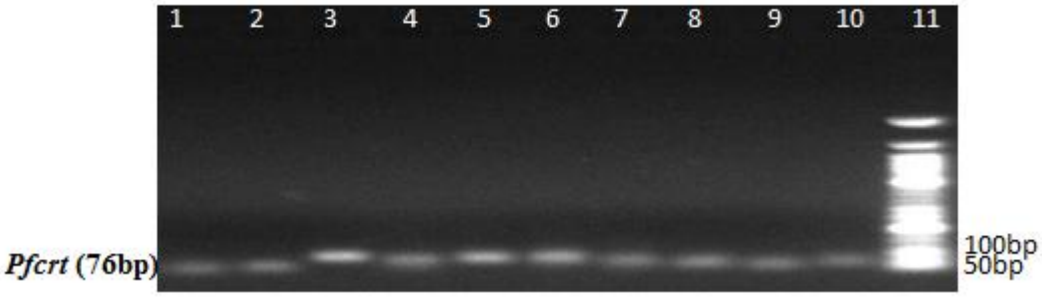


Plate 4.2: Amplification of *Pfcrt* gene from DNA of *P. falciparum* isolates from malaria infested patient's blood in Zaria, Nigeria

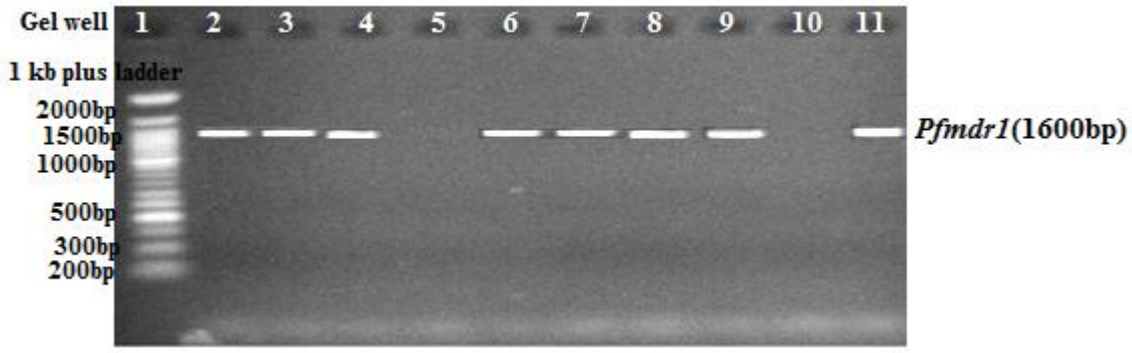


Plate 4.3: Amplification of *Pfmdr* gene from DNA of *P falciparum* isolates from malaria infested patient's blood in Zaria, Nigeria

CHAPTER FIVE

5.0 DISCUSSION

Malaria is the most prevalent tropical disease in the world today and in sub Saharan Africa. It is ranked amongst the most frequent causes of morbidity and mortality especially among children and is often the leading identifiable cause (Greenwood *et al*., 2005). About 50% of Nigerian population experience at least one episode of malaria each year (FMOH, 1997). The complete eradication of the disease is far from reaching completion due to the continuous emergence and spread of single and multi-drug resistance strains of *Plasmodium sp,* particularly *P. falciparum,* which has been identified as the cause of the most serious type of malaria especially in sub Saharan Africa (Olashinde, 2004).

The overall incidence of 2.8% reported in the retrospective study is similar to that reported in Butyira, South Central Ethiopia, Adugna *et al.,* (2012) , reported a prevalence rate of 2.8% .Chand *et al.,* (2009) however reported an incidence of 0.7% amongst children less than 5 years attending a local Health facility in Zambia. A higher prevalence rate of 6.8% was reported by Uko *et al*., (1998) in a study conducted in Calabar. Aina *et al*; zxreported an incidence rate of 14.7% in Ibesle Community in Ikorodu, Lagos State. An even higher incidence rate of 35.7% was reported in General hospital Markarfi, Kaduna State by Umaru and Gabriel (2015). Aribodo *et al*., (2003) in Anambra State South East Nigeria, also reported a high prevalence rate of 76%. The variation in the incidence rates could probably be because of differences in the times at which the studies were conducted. The incidence rate reported in this study was calculated from number of malaria cases that occurred over a three year period and not restricted to the rainy season. A higher incidence/prevalence rate value will be reported for studies conducted during rainy season, when malaria transmission is usually higher,

because of the availability of breeding ground for the mosquitoe in the form of puddles and hoof prints which are more abundant during the rainy season in tropical countries. E. Odongo *et al.,* (2008) observed a direct relationship between malaria transmission and monthly rainfall in Etebbe Municipality in Uganda.

Chloroquine, Quinine, Arthemeter, Fansider and Coartem waa reported in this study as the most commonly prescribed antimalarial in the study centre, with Chloroquine being the most prescribed in 2012 and 2013 and Coartem (Arthemether-lumefantrine) being the most prescribed in 2014. The continuous use of Chloroquine in hospitals, irrespective of reported cases of chloroquine resistance could probably be because of isrelativereported cheapness when compared with other antimalarials such as Coartem and artequine. The shift from single drug therapy (chloroquine) as the most prescribed in 2012 and 2013, to combination drug therapy (Coartem) in 2014 could probably be because of treatment failure observed from the use of Chloroquine.

In this study, *in-vitro* resistance against Chloroquine and quinine was observed amongst the isolates of *P. falciparum* tested. Most of the isolates (90%)of the*P. falciparum*isolates showed *in-vitro* resistance to chloroquine, with a median$IC_{50}$of 5.3µmol/l. In 2003, Abdullahi *et al.,* reported 100% chloroquine resistance in Sokoto while in 2012, Peletri *et al*., reported 88.9% resistance against Chloroquine. Similarly, Sowunmi *et al*., reported 81.4% chloroquine resistance in Ibadan. In another study conducted in Cote d'ivoire, a 56.2% Chloroquine resistance was reported by Brice *et.al.,*(2010). A 45% resistance was reported in Rwanda by Hili dou*et.al.,*(2005). However, Beniti *et. al.,*(1995) reported a resistance of 16% in Equitorial Guinea. The high level of resistance to Chloroquine reported in this study could probably be due to the increase use of Chloroquine evident from the retrospective study where it was the most prescribed in 2012 and 2013 for the treatment of malaria in the study centre.

Increase use of Chloroquine could induce Chloroquine resistance because of lack of compliance to dosage regime of both the oral and parenteral drug. Okomkwo*et. al*., (2001) reported that there was a significant correlation (P<0.0001) between full compliance and successful treatment of malaria cases with the use of Chloroquine.

This study reported a 90% resistance to Quinine. Pradines*et. al*., (1998), reported a 32% resistance to quinine in Gabonese wild isolates of *P. falciparum*. Bruno *et. al*., reported 28% resistance to quinine in Pointe-Noire, Republic of Congo. Hombhanje *et al*., (1998) reported a 10% resistance to quinine in central Province of Papua New Guinea. Benito *et al.*,(2006) reported 9% resistance to quinine. Tinto *et al.*, (2006) reported 0% in-vitro resistance to quinine, Yavo *et. al*., (2010) reported 0% resistance to quinine in a study conducted in Abidjan. Quinine treatment failure was reported in 2007 in a traveler returning from Senegal (Bruno *et al*.2010)

Quinine is the first line treatment for complicated malaria by WHO. High level resistance to quinine as reported in this study could be due to the increased use of Quinine in the study centre as seen in the retrospective study where it came second in line to Chloroquine in 2013 and second in line to Coartem in 2014 as the most prescribed drug in the study centre. Indiscriminate use of quinine as presumptive treatment for uncomplicated malaria often without strict adherence to dosage regimen has been linked to the emergence of quinine resistance.

In this study *P. falciparum* isolates tested were 100% susceptible to Artemether. Chansuda *et al*., 1997 reported 100% susceptibility to artesunate in Vietnam. Basco and Le bras (1994) reported a high invitro activity of artemisinin derivatives against *P. falciparum*Pradines *et al*., (1998), reported 14% decreased susceptibility to Artemether. 100 % full susceptibility of the tested *P.falciparum* isolates to arthemether means that

arthemether and other derivatives of artemisinin are still very effective against *P. falciparum*, and thus will be useful in the treatment of falciparum malaria.

All the 30 *P. falciparum* isolates tested showed full *invitro* susceptibility to Arthemether –lumefantrine. Neils *et al.*, (2013) reported 0% resistance to Arthemether- Lumefantrine in Ghana.Similarly, Zenglei*et al*., (2012) reported 0% resistance to Arthemether-lumefantrine combination in China-Myanmar border. From this result it is evident that that the combination of two or more drugs, rather than a single antimalarial drug, preferably with artemisinin derivative as one of the drug remains the ideal approach to antimalarial treatment (WHO, 2001: Ashley *et al.*, 2007).

Reduction in *in-vitro* susceptibility is not synonymous with diminished therapeutic effectiveness, but it is the probable first step of an alarming cascade and definitely pleads for increased vigilance and a coordinated and rapid deployment of drug combinations (Olashinde *et al*., 2014). A major problem with malaria treatment in developing countries is the illegal production, sale and distribution of fake drugs, which represents more than 50% of the pharmaceutical market in several African countries. These fake medicines increase the risk of resistance developed by the use of sub therapeutic dosage of such antimalarials(Pierre Ambrose-Thomas, 2012)

In this study, two crude extracts of the leaves of *C. papaya* and *P.guajava*used by locals in Nigeria for the treatment of Fever and Malaria were tested invitro against *P. falciparum.* The extracts of *C. papaya*leaves showed promising activity against all the *P. falciparum* isolates tested with median IC50 of 5.4µg/ml. Paula *et al.,(*2012) reported an IC50 value > 50µg/ml for the aqueous extract of *C. papaya,* but an IC50 valueof 16.4µg/ml for the petroleum ether extract. IC 50 of 317.5µg/ml was reported by Protus *et al*., (2014). Similarly the aqueous extracts of *P. guajava* leaves showed

promising activity against all the *P. falciparum* isolates tested with a Median IC50 of 9.2µg/ml, Phillippe *et al*., (2005) reported an IC 50 value of 25.7µg/ml. Based on WHO and other previousreports, *in vitro* antiplasmodial activity is considered as good when IC 50 isless than 50µg/ml (Phillippe *et al*., 2005). This result confirms that *C. papaya* and *P.guajava*leaves used in traditional medicine by locals for malaria treatment show antiplasmodial activity *invitro*due to active constituents in the extract which may be cytotoxic to *P. falciparum*, trophozoites thereby preventing development to schizont stage, and thus justify their use in traditional medicine.

In this study all the Chloroquine resistant (10) *P.falciparum* isolates subjected to Polymerase chain reaction (PCR) carried the *Pfcrt*(76bp) gene and 8 (80%) carried the *Pfmdr1*(1600bp) gene. Olashinde *et al*., 2004 reported that 48 isolates out of 51 *in vitro*chloroquineresistant *P.falciparum*isolates were found to carry the *Pfcrt*76T codon .Similar results were reported by Basco and Ringwald, (2001) in Cameroon, Djimde *et al.,* (2001) in Mali, Mayor *et al*., (2001) in Mozambique, Maguire *et al*.,(2001) in Papua New Guinea, Babiker *et al*., (2001) in Sudan, Ariey *et al*., (2002) in Madagascar, Adagu and Warhurst, (2001) in Nigeria. 8 (80%) of the *P.falciparum*isolates carrying *Pfcrt* mutated allele also carried the *Pfmd*r mutated allele. Adagu and Warhurst (1999) reported that *pfcrt* K76 and *pfmdr 1* Y 86 allelles are closely associated in chloroquine resistant strains of *P. falciparum*. In this study, combination of *Pfcrt* and *Pfmdr 1* mutations in the isolates was observed to be associated with Quinine resistance. Mutant *pfcrt*76 and *pfmdr1*1600 alleles were also seen in 100% and 80% of the samples, respectively. The high prevalence of the mutant *pfcrt*76 allele (100%) and *pfmdr*1 1600 allele (80%) observed in the Health facility in Wusasa Zaria, is consistent with rates ranging from 60% to 100% reported in other malaria endemic zones (Olashinde *et al.,* 2004, Maguire *et al.,* 2003; Basco and Ringwald, 2001). The combination of *pfcrt*76

and *pfmdr*11600 mutations was found to be associated with quinine treatment failure. Similarity in the mode of actions of quinine and chloroquine suggests that the molecular basis of resistance to these drugs may be similar. The high prevalence of *pfcrt*K76 and *Pfmdr*11600, that code for Chloroquine and quinine resistance in Nigeria is a clear indication that the population of *P. falciparum* that circulates in Zaria, Kaduna state has been selected by the long use of chloroquine and quinine evident from the retrospective study. The findings from this study suggests the withdrawal of Chloroquine as first line treatment for Falciparum malaria in Nigeria, and the controlled use of quinine for the treatment of complicated malaria only, are highly required so as to reduce the spread of quinine resistance *P. falciparum* isolates.

5.1 CONCLUSION

This study has shown that:

- Chloroquine and quinine were highly prescribed ina Health facility inWusasa Zaria Nigeria from 2012-2013.
- *Falciparum* isolates from this study centre was resistant to Chloroquine and Quinine.
- Coartem and aqueous extract of *C. papaya* and *P. guajava* were observed to be very potent against Chloroquine resistant *P.falciparum* isolates in a Health facilityin Wusasa, Zaria, Nigeria.
- The Chloroquine resistant *P.falciparum* isolates in this study were found to possess *pfcrt76* and *pfmdr1*1600 alleles.

5.2RECOMMENDATION

Since the complete eradication of the mosquitoes that transmit the parasites responsible for causing the disease appears impossible in Nigeria, there is an urgent need to pay more attention to increase useof preventive measures,such as the use of insecticide treated mosquitoenets, prevention of stagnant waters and other breeding grounds for the vectors. These measures will strongly reduce parasites transmission.

More attention should also be given to developing plants products as the last hope of the ‘’common man’’in treating this disease.

The rationale use of antimalarial agents will also reduce the ever increasing antimalarial resistance development by *P. falciparum.*

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APPENDICES

APPENDIX I

Schizont Count (per 200 parasites) of *P.falciparum* isolates for Chloroquine

Isolates	Control	0.2	0.4	0.8	1.6	3.2	6.4	12.8µmol/l
1	24	14	12	12	8	6	4	4
2	50	23	19	11	5	2	0	0
3	24	21	24	19	18	17	13	7
4	84	42	36	25	35	30	22	17
5	60	19	13	13	7	6	6	3
6	81	50	44	28	31	21	8	3
7	55	20	14	10	7	4	4	2
8	56	17	18	15	15	9	3	2
9	83	30	21	14	10	5	2	2
10	36	33	33	25	9	7	5	5
11	72	26	19	17	9	5	3	3
12	58	2	6	5	7	12	16	21
13	83	30	21	14	10	5	2	2
14	60	22	15	11	8	4	3	2
15	35	17	14	13	8	6	4	4
16	53	19	19	13	9	6	4	1
17	60	32	25	15	15	11	7	3
18	23	23	23	20	19	17	5	3
19	45	45	45	45	43	24	24	20
20	30	29	21	22	17	13	4	4
21	45	43	43	40	37	39	27	30
22	60	30	22	17	15	1	8	3
23	35	17	14	13	8	6	4	4
24	77	53	48	39	37	22	13	8
25	40	30	21	13	7	8	5	5
26	65	38	33	29	25	22	16	10
27	51	48	45	47	38	4	35	34
28	92	49	51	35	27	23	19	16
29	48	37	38	33	31	19	20	26
30	38	36	35	38	37	28	28	24

APPENDIX II

Schizont count (per 200 parasites) of *P. falciparum* isolates for Quinine

ISOLATES	CONTROL	4	8	16	32	64	128	256µmol/l
1	24	24	24	22	20	15	11	8
2	50	32	14	9	3	1	0	0
3	24	18	18	20	20	222	17	15
4	82	18	15	10	5	6	6	3
5	60	24	20	20	21	17	9	3
6	81	45	36	31	24	19	13	10
7	55	22	18	18	16	15	12	4
8	56	25	23	19	15	10	7	4
9	83	21	18	14	12	5	5	3
10	36	29	25	17	9	5	3	3
11	72	20	20	15	13	9	6	4
12	58	21	16	12	7	5	6	2
13	83	21	18	14	12	5	5	3
14	60	21	18	14	12	5	5	3
15	35	29	29	22	13	7	7	4
16	53	14	12	10	8	7	7	1
17	60	33	27	20	15	9	7	7
18	23	21	21	18	19	3	3	1
19	45	45	42	43	43	39	31	27
20	30	8	7	4	4	2	0	1
21	45	5	4	6	0	1	0	0
22	60	21	16	12	7	5	6	2
23	35	29	29	22	13	7	7	4
24	77	31	29	17	17	13	7	4
25	40	2	2	2	3	1	1	0
26	65	7	4	3	4	2	0	1
27	51	2	2	3	1	1	2	0
28	92	11	7	7	3	5	2	2
29	48	31	32	20	15	7	9	4
30	38	7	6	4	5	3	3	2

APPENDIX III

Schizont count (per 200 parasites) of*P. falciparum* isolates for Artemether

Isolates	Control	2	4	8	16	32	64	128µmol/l
1	24	12	12	12	8	10	6	4
2	50	23	16	11	7	0	0	0
3	24	21	18	17	15	13	10	4
4	82	15	10	12	7	5	3	1
5	60	23	21	16	12	4	1	0
6	81	19	15	7	4	2	1	0
7	55	19	15	13	7	4	1	0
8	56	21	18	15	111	4	2	0
9	83	16	15	14	9	2	0	0
10	36	30	30	22	3	1	0	0
11	72	23	25	16	12	5	2	2
12	58	19	14	9	6	2	0	0
13	83	26	21	18	11	7	3	0
14	60	19	16	15	14	9	2	0
15	35	12	9	10	6	4	4	1
16	53	17	15	12	7	4	2	0
17	60	12	9	8	3	1	0	0
18	23	13	13	11	9	5	1	1
19	45	43	41	40	23	6	5	3
20	30	8	7	4	3	0	0	0
21	45	27	21	16	1	1	0	0
22	60	22	17	10	7	3	0	0
23	35	12	9	10	6	4	4	1
24	77	19	21	14	10	9	4	0
25	40	10	7	5	3	0	0	0
26	65	10	10	7	3	0	1	1
27	51	1	1	1	2	0	0	0
28	92	19	19	17	9	1	0	4
29	48	43	43	40	20	5	7	3
30	38	6	4	2	3	1	1	2

APPENDIXIV

Schizontcount(per 200 parasites) of *P. falciparum* isolates for Artemether+lumefantrine(Coartem).

Isolates	Control	Parasite count
1	24	0
2	50	0
3	24	0
4	82	0
5	60	0
6	81	0
7	55	0
8	56	0
9	83	0
10	36	0
11	72	0
12	58	0
13	83	0
14	60	0
15	35	0
16	53	0
17	60	0
18	23	0
19	45	0
20	30	0
21	45	0
22	60	0
23	35	0
24	77	0
25	40	0
26	65	0
27	51	0
28	92	0
29	48	0
30	38	0

APPENDIX V

Schizont count (per 200 parasites) of *P.falciparum* isolates for aqueous extract of *P. Guajava* leaf

ISOLATES	CONTROL	6.25	12.5	25	50µg/ml
1	24	7	3	1	0
2	50	3	1	0	0
3	24	5	1	0	0
4	82	2	2	0	0
5	60	8	3	1	0
6	81	5	2	0	0
7	55	8	6	4	0
8	56	10	3	0	0
9	83	7	3	1	0
10	36	9	7	2	2
11	72	15	8	3	1
12	58	11	7	3	0
13	83	8	5	1	0
14	60	7	3	1	0
15	35	8	5	1	1
16	53	5	3	0	0
17	60	3	1	0	0
18	23	3	1	0	0
19	45	3	0	1	0
20	30	4	1	0	0
21	45	4	1	1	2
22	60	13	7	5	1
23	35	8	5	1	0
24	77	13	9	4	1
25	40	17	12	0	0
26	65	3	1	0	0
27	51	3	1	0	0
28	92	2	2	7	9
29	48	4	0	2	1
30	38	3	1	0	0

APPENDIX VI

Schizont count(Per 200 parasites) of *P.falciparum* isolates for aqueous extract of *C. Papaya*leaf

ISOLATES	CONTROL	6.25	12.5	25	50µg/ml
1	24	18	11	8	5
2	50	5	3	3	0
3	24	4	3	3	0
4	82	25	21	10	2
5	60	20	13	8	1
6	81	17	5	1	0
7	55	20	20	7	2
8	56	11	9	7	1
9	83	5	3	2	1
10	36	19	16	7	7
11	72	53	30	189	16
12	58	30	22	16	7
13	83	7	3	2	2
14	60	5	3	2	1
15	35	8	7	4	1
16	53	10	8	1	0
17	60	7	3	0	0
18	23	7	5	0	0
19	45	13	11	9	5
20	30	16	13	7	4
21	45	28	21	9	7
22	60	23	14	10	4
23	35	8	7	4	1
24	77	18	11	8	5
25	40	18	14	6	6
26	65	9	5	3	3
27	51	11	12	3	3
28	92	21	15	7	7
29	48	16	15	11	7
30	38	13	11	9	5

APPENDIX VII

Concentration of the antimalarial that inhibit 90% of the isolates (IC_{90})

ISOLATES	CHLOROQUINE (µmol/l)	QUININE (µmol/l)	ARTHEMETHER (µmol/l)	*C.PAPAYA (mg/ml)*	*P.GUAJAVA (mg/ml)*
1	2.1	1.3	0.5	0.6	2.4
2	3.1	3.3	3.3	3	4.3
3	1.3	-10	-1.5	2.4	30
4	2.4	2.8	3.7	1.7	1.4
5	2.1	1.4	2.1	1.8	5.4
6	1.7	1.3	3.7	2	4.3
7	2.9	3.1	1.6	1.6	3.0
8	2.0	1.5	2.8	2.1	3.2
9	2.6	3.2	3.7	4.4	3.5
10	1.3	1.6	2.6	0.6	1.3
11	2.2	1.9	2.1	0.7	1.9
12	1.9	2.1	2.4	0.8	2.7
13	2.3	2.3	2.4	3.7	3
14	2.1	2.5	8.5	3.7	2.7
15	1.1	1.2	1.0	1.7	2.5
16	.7	2.3	2.4	2.3	3.4
17	1.4	1.3	2.7	1.8	3
18	0.6	1.4	1.3	1.3	2.6
19	-1.3	-3.9	1.7	0.9	2.4
20	0.9	2.6	2.7	0.9	2.8
21	-2.9	3.4	1.8	0.6	2.2
22	1.6	2	1.8	1.1	1.8
23	1.1	1.2	1.0	1.7	2.5
24	1.0	1.9	2.7	1.5	20
25	1.2	4.8	2.2	0.6	1.3
26	-0.07	2.9	3.6	2.2	5.2
27	-9.2	6	11	1.3	2.7
28	0.6	2.8	2.2	1.2	2.8
29	0.9	1.5	1.8	0	7.1
30	-3.7	1.9	3.4	0.9	

APPENDIX VIII

Concentration of Antimalarial that inhibit 99% (IC_{99}) of the isolates

ISOLATES	CHLOROQUINE (µmol/l)	QUININE (µmol/l)	ARTHMETER (µmol/l)	*C.PAPAYA* (mg/ml)	*P.GUAJAVA* (mg/ml)
1	0.4	2.1	1.8	0.1	1.5
2	2.1	2.3	2.1	1.2	1.3
3	2	-12	-1.4	0.6	1.6
4	-4.2	0.5	1.1	0.8	2.5
5	0.9	0.3	1	0.9	1.7
6	0.7	0.1	1.7	1.2	1.3
7	1.2	1.6	1	1	1.1
8	0.6	0.2	1.5	0.5	1.6
9	1.2	0.8	2.4	0.8	1.3
10	0.9	1.0	2.1	0.1	0.3
11	1.1	0.2	0.7	0.2	1
12	1.1	1.1	1.4	0.1	1.3
13	1.3	1.0	1.3	0.2	1.2
14	1.1	0.8	0.9	0.7	1.2
15	0.2	0.7	0.6	0.8	1.4
16	1.0	-0.3	1.3	1.4	1.5
17	0.7	0.5	1.4	1.1	1.2
18	0.2	1.1	4	1.0	1.2
19	-1.9	-4.7	1.2	0.4	1.1
20	0.5	2	1.6	0.2	1.4
21	-3.8	1.4	1.3	0.2	0.4
22	0.8	0.9	1.1	0.6	0.9
23	0.2	0.7	0.6	0.8	1.4
24	0.1	0.8	1.3	O.5	45
25	0.6	1.2	1.2	0.05	0.9
26	-1.4	0.9	1.6	0.2	1.6
27	-10.9	1.	2	0.7	1.2
28	0.2	1	1.2	0.04	0.5
29	1.4	0.6	1.2	-1.5	0.7
30	-4.5	0.5	2	0.4	1.1