UNIVERSITY OF NAIROBI

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY

EVALUATION OF THE ANTIMALARIAL PROPERTIES AND SAFETY PROFILE OF A POLYHERBAL PRODUCT (*NEFANG*)

PROTUS ARREY TARKANG

MSc, BSc (Hons)

A dissertation submitted to the Board of Postgraduate Studies, University of Nairobi, Kenya, in fulfillment of the award of a Degree of Doctor of Philosophy (PhD) in Pharmacognosy and Complementary Medicine.

2014

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signed by: _____ Date: _____

PROTUS ARREY TARKANG

SUPERVISORS

This dissertation has been submitted with our approval as a requirement for the award of a degree of Doctor of Philosophy.

1. **Signed by:_____ Date:_____**

   PROFESSOR A.N. GUANTAI

2. **Signed by:_____ Date:_____**

   DR. F. A. OKALEBO

3. **Signed by:_____ Date:_____**

   DR. G. A. AGBOR

UNIVERSITY OF NAIROBI PLAGIARISM POLICY

DECLARATION OF ORIGINALITY

This form must be completed and signed for all works submitted to the University for Examination.

Name of Student	PROTUS ARREY TARKANG
Registration Number	U80/95728/2014
College	HEALTH SCIENCES
Faculty/School/Institute	SCHOOL OF PHARMACY
Department	PHARMACOLOGY AND PHARMACOGNOSY
Course Name	DOCTOR OF PHILISOPHY (PhD), PHARMACOGNOSY AND COMPLEMENTARY MEDICINE
Title of the work:	EVALUATION OF THE ANTIMALARIAL PROPERTIES AND SAFETY PROFILE OF A POLYHERBAL PRODUCT (*NEFANG*).

DECLARATION

1. I understand what Plagiarism is and I am aware of the University's policy in this regard

2. I declare that this Thesis (Thesis, project, essay, assignment, paper, report, etc) is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

3. I have not sought or used the services of any professional agencies to produce this work.

4. I have not allowed, and shall not allow anyone to copy my work with the intention of passing it off as his/her own work

5. I understand that any false claim in respect of this work shall result in disciplinary action, in accordance with University Plagiarism Policy.

Signature _____

IN ADVANCE PUBLICATION OF THE DISSERTATION

Parts of the results of this study have been published in advance:

1. **Protus Arrey Tarkang,** Kathrin Diehl Franzoi, Sukjun Lee, Eunyoung Lee, Diego Vivarelli, Lucio Freitas-Junior, Michel Liuzzi, Tsabang Nole, Lawrence S. Ayong, Gabriel A. Agbor, Faith A. Okalebo, Anastasia N. Guantai. *In vitro* Antiplasmodial Activities and Synergistic combinations of differential solvent extracts of the Polyherbal Product, *Nefang. BioMed Research International*, Volume 2014, Article ID 835013, 10 pages, **2014**. doi:10.1155/2014/835013.
2. **Protus Arrey Tarkang,** Achille Parfait Nwakiban Atchan, Jules-Roger Kuiate, Faith Apoelot Okalebo, Anastasia Nkatha Guantai, and Gabriel Agbor Agbor. Antioxidant Potential of a Polyherbal Antimalarial as an Indicator of its Therapeutic Value. *Advances in Pharmacological Sciences*, Volume 2013, Article ID 678458, 9 pages, **2013**. doi:10.1155/2013/678458.
3. **Protus Arrey Tarkang**, Faith A. Okalebo, Gabriel A. Agbor, Nole Tsabang, Anastasia N. Guantai, Geoffrey M. Rukunga. Indigenous Knowledge and folk use of a polyherbal antimalarial by the Bayang Community, South West Region of Cameroon. *Journal of Natural Product and Plant Resources.* **2012**, 2 (3): 372 - 380.

DEDICATION

"Never regard study as a duty but as an enviable opportunity to learn to know the liberating influence of beauty in the realm of the spirit for your own personal joy and to the profit of the community to which your later works belong."

~Albert Einstein

THIS SCIENTIFIC WORK IS DEDICATED TO THE

GLORY OF THE ALMIGHTY GOD

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisors **Professor Anastasia Nkatha Guantai**, **Dr. Faith Apolot Okalebo**, **Dr. Gabriel Agbor Agbor** and **Dr. Geoffrey Rukunga** (RIP†) and Without thier advice, unique support, understanding and kind encouragement, this thesis would never have become a reality.

Further, I would like to thank **Dr. Lawrence S. Ayong** for his assistance and encouragement during the course of this work and and the staff of Institut Pasteur for accommodating me during my internship/training course.

Special thanks to the Chairman and staff of the Department of Pharmacology and Pharmacognosy in particular and School of Pharmacy in general, for their great co-operation, assistance and suggestions throughout this scientific work.

I also wish to thank the co-authors of the publications included in this thesis; Ms Kathrin Diehl Franzoi, Mr. Sukjun Lee, Ms Eunyoung Lee, Mr. Diego Vivarelli, Dr. Lucio Freitas-Junior, Dr. Michel Liuzzi, Dr. Tsabang Nole, Mr. Achille Parfait Nwakiban Atchan and Prof. Jules-Roger Kuiate for their co-operation.

Special thanks go to Mwaura A. Mwaniki, Simiyu Juma and Njihia William (Department of Pharmacology and Pharmacognosy), Mugweru Jackson (Department of Veterinary Anatomy and Physiology), Mbithi Erick, Muthini Felistas (Department of Medical Microbiology) and Dr. Hastings Ozwara (Institute of Primate Research, Nairobi, Kenya).

I would like to thank my Director Dr. Tom Agbor Egbe and colleagues Tchamgoue Deutou Armelle Blanche, Tchokouaha Lauve Rachel Yamthe, Tsabang Nole, Simo David, Mba Romuald and Tengen Edwin Vola for their co-operation, understanding and assistance.

I remain grateful to all those who accepted to participate in the ethnopharmacological survey, the traditional leaders of the villages concerned as well as the health care workers who assisted me in this regard.

I wish to express my sincere and greatest thanks to my family and friends who supported me in all the up's and down's of my life.

Finally, I would like to acknowledge the help and patience of my beloved wife, **Clarice Etchi Ndip Bate-Eya** without which it would be very difficult to achieve this work.

ABSTRACT

BACKGROUND: The emerging resistance of *Plasmodium* species to currently available antimalarials remains a public health concern and necessitates the need to develop new, effective, safe and affordable drugs. Compounds with antioxidant, anti-inflammatory and antipyretic activities are believed to modulate plasmodial infection. Natural products constitute and remain a reliable and alternative source. *Nefang* is a polyherbal preparation composed of *Mangifera indica* (bark-***MiB*** and leaf-***MiL***) and leaves of *Psidium guajava* (***Pg***), *Carica papaya* (***Cp***), *Cymbopogon citratus* (***Cc***), *Citrus sinensis* (***Cs***) and *Ocimum gratissimum* (***Og***). It is used traditionally for the treatment of malaria in the South West Region of Cameroon. There are no documented studies on the efficacy and safety of this product.

OBJECTIVES: The present study aimed at evaluating the efficacy and safety of *Nefang* and its constituents, for antiplasmodial and related biological activities useful for the treatment of malaria, using in vitro and in vivo techniques.

METHODS: An ethnopharmacological survey was carried out to document the folkloric information on formulation and use of *Nefang*. Preliminary phytochemical screening of the constituent plants was carried out. Cytotoxicity was determined by the Resazurin Fluorimetric Cell Viability Assay method using two human cell lines (Hep G2 and U2OS). The in vitro antiplasmodial activity of the extracts was analyzed on CQ-sensitive (3D7) and multi-drug resistant (Dd2) strains of *Plasmodium falciparum* using the SYBR-Green 1 fluorescence-based method. Interactions studies involving the different solvent extracts were further carried out using a variable potency ratio drug/extract combination approach and isobologram analysis. In vivo antiplasmodial activities of *Nefang* and its active components, during early and established infection as well as the prophylactic activity were investigated in rodent models infected with *P. berghei* and *P. chabaudi chabaudi*, using the Peter's 4-day suppressive activity, Rane's curative tests and repository activity methods respectively.

In vivo toxicity of *Nefang* and its constituents was assessed by the single-dose (acute) and repeated dose (sub-acute and sub-chronic) toxicity testing on rodent models. Evaluation of the constituent plants for their antioxidant, antipyretic, anti-inflammatory and antinociceptive

activities that complement in vivo antimalarial activity of *Nefang* was carried out. In vitro antioxidant activities were determined using the radical scavenging activity, total phenolic content estimation and ferric reducing antioxidant power (FRAP) assay methods while in vivo activity of *Nefang* was evaluated in carbon tetrachloride-induced oxidative stressed wistar rats. Antipyretic activities were determined using the D-amphetamine and Brewer's Yeast induced pyrexia methods. Anti-inflammatory activities were determined using the carrageenan-induced rat paw edema method while antinociceptive activities were determined using the tail pressure, tail flick and hot plate methods.

RESULTS: The ethnopharmacological survey study revealed that the respondents had a good knowledge of malaria and its causes. They could also identify the constituent plants and recognized the need for Good Agricultural and Cultivation Practice (GACP) in the harvesting process. Five different formulations used for the preparation of *Nefang* were described: ***MiB****:* ***MiL****:* ***Pg****:* ***Cp****:* ***Cc****:* ***Cs****:* ***Og*** *(w/w)* - (i) 4:2:2:1:1:1:1 (ii) 3:1:1:1:1:1:1 (iii) 2:2:1:1:1:1:1 (iv) 2:1:1:1:1:1:1 (v) 1:1:1:1:1:1:1. Administration was by oral or rectal (enema) routes.

Preliminary phytochemical screening of the constituent plant extracts of *Nefang*, revealed the presence of flavonoids, phenols, triterpenes and sterols in all extracts. Saponins were present in all except ***Cc***, tannins in all except ***Cp*** and ***Cc*** while alkaloids were found only in ***MiB*** and ***Og***.

Cytotoxicity testing of *Nefang* and the solvent extracts revealed no significant toxicity of the extracts relative to their antiplasmodial activities (Selectivity Index>20). The derived EC_{50} values (3D7/Dd2, µg/mL) of the extracts were as follows: ***Nefang***-96.96/55.08; ***MiB***-65.33/34.58, ***MiL***-82.56/40.04, ***Pg***-47.02/25.79, ***Cp***-1188/317.5, ***Cc***-723.3/141, ***Cs***-184.4/105.1, and ***Og***-778.5/118.9. Synergism was obtained with the following pairs: ***MiB/Pg*** (Combination Index=0.351), ***MiL/Pg*** (0.358), ***MiB/Cs*** (0.366), ***MiL/Cs*** (0.482), ***Pg/Cs*** (0.483), and ***Cs/Og*** (0.414) when analyzed at equipotency ratios. This was further confirmed at variable potency combination ratios and by isobologram analysis.

Evaluation of the in vivo antiplasmodial activities revealed that ***Nefang*** and ***MiB/Pg*** exhibited significant ($p<0.05$) suppressive, prophylactic and curative activities when compared to the control with percentage suppression of parasitemia (*P. berghei*/*P.c. chabaudi*) of 82.9/86.3 and 79.5/81.2 respectively. Chemotherapeutic effects of ***Nefang*** ranged from 61.2 – 86.1% with

maximum effect observed at the highest experimental dose (600 $mgkg^{-1}$ bwt). The extracts also prevented loss of body weight and reduction in body temperature in experimental animals.

Evaluation of the in vivo toxicity revealed that no observed adverse effect levels (NOAEL) for all extracts were > 5000 $mgkg^{-1}$ bwt. In the sub-acute and sub-chronic toxicity testing, plasma biochemical analysis revealed slightly significant ($p<0.05$) increase in some renal (blood urea nitrogen) and hepatic (transaminases) parameters after administration of ***Cp***, ***Cs*** and ***Cc*** ethanol extracts. Aqueous extracts exhibited hypolipidemic and hypoglycemic effects in experimental rats. No significant hematological adverse effects were observed.

Evaluation of biological activities: In vitro antioxidant assays revealed that ***Pg***, ***MiL*** and ***MiB*** exhibited greatest radical scavenging activities (>90% inhibition). Their total phenolic contents ranged from 61.7 - 67.2 mg catechine equivalent/g of extract. In vivo activity of ***Nefang*** showed significant ($p<0.05$) increase in superoxide dismutase and decrease in malondialdehyde. Best percentage suppression of pyrexia (amphetamine/brewer's yeast; $p<0.05$) was exhibited by ***Cc*** (95/97) followed by ***Og*** (85/94), ***MiL*** (90/89), ***MiB*** (88/84) and ***Cs*** (82/89), comparable to paracetamol (100/95). Anti-inflammatory studies revealed paw edema inhibition (%) as follows ($p<0.05$): Indomethacin (47), ***MiL*** (40), ***Cp*** (30), ***MiB*** (28) and ***Og*** (22), suggesting best activity by *MiL*. Antinociceptive studies revealed significant ($p<0.01$) pain inhibition (%) as follows: Paracetamol (97), ***Og*** (113), ***MiL*** (108), ***Pg*** (84) and ***MiB*** (88). *Og* and *MiL* exhibited the best activities.

CONCLUSION: This study supports the traditional use of *Nefang* for the treatment of malaria and gives information on its formulation, folk use, efficacy and safety. The demonstrated in vitro and in vivo antiplasmodial activities suggest that *Nefang* has a promising antimalarial activity. The in vivo findings showed that *Nefang* is relatively safe for oral administration at doses tested.

In addition to its antimalarial activity, the constituents of *Nefang* contain biologically active compounds with antioxidant, analgesic, antipyretic and anti-inflammatory activities whose synergism with the antiplasmodial activity could complement its antimalarial effects.

Furthermore, suitable combinations of the constituent plants of *Nefang* with better activities were revealed, suggesting that re-formulation of *Nefang* could yield optimum activity, exploitable towards a rational antimalarial phytotherapeutic drug discovery.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based Combination Therapy
AIDS	Acquired Immuned Deficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
APAD	3-Acetyl Pyridine Adenine Dinucleotide
APADH	3-Acetyl Pyridine Adenine Dinucleotide, Reduced
ART	Artemisinin
AST	Aspartate Transaminase
Aq	Aqueous
BSL-2	Biosafety Laboratory Level-2
BUN	Blood Urea Nitrogen
bwt	Body weight
Ca	Calcium
CAT	Catalase
CBD	Convention on Biodiversity
Cc	*Cymbopogon citratus* leaf
CC_{50}	Concentration required to reduce the cell number by fifty percent
CDC	Centre for Disease Control
CHOL	Cholesterol
CI	Combination Index
CM	Cerebral Malaria
CND3	Centre for Neglected Diseases Drug Discovery
CO_2	Carbon Dioxide
CAM	Complementary and Alternative Medicine
COX-2	Cyclo-oxygenase
Cp	*Carica papaya* leaf
cpm	counts per minute
CQ	Chloroquine
CQS	Chloroquine sensitive
CRE	Creatinine
Cs	*Citrus sinensis* leaf

DAPI	4,6-diamino-2-phenyl indole
DEC	Disease Endemic Countries
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthetase
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl 1,1-diphenyl-2-picrylhydrazyl
DRC	Dose Response Curve
EC_{50}	Concentration of a drug that gives half maximal response
ED_{50}	Dose of a drug that gives half maximal response
EDTA	Ethylene diamine tetra acetate
Em	Emission
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
Ex	Excitation
Fe	Iron
FCR	Folin-Ciocalteau Reagent
FCS	Fecal Calf Serum
FIC_{50}	Fractional Concentration of a drug in combination that gives half maximal response
FRAP	Ferric Reducing Antioxidant Power
GACP	Good Agricultural and Cultivation Practice
GLP	Good Laboratory Practice
GLU	Glucose
HCT	Hematocrit
HEPES	Hydroxyethyl piperazine ethanesulfonic
HEPTA	5-hydroxy-3, 6, 7, 8, 3’.4’-hexamethoxyflavone
HGB	Hemoglobin
HIFCS	Heat Inactivated Fetal Calf Serum
HIV	Human Immunodeficiency Virus
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography

HRP	Histidine rich Protein
IC_{50}	Concentration of an inhibitor at which the response is reduced by half.
IK	Indigenous Knowledge
IL	Interleukin
IFN-γ	Interferon-γ
IMPM	Institute of Medical Research and Medicinal Plants Studies, Yaounde, Cameroon
IPR	Institute for Primate Research, Nairobi, Kenya
K	Potassium
LD_{50}	Half Lethal Dose: Dose of a drug at which half the number of test animals die
LDH	Lactate Dehydrogenase
LMIC	Low and Medium Income Countries
LYM	Lymphocyte
MCH	Mean Cell Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MDA	Malondialdehyde
MDR	Multidrug Resistant
MEM	Minimum Essential Media
Mg	Magnesium
MiB	*Mangifera indica* Bark
MIC	Minimum Inhibitory Concentration
MiL	*Mangifera indica* leaf
Mn	Manganese
MS	Mass Spectroscopy
N_2	Nitrogen
Na	Sodium
NAD	Nicotinamide Adenine Dinucleotide
NCE	New Chemical Entities
nm	nano meter
NBT	Nitro Blue Tetrazolium
NCI	National Cancer Institute
NMRI	Naval Medical Research Institute

NSAID	Non-Steroidal anti-inflammatory Drug
O_2	Oxygen
OECD	Organisation for economic Co-operation and Development
Og	*Ocimum gratissimum* leaf
OMS	*Organisation Mondiale de la Sante* (World Healh Organisation)
PBS	Phosphate Bufferred Saline
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
Pfmdr-1	*Plasmodium falciparum* multidrug resistance-1
Pg	*Psidium guajava* leaf
PIC	Prior Informed Consent
pLDH	parasite Lactate Dehydrogenase
PLT	Platelets
PNLP	*Programme Nationale de Lutte contre le Paludisme* (National Programme for the Fight against Malaria)
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
ROW	Relative Organ Weight
RT-PCR	Real Time Polymerase Chain Reaction
RMPI	Roswell Park Memorial Institute Culture medium developed by Moore *et al.*
RP-HPLC	Reverse-Phase High Pressure Liquid Chromatography
SI	Selectivity Index
SP	Sulphadoxine-Pyrimethamine
spp	species
SOD	Superoxide Dismutase
TDR	Tropical Diseases Research
TGY	Triglyceride
Th	T-Helper Cells (Th1 and Th2)
TNF	Tumour Necrosis Factor
TPC	Total Phenolic Content
TPTZ	2, 4, 6-Tripyridyl-s-triazine
UPOV	Union for the Protection of New Varieties of Plants

UV	Ultra-Violet
v/v	Volume per volume
WBC	White Blood Cell
WHO	World Health Organisation
w/v	Weight per volume
w/w	Weight per weight

1.0 INTRODUCTION

1.1 BACKGROUND

Despite substantial efforts to control malaria in the last few decades, it remains one of the most prevalent infectious diseases globally and emerging resistance of *Plasmodium* species to currently available drugs remains a public health concern (WHO, 2009). Forty percent of the world's populations are exposed to malaria and there is the constant need for new antimalarials in the face of this ever-present and emerging threat. In Cameroon, malaria remains the leading cause of death, with recent statistics showing that thirty-five to forty percent of deaths occur in hospitals and fifty percent of these deaths are children below five years of age. Forty percent of a family's annual expenditure is on malaria treatment (PNLP, 2002). These numbers do not necessarily reflect the reality, since the most vulnerable are the poor with little or no access to modern medical facilities and as such most deaths are not registered. Such people make up the bulk of the estimated eighty percent of the world's population that rely on medicinal plants in managing diseases (Adjanahoun *et al*., 1996).

Historically, plants have had a remarkable role in therapeutics and were the principal source of drugs for many centuries. Quinine, isolated in 1820 from Cinchona species (Rubiaceae) is an illustrative example. It was the first drug introduced for malaria therapy and remained a useful clinical weapon until the 1940's when its 4-aminoquinoline derivative, the efficient and inexpensive chloroquine became available. It was widely used until resistance by *P. falciparum* emerged in the 1960's, causing an increase in mortality in endemic areas and arousing the need for new approaches for the development of antimalarial drugs. Drugs in current use for malaria chemotherapy include artemisinin, from *Artemisia annua* L. (Asteraceae) of Chinese origin, and its semi-synthetic derivatives, artemether, artesunate and arteether (Oliveira *et al*., 2009). A recently introduced plant-derived antimalarial drug is atovaquone, a synthetic naphthoquinone based on lapachol. Lapachol, a prenylnaphtoquinone, was first isolated from *Tabebuia impetiginosa* (Mart. ex DC.) Standl. (synon. T. avellanedeae Lor. ex Griseb.), a South American representative of the Bignoniaceae family (Castellanos *et al.,* 2009). Artemisinin-based combination Therapies (ACTs), are currently the most effective chemotherapy against *P. falciparum* malaria and the emergence of resistance would be a public health disaster in malaria

endemic areas. Therefore, plants continue to represent a valuable tool in antimalarial drug discovery. They do not only provide valuable clues for finding new drugs, but also may help to shift the drug discovery paradigm from 'finding new-entity drugs' to 'combining existing agents' and might even direct the combinations between such agents (Kong *et al.*, 2009; Wagner and Ulrich-Merzenich, 2009).

The modern pharmaceutical industry was born from botanical medicine, but standardized synthetic combinatorial chemistry in drug discovery and high throughput screening (HTS) of potential drug targets have disconnected the historical link between plants and medicines. However, this has been rekindled by the small output of modern antimalarial pharmaceutical research and development which has stimulated new interest in the potential of natural compounds (Ginsburg and Deharo, 2011). Hence natural products continue to provide new starting points in drug discovery.

There is a school of thought that biologically-derived secondary metabolites and synthetic compounds derived from plants perform better as drugs than do randomly synthesized compounds. Since their parent molecules were present in primitive life forms, they co-evolved to interact with one another, thus granting direct ecological benefit to the producing organism, whether in competition for resources, avoiding predation or combating pathogens (Gansesan, 2008). This co-evolution between different plants and/or metabolites within the same plant most at times results in synergy or potentiation which has been proven to achieve favorable results such as enhanced efficacy, decreased dosage at equal or increased level of target inhibition, reduced or delayed development of drug resistance and simultaneous reduction of toxic effects (Ma *et al.*, 2009). Based on this paradigm, drug combination in antimalarial chemotherapy has been adopted and is widely used as a strategy to monitor and prevent resistance (Nosten and White, 2007)

Among the large number of plant species that have been identified as potential sources of therapeutic agents, whole plants or parts of them are prepared and administered as oral decoctions, steam baths, infusion or enemas. Most remedies are a concoction of two or more plants species that work in synergy (Adjanahoun *et al*., 1996), an example being *Nefang*, a polyherbal product composed of *Mangifera indica* (bark and leaf) *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis* and *Ocimum gratissimum* (leaves), used for the

treatment of malaria by the Bayang community in Mamfe Sub-Division, South West Region of Cameroon. Traditionally, a mixture of the freshly-harvested or dried plant parts is used as an oral decoction or oral (enema) administration for the treatment of malaria. However, there is no literature on the formulation and the safety of this product.

Mamfe Sub-Division is one of the four Sub-Divisions of Manyu Division of the South West Region in Cameroon **(Figure 1)**. It is composed of 11 villages as follows; Bachuo-Ntai, Besong-Abang, Egbekaw, Eshobi, Etemetek, Eyanchang, Eyang-Ntui, Mfaitock II (Mile 18), Nchang, Okoyong, Small Mamfe (Presidential Decree N° 2008/376, 2008). The inhabitants speak the Bayang language as their mother tongue, called *Kenyang* (Tanyi, 2000), although English is the official language spoken along with Pidgin, and several other dialects, including Ejagham. Situated in the tropical rain forest zone, Mamfe Sub-Division is in a river valley and its humidity can be over 90% and temperatures sometimes exceed 120^{o}F (49°C) during the Dry Season (February - April). During the rest of the year, temperatures remain around 80-90^{o}F (27 to 37°C) and only drop during the Rainy Season, to approximately 70^{o}F (21°C). Mamfe, its capital is located 60 km (37 miles) from the border of Nigeria, on the Manyu River and is known as a centre for traditional religion and medicine, reflecting the use of medicinal plants for the management of many ailments in the villages of this sub-division in particular and the Division at large (*Encyclopædia Britannica Online*, 2014).

In this area, agriculture is primarily the main stay and could be said to be practiced on a two-pronged approach – subsistence and industrial, though there is no clear cut demarcation between the two because most, if not all farmers practice a combination of both. The last three decades, however, have witnessed an explosion in large-scale agricultural production of cash crops like cocoa, coffee, coconuts (*tamagha*), oranges (*nsukuru*), palms and palm produce, bush mangoes (*nsenghe*), egusi (*nkwai*), plantains (*ekwa*), banana (*nsureh ekwa*) and others, thereby getting plants to respond to human rather than natural selection. The proceeds from these endeavors are used to improvement the quality of life (Annuaire Statistique du Cameroun, 2004).

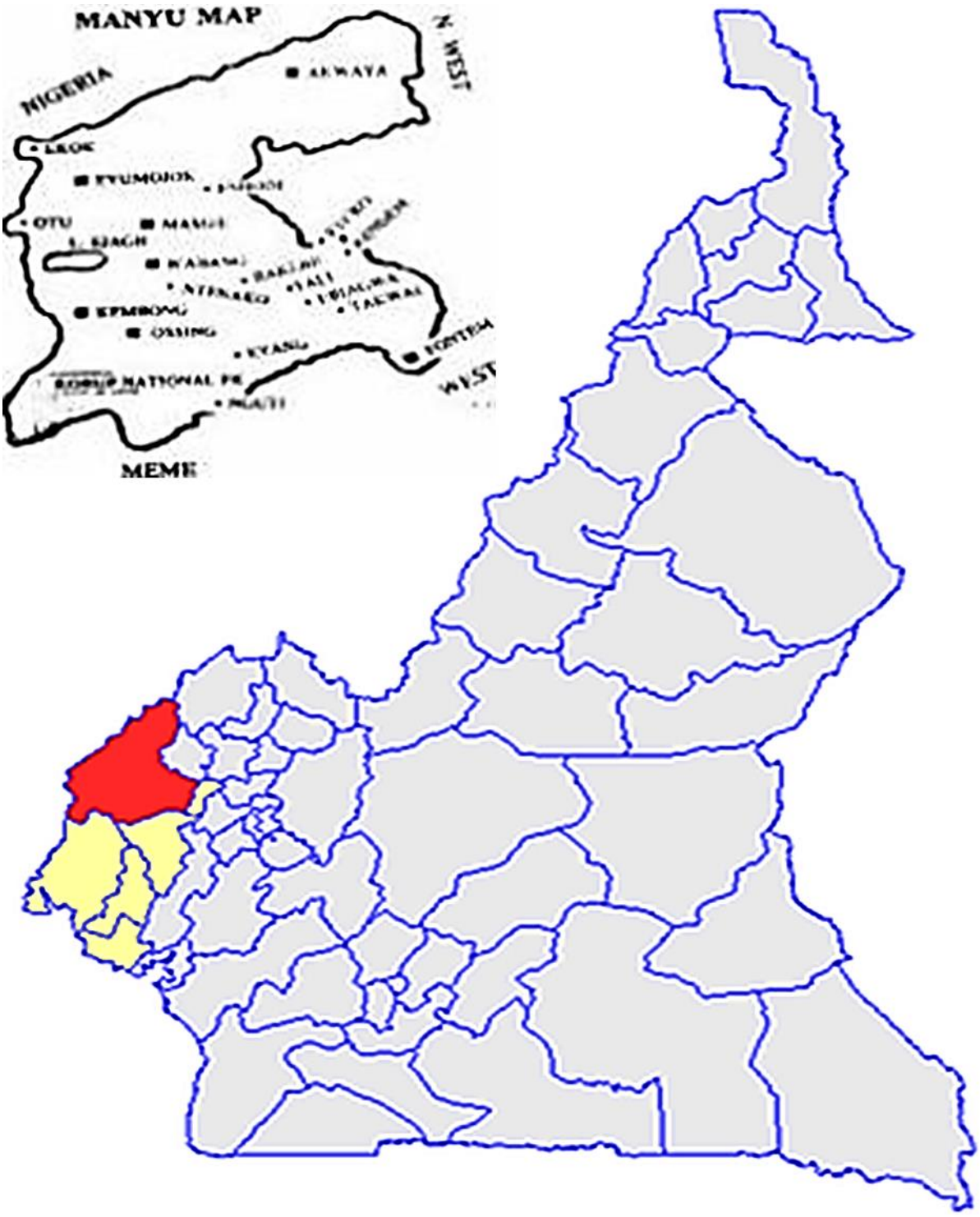

Figure 1.1. Map of the Republic of Cameroon showing the location of Manyu Division (in red) and its constituent sub-divisions (insert)

Source: www.google.com

Among the plant species of this area, medicinal plants constitute a sector of great importance. There is a strong belief by the population in plant medicines reflecting their reliance on this type of treatment. However, local knowledge on medicinal plants is not widespread because the local herbalists and traditional medicine practitioners usually inherit it (Zapfack *et al*., 2001). For most treatments, the number of medicinal plants used is great. In fact many villages have been noted for their reliance on traditional medicine and this can be partly attributed to their culture as well as the distance to affordable health facilities.

As in most tropical rain forests, fever is very recurrent in this area due to the presence of many blood-sucking insects like mosquitoes, tsetse flies, midges, and the absence of mosquito nets and other repellents leading to the frequent use of medicinal plants for treatment. Therefore, the traditional health care system is based on significant local knowledge of medicinal plants just like in all major tropical forest areas (Colfer *et al*., 2006).

The inability of the villagers to distinguish between fevers in the past may have resulted to the death of many, a situation that has changed over time. Presently, most people are aware of at least one plant species used in the treatment of one form of fever.

The growing consensus that synergistic drug combinations are essential to the optimal control of malaria (Fidock *et al.,* 2004) has led to combination therapy becoming the standard of care for emerging *P. falciparum* drug resistance (White and Olliaro, 1996), including the use of polyherbal antimalarials. This has been exploited in certain cases for the design of modern drugs. Consequently, there is the strong need for a valid complementary approach in herbal medicine research ethics (Emanuel *et al.,* 2004) towards the standardization of crude polyherbal antimalarials with demonstrated safety and efficacy (Patwardhan, 2009; Butterweck and Nahrstedt, 2012).

This antimalarial herbal drug discovery study was aimed at evaluating the efficacy and safety of *Nefang* with the view to standardizing, validating and prioritizing new antimalarial herbal drugs for downstream clinical development studies.

1.2 PROBLEM STATEMENT

Traditional medicine otherwise known as Complementary and Alternative Medicine (CAM) has been developed over the years in Cameroon through real life experiences and direct observations of people with diseases. It is a highly complex system. Its practice has been encouraged due to

lack of access to affordable and modern healthcare facilities but its development has been hampered by the lack of defined policies. This systems approach or science of wholeness is the unique philosophy of polyherbal therapy. Herbal extracts represent combinatorial chemistry of nature with vast array of chemical compounds that can deal with multiple targets simultaneously leading to a synergistic systems effect.

The belief that the use of these herbs for therapy is safe because they are natural has led to the ever increasing use of *Nefang.* This belief, however, has hampered the development of phytomedicines in Disease Endemic Countries (DECs). Some of the major factors responsible for this include lack of information and appropriate resources, neglect and inexperience in this area on the part of scientists working in DECs (WHO, 2000).

However, information on the efficacy and safety of *Nefang* is not available but there have been reports of nausea, dizziness and trembling in some patients after administration.

With the view to standardizing *Nefang*, there is therefore the need to acquire the traditional knowledge and folkloric use through interviews. Furthermore, a review of scientific work carried out on some of the constituent plants needs to be carried out with the view to establishing the safety profile and efficacy of this polyherbal antimalarial for the benefit of traditional herbal practitioners, consumers and the scientific community as a whole. There is also the need to understand how the interactions between the constituent plants and their biological activities impact on the antimalarial efficacy of this polyherbal product. The results obtained could be exploited in malaria drug discovery by strengthening the primary healthcare systems and policies.

1.3 STUDY JUSTIFICATION

In the long history of malaria drug discovery, natural products have outlived many synthetic drugs and remain a valuable resource in identifying efficient and long lasting novel antimalarials. Despite CAM rich practices, it remains irreconcilably quite different from modern or Western medicine due to its holistic approach to therapy. In order to exploit the potential of medicinal plants towards a rational phytotherapeutic and evidence-based drug discovery, it is imperative to ethically explore insights and tools of CAM healing practices through rigorous scientific assessment.

Nefang, though commonly used, is yet to be evaluated for its efficacy and safety. For the development of phytomedicines or identification of plant-based drug targets, the profile of the herbal preparation must be ascertained through some pharmacological, drug efficacy and toxicological parameters. Since *Nefang* is in use, its efficacy has first to be evaluated in vitro by high throughput screening of the constituent plants and characterization of the interactions between them to ensure efficacy and ascertain the type of interactions producing its therapeutic effects.

Furthermore, an evaluation of the in vivo antiplasmodial activity of *Nefang*, as well as the biological activities of the constituent plant extracts that contribute to this activity would be of immense importance in confirming its efficacy, contribution of the constituents and possible mechanism of action.

Finally, preclinical safety evaluation, which is a major step towards the development of antimalarial phytomedicines, would be vital. Hence, evaluation of the in vivo toxicity and cytotoxicity of *Nefang* and its constituent plant extracts would be needed to ensure safety.

The assurance of the efficacy and safety profile of this polyherbal antimalarial will enable us standardize, validate and prioritize new antimalarial drugs/compounds and mixtures for downstream clinical development studies as well as facilitate the development of CAM health policies in LMICs.

1.4 OBJECTIVES

1.4.1 Main Objective

The main objective was to evaluate the antimalarial properties and safety of a polyherbal product composed of *Mangifera indica* (bark and leaf) and *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis* and *Ocimum gratissimum* (leaves).

1.4.2 Specific Objectives

The specific objectives of the study were to;

1. Document the folkloric information on the formulation, use and safety of *Nefang*.
2. Evaluate the in vitro and in vivo antiplasmodial activities of *Nefang* and its constituents.
3. Characterise the interactions between the constituent plants of *Nefang*.
4. Determine the cytotoxicity and *in vivo* toxicity of *Nefang*.

5. Evaluate the constituent plants of *Nefang* for the following activities that contribute to antimalarial activity: antioxidant, antipyretic, anti-inflammatory and antinociceptive.

1.5 ETHICAL CONSIDERATION

Approval for the study was obtained from the Kenyatta National Hospital/University of Nairobi Ethics and Research committee, Nairobi-Kenya, through Ref: KNH-ERC/A/324 of 5th December 2012**,** renewed through Ref. No. KNH/ERC/R/25 of 25th March 2014. For studies carried out in Cameroon, approval was obtained from the Institutional Review Board of the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé-Cameroon through N/Ref: 076/82-62/MINRESI/M000 of 1st June 2011. Approval for the research carried out at Institut Pasteur Korea was obtained from Institut Pasteur Korea-Institutional Animal Care and Use Committee (IPK-IACUC) through IACUC No. IPK 12009 of 29th October 2012 **(Annex I).**

The study was conducted in accordance with the protocol and Good Laboratory Practice (GLP) to ensure protection of all aspects of the ethical rights and welfare of research participants and experimental animals.

1.6 INTELLECTUAL PROPERTY STATEMENT

This product is not yet authorized in the market and so does not have a trade mark and utility model. For work to be carried out using these plant species, the traditional knowledge and source of the various plants utilized were ascertained. Measures were taken to respect the provisions of the United Nations Framework Convention on Biodiversity (CBD) at the Earth Summit in Rio de Janeiro, Brazil in 1992, on issues relating to environmental law and policy. These include conservation of biological diversity, sustainable use of the components, fair and equitable sharing of benefits, access to genetic resources, transfer of relevant technologies, consideration of all rights over these resources and technologies and availability of appropriate funding to develop these issues. Assurance of anonymity and confidentiality were also ascertained. These were done using an appended Prior Informed Consent form **(ANNEX II).**

Therefore, all signatories were required to respect, preserve, and maintain the knowledge, innovations, and practices of the indigenous communities.

2.0 LITERATURE REVIEW

2.1 MALARIA

2.1.1 The epidemiology of Malaria

Malaria, also known as impaludism is an infectious disease transmitted by the bite of the female *Anopheles* mosquito. It is the third most important cause of mortality. It is endemic in more than 40% of the world's population and causes more than one million deaths each year, especially in Africa (WHO, 2006), In addition, more than 300 million acute episodes of malaria are experienced each year. Around 90% of these deaths occur in young children. According to WHO in every 30 seconds one African child is killed by malaria (WHO, 2005).

Malaria, along with HIV/AIDS and Tuberculosis, is one of the major public health challenges facing the poorest countries of the world, particularly in terms of morbidity, mortality and deleterious economic consequences (Hunt *et al*., 2006).

In Cameroon, the disease is mesoendemic but hyperendemicity occurs in remote areas located in the forest zone. In urban areas malaria is hypoendemic. Cameroon presents the main African prototypes of malaria epidemiology. In the forest and forest fringe in the southern Cameroon, transmission is perennial. In northern zone which experiences sudanian and sahelian climate, transmission is seasonal; exceptions to this statement are found rice irrigation schemes. Resistance to antimalarial drugs is highly prevalent with most strains showing resistance to chloroquine (50%) and sulfadoxine-pyrimethamine (12%). Cameroon is thus classified as a group IV region, which denotes countries where multidrug resistance is common. (Same-Ekobo, 2008)

2.1.2 Aetiological Agents and vectors

The aetiological agents of malaria are protozoa that belong to the genus *Plasmodium*, phyllum Apicomplexa and family *Plasmodidae*. Five species of *Plasmodium* are pathogenic to humans: *P. vivax, P. ovale, P. malariae, P. falciparum* and *P. knowlesii.* Of these, *P. vivax* and *P. falciparum* are the most important. However, malaria caused by *P. falciparum* is the most severe and potentially fatal and predominates in Africa (Kretti *et al*. 2001; White, 2008). *P. vivax* has a

wider distribution than *P. falciparum* because it is able to develop in the *Anopheles* mosquito vector at lower temperatures, and to survive at higher altitudes and in cooler climates.

Malaria is spread from one person to another by female mosquitoes of the genus *Anopheles* (family Culicidae). There are about 400 diff erent species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance (WHO, 2013).

2.1.3 Geographical distribution

The disease is found mainly throughout the tropical and sub-tropical regions of the world, which include Cameroon and the West and Central African sub-region (**Figure 2.1**). Temperature is particularly critical in the geographical distribution of malaria. At temperatures below 20°C, *Plasmodium falciparum* (which causes severe malaria) cannot complete its growth cycle in the *Anopheles* mosquito, and thus cannot be transmitted. *P. vivax* rarely causes a lethal infection and is widespread in Central and South America, Asia and Oceania. *P. vivax* can exist as a latent infection in the hepatocytes of the liver, re-emerging after many months to several years. Though it causes morbidity and may be an important cause of low birth weight in pregnancy, it is associated with relatively fewer complications (Nosten *et al*., 1999; WHO, 2013). Though commonly transmitted, infections due to *P. ovale* are rare as immunity is established early and parasitaemia remains low. Long term latent infections caused by *P. vivax* and *P. ovale* are established and found principally in Africa, causing less than 0.5% of malaria infections (Breman, 2001). *P. malariae* is found worldwide but with a very patchy distribution. It can cause renal complications or chronic nephropathy and if left untreated patients will remain parasitemic though asymptomatic for years (Hendrickse *et al*., 1972). *P. falciparum* is the most highly virulent species and causes almost all of the 1.7-2.5 million deaths worldwide from malaria (Bray and Sinden, 1979; Ringwald *et al.*, 1993; WHO, 2013).

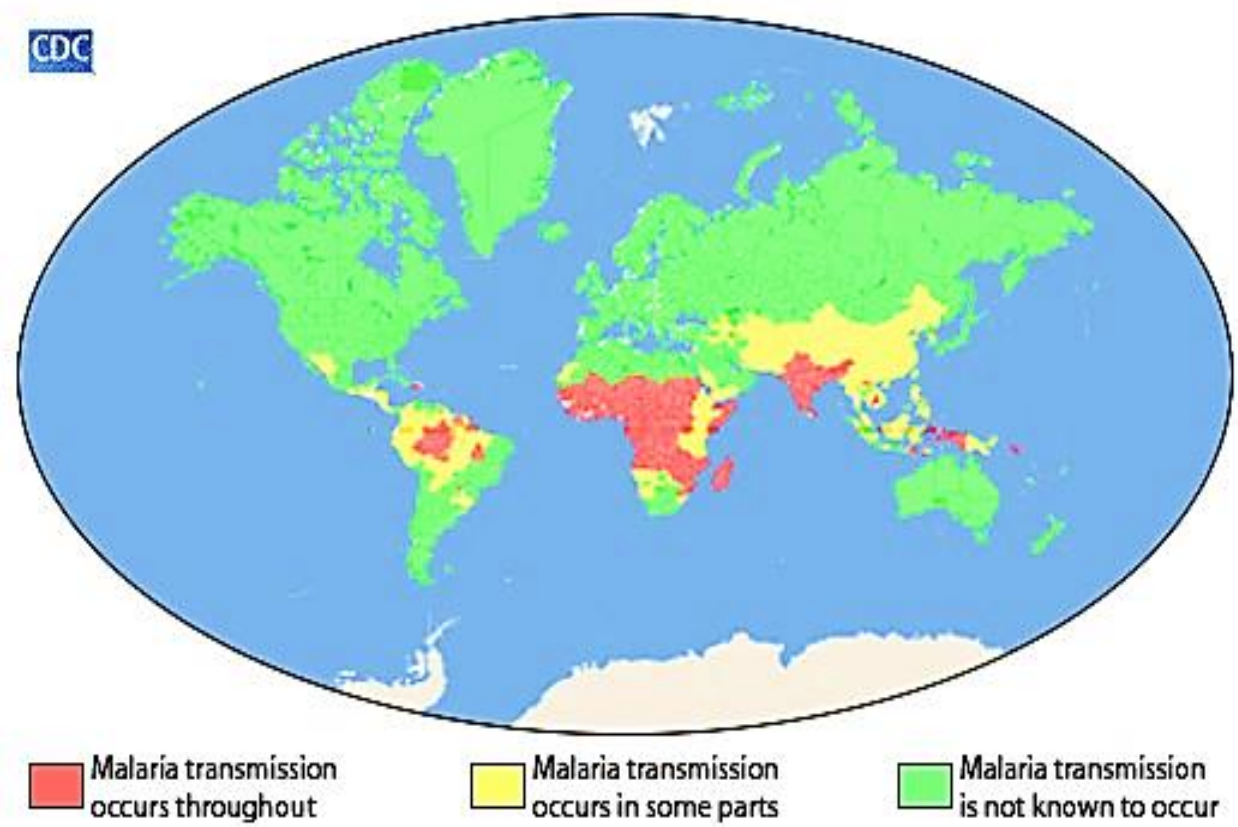

Figure 2.1. The global distribution of malaria in 2010 (CDC)

Reproduced from CDC website (CDC 2010; http://www.cdc.gov/malaria/about/distribution.html)

2.1.4 The Life cycle of the Malaria Parasite

The life cycle of the malaria parasite (*Plasmodium*) is complicated and involves two hosts, humans and *Anopheles* mosquitoes (**Figure 2.2**). The disease is transmitted to humans when an infected *Anopheles* mosquito bites a person and injects the malaria parasites (sporozoites) into the blood. The parasites grow and multiply, first in the liver cells, and then in red blood cells. In the liver cells, there is an initial round of replication (exo-erythrocytic schizogony), after which they undergo asexual multiplication in erythrocytes (erythrocytic schizogony). This process results in the destruction of erythrocytes and the release of daughter parasites (merozoites). The blood stage parasites are responsible for the clinical manifestations of the disease, and are the source of infection to mosquitoes (CDC, 2008). Non-treated or inadequately treated individuals

can be sources of infection to mosquitoes for a period of 2–3 years; the mosquitoes themselves remain infectious until death.

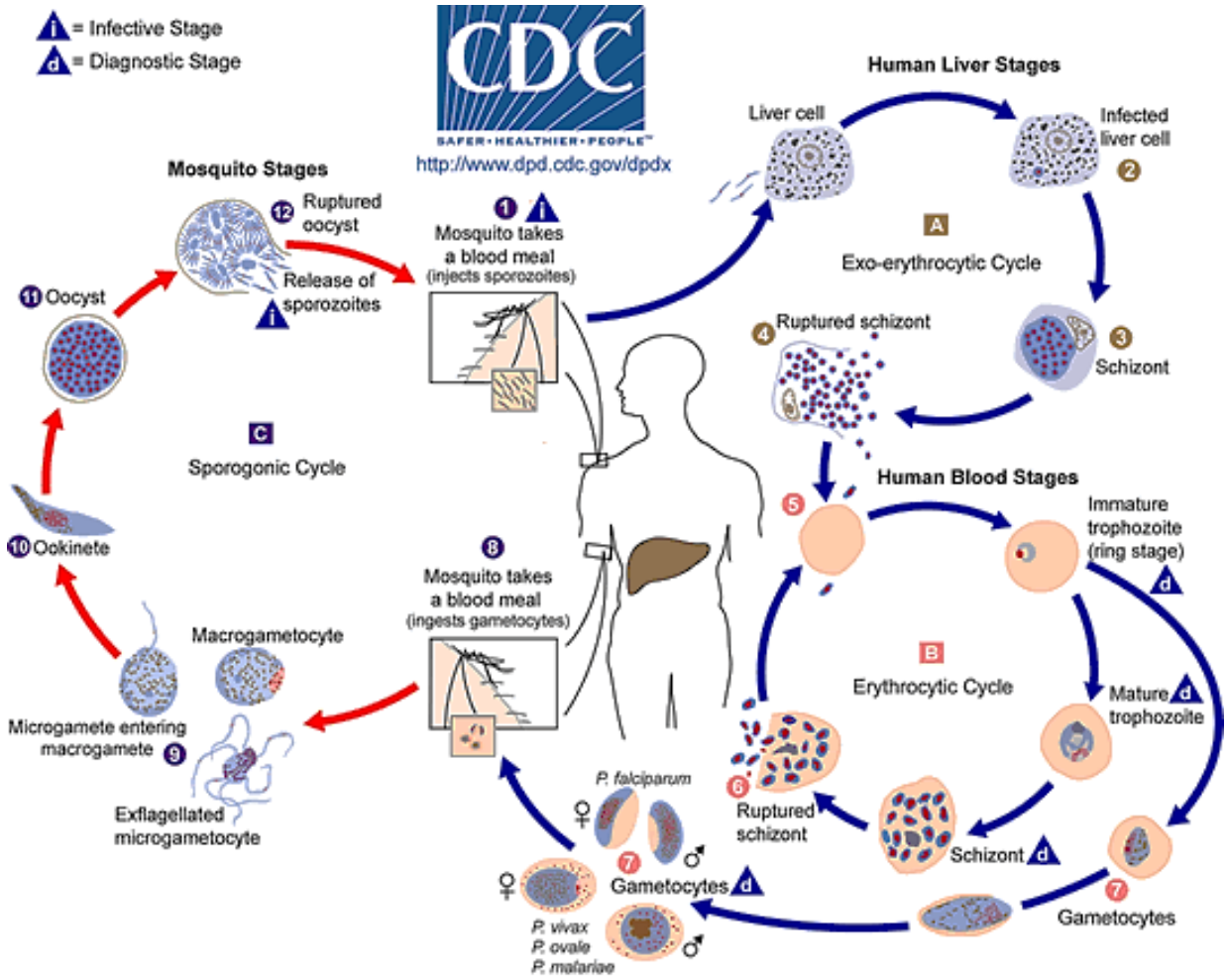

Figure 2.2. CDC illustration of the life cycles of malaria parasites, *Plasmodium* species. Source: CDC (2008)

2.1.5 Clinical Symptoms of Malaria

About one third of infections with *Plasmodium* are forms of mild malaria. These are caused by *P. vivax*, *P. ovale* and *P. malariae*. Symptoms include fatigue, fever, recurrent chills, and haemolytic anaemia. Two third of the cases are forms of severe malaria caused by *P. falciparum*. The symptoms are the result of complex interactions between *Plasmodium* and the immune system of its host. Intense headaches, pain in the joints, splenomegaly and hepatomegaly are accompanied by complications such as cerebral malaria, intravascular haemolysis (bilious fever),

glomerulonephritis caused by immune complexes, leuko- and thrombopenia, hypoglycaemia (especially children) and lung edema (Oliveira *et al*, 2009). Acidosis and hypoglycemia are the most common metabolic complications. Any of these complications can develop rapidly and progress to death within hours or days (WHO, 2000).

Cerebral malaria (CM) is one of the complications that constitute severe malaria. The pathogenic mechanisms underlying CM and reasons why a small percentage of patients develop CM are not fully understood. Accumulation of large numbers of parasites in specific sites such as the brain or placenta (MacPherson *et al*., 1985; Ricke *et al.,* 2000) associated with adverse clinical outcomes suggests that organ-specific accumulation of parasites is important.

Some of the processes that are believed to be involved are illustrated in **Figure 2.3** while the characteristics and clinical presentation of the different types of human malarias are outlined in (**Table 2.1**) (John *et al.*, 2006; Daneshvar *et al.*, 2009).

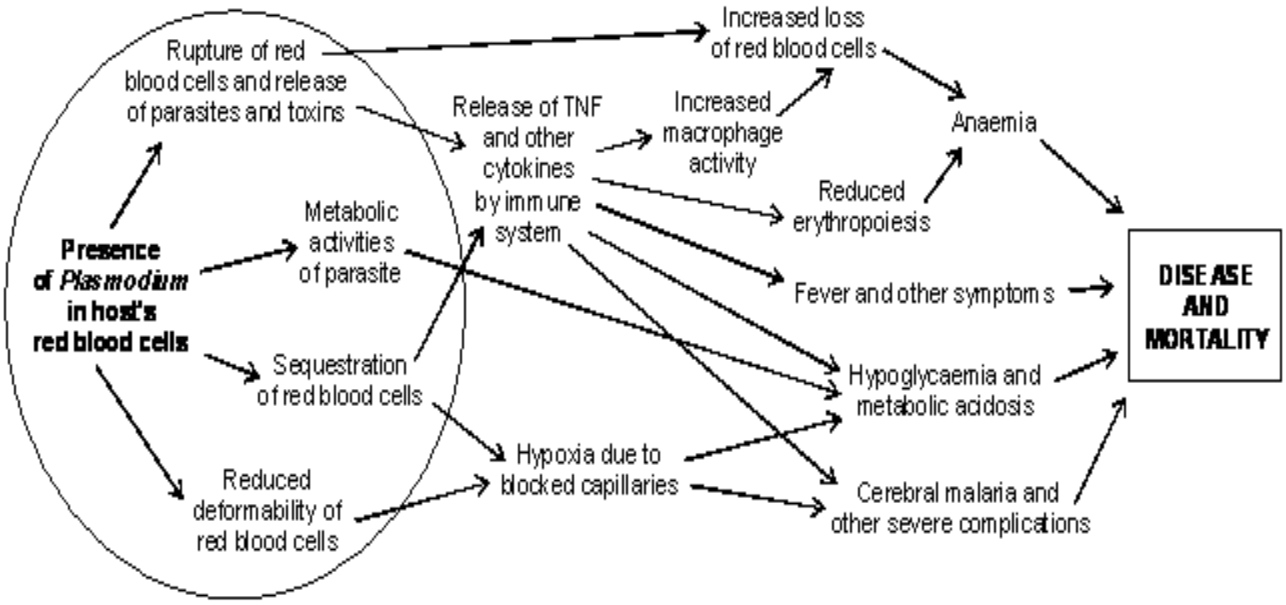

Figure 2.3. Possible mechanisms by which *Plasmodium* causes disease

Source: (Clark and Cowden, 2003).

Table 2.1. Clinical differences between the human malarias

(John and Petri, 2006; Bronner *et al.,* 2009; Müller *et al.,* 2009; Weekley and Smith, 2013)

	P. vivax	***P. ovale***	***P. malariae***	***P. falciparum***	***P. knowlesi***
Other names	**Benign tertian**	**Benign tertian or ovale**	**Benign Quartan**	**Malignant tertian aestivoautumnal**	**Severe Quotidian**
Incubation Period (Days)	14 (8 - 270)	15 (9 - 17)	15 - 30	12 (8 - 25)	11 (10 – 12)
Erythrocytic Cycle (Hours)	48	48	72	48	24
Persistent Exoerythrocytic Stage	Yes	Yes	No	No	No
Parasitemia (mm^3) Average Maximum	20.000 50.000	9.000 30.000	6.000 20.000	50.000 – 500.000 Up to 2.500.000	6.000 20.000
Duration of untreated Infection (Year)	1.5 - 4	Probably 1.5 - 4	1 - 30	0.5 - 2	< 1
Anemia	++	+	++	++++	+
Other Complications	-	-	Renal	Cerebral	Renal
Relative age of infected RBCs	Young and immature	Young and immature	mature	May infect cells of all ages	Young and immature

2.1.6 Host Immune response to the *Plasmodium* infection

The immune system provides different mechanisms to protect organisms against pathogens. Consequently, the course of *Plasmodium falciparum* malaria is characterized by a complex interaction of host immune responses and parasite survival strategies (Winkler *et al*., 1998).

In *Plasmodium* infection, it has been suggested that the balance between Th1 (IL-2, IFN-γ and TNF) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) immune response determines the degree of parasitemia, level of anaemia, clinical severity, presentation and/or outcome through direct or indirect reactions of cytokines and other physiologically active substances (Ho *et al*., 1995). Moreover, in *Plasmodium* infection the cell-mediated-immunity plays a key role, whereas the

humoral (B cell driven) immune response is less important (Yanez *et al*., 1996). Lack of naturally acquired immunity to malaria (premunition) contributes to the severity of the infection. Cytokines are proteins produced and released by monocytes, macrophages, epithelium, fibroblasts, smooth muscles cells (Belec *et al*., 1997). Cytokines have a role in both the innate and the humoral *in vivo* immune response (Abbas, 2000), and are often classified as "pro" and "anti" inflammatory (Pedersen *et al.,* 1998). The pro-inflammatory cytokines include IL-1, IL-6, TNF and INF-γ while some of the anti-inflammatory cytokines are IL2-, IL-4 and IL-10. In malaria, cytokines may be the key determinants of severity and outcomes (Winkler *et al*., 1998). TNF, IL-1 and IL-6 are substantially higher in children with severe malaria than in uncomplicated malaria.

2.1.7 Drugs Used for the Treatment of Malaria

Treatment of human malaria aims to interrupt the blood schizogony (intraerythrocytic asexual multiplication) that causes the pathogenesis and clinical symptoms of the infection. Prompt and effective treatment is probably the most effective method of malaria control. Oral treatment prevents progression to a severe state of the disease and the resultant complications (Guerin *et al.,* 2002). Most of the antimalarial drugs in use belong to the classes aminoquinolones, (chloroquine, amodiaquine, primaquine), quinolinomethanol derivatives (quinine, mefloquine, halofantrine), diaminopyrimidines (pyrimethamine), sulfonamides (sulfadoxine, sulfadiazine), biguanides (proguanil and derivatives), antibiotics (doxycycline, clindamycin), sesquiterpenes (artemisinin, dihydroartemisinin, arteether, artemether, artesunate) and naphtoquinones (atovaquone) (**Table 2.2**).

Table 2.2. Classes of antimalarial compounds and their effect on the life cycle of the malaria parasite (White, 1998; Maselli, 2006)

Chemical class and examples	Stage of life cycle affected	Action on cells	Action on malarial pigment
4-aminoquinolines **Chloroquine** **Mepacrine**	Schizogony All asexual stages	Not clear	Rapid coarse Clumping
8-Aminoquinolines **Quinine** **Mefloquine** **Primaquine**	All stages except mature gametocytes of *P. falciparum*	- Degeneration of nuclei - Vacuolation of cytoplasm	Slow, fine Clumping
Antifolates **Proguanil** **-Pyrimethamine** **- Sulphonamides**	Schizogony	Maturation arrest producing large non-viable parasites	--
Sesquiterpenes **Artemisinin**	Schizogony	--	--

The first developed antimalarial was quinine, isolated in 1820 from *Cinchona* (Rubiceae) bark (Guerra, 1977; de Paula *et al.*, 2009)). It remained the only clinical weapon until the 1940s, when chloroquine, a synthetic 4-aminoquinoline, became available. It was widely used until the 1960s, when resistance to the drug by *P. falciparum* became widespread causing a sharp increase in mortality rates. The antimalarial drugs in current use are artemisinin, the active compound from Artemisia annua L. (Asteraceae), a traditional plant used for millennia in China, and its semisynthetic derivatives artemether, artesunate, and arteether (Oliveira *et al.*, 2009). Artemisinins are currently the most effective drugs for antimalarial chemotherapy and have been globally adopted for the treatment of *P. falciparum* malaria. A recently introduced antimalarial drug is atovaquone, a synthetic naphthoquinone based on lapachol. Lapachol, a prenylnaphtoquinone, was first isolated from *Tabebuia impetiginosa* (Mart. ex DC.) Standl. (synon. *T. avellanedeae* Lor. ex Griseb.), a South American (Castellanos *et al.*, 2009). Parasite resistance of quinine and other antimalarials like proguanil has prompted the development of new and effective drugs against existing targets.

The antimalarial drug combination therapy that was proposed by the WHO in 2001 includes **A**rtemisinin-based **C**ombination **T**herapy (ACT). It combines artemisinin and its derivatives with existing antimalarial drugs. Several artemisinin-based pharmaceutical variants, which include artesunate-amodiaquine, artemether-lumefantrine (Coartem™), artesunate-mefloquine, and dihydroartemisinin-piperaquine (Artecom™), are available. Artemether-lumefantrine (Coartem™) is currently the most effective treatment for human falciparum malaria. The drug has passed extensive efficacy and safety trials and is recommended as a first- or second-line treatment for uncomplicated falciparum malaria.

Atovaquone-proguanil (Malarone) is one of the non-artemisinin combination therapies (WHO 2005, 2008). However, there are concerns regarding the affordability and accessibility of this high cost artemisinin and atovaquone combinations for communities in poor countries.

The characteristics of current treatment options of both single drug and combination therapies are listed in **Table 2.3** (Bloland, 2001; Lindberg, 2004).

Table 2.3. Summary of the characteristics of current anti-malarial drugs (Bloland, 2001; Lindberg, 2004)

Drug	High prevalence of Resistance	Severity and frequency of Side Effects	Mean Cost/Dose (US$)
Chloroquine	Yes	+	0.070
Sulfadoxine-pyrimethamine	Yes	++	0.083
Quinine	Yes	++	1.350
Amodiaquine	Yes	+++	0.150
Artesunate	None	++	2.160
Mefloquine	Yes	++	3.220
Halofantrine	None	+++	4.750
Atovaquone	Yes	+	35.00

+ Mild and low frequency; ++ Moderate serverity; +++ High frequency and servirity

2.1.8 Drug Resistance of *P. falciparum*

Drug resistance can be defined as the ability of a cell or an organism to survive in the presence of a high concentration of a drug that normally destroys cells or organisms of the same species or prevent their multiplication. The development of resistance to antimalarial drugs poses one of the greatest threats to malaria control and is the main cause of recent increases in malaria morbidity and mortality (Stepniewska, 2007). Monitoring the emergence and spread of drug resistance will influence drug policies so that public health bodies can take appropriate action. This may involve switching to a second line drug when resistance arises or the use of combination therapies (White, 1998). Resistance to antimalarials has been documented for *P. falciparum*, *P. vivax* and recently, *P. malariae.* Resistance has been observed to almost all currently used antimalarials (amodiaquine, CQ, mefloquine, quinine and sulfadoxine–pyrimethamine) (WHO, 2006) and recently pockets of resistance have been detected for artemisinin and its derivatives in Vitenam (Thanh *et al.*, 2010). Unfortunately, resistance is now widely disseminated throughout malaria-endemic regions (**Figure 2.4**) (Fidock *et al*., 2004).

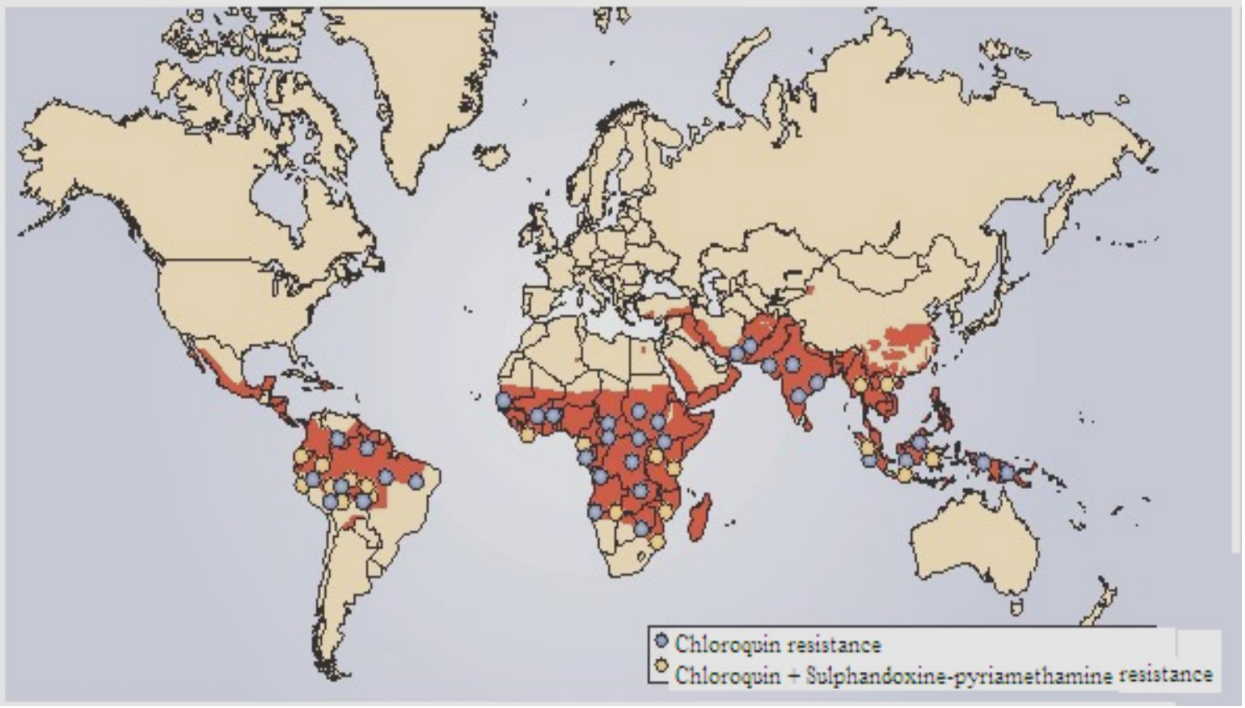

Figure 2.4. The global distribution of drug resistant malaria (Fidock *et al.*, 2004).

At present major drug resistance markers involved in CQ and sulfadoxine- pyrimethamine (SP) resistance are known (Djimde *et al*., 2001; Hayton and Su, 2004), these are *P. falciparum* multi-drug resistance-1 (pfmdr1) and *P. falciparum* chloroquine resistance transporter (pfcrt), encoding membrane transporter proteins, which are localized in the membrane of the parasite's food vacuole (Cowman *et al*., 1991; Fidock *et al*., 2000). In addition, the molecular basis for resistance to antifolates, such as SP has been well characterized. *P. falciparum* resistance to SP is primarily conferred by successive single-point mutations in parasite *dhfr*, the gene that encodes the target enzyme dihydrofolate reductase (*DHFR*), and by additional mutations in *DHPS*, which encodes for the enzyme dihydropteroate synthetase (*DHPS*) (Triglia *et al.,* 1997).

In fact, part of the reason for the failure to control malaria is the emergence and spread of resistance to first-line antimalarial drugs and the cross-resistance between drugs of the same class such as mefloquine and quinine (Brasseur *et al.* 1992), as well as cross-resistance between the members of the limited number of drug families available. However, cross resistance between artemisinin and other antimalarials has not yet been reported to date. Even though resistance of *P. falciparum* to artemisinin and its derivatives has not been reported worldwide in humans (WHO, 2006), recently pockets of resistance of *P. falciparum* to artemisinins have been detected in four countries (Cambodia, Thailand, Myanmar, and Vietnam) in the Greater Mekong subregion in Southeast Asia (WHO, 2013). Artemisinin resistance could spread from these countries to other regions, including sub-Saharan Africa, where the incidence of malaria is highest, and where artemisinin resistance would have devastating consequences. Afonso *et al.,* (2006) had earlier reported a murine malaria parasite that is genetically stable and has transmissible resistance to artemisinin and artesunate. There have been other reports on development of the rodent malaria parasite strains resistant to the drug (Chawira, 1986).

Over the last 30 years, the situation worsened and the increasing prevalence of resistant strains of *P. falciparum* was the major factor responsible for the increase in mortality that occurred mainly in Africa (WHO 2005). Treatment of chloroquine-resistant malaria was done with alternative drugs or drug combinations, which were rather expensive and sometimes toxic. Furthermore, these combinations were not always based on pharmacokinetic principles due to inadequate knowledge of metabolism and mechanism of action of most antimalarial drugs (Mukherjee, 1991).

Taken together, drug resistance in *P. falciparum* has become an extremely important concern and is an established fact, which should be faced. This has led to new strategies of finding new drug targets.

2.2 PLANTS AS A SOURCE OF NEW ANTIMALARIAL AGENTS

The first antimalarial drug was quinine, isolated from the bark of *Cinchona* species (*Rubiaceae*) in 1820 (Beckman, 1958). It is one of the oldest and most important antimalarial drugs that is still used today. In 1940, using the quinine template, another antimalarial drug chloroquine was synthesized. Despite the prevalence of CQ-resistant *P. falciparum*, it was until recently, the only drug used for the treatment of malaria (Bharel *et al*., 1996.).

Despite intensive efforts to produce new synthetic antimalarial drugs, the most significant recent contribution to new antimalarial drugs came from research on medicinal plants that contained artemisinin. In 1972, this compound was isolated from *Artemisia annua*; this plant species has had many traditional uses in China for several millennia. Artemisinin represents a new structure of antimalarial pharmacophore that comprises an endoperoxide sesquiterpene lactone. Semi-synthetic derivatives, including arteether, artemether and sodium artesunate, are used increasingly more often. Although they are effective against CQ-resistant *P. falciparum* as single therapeutic agents, to minimize the risks of recrudescence and the development of resistance, combination treatment with a second antimalarial drug is recommended (WHO 2005).

The newest antimalarial of plant source is atovaquone, a prenylnaphthoquinone from the *Tabebuia* species (*Bignoniaceae*). Though effective in combination with proguanil, its high cost precludes its wide scale use (Fidock *et al.*, 2004).

Therefore, plants may well prove to be the source of the next antimalarial drugs in view of the success with the two important chemotherapeutic agents, quinine and artemisinin, both of which are derived from plants. There have been reports of crude extracts, essential oils and some secondary metabolites with diverse chemical structures from higher plants possessing significant antimalarial activity (Sudhanshu *et al*., 2003).

The ethnopharmacological approach for the search of antimalarials from plant source has proved to have good predictive validity, and hence necessitates some scientific attention for the search of new chemical entities (NCE) from natural origin and the development of phytochemicals. It

should be stressed that the basic requirement for the validation of a medicinal plant is the standardization of the extracts that are to be evaluated.

2.3 CONSTITUENTS OF NEFANG

Nefang is constituted of *Mangifera indica* (bark and leaf), *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis* and *Ocimum gratissimum* (leaves).

2.3.1 *Mangifera Indica* Linnaeus (Mango)

Mango as it is commonly called, belong to the genus *Mangifera* of the family Anacardiaceae. The genus *Mangifera* constitutes several species that bear edible fruits. Most of these fruit trees belong to the species *Mangifera* (Ian, 2006). It is a cultivated fruity tree with a short-stocky bole and dense-ovate crown. The leaves are alternate-lanceolate, 20-30 cm long and 3-6 cm broad with long petioles. Mango flowers are borne on a terminal inflorescence (panicles) with numerous little pale green to pink or red flowers. Fruits are ovate, drupe, slightly reniform with more or less fibrous pulp depending on the variety. Being of India origin, the plant has been dispersed in Africa during the last two centuries (Hutchinson and Dalziel, 1954-1972; Keay, 1989).

Mango, besides being eaten as a ripe fruit has many ethnomedicinal uses. The root, bark, leaves, flowers, unripe and ripe fruits are acrid, cooling and astringent to the bowels and have been employed for therapy in ayurvedic medicine. These parts have also been utilized in treating piles, bronchitis, urinary discharge and vaginal troubles. *M. indica* is also used as aphrodisiac, appetizer, laxative, diuretic and for tanning purposes in various parts of the world (Kirtikar and Basu, 2005) The bark is used for the treatment of dental caries and toothache. The leaves together with the bark and leaves of *Psidium guajava*, *Cymbopogon citratus*, *Carica papaya*, citrus fruits and *Ocimum gratissimum* are used for preparing concoctions for the treatment of malaria and yellow fever by enema or wash-in bath. Leaves have been used as a laxative and an expectorant (Dalziel, 1995).

Phytochemical screening of active compounds from the leaves extracts revealed the presence of saponins, steroids, tannins, flavonoids, reducing sugars, cardiac glycosides and anthraquinones (Aiyelaagbe and Osamudiamen, 2009) while xanthones, amino acids, plant acids, triterpenoids, sesquiterpenes, triterpenoidal saponins and steroids are amongst the multiple chemical

constituents isolated from the stem bark extracts (Singh *et al*., 2012). Mangiferin, the major xanthone glucoside isolated from both the leaves and the stem bark has been shown exhibit antioxidant, radioprotective, antitumor, immunomodulatory, anti-allergic, anti-inflammatory, antidiabetic, lipolytic, antibone resorption, antiviral, antifungal antibacterial and antiparasitic properties. These are similar to the pharmacological properties exhibited by the crude extracts (Wauthoz *et al*., 2007). It has also been used in many herbal formulations.

Mass spectroscopy and HPLC characterization of various mango cultivars revealed the presence polyphenols which comprised of galloyl glycosides, flavanol glycosides, mangiferin, and gallotannins. Most of these compounds have been shown to exhibit antioxidant, anti-cancer, antiviral, antiseptic, anti-inflammatory, cardiotonic, hypotensive, hypoglycemic and emetic activities (Talcott and Talcott, 2009). Numerous toxicological studies have been carried out on both water and alcohol extracts of the leaves and bark of mango and they have proven to be non-toxic (Nwinuka *et al*, 2008).

2.3.2 *Psidium guajava* Linnaeus (Guava)

Guava belongs to the genus *Psidium* of the Myrtaceae family and the species *Psidium guajava*. It is a low evergreen shrub, 5 – 8 metres high. Leaves are simple, opposite, entire, ovate, 3 – 5 cm long and 2 - 4cm broad, glaborous with eight to fifteen prominent lateral nerves beneath. It is an influorescent with an axillary cyme or solitary flowers. Flowers are white, pedunculate, 1.5 – 2 cm in diameter. Fruits are spherical berries with persistent sepals on top with numerous small seeds embedded in a white or pink pulp (Hutchinson and Dalziel, 1954; Smith *et al.*, 1992). The species is a native of South America but is now cultivated in all tropical regions.

The leaves and bark of guava are astringent. Leaves are used for the treatment of diarrhea, amoebic dysentery, dermatitis and malaria. Tender leaves are chewed for bleeding gums, mouth sores and bad breath (Burkill, 1977). Phytochemical screening of the aqueous and alcohol extracts of different plant parts revealed the presence of alkaloids, cardiac glycosides, flavonoids, tannins, saponins, terpenoids, reducing sugars, cardenolides with steroidal rings and cardenolides with deoxy sugars (Elekwa *et al.*, 2009; Ayoola *et al.*, 2008). Of all these, guava's major plant chemicals are the flavonoids, tannins and isoprenoids. Quercetin, its glycosides and morin-3-O-α-L-lyxopyranoside, morin-3-O-α-L-arabinopyranoside, kæmpferol, luteolin-7-O-glucoside and apigenin-7-O-glucoside constitute the major flavonoids while the tannins include amritoside

(ellagic acid 4-gentiobioside), guavin, the antidiabetic agents (isostrictinin, strictinin, pedunculagin) and gallocatechin. The isoprenoids include many monoterpenes (examples include caryophyllene, d-limonene, myrcene and eugenol) and terpenoids (examples include guavanoic acid, guajavanoic acid, guajavolide and jacoumaric acid) (Beckstrom-Stenberg *et al*., 1994; Metwally *et al*., 2011)).

These components have been shown to exhibit amebicide, analgesic (pain-reliever), antibacterial, anticandidal, antidysenteric, antifungal, antimalarial, antioxidant, antispasmodic, antiulcerous, cardiodepressant, cardiotonic, central nervous system depressant, cough suppressant, gastrototonic, hypotensive, sedative and vasoconstrictor activity (Arima *et al*., 2002; Conde *et al*., 2003; Egharevba *et al*., 2010). The LD_{50} of the aqueous and alcohol extract of *Psidum guajava* is > 5 g/kg body, thus showing that it is not toxic.

2.3.3 *Carica papaya* L. papaya (Paw-paw)

Paw-paw is of the genus *Carica* of the Caricaceae family and of the species *Carica papaya* Linn. It is an herbaceous succulent plant that possesses a self-supporting stem (Gross, 2003). The plant is usually short-lived but can produce fruits for up to 20 years. It can grow up to 10 m high. The leaves are alternate, palmate and clustered at the top of the stalk. Petioles are very long and hollow. It possesses male and female white flowers separated on different plants; the male ones are smaller and numerous, clustered in cymes while the female ones are subsessile, larger and less numerous, fixed at the axial of leaves. It bears a big, ovoid berry polysperm fruit which change from green to yellow upon maturity (Adjanahoun and Aké, 1973).

Apart from being used as food, its ethnomedical uses include: the use of the fruits to treat anemia, diabetis mellitus and intestinal helminthiasis, while the leaves are used for treating malaria, gonorrhea, jaundice and headache. The seeds of the unripe fruit are used for the treatment of dysentery (Dalziel, 1995). Fresh leaves are also used as an analgesic, emenagogue, febrifuge and as a laxative.

Preliminary phytochemical screening revealed the presence saponins, cardiac glycosides and alkaloids. Nutrient evaluation revealed that green plant leaves contained thiamine (vitamin B1), riboflavin (vitamin B2), and ascorbic acid (vitamin C). Mineral analysis showed high values (mg/kg) of Ca, 8612.50; Mg, 67.75; Na, 1782.00; K, 2889.00; Mn, 9.50 in the green leaves, and

Fe, 147.50 in yellow leaves. Therefore pawpaw leaves are a potential source of useful elements for drugs formulation (Ayoola and Adeyeye, 2010).

Biofractionation of leaf extracts revealed the presence of glycosides, carposide, and the alkaloid, carpaine (Sharma and Ogbeide, 1982). Fresh leaf latex contains 75% water, 4.5% caoutchouc-like substances, 7% pectinous matter and salts 0.44% malic acid, 5.3% papain, 2.4% fat, and 2.9% resin. These active principles have shown analgesic, amebicide, antibiotic, antibacterial, cardiotonic, hypotensive, laxative, pectoral, antiseptic, stomachic, vermifuge activity by plant extracts (Krishna *et al*., 2008).

Though the *in vivo* toxicity studies of plant extracts have reported *Carica papaya* leaves extract to be non-toxic (Afzan *et al*., 2012), on external application, the leaf latex is irritant, dermatogenic, and vescicant. On ingestion, it causes severe gastritis (Morton, 1977).

2.3.4 *Cymbopogon citratus* (DC. ex Nees) Stapf (Fever Grass or Lemon Grass)

It is commonly called fever grass or lemon grass. *Cymbopogon citratus* is of the Poaceae family. It is a perennial robust herb, growing in dense tufts. Its leaves are fragrant, up to 70 cm long and 5-15 cm broad, with scabrid margins and a prominent midrib beneath. Its inflorescence is in panicles, 30-60 cm long, with sessile spikelets and linear or linear-lanceleote (Adjanahoun *et al*., 1996). It probably originates from India and is widely spread in the tropical regions. It is cultivated in Africa, Central America and other tropical countries.

The whole plant is used medicinally to treat typhoenteritis and to repel mosquitoes, flies and other insects. It is also employed as an ingredient in many malaria remedies (Tchoumbougnang *et al*., 2005). The leaves of *C. citratus* which contain volatile oils which are usually used in the form of tea to serve as a febrifuge while the roots are used as chewing sticks to clean the teeth (Sawyerr, 1982).

Preliminary phytochemical screening revealed the presence of two new triterpenoids, cymbopogone and cymbopogonol, the flavonoids, luteolin and its 6-C and 7-O –glycosides, (Guanasingh and Nagarajan, 1981), isoorientin 2'-O-rhamnoside and the flavonoids, quercetin, kaempferol and apiginin. The phenolic compounds, elimicin, catechol, chlorogenic acid, caffeic acid and hydroquinone, were also isolated from the plant. The essential oil of *Cymbopogon citratus* contains citral α (40%), citral β (32%), nerol (4.18%), geranicol (3.04%), citronellal (2.10%), terpinolene (1.23%), geranyl acetate (0.83%), myrecene (0.72%), terpinol (0.45%),

methylheptenone (0.2%), borneol (0.1-0.4%), lanilyl acetate (0.1%), α-pinene (0.07%) and β-pinene (0.04%) and all are important raw material used in the pharmaceutical, perfumery and cosmetics industries, especially for the synthesis of Vitamin A and ionones. Citral possesses various biological activities. It has neurobehavioral effects, larvicidal, hypoglycemic and hypolipidemic, hypocholesterolaemic, free radical scavenging and antioxidant, ascaricidal, antiprotozoan, antinociceptive, antimycobacterial, antimalarial, anti-inflammatory, antifungal, antifilarial, antidiarrhoeal, antibacterialand anti-amoebic activities (Kumar *et al*., 2010).

In vitro cytotoxicity studies revealed that the essential oil is toxic at very high concentrations, though it has the ability to suppress oxidative stress (Koba *et al*., 2009).

2.3.5 *Citrus sinensis* (Linnaeus) Osbeck (pro sp.) [maxima reticula] (Sweet Orange)

Citrus sinensis, commonly called sweet orange, is of the Rutaceae family. It is a spreading evergreen tree with slender blunt spines which grows to about 1.5 m. Its leaves are narrow and rounded at the base, 2 cm long, ovate-oblong, pointed at the tip, green on the upper surface and deep green on the lower surface. Petioles are short with very narrow wings. Its inflorescence is small and white. It bears sweet smelling rounded fruits which are deep yellow to orange in colour (Sofowora, 1993). Though of Asian and Indian origin, cultivation is carried all around the world.

Fruits are used basically as a food due to presence of many vitamins. Extracts obtained from the leaves and fruit peels are used to kill mosquito larva and mites and as antimicrobial and antifungal agents. Leaf extracts have also been used in folk medicine to treat neurological disorders and to facilitate digestion (Mwaiko, 1992; Fan *et al*., 1995).

Preliminary phytochemical screening of leaf and fruit peel extracts revealed the presence of alkaloids, phenols, flavonoids, tannins and saponins, with fruit peel extracts showing a higher quantity of all observed phytochemicals than the leaf extracts (Okwu *et al*., 2007). Many substances have been isolated from *C. sinensis* leaves: glycosides (apigenin and diosmetin), ruteosides (luteolin), caffeine, hydroxyproline, flavonoids (natsudaidaine, HEPTA (5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone), hesperidin, and diosmin), and the triterpene linomin (Duke, 1992; Stewart, 1985). Hesperidin and diosmin possess anti-inflammatory, antihypertensive, diuretic, analgesic, and hypolipidemic properties (Gil-Izquierdo *et al*., 2001). Natsudaidaine and

HEPTA showed a positive inotropic effect on the guinea pig right ventricle; hence it could be of importance in the future as pharmacological tools for the treatment of cardiac disorders (Oliveira *et al*., 2005).

2.3.6 *Ocimum gratissimum* Linnaeus (Wild basil or Mosquito Plant)

Ocimum gratissimum commonly called Wild Basil or Mosquito Plant is of the Lamiaceae family. It is an aromatic, perennial herb, 1-3 m tall; stem erect, round-quadrangular, much branched, glabrous or pubescent, woody at the base, often with an epidermis peeling in strips. Its leaves are opposite; petiole 2 - 4.5 cm long, slender, pubescent; blade elliptical to ovate, 1.5-16 cm x 1-8.5 cm, membraneous, sometimes glandular punctate, base cuneate, entire, margin elsewhere coarsely crenate-serrate, apex acute, puberulent or pubescent. The inflorescence is a verticillaster, arranged in a terminal, simple or branched raceme 5-30 cm long. Flowers are formed in 6-10-flowered verticillasters, small, hermaphrodite; calyx 2-lipped, 2-3 mm long, in fruit 5-6 mm; corolla campanulate, 3.5-5 mm long, 2-lipped, greenish white. The fruit consists of 4, dry, 1-seeded nutlets enclosed in the persistent calyx (the lower lip closing the mouth of the fruiting calyx); nutlet subglobose, 1.5 mm long, rugose, brown; the outer pericarp does not become mucilaginous in water (Orwa *et al*., 2009). It is probably a native of Asia but is now cultivated in the tropics.

The whole plant and its essential oils have many applications in traditional medicine. Preparations from the whole plant are used as stomachic and in treating stroke, diabetes, headache and influenza. The seeds have laxative properties and are used against gonorrhea. The plant is mostly cultivated for its essential oils, eugenol and thymol. The essential oil is used to treat malaria fever (Tchoumbougnang *et al.,* 2005), inflammation of the throat, ears, eyes and skin diseases. It is also a strong insect repellant. The leaf is used as a flavouring agent and in ceremonial washing of corpses (Awuah, 1994; Sulistiarini, 1999). Phytochemical screening of different *Ocimum* extracts revealed the presence of carbohydrates, reducing sugars, lipids, flavonoids, alkaloids, steroids, tannins and saponins. Extracts have strong antifungal, antimicrobial and antibacterial activity (Amadi *et al*., 2010). The leaf concoction is used to control illnesses such cholera, diarrhea, dysentery, typhoid fever, malaria, headache, pains, stomach upset and other domestic and acute illness (Nargarajun *et al.,* 1989; Sofowora, 1993). HPLC quantitative analysis of the leaf extract revealed the presence phenolic acids,

hydroxycinnamates and flavonoids. Extracts contain rosmarinic acid, lithospermic acid, vanilic acid, *p*-coumaric acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid, cinnamic acid, dihydroxy phenyllactic acid, and sinapic acid. These components explain the strong antioxidant and free radical scavenging activity of the extract (Lukmanul *et al*., 2008). The LD_{50} in rats was > 2000 $mgkg^{-1}$ bwt making it non-toxic (Mohamed *et al*., 2007).

2.4 METHODS OF EVALUATING ANTIMALARIAL EFFICACY OF TEST COMPOUNDS

As malaria continues to be a worldwide problem due to increasing drug resistance, several drug susceptibility techniques have been reported in the literature. As with other pathogens, several methods are available to measure drug susceptibility but due to the complicated lifestyle of *P. falciparum*, assay development has been complicated. A particularly confounding problem is the lack of standardization between methods that result in differences in sensitivities, however, therapeutic efficacy or *in vivo* tests are the accepted gold standard for measuring antimalarial drug susceptibility. As the need to develop safe and affordable antimalarials, various conventional and modern *in vitro* and *in vivo* screening methods are used for the evaluation of antimalarial compounds (Co *et al*., 2010).

2.4.1. In vitro Methods

In vitro screens for activity, which constitute a key component of a critical path for antimalarial drug screening, is based on the ability to culture *P. falciparum* in vitro in human erythrocytes (Fidock *et al*, 2004). Continuous cultivation of *P. falciparum* is a reliable source of stock culture of parasite. Cultures of *P. falciparum* are used to study the mode of entry of parasites into erythrocytes, screening for new drugs, isolation and characterization of strains and clones, identification of immunogenic antigens and parasite genomic studies (Kaira *et al*, 2006). Some of the *in vitro* screening methods include the hypoxanthine uptake, Giemsa stained slide or the Minimum Inhibitory Concentration (MIC) test, Micro-test (Mark III), flow cytometry, measurement of lactate dehydrogenase activity and SYBR Green 1 methods.

In vitro methods are precise, fast and efficient. Using these methods, large number of compounds can be evaluated at the same time, the synergism or antagonism of drug combinations can be studied and the intrinsic activity of a drug can be well assessed. However, these methods have

limitations in that expertise and infrastructure is needed. More to that, drugs acting through active metabolite cannot be studied and there is non-reproducibility of pharmacokinetic effects. In carrying out these methods, there may be the lack of correlation between in vitro activity and clinical efficacy. These assays do not give an indication of the toxicity of drug candidates and toxic compounds may be selected for further investigation. Hence the need to include toxicity assays during the screening phase.

2.4.1.1 Hypoxanthine uptake Method

Hypoxanthine uptake is a standardized method in which hypoxanthine (which is taken up by the parasite for purine salvage and DNA synthesis) is used to determine the level of *P. falciparum* growth inhibition. Radio-labeled hypoxanthine uptake by the parasite is an indicator of its growth and multiplication. Parasites are cultured in the presence of different concentrations of the test compound in media containing a reduced concentration of hypoxanthine, after which radio-labeled hypoxanthine is added for an additional incubation period before harvesting and measurement of radioactivity. Mean counts per minute (cpm) are generally in the range 20000-60000, with an acceptable minimum of 10000. Percent reductions in radio-labeled hypoxanthine uptake are used to plot percentage inhibition as a function of drug concentration and IC_{50} values determined by linear regression analysis of the linear segment of the dose-response curve. This method is the most common method but its shortcoming is that it is expensive, complicated and involves the use of radioactive material (Fidock *et al*., 2004).

2.4.1.2 Giemsa Stained Slide Method (MIC)

In this method, parasites are incubated in 5% erythrocyte suspension with an initial parasite density of 1-2%, at different concentrations of the test compound at 37^{o}C, for a period of 72 hours. The chamber must be closed and be continuously gased with a mixture of 2% O_2, 8% CO_2 and 90% $N_{2.}$ Parasitaemia of control and treated groups are compared by counting Giemsa stained parasites by light microscopy. This method is a low cost alternative for testing small amounts of test compounds and relies on a morphological response and reports a single concentration as the end point. This measurement is known as the Minimum Inhibitory Concentration (MIC) which is suitable for distinguishing susceptible and resistant isolates (Desjardins, 1984).

2.4.1.3 Flow Cytometry Method

This method takes advantage of the fact that human erythrocytes lack DNA. Parasites are fixed after an appropriate incubation period with test compounds, after which the parasitized cells are either stained with hydroethidine (which is metabolized to ethidine 53) or with 4,6-diamidino-2-phenylindole (DAPI). Counts of treated and control culture can then be obtained by flow cytometry. Appropriate gating can also allow one to distinguish different parasite stages in erythrocytes. This relatively simple assay provides high throughput but requires expensive material (Kaira *et al*, 2006).

2.4.1.4 Measurement of Parasite Lactate Dehydrogenase (pLDH) Activity Method

Measuring LDH activity of *P. falciparum* as an assessment of parasitaemia by colorimetry is a less sensitive method. This assay is based on the observation that LDH enzyme of *P. falciparum* has the ability to rapidly use 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction leading to the formation of pyruvate from lactate. Presence of *P.falciparum* at parsitaemia levels of above 0.02% cultures can be detected by measuring the level of APADH (Mackler and Hinrichs, 1993). This enzyme-linked immunosorbent assay (ELISA) method depends on the availability of monoclonal antibodies.

2.4.1.5 Micro-test (Mark III) Method

This is the most commonly used method for antimalarial *in vitro* testing of resistance. It provides information on the quantitative drug response of *P. falciparum* irrespective of the patients' immune system. The test can be carried out with several drugs, in a 96 well micro test kit pre-dosed with standards. The patient's blood is inoculated in the wells and incubated with suitable medium. The number of schizonts with three of more nuclei out of a total of two hundred parasites is counted and compared with the control (WHO, 2004).

2.4.1.6 Molecular Methods for detection of resistant parasites

Molecular diagnostic methods are used for detecting resistant parasites in the process of monitoring the level and spread of resistance (Kaira *et al*, 2006). They include direct sequencing, pulse-rate electrophoresis, real-time polymerase chain reaction (RT-PCR) and micro-array techniques. These molecular tools are based on the detection by PCR of point mutation in the parasite genome responsible for in vitro resistance. These methods are suitable for use on a large

number of samples and have an advantage over in vitro techniques that require parasite cultivation that takes days to perform. However, some of the shortcomings are that it is difficult to identify drug targets because the drug mechanisms of action are yet to be elucidated and some of the drugs act on multiple targets and thus may have multiple mechanisms of action. In addition, some resistant phenotypes are a result of altered gene expression while multiplicity of genetic changes in identified drug targets makes development of an easy-to-use assay difficult (Co *et al*, 2010).

2.4.1.7 SYBR Green 1 based Fluorescence Method

This is a microfluorimetric method using SYBR Green I for assessing susceptibility of parasites to antiplasmodial compounds (Bennett *et al*., 2004). Parasitemia can be quantified by this method involving this dye whose fluorescence is enhanced upon contact with nucleic acids. It is a one-step procedure, relatively simple and quick to perform, does not require highly specialized equipment and has the potential to replace traditional assays in the routine monitoring of susceptibility of *P. falciparum* isolates to antimalarial drugs. Previously described methods have the advantage of their sensitivity, enabling their use for any fresh isolate irrespective of parasite density (0.002% for HRP-2 and 0.005% for pLDH) and the possibility to be performed in isolated laboratories. However, SYBR Green I assay still has better advantages and has become a method of choice, especially in malaria-endemic countries, where there is massive implementation of new artemisinin-based combination therapies (Smilkstein *et al*., 2004; Bacon *et al*., 2007).

2.4.2 In vivo Methods for evaluation of antimalarial activity

In vivo tests are the oldest approach in the assessment of therapeutic responses and have enabled determination of the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies (WHO, 2003). Compounds effective in in vitro tests (those with $IC_{50} < 1\mu M$) are taken up for in vivo evaluation. Since *Plasmodium* species that cause human malaria are essentially unable to infected non primate animal models, in vivo evaluation of antimalarial compounds begins with the use of rodent malaria parasites. These include *Plasmodium berghei*, *P. yoelii, P. chabaudi,* and *P. vinckei* (Fidock *et al*., 2004). These in vivo methods are classified into primary, secondary and tertiary biological assessments. Some of these methods include the

P. berghei 4-Day suppressive test, Hill's test for causal prophylaxis, residual activity and the sporonoicidal activity tests.

2.4.2.1 Primary Biological Assessment using *P. berghei* 4-Day Suppressive Test

This is the most widely used primary biological test, in which the efficacy of a compound is assessed by comparison of blood parasitaemia and mouse survival time in treated and untreated mice. Naval Medical research Institute (NMRI) mice are infected with *P. berghei*, ANKA strain on day 0 and 2 - 4, 24, 48 and 72 hours post-infection. The mice are treated with the test compound while using chloroquine and artemisinin as standards. On day four, blood smears from all groups of animals are prepared and stained with giemsa stain. Parasitaemia is determined from day 4 to 6 and compounds that suppress parasitaemia by 90% are considered effective (Kaira *et al*, 2006). Compounds identified as active are further subjected through several secondary biological assessments.

2.4.2.2 Secondary Biological Assessments

i) Dose Ranging Test: In this procedure, compounds are tested at a minimum of four different doses (the "dose ranging, full 4-day test"). ED_{50} and ED_{90} values are calculated by plotting the log dose against probit activity. These reflect the drug concentrations at which 50% and 90% of suppression of parasitaemia is achieved. This test also leads to information about the relative potency compared to an appropriate standard drug. Information about oral bioavailability is inferred by comparison of activity on oral and parental administration (Fidock *et al.*, 2004).

ii) Onset/Recrudescence test: In this test mice are administered with a single dose of the test compound on day three of post-infection, subcutaneously. Control mice receive the suspension vehicle alone. Tail blood smears are prepared at intervals of 12 and 24 hours and extending to day 33 of the study. The slides are giemsa stained and assessed for parasitaemia. Results are expressed as the rapidity of onset of activity, time to onset of recrudescence, increase in parasitaemia and survival in number of days. (Fidock *et al.*, 2004).

iii) Prophylactic Test: Compounds with a therapeutic lead are tested for prophylactic activity by administering them prior to infection and smears continually examined to assess suppression of parasitaemia and survival times are measured in days (Fidock *et al*., 2004).

a) Hill's Test for Causal Prophylaxis and Residual Activity

In this method mice are inoculated with *P. yoelii*, N67 strain sporozoites and test compounds administered, using chloroquine as the standard. For a compound to be considered a truly causal prophylactic it must pass through four different phases, the last of which meticulously tests for residual effects upon blood stage parasites (Kaira *et al,* 2006).

Phase 1. This basic procedure involves detection of causal prophylactic activity of the test compound in mice. Test compound is administered three hours after a sporozoite inoculation. During the ensuing 14 day period, blood films are taken until 2% parasitaemia is achieved. If parsitaemia is not detected for 14 days the compound is considered to be fully protective.

Phase 2. Compound is tested for residual activity directed against blood stage parasites by administering a single dose of the test compound 48 hours before trophozoites are injected intravenously. If the time interval to reach 2% parasitaemia is similar to that of the control group, then it is considered that no residual activity has occurred.

Phase 3. Compounds suspected of prolonged residual activity are tested by administering sporozoites followed by the test compound 3hrs later. After an additional 48 hour period, blood is obtained from each mouse and injected intraperitoneally into an uninfected mouse. Blood films from the recipient are examined for 14 days or until patency develops. Residual activity is confirmed if less than 50% of the recipients develop parasitaemia. A compound has no residual activity if 75% or more recipients develop patent infection.

Phase 4. This procedure is used to determine whether a test compound has residual effect on erythrocytic stages during the 48 hour period of drug exposure in vivo. If no residual effect is seen then parsites remain infective. To determine if the erythrocytic parasites are still viable, mice are injected intravenously with trophozoites 48 hours after the test compound administration. After an additional 3-4 hours blood is harvested and injected into clean recipient mice. Blood films are taken and a comparison of the time interval to reach 2% parasitaemia is

compared with control mice. If the time interval is similar, it reflects that no permanent damage has been done to the parasites and thus no residual activity present.

2.3.2.3 Tertiary Biological Assessment

i) Cross-Resistance: Cross resistance studies employ the 4-day suppressive test in order to determine whether the same ED_{50} and ED_{90} values are observed in drug-resistant strains of rodent malaria when compared to *P. berghei* ANKA infection (Fidock *et al*., 2004).

ii) In vivo generation of drug resistance: To determine the potential of parasites to develop resistance to a new compound in vivo, the 2% relapse method is the most widely used for most classes of compounds (Peters and Robinson, 1999). This entails administering a dose of compound that when given 1 hour before infection, delays the development of 2% parasitaemia until about 7 - 10 days post-infection. When this parasitaemia is attained, the procedure is repeated and the time to reach 2% parasitaemia is monitored on a daily basis. The degree of resistance is expressed as a reduction in "delay time to 2 %". This method can be extended to demonstrate the stability of resistance following the removal of drug pressure and to select a partner compound that can slow the rate of development of resistance.

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All reagents used for in vitro and in vivo bioassays in this study were of biological and analytical grade. Adrenaline, albumax and catechine were sourced from Invitrogen, USA. SYBR Green I dye was sourced from Roche, Germany while gentamycin was purchased from Cambrex, USA. Milli-Q water was obtained from Ambion, UK. In addition, Tris base and sodium potassium tartrate were purchased from Roth, Germany. Fecal Calf Serum was purchased from PAA, Österreich, Austria, Plasma biochemical diagnostic kits were bought from Fortress Diagnostics Ltd, UK. All other chemicals and reagents were purchased from Merck and Sigma-aldrich, Germany.

3.1.2 *Plasmodium* strains used for in vitro assay

Plasmodium falciparum/3D7 (MRA-102, CQ sensitive) and *Plasmodium falciparum*/Dd2 (MRA-156, Multi-drug resistant) were obtained from the Biodefense and Emerging Infections (BEI) Research Resources (Manassas, Virginia, USA) and maintained as cryo-frozen stock of infected red blood cells (iRBCs) cultured in vitro.

3.1.3 *Plasmodium* strain for in vivo drug assay

Plasmodium chabaudi chabaudi (PccAS-Drug sensitive) was provided by Institut Pasteur, Korea while *P. berghei* ANKA (PbANKA - CQS) was generously donated by Institute of Primate Research, Nairobi, Kenya. Parasites were preserved as cryo-frozen stock of iRBCs, prepared from donor mice infected with sporozoites and maintained by continuous sub-passage in mice and rats.

3.1.4 Cell Lines for cytotoxicity

Hep G2 hepatoma and U2OS osteosarcoma epithelial cell lines were provided by Institut Pasteur, Korea, maintained in DMEM glutamax-1 containing sodium pyruvate, glucose, pyridoxine and supplemented with FCS and cryo-preserved in liquid nitrogen at -80^{o}C.

3.1.5 Experimental Animals

3.1.5.1 In vivo antiplasmodial Studies

Male and female BALB/c mice (6-9 weeks old) were obtained from the animal facility of Institut Pasteur Korea for *P. chabaudi chabaudi* infection, while BALB/c mice (6-10 weeks old) and wistar rats were obtained from the animal breeding facility of the Department of Pharmacology and Pharmacognosy, School of Pharmacy of the University of Nairobi, Kenya for *P.berghei* infection.

3.1.5.2 In vivo toxicity and evaluation of biological activities

Male and female Swiss albino mice (6-9 weeks old) and wistar rats (8-12 weeks old) were obtained from the animal house of the Institute of Medical research and Medicinal Plants Studies (IMPM), Yaounde-Cameroon, for in vivo toxicity and antioxidant studies.

Male and female Swiss albino mice (6-9 weeks old) and wistar rats (8-12 weeks old) were obtained from the animal breeding facility of the Department of Pharmacology and Pharmacognosy, School of Pharmacy of the University of Nairobi, Kenya, for evaluation of antipyretic, anti-inflammatory activities and antinociceptive.

3.1.6 Instruments

Air oven	Zhengzhou Amisy, China
Animal cages (rats and mice)	North Star Rescue, USA
Analgesy meter	Ugo Basile, USA
Analgesiometer	Inco, India
Automatic blood analyser (Hema Screen 18)	Hospitex Diagnostics. Italy
Centrifuge	Eppendorf, Germany
	Beckman Coulter®, VWR, USA
Biosafety cabinet	ThermoFisher Scientific, USA
CO_2 incubator	ThermoFisher Scientific, USA
Conical percolator	HARRISON'S, India
Culture flasks (Greiner Bio)	ThermoFisher Scientific, USA
Cuvettes	Eppendorf, Germany
DRC plate (Greiner Black; 384-well format)	SIGMA-aldrich, Germany

DRC plate sealing film (Platemax)	SIGMA-aldrich, Germany
EDTA tubes	KABE Labortechnik, Elsenroth, Germany
Erlenmeyer flask	ThermoFisher Scientific, USA
Eppendorf tubes	Eppendorf, Germany
Animal Feeding needles	Kent Scientific Corporation, USA
Hemocytometer (Brightline^{TM})	SIGMA-aldrich, Germany
Immersion oil	FLUKA, Germany
Infra-red thermometer	Shenzen Northvision Tech. Co. Ltd, China
Light microscope (CX31 Binocular/LCD screen)	Olympus, USA
Liquid handler (WellMate)	ThermoFisher Scientific, USA
Manual Blood Cell counter	Miniscience Inc, USA
Microscopic slides	SIGMA-aldrich, Germany
Micro-titer plate reader (VICTOR)	PerkinElmer, USA
Modular Culture chambers	BioSpherix, USA
Mortexer Multi-Head Vortexer	SIGMA-aldrich, Germany
Plethysmograph	SINGHLA Scientific Industries, India
Buchi Rotary evaporator (Rotavapor)	SIGMA-aldrich, Germany
Spectrophotometer (Jenway)	CAMLAB, UK
Vortexer (MixMate)	ThermoFisher Scientific, USA
Water bath with thermostat	Anova Inc, USA.

3.1.7 Preparation of Media and Buffer

Acetate buffer (pH 3.6): Sodium acetate trihydrate (0.31 g) was weighed and 1.6 mL of glacial acetic acid was added. The volume was made up to 100 mL with distilled water and pH checked to make sure it was at 3.6, using a pH meter.

Bicarbonate buffer (pH 10.2): Prepared by mixing 30 mL of 0.1 M $NaHCO_3$ and 70 mL of 0.1 M Na_2CO_3 and adjust pH to 10.2 using a pH meter.

HEPES buffer (pH 7.4): HEPES (23.3 g) was dissolved in 100 mL of distilled water and the pH was adjusted to 7.4 with potassium hydroxide (KOH), using a pH meter. It was then stored at 4^oC until used.

Phosphate buffer (pH 7.0): Prepared by adding 40.5 mL of $Na_2HPO_4.2H_2O$ (0.2 M) to 9.5 ml of $NaH_2PO_4.H_2O$ (0.2 M) and making up the volume to 100 mL.

Phosphate-buffered saline solution (PBS): Prepared by dissolving 160 g NaCl, 23.6 g NA_2HPO_4, 4.0 g KH_2PO_4 and 4.0 g KCl in 1 L of distilled water. The pH was adjusted to 7.4.

Freezing solution for the *Plasmodium* cryopreservation: It was composed of 28% glycerol, 3% sorbitol and 0.65% NaCl. For 250 mL, 180 mL of 4.2% sorbitol was mixed in 0.9% NaCl with 70 mL glycerol, filtered, sterilized and then stored at 8º C.

Thawing solution: Composed of 3.5% sterilized NaCl.

Giemsa Stain: One mL of Giemsa solution was dissolved in 19 mL buffer solution, pH 7.2.

3.2 METHODS

3.2.1 Ethnopharmacological survey of the preparation and use of *Nefang*

3.2.1.1 Ethical considerations

This was a minimal risk study and ethical approval was sought and obtained from the Institutional Review Board of the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé-Cameroon Cameroon through letter N/Ref: 076/82-62/MINRESI/M000 of 1st June 2011 and the Kenyatta National Hospital/University of Nairobi Ethics and Research committee, Nairobi-Kenya through Ref: KNH-ERC/A/324 of 5th December 2012 **(Annex I).**

3.2.1.2 Intellectual Property Agreement

Informed consent to participant in the survey was obtained using an appended prior informed consent (PIC) form (**Annex I**). All information obtained was treated confidentially.

This PIC form gave details on the objectives of the interview, fair and equitable sharing of benefits, contact of all the researchers concerned and assurance of anonymity and confidentiality, in accordance with the provisions of the United Nations Framework Convention on Biodiversity at the Earth Summit in Rio de Janeiro, Brazil in 1992.

3.2.1.3 Study Design

The study design was an exploratory survey. The interviews were conducted in Bayang community, Mamfe Central Sub-Division in the South West Region of Cameroon in the month of January and June, 2011. The objectives of this study were explained to traditional leaders of

some selected villages in the Bayang community of Mamfe Central Sub-Division, Manyu Division of the South West Province. Following their approved consent to carry out this research, interviews were conducted with the help of a physician working in the locality.

3.2.1.4 Study population

The study population consisted of adult local inhabitants of the villages in Mamfe Sub-division who had prepared and used *Nefang*. This included herbalists, traditional medicine practitioners (TMP: use other natural means of therapy in addition to herbs), herbs sellers, mothers, community leaders and village elders.

3.2.1.5 Eligibility criteria

Those who met the following criteria were included in the study:

- Agreed to participate in the study.
- Have prepared and used *Nefang* for at least one year.
- Resident in Mamfe sub-division

3.2.1.6 Sampling and participant recruitment

With Prior Informed Consent (PIC), authentic and well known stakeholders were identified through a Participatory Rural Appraisal (PRA) approach, with the assistance of local administrative officers and health workers. Twenty healthy eligible participants were recruited for the study.

3.2.1.7 Data collection

Interviews were considered the best qualitative method of data collection since they provided the best "real-life" experiences. These were done using a semi-structured questionnaire (**Annex III**) which was administered randomly to participants. Pre-determined questions with wordings that could be varied were asked from respondents, and extended according to how the interviewee response was perceived (Laforest, 2009). 20 traditional medicine practitioners were interviewed. Information on their traditional knowledge about malaria and their understanding of the cause was obtained, as well as the parts of these plants used for the treatment, method of collection, preparation, optimal composition, administration and side effects of this antimalarial remedy.

3.2.1.8 Data analysis

The guidelines for analysis of the questionnaires were by the inductive and deductive analysis method described by Harrel and Bradley (2009). Questionnaires were read a couple of times to identify different themes and differences in response among the respondents as follows. The key themes were:

i) Their understanding of the causes and physical manifestation of malaria.
ii) Methods of herbal antimalarial treatment using plant parts and how they are harvested.
iii) Aspects on the preparation of *Nefang*, method of administration and side effects.
iv) Conservation of *Nefang*.

3.2.2 Extraction and Phytochemical screening of the constituent plants of *Nefang*

3.2.2.1 Plant Extractions

The constituent plants of *Nefang* were identified by a botanist in the areas where they are abundant and frequently used in Cameroon. Fresh plant parts were harvested from their natural habitat between July and August 2011, in accordance with Good Agricultural and Collection Practice (GACP) (WHO, 2003). Identity confirmation and voucher specimen referencing was done by a botanist at IMPM, Yaoundé, Cameroon.

The freshly harvested plants parts were weighed, air-dried, pulverized, and conserved in sealed plastic bags for subsequent extractions.

Aqueous extraction was performed because this is the method used traditionally. Research evidence also shows that the ethanol extract may be a good alternative (Wilcox, 2011). Therefore, ethanol extraction was also performed. The extraction procedure was performed using the cold percolation method (Handa *et al*., 2008). Weighed quantities were immersed in water and ethanol (80% v/v) respectively for 4 h. Each of the macs was transferred to a conical percolator for 72 h and the extracts were filtered with a sieve of 80 µm pore size. The ethanol filtrate was first concentrated using a rotary evaporator. Both filtrates were then concentrated in an air oven at 60^{o}C. The extracts were weighed, yields appropriately recorded and stored in sealed plastic containers at 4^{o}C for subsequent use.

For in vitro antioxidant evaluation, 10 mL of distilled water was added to 100 mg of each pulverized plant and heated in a water bath at 100^{o}C for 90 min. This was allowed to cool down

overnight and the aqueous supernatant was pipetted and stored in sealed plastic containers at $4^{o}C$ for subsequent use.

3.2.2.2 Preliminary Phytochemical Screening

The constituent plant extracts were analyzed for the presence of alkaloids, anthocynanins, flavonoids, phenols, saponins, tannins, triterpenes and sterols according to standard methods (Harborne, 1973; Odebiyi and Sofowora, 1978).

a) Test for Alkaloids: The test extract (3 g) was stirred with ethanol containing 3% tartaric acid. The filtrate was aliquoted into three beakers and tested for alkaloids using the following: Hagar's reagent (saturated solution of picric acid), Mayers reagent (for narcotic alkaloids) and Marquin's reagent respectively. Precipitation in any of the three tests indicated the presence of alkaloids.

b) Test for Anthocyanins: Five drops of concentrated hydrochloric acid were added to the aqueous extract in a test tube and the change in colour observed; a red colour indicated the presence of anthocyanins.

c) Test for Flavonoids: Powdered plant material (2 g) was completely detanned with acetone. The acetone extract was evaporated in a warm water bath. The mixture was filtered while still hot, the filtrate cooled and then 5 mL of 20% NaOH was added to equal volume of the detanned aqueous extract. A yellow solution indicated the presence of flavonoids.

d) Test for Phenols: The test extract (100 mg) was dissolved in 3 mL of 70% ethanol. Three drops of 10% ferric (III) chloride was then added and the colour change observed. Appearance of a blue-violet colour indicated the presence of phenols.

e) Test for Saponins: The plant extract (5 g) was shaken with water in a test tube. Frothing which persisted on warming was taken as a preliminary evidence for the presence of saponins. A few drops of olive oil was added to 0.5 g of the extract and vigorously shaken. Formation of soluble emulsion in the extract indicated the presence of saponins.

f) Test for Tannins: Water extract of the sample was treated with 15% ferric chloride test solution. The resultant colour was observed. A blue indicated the presence of hydrolysable tannins. A second confirmatory test was carried out: 0.5 g of the extract was added into 10 mL of freshly prepared potassium hydroxide in a beaker and shaken to dissolve. A dirty precipitate indicated the presence of tannins.

g) Test for Triterpenes and Sterols (Liebermann Burchard Test): The extract (100 mg) was dissolved in 3 mL of methanol and then 0.2 mL each of chloroform, glacial acetic acid and concentrated sulphuric acid was added. The solution was then observed for colour change; the appearance of a greenish blue or purple pink colour indicated the presence of sterols or triterpenes respectively.

3.2.3 Cytotoxicity Screening

Cytotoxicity screening of each plant extract was carried out using the Resazurin Fluorimetric Cell Viability Assay method (Ahmed *et al*., 1994; Nociari *et al*., 1998) on Hep G2 hepatoma and U2OS osteosarcoma epithelial cell lines (provided by Institut Pasteur, Korea).

The cells were maintained in Dulbecco's MEM glutamax-1 containing sodium pyruvate, glucose, pyridoxine and supplemented with 10% FCS. For each ethanol extract, 200 mg/mL was prepared in 100% DMSO and diluted 1/10 in DMEM to obtain 20 mg/mL in 10% DMSO. Meanwhile 20 mg/mL of each aqueous extract was prepared in DMEM. Serial dilutions were prepared for each extract in an intermediate DRC plate (384-well format) containing 25 µL of 10% DMSO (ethanol extracts) or 25 µL of DMEM (aqueous extracts) to obtain concentrations of 2000 µg/mL – 0.061 µg/mL. Each dose response experiment comprised a 2-fold dilution of the extracts (2000 µg/mL max concentration, 16 dose-response points) in DMSO (Cf<1% ethanol extracts) or plain DMEM. Hep G2 hepatoma and U2OS osteosarcoma epithelial cells in log phase of growth were harvested by trypsinization (0.05% trypsin-treatment for 10 min) and then seeded at 5 $x10^3$ cells/per 100 µL of media in a DRC 384-well plate followed by a 24-h culture at 37^oC in a 5% CO_2 incubator to allow for cell attachment. To each well, 10 µL of each concentration of plant extract was added in triplicate. Each plate contained an untreated cell control, a blank control and puromycin standard. Prepared plates were incubated at 37^oC for 72 h in a 5% CO_2 environment. After incubation, 10 µL of Resazurin solution was added to each well and plates incubated for further 12 h. Fluorescence of the formed resorufin product in each well was measured at an excitation wavelength at 530 nm and emission wavelength at 590 nm using a VICTOR micro-titer plate reader. Fluorescence signal from each sample was obtained after background fluorescence subtraction.

3.2.4 Evaluation of in vitro antiplasmodial activity

In vitro susceptibility assays of *Nefang* and its constituent plant extracts were performed on cultured 3D7 (CQ sensitive) and Dd2 (MDR) strains of *Plasmodium falciparum* (Brandao *et al*., 1997). Both the 3D7 (MRA-102) and Dd2 (MRA-156) strains were obtained from the Biodefense and Emerging Infections (BEI) Research Resources (Manassas, Virginia, USA).

Human serum was obtained from Gyeonggi Blood Center, Korean Red Cros. The serum was inactivated in a water bed at 56^{o}C with disinfectant (Ethanol) for 60 min. Each 4-5 packets were mixed in 500 mL glass bottle and aliquots of 50 mL were stored at -20^{o}C in falcon test tubes until used.

Blood samples (blood type A+) obtained from Gyeonggi Blood Center, Korean Red Cross, were collected in 10 ml heparinized tubes. These samples were centrifuged for 5 min at 3000 rpm. The supernatant serum was discarded and the rest was washed, 2-3 times, with RPMI-1640 by centrifugation for 5 min at 3000 rpm. Serum (2-3 mL) was added to the finally obtained Red blood cells (RBCs) in each sample which were then stored at 8^{o}C until used.

Crypopreserved parasites were thawed by adding an equal volume of thawing solution. The thawed parasites were centrifuged at 3000 rpm for 5 min, the supernatant was discarded and the iRBCs were added to 0.8 mL of uninfected RBCs (uiRBCs) in 5 cm Petri dishes and cultured under microaerophilic conditions as described by Trager and Jensen (1976), with the following modifications. Parasites were maintained at 3% hematocrit in human red blood cells in media comprising RPMI 1640, 25 mM HEPES buffer (pH 7.4), 100 μM hypoxanthine, 16 μM thymidine, 20 μg/mL gentamycin and 0.5% Albumax. Cultures were grown at 37^{o}C in 75-cm^{2} flasks after gassing with a mixture of 4% CO_2, 1% O_2, and 95% N_2. Parasites were synchronized twice at an 8-hour interval by 5% sorbitol-treatment at the ring stage and then cultivated for one complete developmental cycle prior to the assays (Lambros and Vanderberg, 1979).

In vitro extract activities on 3D7 and Dd2 strains of *P. falciparum* were determined using the SYBR-Green 1 fluorescence-based method (Izumiyama *et al*., 2009). Each ethanol (200 mg/mL) and aqueous (20 mg/mL) extract was prepared as earlier described, replacing DMEM with RPMI 1640 in dissolving the aqueous extracts. The ring-stage parasitized erythrocytes (~10hpi) were then diluted in complete medium to 0.5% parasitaemia and 1.5% hematocrit, and 45 μL added to 5 μL extract preparation in a 384-well micro-titer plate (Greiner, black) in triplicate, using a

WellMate liquid handler. Control wells comprised of infected RBC (positive growth controls), uninfected RBC at 1.5% hematocrit (negative growth controls) and the antimalarial drugs chloroquine and artemisinin (Cmax = 1 μM) as treatment controls. The plates were then assembled in culture chambers, gassed, and incubated at 37^{o}C for 72 h prior to SYBR Green I fluorescence-based assay. Parasite growth was monitored microscopically with a Giemsa stained thin blood smear from a tracking culture and the experiment was terminated when the untreated parasites had reached the early trophozoite stage of the second cycle.

SYBR-Green 1 assay: Lysis solution (for 12mL) was prepared by adding 300 μL of Tris base (1M), 180 μL of EDTA (500mM), 9.6 μL of Saponin (15%) and 14.4 μL of Triton X-100 (100%) to 11.436 mL of Milli-Q water. Just before use, 0.3 μL of SYBR Green I (10,000x) was added per mL of Lysis solution.

Using the WellMate liquid handler, 25 μL of lysis /SYBR Green I solution was added directly to each 50 uL culture in the 384-well micro-titer plates and sealed with Platemax sealing film. Each plate was vortexed using a MixMate vortexer for 45 sec at 1700 rpm and then wrapped with aluminum foil and incubated at room temperature for 1 h prior to fluorescence reading using a VICTOR micro-titer plate reader (Ex 485 nm/Em= 530 nm).

3.2.5 Characterisation of the interactions between the components of *Nefang*

3.2.5.1 Equipotency Extract combination ratios

In vitro susceptibility assays of paired constituent plant extracts of *Nefang* were performed on cultured Dd2 (MDR) strain of *Plasmodium falciparum* using the fixed ratio method (Ohrt *et al*., 2002). The parasites were maintained in culture and synchronized as earlier described in the in vitro antiplasmodial studies.

Paired combinations of aqueous and ethanol extracts of the constituent plants of *Nefang* were prepared at equipotency ratios ($5EC_{50}A$:$5EC_{50}B$) from a stock of 20 mg/mL. Two-fold serial dilutions were then prepared in an intermediate 384-well DRC plate containing 5% DMSO (ethanol-containing extract pairs) or plain RPMI 1640 (aqueous extract pairs) to obtain 16 dose-response points. The ring-stage parasitized erythrocytes (~10hpi) were then diluted in complete medium to 0.5% parasitaemia and 1.5% hematocrit and 45 μL added in triplicate to 5 μL extract pair preparation in a 384-well plate (Greiner, black), using a WellMate liquid handler. Control wells comprised of infected RBC (positive growth controls), uninfected RBC at 1.5% hematocrit

(background controls) and the antimalarial drug combinations chloroquine/chloroquine and chloroquine/artemisinin at equipotency ratios as drug-drug interaction controls. The plates were then assembled in culture chambers, gassed, and incubated at 37^{o}C for 72 h prior to SYBR Green I fluorescence-based assay. Parasite growth was monitored microscopically with a Giemsa stained thin blood smear from a tracking culture and the experiment was terminated when the untreated parasites had reached the early trophozoite stage of the second cycle.

3.2.5.2 Variable Potency Extract combination ratios

Following the above assays, extract combinations that demonstrated promising synergistic or additive interactions were selected and further analyzed using a variable potency ratio drug combination approach starting at $5EC_{50}A{:}0EC_{50}B$ to $0EC_{50}A{:}5EC_{50}B$ paired combinations. Parasite growth in the plate wells was then assessed using the SYBR Green I fluorescence-based assay as described earlier.

3.2.5.3 Isobologram Analysis

The selectivity index ($SI=CC_{50}/EC_{50}$) (Benoit-Vical *et al*., 1999) was calculated for each extract as an indication of its toxicity relative to the observed antimalarial activity.

Furthermore, the obtained EC_{50}s were used to calculate 50% fractional inhibitory concentrations (FIC_{50}) and combination indices as previously described (Berenbaum, 1978; Saiman *et al.*, 1996). That is $FIC_{50}A = EC_{50}$ of drug A in combination/EC_{50} of drug A alone.

The sums of the FIC_{50} gave the combination index (CI) of the pair ($CI_{A/B} = FIC_{50}A + FIC_{50}B$). For CI values, sums of less than 1.0 ($CI < 1$) represented a trend toward synergy, and greater than 1.0 ($CI > 1$) represented a trend toward antagonism.

FIC_{50}s of drug A and drug B at different combination ratios were used to plot isoboles, with the line of additivity running from point (0,1) of the vertical axis to point (1,0) of the horizontal axis. Synergy or antagonism was revealed when the plotted FIC_{50} values was below or above the line of additivity respectively.

3.2.6 In vivo Antiplasmodial Activity of *Nefang*

3.2.6.1 Experimental Animals

Ethical approval for the use of experimental animals was sought and obtained from Institut Pasteur-Institutional animal Care and Use Committee (IPK-IUCAC), Korea) through IACUC

letter No. IPK 12009 of 29th October 2012 and the Kenyatta National Hospital/University of Nairobi Ethics and Research committee (KNH/UON-ERC), Nairobi-Kenya through Ref: KNH-ERC/A/324 of 5th December 2012 **(Annex I)**.

The care and use of experimental animals described in the rationale and methodology of this research are in accordance with the goals, outcomes and considerations defined in the Guide for Care and Use of Laboratory Animals, by the Committee for the update of this guide, National Research Council of the National Academies (2010). BALB/c mice (20 – 25 g) and wistar rats (160 – 180 g) used for the experiment were obtained from the animal facility at Institut Pasteur Korea and University of Nairobi, Kenya respectively. They were housed in standard cages and were maintained on a standard pelleted feed.

3.2.6.2 Parasite infection of experimental animals

The chloroquine sensitive strain of *P. berghei* (ANKA) was generously donated by the Institute of Primate Research (IPR), Nairobi, Kenya while the *P. chabaudi chabaudi* was obtained from the Centre for Neglected Diseases Drug Discovery (CND3), Institut Pasteur Korea, as cryo-frozen stock of parasitized red blood cells (PRBCs). The parasites were prepared through two cycles of passage of the PRBCs in rats and mice. Donors with parasitemia level of 20-30% were sacrificed and blood collected by cardiac puncture into heparinized tubes. The blood was then diluted with phosphate buffered saline (PBS) based on parasitemia level of each donor and the RBC count of normal mice and rats, such that 1 mL blood contained 5 × 107 parasites. The experimental animals were each treated with 1×10^7 PRBCs by intraperitoneal injection (Basir *et al*., 2012).

3.2.6.3 Evaluation of the Suppressive Activity (Peter's 4-Day Test)

Following the in vitro antiplasmodial assay, *Nefang*, ***Pg*** (the most active constituent solvent extract) and ***MiB/Pg*** (the solvent extract combination that showed the most promising synergistic activity) were chosen for *in vivo* studies. Suppressive activity of *Nefang* was evaluated in early *P. chabaudi chabaudi* and *P. berghei* (ANKA) infection in BALB/c mice and wistar rats respectively, using the method described by Knight and Peters (1980). Forty-five mice and forty-five rats were each randomly divided into fifteen groups of three each. On the first day (D0), the mice were each infected with 10^7 *P. chabaudi chabaudi* while the rats were each infected with *P.*

berghei (10^7) infected RBCs. Three hours later, the experimental animals in each case were administered 10 $mLkg^{-1}$ body weight (bwt) of chloroquine (10 $mgkg^{-1}$) (Group 2 - positive control) and pyrimethamine (30 $mgkg^{-1}$) (Group 3 – positive control) while *Nefang* (Group 4 – 7), *Pg* (Group 8 – 11) and *MiB/Pg* (Group 12 – 15) aqueous extracts were administered orally at a dose of 75, 150, 300 and 600 $mgkg^{-1}$ bwt respectively for four consecutive days (D0 – D3). Group 1 (negative control) received PBS. The body weight of each mouse were measured before infection (D0) and on the fifth day (D4) using a sensitive digital analytical weighing balance, while the body temperature was measured before infection and three hours after infection (D0) and then monitored daily to the fifth day (D4).

On the fifth day (D4), a thin blood film was made from the tail blood of each experimental animal, fixed in methanol and stained with giemsa to reveal parasitized erythrocytes out of 500 in a random field of the microscope. Parasitemia was determined by light microscopy using a 100X objective lens and the following equation:

$$\%\text{parasitaemia} = \frac{\text{No. of parasitized RBC}}{\text{Total no. of RBC counted}} \times 100$$

Average percentage chemosuppression was calculated as

$$100\left[\frac{A-B}{A}\right]$$

where *A* is the average percentage parasitemia in the negative control group and *B* is the average percentage parasitemia in the test group.

3.2.6.4 Evaluation of the Prophylactic Activity

The repository activity of *Nefang* was assessed using the method described by Peters (1965). The mice were randomly divided into seven groups of three BALB/c mice each. Group 1 (negative control) was treated with 10 $mLkg^{-1}$ of PBS, group 2 and 3 (positive controls) – 10 $mgkg^{-1}$ of CQ and 30 mg/kg^{-1} of pyrimethamine respectively, group 4 – 7 (test groups) – 75, 150, 300 and 600 $mgkg^{-1}$ of *Nefang*. Administration of the extract and standard drugs continued for three consecutive days (D0 – D2). On the fourth day (D3), the mice were inoculated with 10^7 *P. berghei* and parasitemia level was assessed by blood smear 72 h later.

3.2.6.5 Evaluation of the Curative Activity (Rane's Test)

The schizontocidal activity of *Nefang* during established infection was evaluated using the method described by Ryley and Peters (1970). Ten million *P. berghei* (ANKA) parasitized RBCs were injected intraperitoneally into each of twenty-one rats on the first day (D0). Seventy-two hours later (D3), the rats were randomly divided into seven groups of three rats each. Different doses of *Nefang*, 75, 150, 300 and 600 $mgkg^{-1}$, were orally administered to rats in groups 4 – 7, CQ (10 $mgkg^{-1}$) to group 2 (positive control), artesunate (5 $mgkg^{-1}$) to group 3 (positive control) and group 1 (negative control) was treated with PBS. *Nefang* and standard drugs were administered once daily for 5 days. Giemsa stained thin smears were prepared from tail blood samples collected on each day of treatment to monitor parasitemia level. The body weight and temperature were measured before infection (D0) and from the fourth day (D3) to the eighth day (D7) while the mean survival time (MST) of the rats in each treatment group was determined over a period of 29 days (D0 – D28) as follows:

$$\text{MST} = \frac{\text{Number of days survived}}{\text{Total number of days (29)}} \text{ X } 100$$

3.2.7 In vivo toxicological profile of *Nefang* and its constituents

3.2.7.1 Experimental animals

Ethical approval for the use of experimental animals was sought and obtained from the Institution Review Board of the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaounde-Cameroon through letter N/Ref: 076/82-62/MINRESI/M000 of 1st June 2011 and the Kenyatta National Hospital/University of Nairobi Ethics and Research committee, Nairobi-Kenya through Ref: KNH-ERC/A/324 of 5th December 2012 **(Annex I)**.

Swiss albino mice and wistar rats used for the experiment were obtained from the animal facility of IMPM. They were housed in standard cages and were maintained on a standard pelleted feed.

3.2.7.2 Acute Toxicity Testing (Single Dose Toxicity Testing)

The acute oral toxicity of the aqueous and ethanol extracts was evaluated in Swiss albino mice according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD, 2001; Shetty Akhila *et al*, 2007). Following the fasting period, the mice

were weighed and the dose was calculated in reference to the body weight. Volume of the extracts given to the mice was 10 $mLkg^{-1}$ bwt. The crude extract was suspended in a vehicle (distilled water and corn oil for the aqueous and ethanol extracts respectively). Single male and female adult Swiss albino mice (25-30 g) were dosed in a stepwise procedure using the fixed doses of 5, 50, 300, 1200 and 2000 $mgkg^{-1}$ bwt of the aqueous and ethanol extracts respectively and animals were observed for signs of toxicity. If there was no mortality or signs of toxicity at the highest dose, then an upper limit dose was used for the main test. For the main test, a single high oral dose of 5000 $mgkg^{-1}$ bwt of each crude extract was administered to three male (Test 1) and three female (Test 2) mice in the treatment groups, whereas the control groups received the vehicle. Food was provided to the mice approximately an hour after treatment. The animals were observed 30min after dosing, followed by hourly observation for 8 h and once a day for the next 13 days. All observations were systematically recorded with individual records being maintained for each animal. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

3.2.7.3 Sub-acute and Sub-chronic Toxicity Testing (Repeated Dose Toxicity Testing)

Sub-acute and sub-chronic toxicity of the aqueous and ethanol extracts was evaluated in Wistar rats (170 – 210 g). For each aqueous extract the rats were divided into 4 groups (A, B, C, D) of 12 rats each, while for each ethanol extract the rats were divided into 4 groups (E, F, G, H) of 6 rats each. Groups A and E served as control and received the vehicle only (water and corn oil for aqueous and ethanol extracts respectively), while groups B, C, D and F, G, H served as test groups and were administered graded doses of 250, 500 and 1000 $mgkg^{-1}$ bwt of each extract respectively. At the end of 28 days (sub-acute toxicity), blood was collected through the jugular vein and then 6 rats in each group of A, B, C, D and all the rats of E, F, G, H were sacrificed after an overnight fast, under diethyl ether anaesthesia, whereas the remaining 6 rats of each of groups A, B, C and D (labelled B1, C1, D1, E1) were sacrificed in like manner at the end of 90 days (sub-chronic toxicity). Prior to killing the animals, blood was collected through the jugular vein after an overnight fast into separate EDTA tubes, one for immediate hematological analysis and the other for collection of plasma after centrifugation (3000 rpm × 10 minutes), to be used for biochemical assays respectively. The liver, kidney and heart were harvested immediately

clean of blood using physiological saline and weighed. The liver and kidney were then fixed in 10% formalin for histopathological examination

Hematological analysis was done using a Hospitex Diagnostics Hema Screen 18 automatic analyser and parameters analysed were full white blood cell (WBC) count, red blood cell count and platelet count as well as their indices.

Safety endpoints for biochemical assays included total proteins (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), urea (BUN), uric acid (URIC), creatinine (CRE), triglycerides (TGY), cholesterol (CHOL) and glucose (GLU). All plasma biochemical assays were performed using standard analytical diagnostic kits from Fortress Diagnostics Ltd, U. K., while optical densities were read using a light spectrophotometer.

The fixed tissues were then dehydrated with 100% ethanol solution and embedded in paraffin. They were then processed into 4-5 µm thick sections and stained using hematoxylin-eosin and observed under light microscope as earlier described by Gabe (1968).

3.2.8 Biological activities of the constituent plants of *Nefang* that complement in vivo antimalarial effects

Ethical approval for the use of laboratory animals was sought and obtained from the Institutional Review Board of the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé-Cameroon through N/Ref: 076/82-62/MINRESI/M000 of 1st June 2011 and the Kenyatta National Hospital/University of Nairobi Ethics and Research committee, Nairobi-Kenya through Ref: KNH-ERC/A/324 of 5th December 2012 **(Annex I)**.

3.2.8.1 Test for Antioxidant Activity

3.2.8.1.1 In vitro 2,2-Di-Phenyl-1-Picryl-Hydrazyl (DPPH) Radical Scavenging Activity Assay

DPPH radical scavenging activity was measured using the method described by Yen and Duh (1994). Twenty µL of the aqueous plant extract was introduced to 2 mL methanol solution of DPPH (0.3 mM) and incubated at 37^{o}C in the dark for 30 minutes. The extract was replaced by methanol for the control and catechine for the standard. Absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. All the tests were performed in triplicate and the results averaged. The percentage DPPH radical scavenging activity was calculated by comparing

the results of the test with those of the control (not treated with extract) using the following equation:

Percentage radical scavenging activity = (1– absorbance of test/absorbance of control) × 100.

3.2.8.1.2 In vitro Estimation of Total Phenolic compounds

Total soluble phenolic in each extract was determined using the Folin-Ciocalteau reagent (FCR) according to the method described by Slinkard and Singleton (1977). Each aqueous plant extract (0.1 mL) was transferred to 100 mL Erlenmeyer flask then final volume adjusted to 46 mL by addition of distilled water. After 3 min, 1 mL of FCR and 3mL of Na_2CO_3 (2%) was added to this mixture. The mixture was then incubated for 2 hours at room temperature then the absorbance measured at 760 nm. All the tests were performed in triplicate and the results averaged. The concentration of total phenolic compounds in each extract was estimated as milligram of catechine equivalent by linear interpolation of a catechine standard curve.

3.2.8.1.3 In vitro Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric Reducing Antioxidant Power of each extract was determined according to the method of Benzie and Strain (1996). The FRAP reagent consisted of ten parts acetate buffer (300 mM pH 3.6), one part of TPTZ (10 mM in 400 mM of HCl) and one part of ferric chloride (10 mM). Two mL of freshly prepared FRAP solution was transferred to a cuvette in a spectrophotometer and absorbance read after 6 mins at 593 nm. Each extract (75 µL) was transferred to a cuvette containing 2 ml of FRAP solution, agitated for 10 min and absorbance read after 12 min at 593 nm. The ferric reducing antioxidant power in each extract was determined as milligram of catechine equivalent by linear interpolation of a catechine standard curve.

3.2.8.1.4 Evaluation of in vivo Antioxidant Activity

From previous in vivo antiplasmodial studies, we observed minimum and maximum activity at doses of 100 and 500 $mgkg^{-1}$ bwt respectively, and these doses were used in the in vivo study. Wistar rats weighing 170-200 g were divided into four groups (A, B, C, D) of 5 animals each. The first group was treated with the vehicle (corn oil). Oxidative stress was induced in the other three groups of animals by oral administration of 10 $mLkg^{-1}$ bwt carbon tetrachloride in corn oil

(1:5 v/v). Two of these groups were treated with 100 and 500 $mgkg^{-1}$ bwt of *Nefang* aqueous extract by daily oral administration for 14 days. Twenty-four hours after the last dose, all the animals were anesthetized by intramuscular administration of Zoletil 50 (30 mg/Kg) + xylazine (5 mg/kg) for 5-10 minutes, blood was harvested by cardiac puncture into EDTA tubes after which they were sacrificed by cervical dislocation. The blood sample was centrifuged at 3000 rpm for 10 min. Supernatant plasma was collected for biochemical analysis. Physiological saline (0.9%) was then added to the packed cellular layer to double the volume. After mixing, it was centrifuged at 3000 rpm and the supernatant discarded. This procedure was repeated thrice and the erythrocytes were then isolated and stored at -20^{o}C for subsequent use. The liver, kidneys and heart were harvested, cleaned of blood and weighed.

3.2.8.1.5 Determination of Biochemical Parameters

The plasma was subjected to biochemical analysis using standard analytical kits from Fortress Diagnostics Ltd, UK. The parameters that were determined included glucose (GLU), cholesterol (CHOL), triglycerides (TGY), alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine (CRE).

3.2.8.1.6 Analysis for Oxidative Stress Markers

Red blood cells were used as a cellular model for evaluating the ability of antioxidants to cross the plasma membrane into living cells. Oxidative stress markers analyzed in the RBC hemolysate;

a) Superoxide Dismutase (SOD) activity was determined using the method described by Misra and Fridovich (1972). An aliquot of 0.2 mL of the haemolysate was added to 2.5 mL 0.05 M carbonate buffer (pH 10.2). This was allowed to equilibrate in a spectrophotometer. The reaction was started by adding 0.3 mL of freshly prepared 0.3 mM adrenaline to the buffered sample mixture. This was quickly mixed by inversion and placed in the spectrophotometer. The reference cuvette contained 2.5 mL of the buffer, 0.3 mL of the substrate and 0.2 mL distilled water. Increase in absorbance against a blank at 480 nm was monitored every 30 seconds for 150 seconds.

SOD was calculated in units as the amount necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during one minute as shown below:

Calculation

$$\Delta A \min = \frac{Abs_2 - Abs_1}{1.5}$$

$$\% \text{ inhibition} = \frac{100 - [\text{Abs} \times 100]}{\text{Abs blank/min}}$$

50% inhibition = 1 unit

X% inhibition = Y units

$$\text{SOD units/mg protein} = \frac{SOD \text{ unit/mL}}{\text{Protein mg/mL}} \times dil. \text{factor}$$

b) Catalase activity was determined using the method described by Sinha (1972). In six different tubes containing increasing concentration of hydrogen peroxide (0-640 µM), 2 mL of acidified potassium dichromate was added. Each tube was shaken and heated at 100^{o}C for 10 minutes then allowed to cool and optical density read at 570 nm. A standard curve of absorbance against concentration of hydrogen peroxide was then plotted.

One mL of diluted haemolysate (1:10 v/v) was added to a tube containing 2 mL of hydrogen peroxide and 2.5 mL of 0.01 M phosphate buffer (pH 7.0) and mixed. From this mixture 1 mL of solution was withdrawn after every 30 seconds and transferred into 2 mL of acidified potassium dichromate for 120 seconds. The mixture was shaken and heated for 10 minutes at 100^{o}C. After cooling the optical density was measured at 570 nm against a blank.

The activity was then calculated in units of catalase as the amount required to catalyze the reduction of 1 µM of H_2O_2 in 1 minute.

c) Lipid peroxidation was measured by applying the malondialdehyde (MDA) assay method earlier described by Biswas *et al.* (1995). To a tube containing 2 mL of acetic acid and 2 ml of a working reagent (NaOH + Thiobarbituric acid), 0.4 mL of diluted haemolysate (1:10 v/v) was added. The tubes were incubated in an oven at 100 °C for 20 minutes; the absorbance was then

read at 532 nm against the blank. The concentration of lipid peroxides was then calculated from the molar extinction coefficient of 1.56×10^5 $mole^{-1}$ cm^{-1}.

$$\text{Concentration of MDA} = OD/\ l\varepsilon\ (\varepsilon = 1.56 \times 10^5\ mole^{-1}\ cm^{-1})$$

Where ε is the molar extinction coefficient and l is the length of the cuvette (cm).

d) **Total proteins** were estimated using the method described by Lowry *et al*. (1951). To different tubes containing 0.2 mL of diluted haemolysate and 0.2 mL bovine albumin (2 g/dL) standard, 2 mL of distilled water and 1mL of working reagent 1 [1 vol ($CuSO_4$+NaK) + 100 vol (Na_2CO_3 + NaOH)] were added. The contents were mixed by inversion and incubated at room temperature for ten minutes after which 0.25 µL of 50% Folin Ciocalteau reagent was added, mixed and incubated at room temperature for ten minutes. Absorbance was then read at 570 nm against a blank.

The concentration of total proteins in the RBC haemolysate was determined from the equation below:

Protein (g/dL) = (Concentration of Standard/OD of Standard) × OD of Test Sample.

3.2.8.2 Test for Antipyretic Activity

3.2.8.2.1 D-Amphetamine induced pyrexia method

The antipyretic activity of the constituent aqueous plant extracts of *Nefang* was determined by the D-Amphetamine induced pyrexia method (Mbagwu *et al*., 2007). Adult wistar rats (170-200 g) of both sexes were fasted for 24 h but allowed water *ad libitum* and used for this experiment. At zero hour, the basal temperature of all the animals was taken by using an infra-red thermometer. D-amphetamine (5 $mgkg^{-1}$) was then administered to all the animals. After 30 minutes, sixty four animals with an increase in body temperature of 0.5 - 1^oC were selected for the study.They were randomized into sixteen groups of four rats each and Group 1 was treated with 10 $mLkg^{-1}$ of the vehicle (normal saline), group 2 - the reference drug (Paracetamol, 150 $mgkg^{-1}$) and two groups for each extract were treated orally with 200 and 400 $mgkg^{-1}$ bwt of each extract. Body temperatures were obtained at 60, 120, 180 and 240 minutes after drug administration. The temperatures of the extract-treated groups were compared with those of the control to evaluate the activity of each extract.

3.2.8.2.2 Brewer's yeast-induced hyperpyrexia method

The method used to determine the antipyretic activity of the constituent plants of *Nefang* was as previously described by Turner (1965). Adult wistar rats (170 – 200 g) of both sexes were fasted for 24 h but allowed water ad libitum, were used for this experiment. They were then randomized into sixteen groups of three rats each. At zero hour, the basal temperature of each rat was taken by using an infra-red thermometer. Thereafter, each animal was administered subcutaneous injection of 10 $mLkg^{-1}$ of aqueous yeast suspension (20 % w/v) for elevation of body temperature of rats. Eighteen hours post yeast injection, 10 $mLkg^{-1}$ of the standard drug (aspirin, 100 $mgkg^{-1}$), the vehicle (normal saline), 200 and 400 $mgkg^{-1}$ of each aqueous extract were administered orally to different groups of rats. Body temperature of each animal was recorded at 0, 60, 120 and 180 minutes after drug administration. The pre-test and post-test temperatures of the extract-treated groups were compared with those of the standard drug-treated group to evaluate the activity of each extract.

3.2.8.3 Test for Anti-inflammatory Activity

In vivo anti-inflammatory activity of the constituent plants of *Nefang* was investigated using the carrageenan-induced rat paw edema method as described by Mohamed Saleem *et al*. (2011). Ninety-two adult wistar rats were randomly divided into twenty-three groups of four rats each. Paw volumes were measured at zero minute using the Archimedes principle of mercury displacement in a plethysmograph. Thereafter, 10 $mLkg^{-1}$ of the vehicle (normal saline), the reference drug (Indomethacin, 10 $mgkg^{-1}$), and 100, 200 and 400 $mgkg^{-1}$ bwt of each extract were administered orally to different groups of rats. Thirty minutes later, paw edema was induced in each rat by injecting 0.1 mL of carrageenan (1% in normal saline) to the right hind paw. Paw volumes were measured at 60, 120, 180 and 240 min. The difference between the paw volume at zero minute and each time point was taken as a measure of edema. The percentage inhibition of paw volumes in extract-treated groups was compared with the standard drug to evaluate the activity of the extracts.

3.2.8.4 Tests for Antinociceptive Activity

3.2.8.4.1 Tail Pressure method

The method described by Randall and Selitto (1957) and modified by Kitchen (1984) was used for the evaluation of analgesic activity of the extract using an analgesimeter. Twenty-seven Swiss albino mice (20 – 25 g) were randomly divided into nine groups of four each. At zero minutes, the tail of each mouse was put on the tip of the Analgesy Meter and pressure gradually increased up to a maximum of 25 units. The pressure at which the mouse began to struggle was noted and recorded. Mice in group 1 (positive control) were treated by intraperitoneal route with 0.1 mL of the standard drug (morphine, 5 $mgkg^{-1}$); group 2 – PBS; while group 3 – 7 were each treated with 10 $mLkg^{-1}$ of 1000 $mgkg^{-1}$ bwt of each aqueous plant extract. After 30 min, mechanical pain was induced on the tail of each mouse in turn by the use of analgesy meter which exerted force at a constantly increasing rate, while the mouse was restrained. The force was continuously monitored by a pointer moving along a linear scale. Pain response was taken to be the point at which the mouse struggles to set itself free or its tail slips off the plinth of the instrument. The weight causing pain before treatment (0) and then at 30, 60, 90 and 120 minutes after the extract and drug treatment to individual mouse in all the groups was recorded. Percentage pain inhibition (increase in pain threshold) produced relative to the control group was calculated for each extract-treated group and compared with the standard drug-treated group to evaluate the activity of each extract.

$$\text{Pain inhibition (\%)} = \frac{Ft - Fo}{Fo} \text{ X } 100$$

Where Ft = Force at which the animal tries to free its tail after drug administration.
Fo = Force at which the animal tries to free its tail before drug administration.

Extracts that showed activity were then subjected to further dose-response testing.

3.2.8.4.2 Tail Flick response method

The dose-response analgesic activity of each of the constituent plants of *Nefang* was determined by radiant heat tail-flick method in Swiss albino mice as described by Ramesh (2010). The tail flick latency was assessed by an analgesiometer (Inco, India). Before selecting each mouse for

the test, a baseline reaction was taken by placing the tip of the tail of each mouse on the radiant heat source, from an analgesiometer. The strength of the current passing through the naked nichrome wire was kept constant at 5 amperes. The distance between the heat source and the mouse tails was 1.5 cm and the application site of the heat on the tail was maintained within 2 cm. Each mouse in turn was held in a suitable re-strainer with tail protruding out. Radiant heat from the analgesiometer was applied on a designated spot on the tail. The time interval between the onset of stimulus and withdrawal (flicking) of the tail was taken as the reaction time for each mouse. Cut-off reaction time was 10 sec to avoid tissue injury during the process. The initial flicking time of each group was considered as the control readings.

One hundred and ten Swiss albino mice (20 – 25 g) that responded positively were randomly divided into twenty-two groups of five mice each. Group 1 (positive control) was treated with 0.1 mL of the standard drug (Paracetamol, 10 $mgkg^{-1}$) while the rest of the twenty-one groups were divided into three groups per aqueous extract and administered 10 $mLkg^{-1}$ of 300, 600 and 1200 $mgkg^{-1}$ bwt of each aqueous extract by intraperitoneal injection. The tip of the tail of each mouse was then exposed to the heat source from the analgesiometer and reaction time recorded at intervals of 30 minutes till 120 minutes. The percentage of pain inhibition was calculated for each extract –treated group and compared with that of the standard drug-treated group to evaluate the activity of each extract.

$$\text{Percentage inhibition} = \frac{(\text{Test mean})_t - (\text{Control mean})_t}{(\text{Control mean})_t} \text{ X } 100$$

Where t = time

3.2.8.4.3 Hot Plate method

The dose-response analgesic activity of each of the constituent plants of *Nefang* was tested in Swiss albino mice using the hot plate method described by Janssen and Jagenea (1957), with modifications. Ninety-two mice were randomly divided into twenty three groups of four mice each. Group 1 was treated with 0.1 mL of normal saline, group 2 - the reference drugs (piroxicam, 20 $mgkg^{-1}$ and Pethidine, 50 $mgkg^{-1}$), while the rest twenty-one groups were divided into three groups per extract and administered 10 $mLkg^{-1}$ of 300, 600 and 1200 $mgkg^{-1}$ bwt of each extract respectively. A 500 mL inverted glass conical flask, on a clamp and stand was

connected by rubber tubing to a hot water bath with thermostat and pump and the temperature regulated to 55^{o}C, such that the temperature on the surface of the inverted conical flask is same as that of the water bath. Each mouse was then placed in the conical flask in order to obtain its response to heat-induced nociceptive pain stimulus. Licking of the forepaws and eventually jumping off the conical flask was taken as an indicator of the animal's response to heat-induced nociceptive pain stimulus. The reaction time for each mouse was recorded in seconds. Readings were taken at time zero before administration of drugs/extracts and after that at intervals of 30, 60 and 120 minutes after extract administration. Cut off time in the absence of response was 60 s to avoid tissue damage to the mice paws (Sharma *et al.,* 1982). The percentage of pain inhibition was calculated for each extract-treated group and compared with that of the standard drug-treated group to evaluate the activity of each extract.

$$\text{Percentage inhibition} = \frac{(\text{Test mean})_t - (\text{Control mean})_t}{(\text{Control mean})_t} \text{ X } 100$$

Where t = time

3.2.9 Statistical Analysis

All data was entered and stored in Excel spreadsheet. The data was examined regularly for consistency and for errors. Data was backed up at regular intervals. Access to the data was restricted to the supervisors and the researcher.

All variables were subjected to descriptive data analysis (SPSS 20.0 Inc., USA). All continuous variables were expressed as the mean and the standard deviation from the mean. The results were analysed statistically using one-way ANOVA followed by Neuman-Keuls multiple comparison test to identify the differences between treated groups and controls. The data was considered significant at $P < 0.05$.

To determine 50% cytotoxic (CC_{50}) and effective (EC_{50}) concentration values for each extract or extract combinations, the obtained data were analyzed using GraphPad Prism 6.0. The logarithm of the extract concentration was plotted against its activity represented by the fluorescence

reading to obtain a nonlinear regression curve-fitting and a variable slope sigmoidal dose-response curve.

For experiments involving animals, the cumulative deaths and survivals in each group were calculated. The cumulative deaths were a progressive sum of dead animals for low to high doses. The cumulative survivals are the cumulative sum of survivors for high to low doses. Statistical significance testing was done using one way ANOVA followed by Neuman-Keuls multiple comparison test.

4.0 RESULTS

4.1 ETHNOPHARMACOLOGICAL SURVEY OF THE PREPARATION AND USE OF NEFANG

4.1.1 Data Generation

Among the 20 people interviewed from 7 villages, were 5 elders/community leaders, 4 herbalists, 5 herb sellers, 3 mothers and 3 traditional medicine practitioners as shown on **Table 4.1**.

Table 4.1. Demographic characteristics of the respondents

Designation	Name/Description	Number of Respondents (%)
Village/localitity	Bachuo-ntai	3 (15)
	Besongabang	2 (10)
	Egbekaw	4 (20)
	Eyanchang	2 (10)
	Mamfe/Small Mamfe	6 (30)
	Nchang	1 (5)
	Okoyong	2 (10)
Occupation	Community Leaders/ Village Elders	5 (25)
	Herbalists	4 (20)
	Herb Sellers	5 (25)
	Mothers	3 (15)
	Tradional Medicine Practitioner	3 (15)
Gender	Male	13 (65)
	Female	7 (35)

Amongst the people interviewed, 80% were above fifty years old. All respondents spoke and understood *Kenyang* language spoken by the scientist for easy communication and the pre-determined questions only provided guidelines for the research but further questions naturally arose between interviewer and respondents to provide valuable insights into methods of herbal practice. However, the questions were modified or discontinued to respect the wishes of the respondents especially on sensitive issues.

4.1.2 Traditional Knowledge about Malaria

Knowledge of malaria among the respondents was good and they even recognized the importance of sleeping under impregnated mosquito bed nets. All respondents knew that malaria was caused by mosquito bites, though some mentioned indirect causes like hot weather (more mosquitoes), stagnant water, dirty environment and unhygienic living conditions. They also recognized some of the frequent symptoms of malaria like high body temperature, headache, shivering, weakness, loss of appetite, diarrhea and vomiting as shown in **Table 4.2.**

Table 4.2. Malaria symptoms mentioned by respondents

Symptom	No of Respondents	%
High Body Temperature	20	100
Shivering	20	100
Headache	19	95
Vomiting	18	90
Loss of appetite	15	75
Weakness	15	75
Abdominal Pain	12	60
Diarrhea	10	50
Blisters on mouth	8	40
Dizziness	2	10
Sweating	12	60
Anemia	4	20
Nausea	6	30

4.1.3 Plant parts used for the preparation of *Nefang*

All respondents could identify the constituent plants of *Nefang* and the plant parts used in its preparation. The plants were identified by species, family, common and vernacular name. Voucher specimens of the plants were then deposited at the IMPM herbarium.

All respondents recognized the importance of harvesting these plants species from their natural habitat or from well-kept nurseries or botanical gardens. The plant parts to be used were either harvested in the evening or early in the morning and the method of harvest was in accordance with the preservation of biodiversity as handed down to them through folklore. The harvested plant parts were either used immediately or dried either under a shade or sun and then stored in a

cool dry place separately for eventual use. The role of each plant was not ascertained but all respondents confirmed the fact that the plants acted together for maximum efficacy. Eighty percent of the respondents confirmed that the method of administration was either by enema or oral while 20% confirm oral administration only.

4.1.4 Method of preparation and use of *Nefang*

The method of mixing, preparation and use of *Nefang* was handed down to users through folklore as ascertained by the respondents. There was, however, a difference in the proportions of the components of *Nefang*, used by the respondents. These are presented in **Table 4.3**.

Table 4.3. Formulation of *Nefang*

Constituent Plant Proportion in *Nefang* [*MiB: MiL: Pg: Cp: Cc: Cs: Og w/w*]	**No. of Respondents**	**%**
4:2:2:1:1:1:1	1	5
3:1:1:1:1:1:1	2	10
2:2:1:1:1:1:1	7	35
2:1:1:1:1:1:1	5	25
1:1:1:1:1:1:1	5	25

Freshly harvested tree bark was cut into small pieces while freshly harvested leaves were sliced into equal sizes and mixed in their given proportions, whereas the dried plant parts were crushed before mixing in same proportions. From the method of preparation given by the respondents, we estimated that approximately 2 kg of a mixture of the freshly harvested plants or 500 g of the crushed dried plants was boiled in 4 litres of water for 5-10 minutes and taken orally or administered through enema. Four respondents (20%) reported administration by oral only route while 16 (80%) reported both by oral route and enema. Respondents stated that there was no difference in efficacy between using freshly harvested plant parts and dried plants, apart from the fact that freshly harvested plant parts were used immediately after harvest whereas crushed dried plant parts could be preserved in a cool dry place for eventual use. The administration of this product did not involve any rituals and there were no taboos in relation to its preparation.

Oral administration required taking one glass full (0.33 litre) daily before eating for 6 days for adults and half a glass for children, whereas administration by enema required repeated rectal

insertion of mild warm enema early in the morning before eating at a unique dose of 1 litre × 3 times for adults and 0.5 litre × 2 times for children. The repeat of rectal insertion was done after emptying of the bowels of the patients. This treatment dose was reportedly taken once in 3 - 6 months. The product was immediately disposed of after use in the case of enema. After the administration of enema, patients could only eat after 2-3 hours. When taken orally, the period of conservation of this product within which it remained active was reported to be one week, after which it had to be disposed of and a new product prepared.

Reported side effects included dizziness, over sleeping and nausea, though 11 respondents (55%) reported no side effects.

4.2 ANTIPLASMODIAL ACTIVITY AND CYTOTOXICITY OF NEFANG AND ITS CONSTITUENTS

4.2.1 Extraction of the constituent plants of *Nefang*

The common names, place of collection, voucher specimen number and yields of the aqueous and ethanol extracts of the constituent plants of *Nefang* are shown in **Table 4.4**. The ethanol extract of *Mangifera indica* leaves had the highest yield while the aqueous extract of *Citrus sinensis* had the lowest. However, for each plant part, the ethanol extract had a higher yield than the corresponding aqueous extract.

Table 4.4. Constituent Plants of *Nefang*: voucher numbers, common names, parts used, collection and extraction yield

Plant Family and Species (Voucher specimen number)	Common Name (Part Used)	Place of Harvest	Extraction	
			Ethanol Yield (%)	Aqueous Yield (%)
Anacardiaceae *Mangifera indica* Linnaeus (TN6225)	(Bark) Mango (Leaves)	Mballa II, Yaoundé	5.40 8.05*#	5.52 6.20
Myrtaceae *Psidium guajava* Linnaeus (TN6226)	Guava (Leaves)	Nkomo, Yaoundé	7.88	5.84
Caricaceae *Carica papaya* L. papaya (TN6227)	Pawpaw (Leaves)	Nkoabang, Yaoundé	7.94	6.59**
Poaceae *Cymbopogon citratus* (DC. Ex Nees) Stapf (TN6228)	Lemon Grass or Fever Grass	Kombone, Kumba	6.70	5.80
Rutaceae *Citrus sinensis* (Linnaeus) Osbeck (pro sp.) [maxima reticula] (TN6229)	Sweet Orange (Leaves)	Mamfe	4.85	3.28
Lamiaceae *Ocimum gratissimum* Linnaeus (TN6230)	Wild Basil or Mosquito Plant (Leaves)	Buea	5.63	4.64

*#=Highest extraction yield; *=Highest ethanol extraction yield; **=Highest aqueous extraction yield.*

4.2.2 Preliminary Phytochemical screening

Preliminary phytochemical screening of the constituent plant extracts of *Nefang*, revealed the presence of flavonoids, phenols, triterpenes and sterols in all extracts, saponins in all except ***Cc***, tannins in all except ***Cp*** and ***Cc*** and alkaloids in ***MiB*** and ***Og*** only **(Table 4.5).**

Table 4.5. Phytochemical screening of the constituent plants extracts of *Nefang*

Phytochemical constituent	Plant						
	MiB	***MiL***	***Pg***	***Cp***	***Cc***	***Cs***	***Og***
Alkaloids	+	-	-	-	-	-	+
Anthocyanins	+	+	+	-	-	-	+
Flavonoids	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+
Saponins	+	+	+	+	-	+	+
Tannins	+	+	+	-	-	+	+
Triterpenes and Sterols	+	+	+	+	+	+	+

+ = presence; - = absence

4.2.3 Cytotocicity screening

All the aqueous and ethanol extracts of *Nefang* and its constituents were screened against Hep G2 hepatoma and U2OS osteosarcoma epithelial cell lines and the results showed no significant or toxic activity (SI>20) of *Nefang* and the majority of its component extracts **(Table 4.6).**

4.2.4 Evaluation of *in vitro* antiplasmodial activities

The *in vitro* antiplasmodial activity of all the aqueous and ethanol extracts of *Nefang* and its constituents against cultured CQ-sensitive (3D7) or MDR (Dd2) strains of *Plasmodium falciparum* are summarized in **Table 4.6**. Nine out of the sixteen extracts tested showed significant antiplasmodial activity at concentrations less than 50 µg/mL. EC_{50}s (3D7/Dd2) of the ethanol extracts exhibiting good antiplasmodial activities against both parasite strains were: ***MIB***-24.46/14, ***MIL***-24.32/16.34, ***Pg***-37.28/23, ***Cc***-28.75/54.84, ***Nefang***-51.10/29.99 (µg/mL) whereas the only aqueous extract with a similarly promising antiplasmodial activity was ***Pg***-47.02/25.79 µg/mL. All other extracts revealed weak activities (EC_{50} > 100 µg/mL) against one or both parasite strains, indicating that not all solvent extracts exhibited antimalarial properties.

Table 4.6. Cytotoxicity profile (CC_{50}), in vitro antiplasmodial activity (EC_{50}) and selectivity index (SI) of *Nefang* and constituent plant extracts

Nature of Extract	Extract	CC_{50} (µg/mL) U2OS	CC_{50} (µg/mL) Hep G2	EC_{50} 3D7 (µg/mL)	EC_{50} Dd2 (µg/mL)	Selectivity Index (SI)	Resistance Index (RI)
Ethanol (EtOH)	*Mangifera indica bark*	> 2000	> 2000	24.46 ± 0.03	14.00 ± 0.03**	> 142.85*	1.75
	Mangifera indica leaf	> 2000	> 2000	24.32 ± 0.03	16.34 ± 0.04	> 122.39*	1.49
	Psidium guajava	> 2000	> 2000	37.28 ± 0.02	23.00 ± 0.03	> 86.95*	1.62
	Carica papaya	> 2000	> 2000	76.03 ± 0.04	121.60 ± 0.11	> 16.44	0.63
	Cymbopogon citratus	> 2000	> 2000	28.75 ± 0.04	54.84 ± 0.01	> 36.47	0.52
	Citrus sinensis	> 2000	> 2000	39.34 ± 0.04	86.08 ± 0.14	> 23.34	0.46
	Ocimum gratissimum	> 2000	> 2000	81.46 ± 0.04	121.50 ± 0.08	> 16.46	0.67
	Nefang	**> 2000**	**> 2000**	**51.10** ± 0.02	**29.99** ± 0.04	**> 68.44**	**1.70**
Aqueous (Aq)	*Mangifera indica bark*	> 2000	> 2000	65.33 ± 0.02	34.58 ± 0.03	> 57.84*	1.89
	Mangifera indica leaf	> 2000	> 2000	82.56 ± 0.02	40.04 ± 0.03	> 49.95*	2.06
	Psidium guajava	> 2000	> 2000	47.02 ± 0.03	25.79 ± 0.03	> 77.56*	1.82
	Carica papaya	> 2000	> 2000	1188.00 ± 0.03	317.50 ± 0.09	> 6.29	3.74
	Cymbopogon citratus	> 2000	> 2000	723.30 ± 0.01	141.00 ± 0.07	> 14.18	5.13
	Citrus sinensis	> 2000	> 2000	184.40 ± 0.04	105.10 ± 0.08	> 19.03	1.75
	Ocimum gratissimum	1872.5	> 2000	778.50 ± 0.10	118.90 ± 0.09	> 16.82	6.54
	Nefang	**> 2000**	**> 2000**	**96.96** ± 0.03	**55.08** ± 0.03	**> 36.31**	1.76
Standard Drugs	**Chloroquine**	-	-	21.0 ± 0.01 nM	139.60 ± 0.05 nM	-	0.15
	Artemisinin	-	-	20.63 ± 0.01 nM	18.20 ± 0.04 nM	-	1.13

EC_{50} 3D7/Dd2 expressed as Mean ± SEM, n=3. *= *SI (Extract) > SI (Nefang): Potentially safer and promising Therapy;* **=*Best antiplasmodial activity.*
SI=CC_{50}-Hep G2/EC_{50}-Dd2; RI=EC_{50}3D7/EC_{50}Dd2.

4.2.5 Characterisation of the interaction between the constituents of *Nefang*

For stringency reasons, the interactions between the constituent plant extracts of *Nefang* evaluated in this study at equipotency ratios, were classified as synergistic (CI < 0.7), additive (0.7 < CI < 1.5) or antagonistic (CI > 1.5). Additionally, fold increases in the extracts' activities in a pair, relative to the activities when tested alone were determined and used to identify pairs not exhibiting synergistic, additive, or antagonistic interactions. Thus, ***Cp/Og***-(EtOH) - (CI=0.36), ***MiB/Pg***-(Aq) - (CI=0.35), ***MiB/Cs*** - (0.36), ***MiL/Pg*** - (0.36), ***Cp/Cs*** - (0.30) and ***Cc/Cs*** - (0.32), were identified as exhibiting strong apparent synergism with antiplasmodial activities >5-fold that of *Nefang* or the respective activities when tested alone (**Table 4.7**). The combinations with apparent additive or antagonistic interactions are indicated in **Table 4.8** and **Table 4.9** respectively.

The *in vitro* antiplasmodial activities at variable potency ratios of the six extract pairs that were identified as exhibiting the best synergistic interactions are summarized in **Table 4.10** and the ratio exhibiting the best synergistic activity identified. Thus, ***Cp/Og***-EtOH (ratio 1:4) - (CI=0.61), ***MiB***/***Pg***-Aq (2:3) - (0.76), ***MiB***/***Cs***-Aq (1:4) - (0.68), ***MiL***/***Pg***-Aq (1:4) - (1.0), ***Cp***/***Cs***-Aq (2:3) - (0.85), ***Cc***/***Cs***-Aq (1:4) - (1.27).

Isobole analyses of the extract pairs with best synergistic interactions confirmed the occurrence of synergism over a wide range of combination ratios in 4 of the 6 identified pairs. These include ***Cp/Og***-EtOH (ratios of 2:3 to 1:4), ***MiB/Pg***-Aq (4:1 to 1:4), ***MiB/Cs***-Aq (3:2 to 1:4), and ***Cp/Cs***-Aq (2:3 to 1:4), as shown in **Figure 4.1.**

Aqueous extract pairs exhibited better activities than their ethanol counterparts.

Table 4.7. In vitro antiplasmodial activity (EC_{50}) of paired extracts exhibiting synergistic interaction (CI < 0.7) at equipotency ratios

Extract Combination	EC_{50} Ratio (µg/mL)	EC_{50} Ratio in Combination (µg/mL)	FIC_{50} A	FIC_{50} B	Fold Increase	Combination Index (CI)
Cp/Cc-EtOH	121.60/54.84	28.45/12.83	0.24	0.24	4.27/4.27	0.48
Cp/Cs-EtOH	121.60/86.08	37.69/26.68	0.31	0.31	3.23/3.23	0.62
Cp/Og-EtOH	**121.60/121.5**	**21.39/21.46**	**0.18**	**0.18**	**5.68/5.66**	**0.36***
Cc/Og-EtOH	54.85/121.5	13.68/30.43	0.25	0.25	4.01/3.99	0.50
Cs/Og-EtOH	86.08/121.5	21.99/30.17	0.26	0.26	3.91/4.02	0.52
MiL-EtOH /MiL-Aq	16.34/40.04	5.26/12.88	0.32	0.32	3.11/3.11	0.64
Pg-EtOH /Pg-Aq	23/25.79	5.99/6.72	0.26	0.26	3.84/3.84	0.52
MiB/Pg-Aq	**34.58/25.79**	**6.07/4.53**	**0.17**	**0.17**	**5.70/5.70**	**0.35***
MiB/Cp-Aq	34.58/317.5	10.24/93.99	0.29	0.29	3.38/3.38	0.59
MiB/Cs-Aq	**34.58/105.1**	**6.34/19.25**	**0.18**	**0.18**	**5.45/5.46**	**0.36***
MiL/Pg-Aq	**40.04/25.79**	**7.18/4.62**	**0.18**	**0.18**	**5.58/5.58**	**0.36***
MiL/Cp-Aq	40.04/317.5	11.59/91.91	0.29	0.29	3.45/3.45	0.58
MiL/Cs-Aq	40.04/105.1	9.63/25.27	0.24	0.24	4.16/4.16	0.48
Pg/Cp-Aq	25.79/317.5	6.88/84.64	0.27	0.27	3.75/3.75	0.54
Pg/Cc-Aq	25.79/141	8.26/45.13	0.32	0.32	3.12/3.12	0.64
Pg/Cs-Aq	25.79/105.1	6.22/25.36	0.24	0.24	4.15/4.14	0.48
Pg/Og-Aq	25.79/118.90	7.52/34.66	0.39	0.39	3.43/3.43	0.58
Cp/Cc-Aq	317.50/141	80.11/35.57	0.25	0.25	3.96/3.96	0.50
Cp/Cs-Aq	**317.50/105.10**	**46.50/15.39**	**0.15**	**0.15**	**6.83/6.83**	**0.30***
Cp/Og-Aq	317.50/118.90	107.4/40.21	0.34	0.34	2.96/2.96	0.68
Cc/Cs-Aq	**141/105.10**	**22.19/16.54**	**0.16**	**0.16**	**6.35/6.35**	**0.32***
Cc/Og-Aq	141/118.90	48.93/41.26	0.35	0.35	2.88/2.88	0.70
Cs/Og-Aq	105.10/118.90	21.78/24.64	0.21	0.21	4.83/4.83	0.42

Data presented as mean, n=3

**= Extract pairs exhibiting strong synergistic interactions.*

EtOH = Ethanol Extracts; Aq = Aqueous Extracts

Table 4.8. In vitro antiplasmodial activity (EC_{50}) of paired extracts exhibiting additive interaction (0.7 < CI < 0.7) at equipotency ratios

Extract Combination	EC_{50} Ratio (µg/mL)	EC_{50} Ratio in Combination (µg/mL)	FIC_{50} A	FIC_{50} B	Fold Increase	Combination Index (CI)
MiB/MiL-EtOH	14/16.34	6.88/8.02	0.49	0.49	2.03/2.03	0.98
MiB/Cp-EtOH	14/121.60	7.99/69.36	0.57	0.57	1.75/1.75	1.14
MiB/Og-EtOH	14/121.50	5.20/45.32	0.37	0.37	2.69/2.68	0.74
MiL/Pg-EtOH	16.34/23	7.94/11.18	0.48	0.48	2.06/2.06	0.96
MiL/Cc-EtOH	16.34/54.84	11.01/36.94	0.67	0.67	1.48/1.48	1.34
MiL/Cs-EtOH	16.34/86.08	9.22/48.55	0.56	0.56	1.77/1.77	1.12
MiL/Og-EtOH	16.34/121.50	5.89/43.95	0.36	0.36	2.77/2.76	0.72
Pg/Cp-EtOH	23/121.60	10.85/57.38	0.47	0.47	2.12/2.12	0.94
Pg/Cs-EtOH	23/86.08	13.77/51.52	0.59	0.59	1.67/1.67	1.19
Pg/Og-EtOH	23/121.50	11.31/59.97	0.49	0.49	2.03/2.03	0.98
Cc/Cs-EtOH	54.84/86.08	35.03/54.99	0.64	0.64	1.57/1.57	1.28
MiB-EtOH /MiB-Aq	14/34.58	7.75/19.13	0.55	0.55	1.81/1.81	1.10
MiB/MiL-Aq	34.58/40.04	19.68/22.78	0.57	0.57	1.76/1.76	1.14
MiB/Cc-Aq	34.58/141	16.87/68.79	0.49	0.49	2.05/2.05	0.98
MiB/Og-Aq	34.58/118.90	14.91/51.28	0.43	0.43	2.32/2.32	0.86
MiL/Cc-Aq	40.04/141	14.31/50.39	0.36	0.36	2.80/2.80	0.72
MiL/Og-Aq	40.04/118.90	16.95/50.32	0.42	0.42	2.36/2.36	0.84

Data presented as mean, n=3

EtOH = Ethanol Extracts; Aq = Aqueous Extracts

Table 4.9. In vitro antiplasmodial activity (EC_{50}) of paired extracts exhibiting antagonistic interaction (CI > 1.5) at equipotency ratios

Extract Combination	EC_{50} Ratio (µg/mL)	EC_{50} Ratio in Combination (µg/mL)	FIC_{50} A	FIC_{50} B	Fold Increase	Combination Index (CI)
MiB/Pg-EtOH	14/23	16.77/27.55	1.20	1.20	0.83/0.83	2.40
MiB/Cc-EtOH	14/54.84	24.85/97.33	1.78	1.78	0.56/0.56	3.56
MiB/Cs-EtOH	14/86.08	10.86/66.79	0.78	0.78	1.29/1.29	1.56
MiL/Cp-EtOH	16.34/121.6	12.33/91.78	0.75	0.75	1.33/1.33	1.50
Pg/Cc-EtOH	23/54.84	24.47/58.34	1.06	1.06	0.94/0.94	2.12

Data presented as mean, n=3

EtOH = Ethanol Extracts; Aq = Aqueous Extracts

Table 4.10. In vitro antiplasmodial activity (EC_{50}) of best synergistic extract pairs at variable potency ratios

Extract Combination (A/B)	Ratio (EC_{50})	EC_{50} Ratio (µg/mL)	EC_{50} A(µg/mL)	EC_{50} B(µg/mL)	FIC_{50} A	FIC_{50} B	Combination Index (CI)	Apparent EC_{50} (SUM EC_{50})
Cp/Og-EtOH	5 v 0	608/0	64.09	0	1.00	0	1.00	64.09
	4 v 1	486.4/121.5	69.58	17.38	1.09	0.21	1.30	86.96
	3 v 2	364.8/243	47.44	31.6	0.74	0.38	1.12	79.04
	2 v 3	243.2/364.5	24.51	36.73	0.38	0.44	0.83	61.24
	1 v 4	**121.6/486**	**9.60**	**38.38**	**0.15**	**0.46**	**0.61**	**47.98***
	0 v 5	0/607.5	0	82.95	0.00	1.00	1.00	82.95
MiB/Pg-Aq	5 v 0	172.9/0	44.46	0	1.00	0.00	1.00	44.46
	4 v 1	138.3/25.8	21.75	4.06	0.49	0.32	0.81	25.80
	3 v 2	103.7/51.6	18.85	9.37	0.42	0.35	0.77	28.22
	2 v 3	**69.2/77.4**	**6.83**	**7.64**	**0.15**	**0.61**	**0.76**	**14.46***
	1 v 4	34.6/103.2	3.30	9.85	0.07	0.79	0.86	13.15
	0 v 5	0/128.9	0	12.51	0.00	1.00	1.00	12.51
MiB/Cs-Aq	5 v 0	172.9/0	30.29	0	1.00	0.00	1.00	30.29
	4 v 1	138.3/105.1	21.35	16.04	0.71	0.46	1.17	37.39
	3 v 2	103.7/210.2	12.23	24.78	0.41	0.47	0.88	37.01
	2 v 3	69.2/315.3	7.26	33.08	0.24	0.55	1.19	40.34
	1 v 4	**34.6/420.4**	**1.77**	**21.47**	**0.06**	**0.62**	**0.68**	**23.23***
	0 v 5	0/525.5	0	34.73	0.00	1.00	1.00	34.73
MiL/Pg-Aq	5 v 0	200.2/0	40.61	0	1.00	0.00	1.00	40.61
	4 v 1	160.1/25.8	36.61	5.89	0.90	0.46	1.36	42.50
	3 v 2	120.1/51.6	20.31	8.72	0.50	0.68	1.18	29.03
	2 v 3	80.1/77.4	12.44	12.02	0.31	0.94	1.25	24.46
	1 v 4	**40.1/103.2**	**4.42**	**11.39**	**0.11**	**0.89**	**1.00**	**15.81***
	0 v 5	0/128.9	0	12.8	0.00	1.00	1.00	12.8
Cp/Cs-Aq	5 v 0	1587.5/0	120.80	0	1.00	0.00	1.00	120.8
	4 v 1	1270/105.1	97.45	8.06	0.81	0.35	1.16	105.5
	3 v 2	952.5/210.2	59.49	13.13	0.49	0.57	1.06	72.62
	2 v 3	**635/315.3**	**28.60**	**14.20**	**0.24**	**0.62**	**0.86**	**42.8***
	1 v 4	317.5/420.4	13.38	17.72	0.11	0.77	0.88	31.1
	0 v 5	0/525.5	0	22.97	0.00	1.00	1.00	22.97
Cc/Cs-Aq	5 v 0	705/0	76.95	0	1.00	0.00	1.00	76.95
	4 v 1	564/105.1	58.46	10.89	0.76	0.69	1.45	69.35
	3 v 2	423/210.2	37.47	18.62	0.49	1.18	1.67	56.09
	2 v 3	282/315.3	18.09	20.22	0.24	1.28	1.52	38.31
	1 v 4	**141/420.4**	**6.24**	**18.59**	**0.08**	**1.18**	**1.27**	**24.82***
	0 v 5	0/525.5	0	15.7	0.00	1.00	1.00	15.7

Data presented as mean, n=3

**=Ratio of extract pairs exhibiting best synergistic interactions.*

EtOH = Ethanol Extracts; Aq = Aqueous Extracts

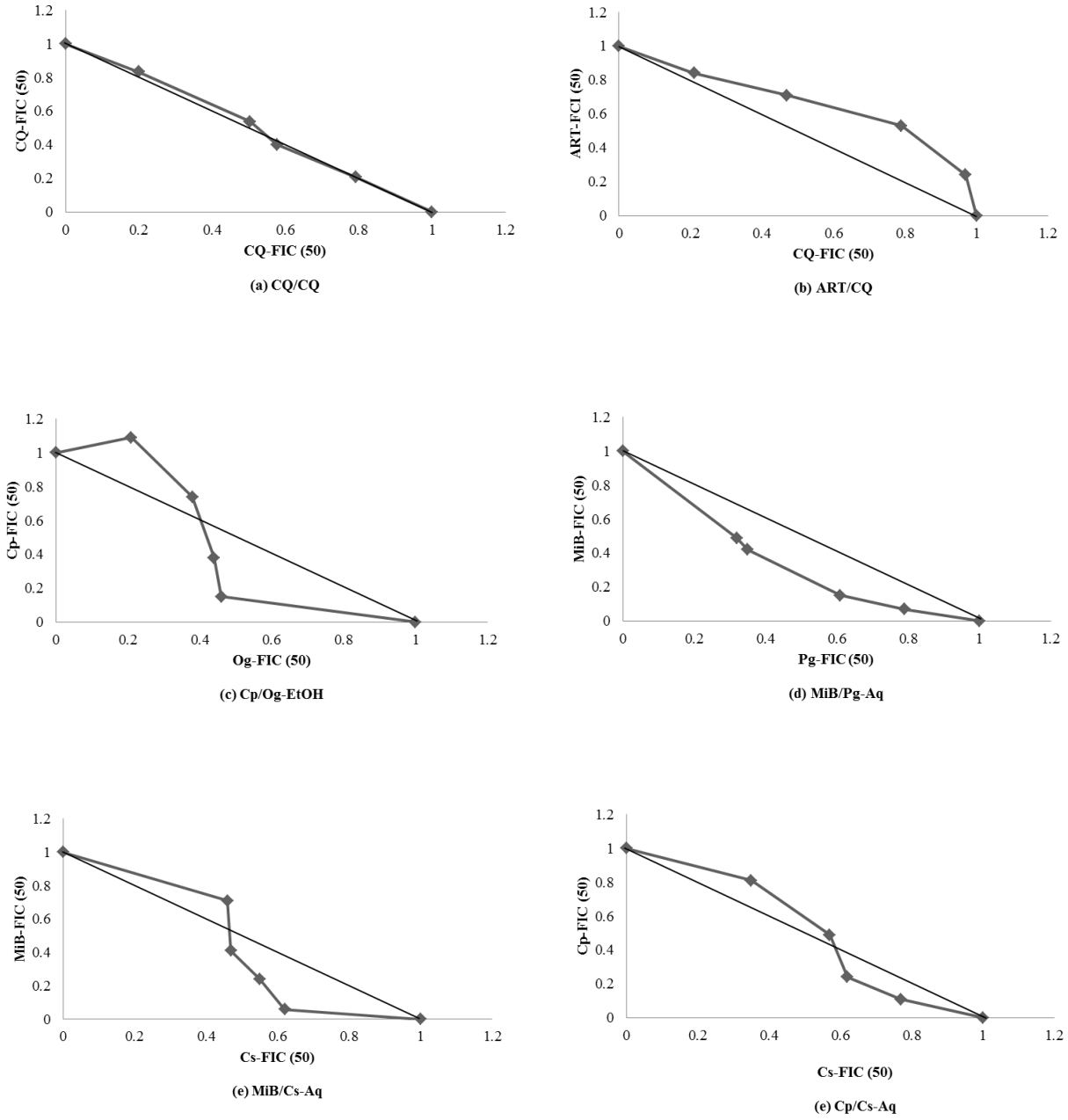

Figure 4.1. Isobolograms of the in vitro interactions between differential solvent extracts of *Nefang* at variable potency ratios.

(a) Control CQ/CQ **(b)** Control ART/CQ **(c)** Cp/Og-EtOH **(d)** MiB/Pg-Aq **(e)** MiB/Cs- Aq **(f)** Cp/Cs-Aq.
CQ: Chloroquine; ART: Artemisinin; FIC_{50}=Fractional Inhibitory Concentration 50. A concave isobologram is consistent with synergy, a convex one is consistent with antagonism, and a straight line is consistent with additivity. Axes are EC_{50}s normalized to 1.

4.2.6 Evaluation of the in vivo antiplasmodial activity of *Nefang*

4.2.6.1 Evaluation of the Suppressive Activity (Peter's 4-Day Test)

Evaluation of the suppressive activity of *Nefang, Psidium guajava* (the most active constituent aqueous extract) and *MiB/Pg* (the solvent extract combination that showed the most promising synergistic activity) in *P. berghei* infected rats and *P. c. chabaudi* infected mice revealed that *Nefang*, *Pg* and *MiB/Pg* all showed a dose-dependent chemosupressive activity on parasitemia. These effects were statistically significant ($p<0.001$) relative to the control. At all doses, the suppressive activity between *P. berghei* **(Figure 4.2)** infection in rats and *P. c. chabaudi* **(Figure 4.3)** infection in mice were comparable in all experimental cases. However, on day 6, at the highest experimental dose of 600 $mgkg^{-1}$ bwt, percentage chemosuppression were as follows (*P. berghei*/*P.c. chabaudi*): *Nefang* - 82.9/86.3, *MiB/Pg* - 79.5/81.2, *Pg* - 58.9/67.4 as against chloroquine - 92.1/97.7 at 10 $mgkg^{-1}$ and pyrimethamine - 85.5/88.6 at 30 $mgkg^{-1}$. Though significantly ($p<0.05$) lower than for chloroquine, the in vivo activity of *Nefang* was as good as that of pyrimethamine and better than *MiB/Pg* and *Pg*.

Analysis of the body temperature revealed that *Nefang* and *MiB/Pg* caused significant attenuation of reduction in body temperature in both *P. berghei* infected rats and *P. c. chabaudi* infected mice in a dose-dependent manner ($p<0.001$ for *Nefang* 300 and 600, *MiB/Pg* 600; $p<0.05$ for *MiB/Pg* 600, *Pg* 600 $mgkg^{-1}$) **(Table 4.11).** Both *Nefang* and *MiB/Pg* had activities comparable to those of the standard drugs CQ and PYR. The extracts averted the loss of body weight associated with *Plasmodium* infection at same doses when compared to the control **(Table 4.12).** No significant increases in weight were observed.

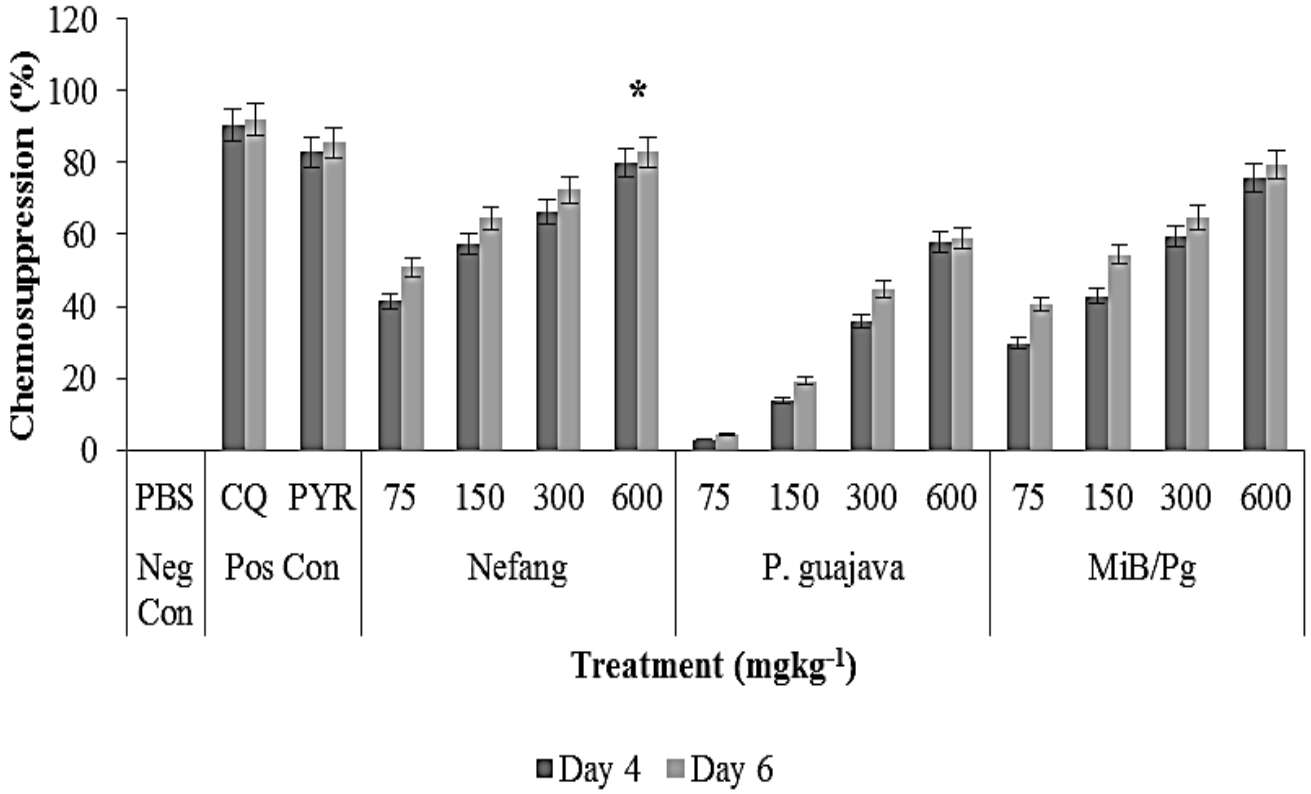

Figure 4.2. Suppressive activity of *Nefang* and active solvent extracts on *P. berghei* infection in rats.

Each bar represents the Mean ± SD for each group of rats, n = 3.
***Neg Con** – negative control; **Pos Con** – positive control; **CQ** – chloroquine; **PYR** – pyrimetahmine*
*= Best chemosuppression compared to control.

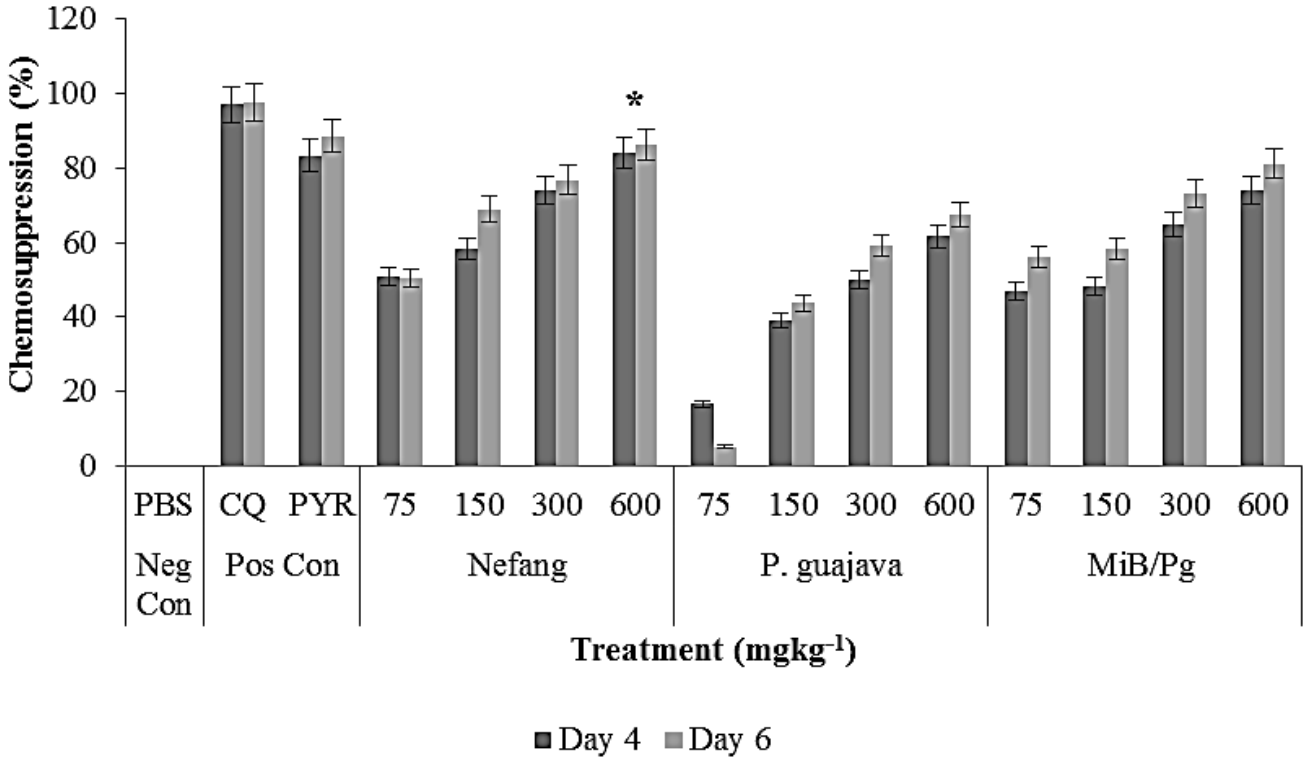

Figure 4.3. Suppressive activity of *Nefang* and active solvent extracts on *P. chaubaudi chabaudi* infection in mice.

Each bar represents the Mean ± SD for each group of rats, n = 3.
***Neg Con** – negative control; **Pos Con** – positive control; **CQ** – chloroquine; **PYR** –pyrimethamine*
*= Best chemosuppression compared to control.

Table 4.11. Body temperature (Day 0 and Day 4) of *Plasmodium* infected animals treated with aqueous extract of *Nefang* and its active components in the 4 day suppressive test

Extract	Dose ($mgkg^{-1}$)	Body Temperature (^oC) ($\bar{x} \pm SD$, n=3)			
		***P. berghei* infection in rats**		***P. c. chabaudi* infection in mice**	
		D0	**D4**	**D0**	**D4**
Negative Control	0	37.20 ± 0.16	35.41 ± 0.38	37.10 ± 0.41	34.91 ± 0.17
Positive Control (CQ)	10	37.10 ± 0.13	37.30 ± 0.21***²**	37.50 ± 0.31	37.40 ± 0.09***²**
Positive Control (PYR)	30	37.40 ± 0.19	37.40 ± 0.26***²**	37.10 ± 0.11	37.30 ± 0.25***²**
Nefang	75	37.20 ± 0.18	35. 96 ± 0.35**#2**	37.20 ± 0.24	35.85 ± 0.31**#2**
	150	37.50 ± 0.47	36.46 ± 0.31**#1**	37.20 ± 0.44	36.30 ± 0.53***¹**
	300	37.40 ± 0.31	37.10 ± 0.16***²**	37.30 ± 0.41	36.90 ± 0.18***²**
	600	37.40 ± 0.44	37.30 ± 0.39***²**	37.10 ± 0.30	36.90 ± 0.29***²**
Pg	75	37.40 ± 0.36	35.31 ± 0.48**#2**	37.30 ± 0.32	35.56 ± 0.44**#2**
	150	37.40 ± 0.51	35.67 ± 0.52**#2**	37.20 ± 0.52	35.82 ± 0.38**#2**
	300	37.60 ± 0.29	36.21 ± 0.52**#1**	37.20 ± 0.33	36.42 ± 0.46**#2**
	600	37.40 ± 0.47	36.59 ± 0.15***¹**	37.30 ± 0.22	36.68 ± 0.26***¹**
MiB/Pg	75	37.10 ± 0.21	36.01 ± 0.33**#1**	37.20 ± 0.21	36.31 ± 0.33
	150	37.30 ± 0.24	36.54 ± 0.29**#1**	37.20 ± 0.41	36.75 ± 0.16***¹**
	300	37.30 ± 0.39	36.86 ± 0.37***¹**	37.30 ± 0.38	36.84 ± 0.19***¹**
	600	37.20 ± 0.22	36.95 ± 0.26***²**	37.20 ± 0.18	37.00 ± 0.23***²**

******* *= compared to negative (-ve) control,* ***#*** *= to positive (+ve) control;*
Significant difference: ***¹****= p<0.05,* ***²****=p<0.001.*

Table 4.12. Body weight (Day 0 and Day 4) of *Plasmodium* infected animals treated with aqueous extract of *Nefang* and its active components in the 4 day suppressive test

Extract	Dose ($mgkg^{-1}$)	Body Weight (g) ($\bar{x} \pm SD$, n=3)			
		P. berghei infection in rats		*P. c. chabaudi* infection in mice	
		D0	D4	D0	D4
Negative Control	0	175.00 ± 2.81	169.50 ± 2.26	24.80 ± 0.12	23.20 ± 0.18
Positive Control (CQ)	10	176.50 ± 3.32	176.40 ± 3.48*[2]	24.60 ± 0.16	24.70 ± 0.21*[2]
Positive Control (PYR)	30	174.50 ± 2.43	175.00 ± 1.89*[2]	24.80 ± 0.19	24.80 ± 0.27*[2]
Nefang	75	175.40 ± 4.81	170.34 ± 6.21[#2]	24.85 ± 0.11	23.40 ± 0.18[#1]
	150	174.34 ± 5.39	171.60 ± 4.32[#1]	24.60 ± 0.29	24.10 ± 0.32*[1]
	300	174.50 ± 3.96	173.50 ± 2.73*[1]	24.90 ± 0.23	24.70 ± 0.16*[2]
	600	172.00 ± 2.85	172.50 ± 3.17*[2]	25.00 ± 0.18	24.90 ± 0.23*[2]
Pg	75	178.46 ± 9.37	173.86 ± 7.45[#2]	24.50 ± 0.33	23.10 ± 0.36[#2]
	150	175.48 ± 6.59	171.65 ± 5.59[#2]	24.65 ± 0.27	23.50 ± 0.41[#1]
	300	176.39 ± 9.38	173.40 ± 6.71[#1]	24.80 ± 0.38	23.70 ± 0.25[#1]
	600	175.30 ± 7.21	173.65 ± 5.49*[1]	24.80 ± 0.22	24.15 ± 0.34*[1]
MiB/Pg	75	178.40 ± 6.73	175.50 ± 4.28[#1]	24.60 ± 0.22	23.80 ± 0.47
	150	176.28 ± 7.69	174.10 ± 7.12[#1]	24.70 ± 0.35	23.95 ± 0.51
	300	176.80 ± 7.16	174.39 ± 5.49*[1]	24.80 ± 0.44	24.10 ± 0.28*[1]
	600	175.50 ± 4.65	174.42 ± 8.19*[2]	24.50 ± 0.17	24.30 ± 0.35*[2]

***** = compared to negative (-ve) control, [#] = to positive (+ve) control;*
Significant difference: [1]= $p<0.05$, [2]=$p<0.001$

4.2.6.2 Evaluation of the Prophylactic Activity

Evaluation of the prophylactic activity of *Nefang* aqueous extract during early *P. berghei* infection in mice revealed that *Nefang* showed a dose-dependent reduction of parasitemia in experimental groups of mice. These reductions were statistically significant relative to the control. At the highest experimental dose of 600 $mgkg^{-1}$, *Nefang* demonstrated a high antiplasmodial activity of 79.5%, which was highly significant ($p<0.001$) when compared to the control. Though slightly lower than that exhibited by chloroquine (86.9%) at 10 $mgkg^{-1}$, it was comparable to that exhibited by pyrimethamine (78.36%) at 30 $mgkg^{-1}$ **(Figure 4.4).**

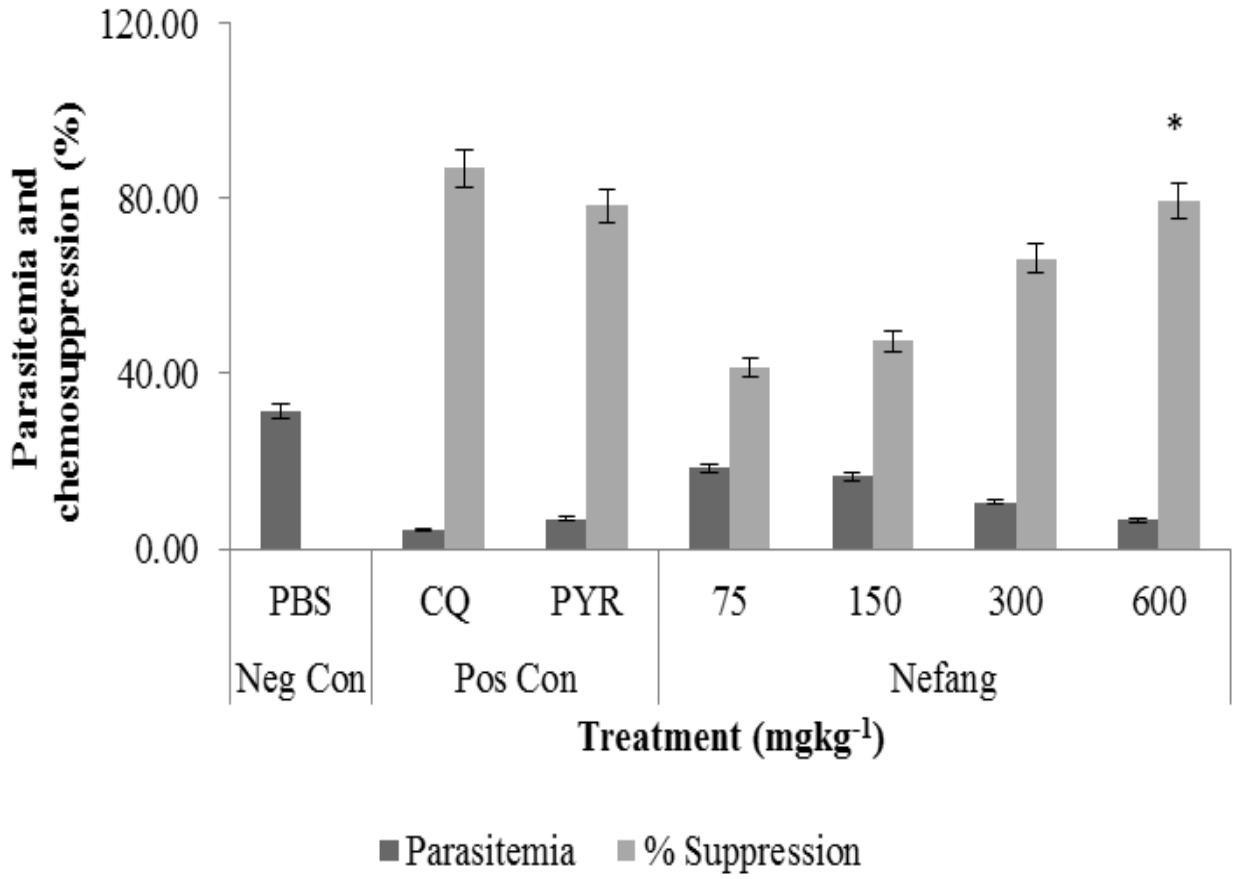

Figure 4.4. Repository/Prophylactic activity of *Nefang* against early *P. berghei* infection in BALB/c mice.

Each bar represents the Mean ± SD for each group of rats, n = 3
**= Best Prophylactic activity*

4.2.6.3 Evaluation of the Curative Activity (Rane's Test)

Evaluation of the curative activity of *Nefang* during established *P. berghei* infection in rats revealed that there was a progressive dose-dependent statistically significant ($p<0.001$) reduction of parasitemia by *Nefang* relative to the control. The dose-dependent chemosuppression exhibited by *Nefang* was comparable to that of the standard drugs **(Figure 4.5).** *Nefang* also demonstrated a significant ($p<0.05$) protective potential on the experimental rats as was observed in the mean survival time of the animals, especially at the highest dose which was comparable to that of the standard drugs, with all animals surviving all through the experiment **(Figure 4.6).** Determination of body weight of infected animals revealed that the reduction was significantly ($p<0.05$) prevented by *Nefang* at doses of 300 and 600 $mgkg^{-1}$ **(Table 4.13).** This activity was comparable to that of CQ and ART. At same doses, *Nefang* also significantly ($p<0.001$) prevented the reduction in body temperature of inoculated animals at a rate comparable to that of the standard drugs **(Table 4.14).**

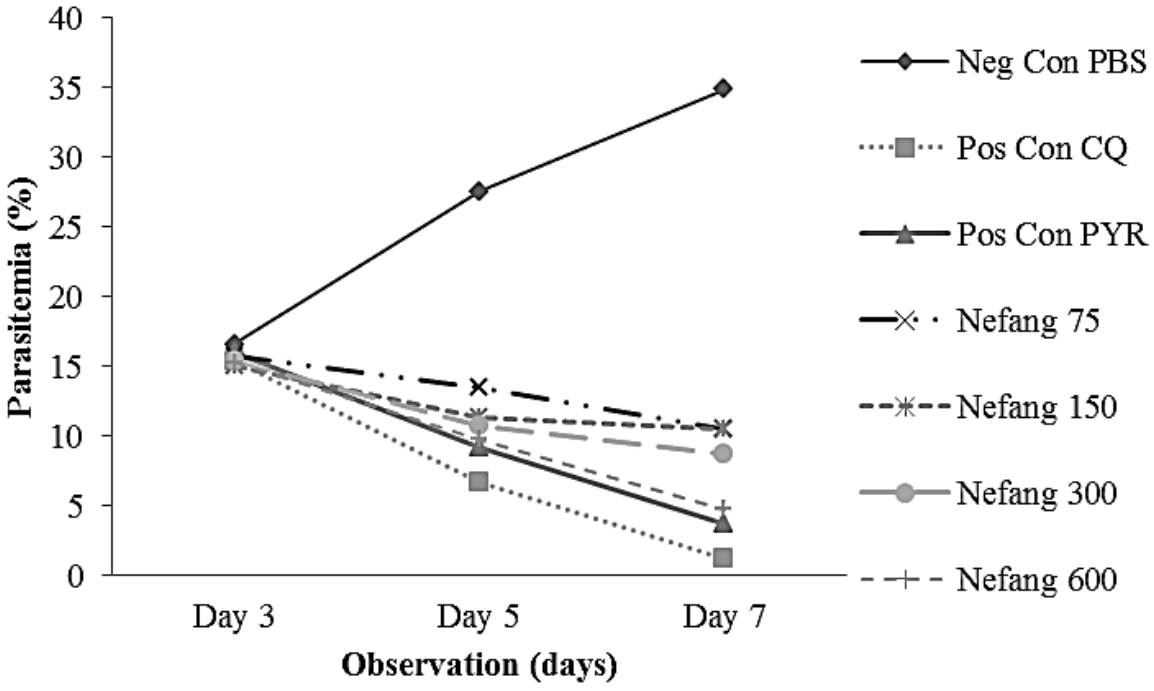

Figure 4.5. Curative effect of *Nefang* aqueous extract against established *P. berghei* infection in wistar rats.

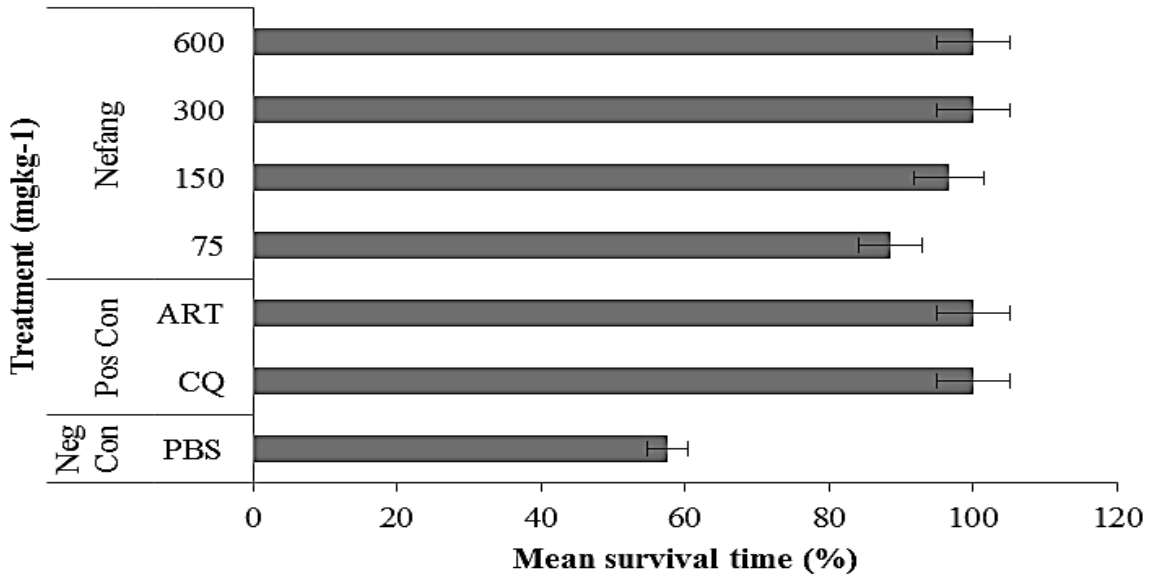

Figure 4.6. Mean survival time of wistar rats treated with *Nefang* aqueous extract during established *P. berghei* infection.

Each bar represents the Mean ± SD for each group of rats, n = 3

Table 4.13. Effect of *Nefang* aqueous extract on the body weight of *P. berghei* infected rats during established infection (Rane's Test)

Treatment	Dose ($mgkg^{-1}$)	Experimental Period (Days)	
		Body weight (g) ($\bar{x}$ ± SD, n=3)	
		D0	**D7**
Negative Control	-	172.48 ± 4.69	161.24 ± 4.31
Positive Control (CQ)	10	174.21 ± 2.98	172.48 ±1.29*[1]
Positive Control (ART)	5	175.44 ± 3.24	172.95 ±3.21*[1]
Nefang	75	172.98 ± 4.37	165.49 ± 5.72[#1]
	150	174.38 ± 6.38	166.38 ± 4.38[#1]
	300	173.92 ± 3.87	169.49 ± 3.25*[1]
	600	174.64 ± 5.40	171.28 ± 2.93*[1]

** = compared to negative control, [#] = to positive control;*
Significant Difference - [1] = p<0.05; [2] =p<0.001; CQ = chloroquine, ART = Artemisin

Table 4.14. Effect of *Nefang* aqueous extract on the body temperature of *P. berghei* infected rats during established infection (Rane's Test)

Treatment	Dose ($mgkg^{-1}$)	Experimental Period (Days)					
		Body Temperature (^oC) ($\bar{x} \pm SD$, n=3)					
		D0	D3	D4	D5	D6	D7
Negative Control	-	37.30 ± 0.31	33.94 ± 0.98	33.61 ± 1.08	33.15 ± 0.74	32.96 ± 0.53	32.84 ± 0.29
Positive Control (CQ)	10	37.20 ± 0.86	34.01 ± 0.83	34.52 ± 0.28	35.36 ± 0.38	36.41 ± 0.33	37.12 ± 0.24[2]
Positive Control (ART)	5	37.50 ± 0.92	33.86 ± 0.17	34.48 ± 0.34	35.28 ± 0.27	36.58 ± 0.41	37.38 ± 0.21*[2]
Nefang	75	37.20 ± 1.12	33.91 ± 0.71	34.12 ± 0.32	34.86 ± 0.24	35.04 ± 0.28	35.37 ± 0.18[#1]
	150	37.42 ± 0.49	34.06 ± 0.53	34.26 ± 0.22	35.02 ± 0.17	36.12 ± 0.30	36.65 ± 0.26[#1]
	300	37.38 ± 0.94	34.03 ± 0.55	34.41 ± 0.74	35.21 ± 0.54	36.07 ± 0.15	36.74 ± 0.11*[2]
	600	37.32 ± 1.26	33.98 ± 0.26	34.72 ± 0.23	35.42 ± 0.18	36.71 ± 0.62	37.16 ± 0.35*[2]

** = compared to negative control, [#] = to positive control;*
Significant Difference - [1] = p<0.05; [2] =p<0.001; CQ = chloroquine, ART = Artemisinin

4.3 EVALUATION OF THE IN VIVO TOXICOLOGICAL PROFILE OF NEFANG AND ITS CONSTITUENTS

4.3.1 Acute (Single Dose) Oral Toxicity Testing

The sighting study did not result in any signs of toxic effect at all oral dose levels tested; 5, 50, 300, 2000 $mgkg^{-1}$ *BW* for all the aqueous and ethanol extracts as well as a combination of all the aqueous and ethanol extracts respectively. All the mice survived.

For the main test at 5000 $mgkg^{-1}$ *BW*, the acute (single dose) oral toxicity study did not result in any mortality and no toxic effect was observed throughout the 14 days study period in mice administered the various aqueous and ethanol extracts. Physical observation of all extract treated mice revealed that none of them showed any signs of toxic effect such as changes on skin, eyes and mucus, behaviour patterns, trembling, diarrhea, falling of the fur, sleep or coma. No significant changes were observed in their body weights.

4.3.2 Sub-acute and Sub-chronic (Multiple Dose) Oral Toxicity Testing of *Nefang* and its constituents

For stringency reasons and a better understanding, the sub-acute and sub-chronic oral toxicity testing of *Nefang* and its constituent plants was appreciated from two dimensions; the toxicities of the constituent plants that showed moderate to good antiplasmodial activity (*MiB*, *MiL* and *Pg*) and those that showed weak activity (*Cp*, *Cc*, *Cs* and *Og*), alongside that of *Nefang*. The effects of short and long term oral administration of the ethanol and aqueous extracts on some plasma biochemical parameters analysed in experimental animals were summarized using the organ system approach.

4.3.2.1 Hepatotoxicity/ Effects on the liver

After sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents, the effects on the liver were evaluated using some physiological, biochemical and histopathological parameters.

The liver: body mass ratio (Relative Organ Weight) of each group of animals was calculated and no significant differences were observed in weights of the liver of test animals when compared to the control **(Figure 4.7).**

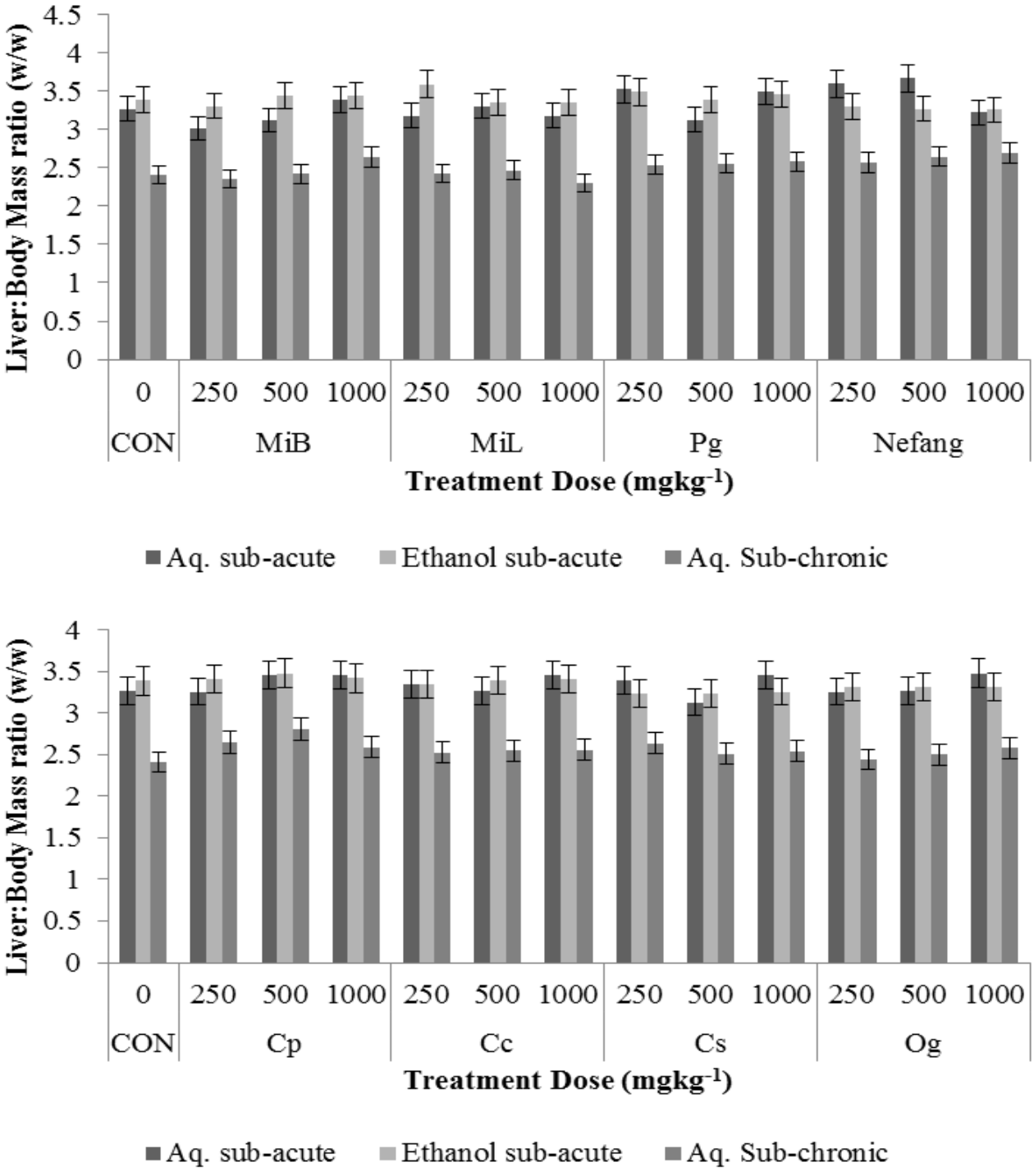

Figure 4.7. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the liver weight of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n=6.

Biochemical parameters for hepatotoxicity evaluated included plasma AST (**Figure 4.8**), ALT (**Figure 4.9**) and ALP (**Figure 4.10**).

After sub-acute administration of *Nefang* aqueous and ethanol extracts, we observed a significant ($p<0.05$) decrease in AST and ALT values when compared to the control, whereas the values of all the liver profile parameters analysed remained within low to normal after sub-chronic administration of the aqueous extract.

Among the constituent plants that showed good to moderate antiplasmodial activity, determined AST values in experimental animals revealed a significant ($p<0.05$) decrease after sub-accute *MIB* ethanol and *Pg* aqueous extract administration as well as a significant ($p<0.05$) increase and a dose-dependent significant increase after sub-acute *Pg* ethanol and sub-chronic *MiL* aqueous extracts administration respectively. Meanwhile, among the constituents that exhibited poor antiplasmodial activities, plasma AST analysis revealed a significant ($p<0.05$) decrease in values in sub-acute/sub-chronic *Cp*, *Cs*, *Og* and sub-acute Cc aqueous extracts-treated animals when compared to the control. However, we observed a significant ($p<0.05$) increase in AST values in *Cp*, *Cc* and *Cs* ethanol extracts-treated animals at a dose of 1000 $mgkg^{-1}$ **(Figure 4.8).**

ALT analysis revealed a dose-dependent significant ($p<0.05$) decrease in values after sub-acute *Pg* aqueous extract administration whereas significant ($p<0.05$) increases were observed in sub-acute *Cc* and *Cs* ethanol and sub-chronic *Cc* aqueous extracts-treated animals when compared to the control. ALT values in all other experimental groups stayed within normal ranges **(Figure 4.9).**

In plasma ALP analysis, we observed a slight but insignificant increase in values after sub-acute *MiB* aqueous, sub-acute and sub-chronic *Cc* ethanol and aqueous extracts-treated animals, at a dose of 1000 $mgkg^{-1}$. Values in all other experimental groups ranged from low to normal when compared with the control **(Figure 4.10).**

The increase in values of some of the liver profile parameters observed after administration of some of the constituent plant extracts was absent after *Nefang* administration, which demonstrated no signs of hepatotoxicity.

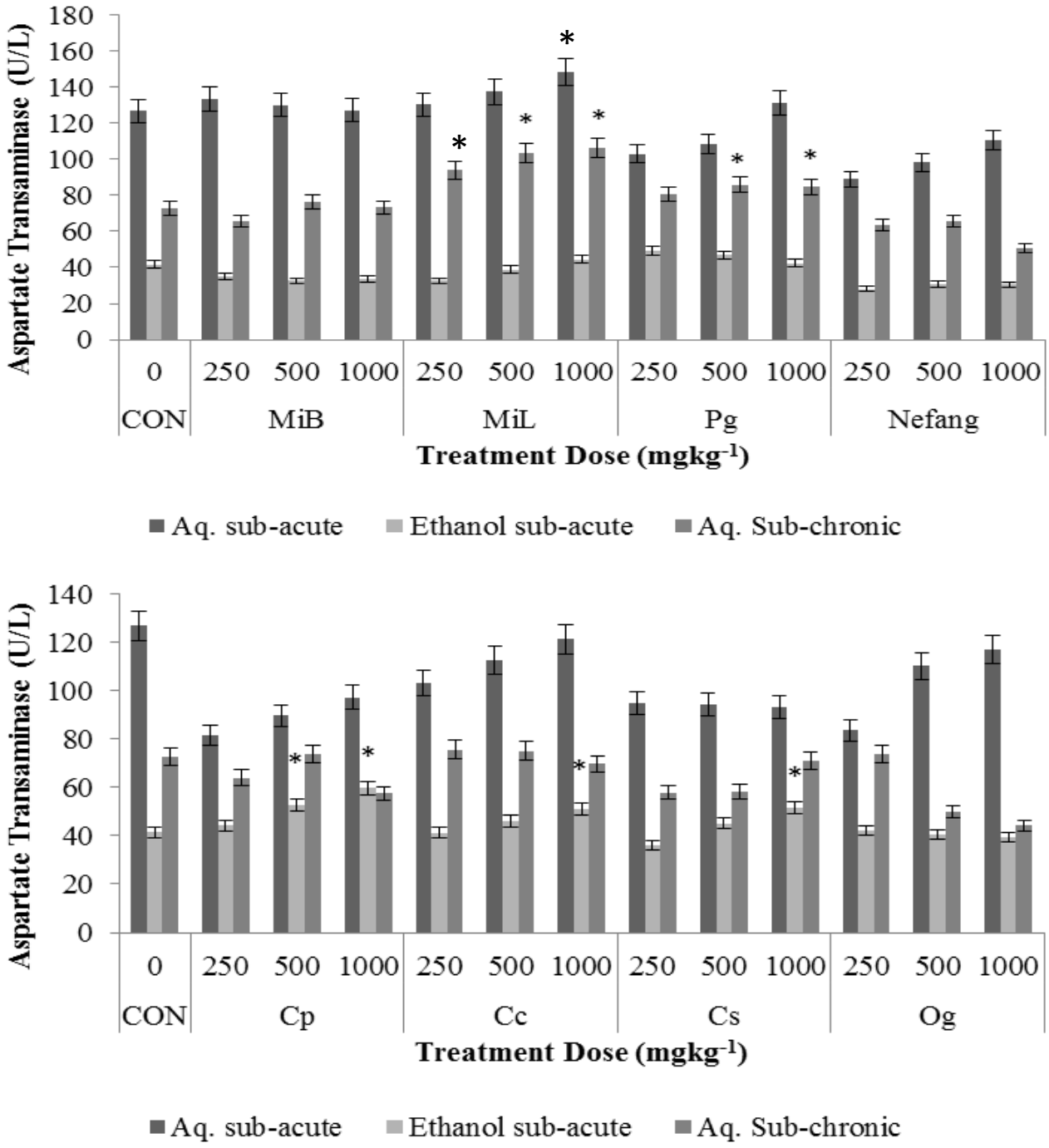

Figure 4.8. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma aspartate transaminase (AST) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = AST levels significantly ($p<0.05$) higher than the control*

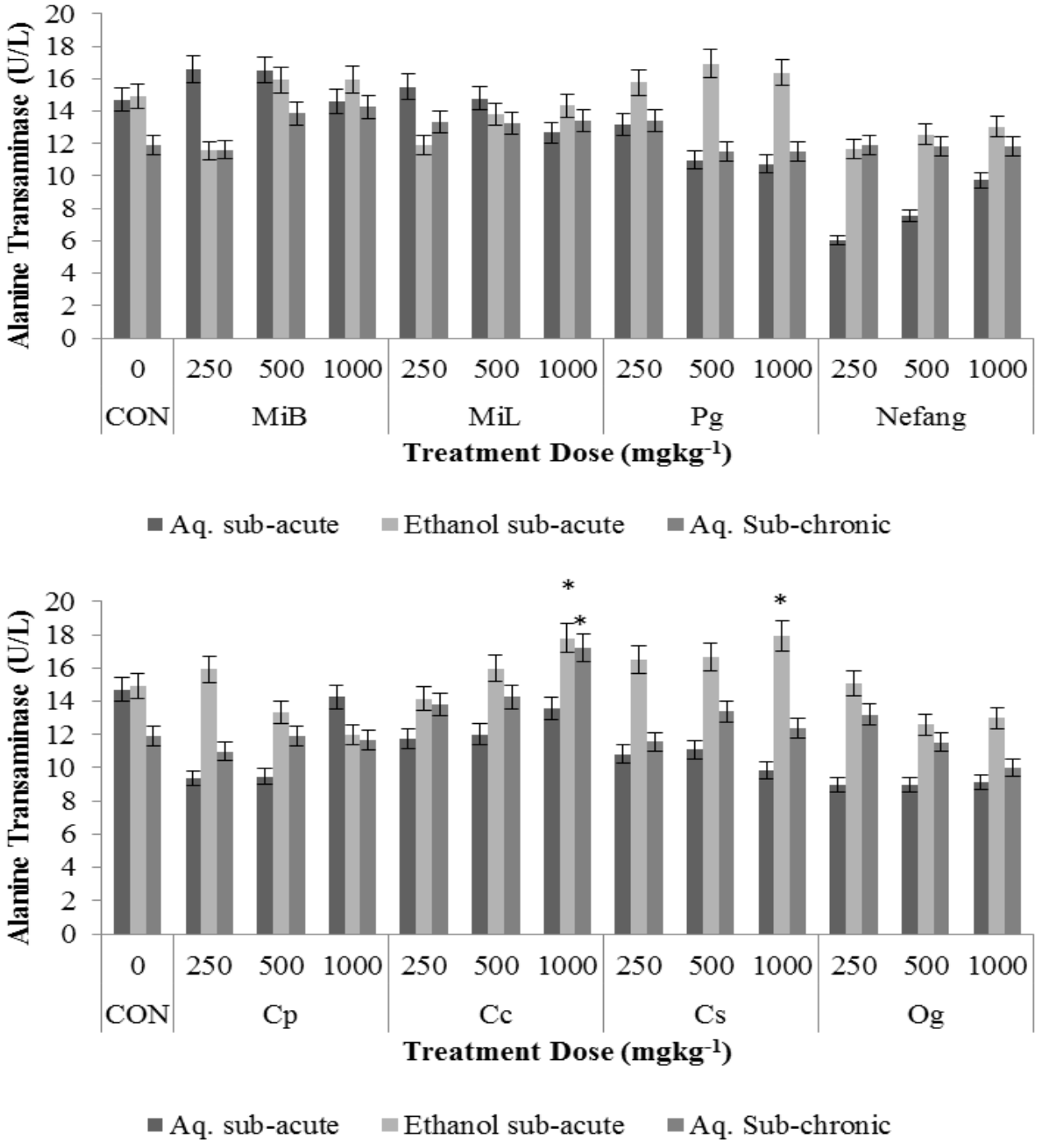

Figure 4.9. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma alanine transaminase (ALT) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = ALT levels significantly ($p<0.05$) higher than the control*

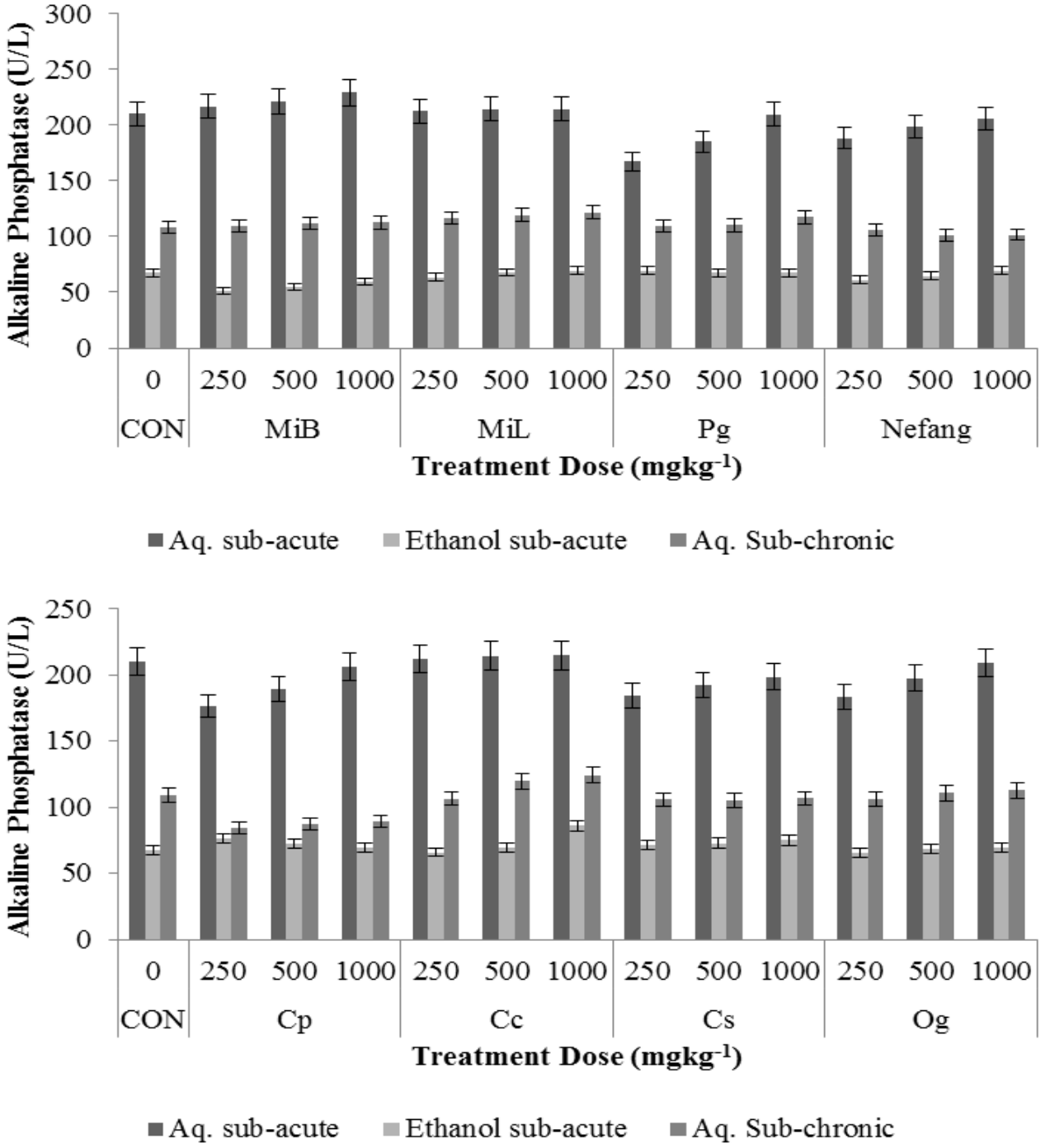

Figure 4.10. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma alkaline phosphatase (ALP) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = ALP levels significantly ($p<0.05$) higher than the control*

Histopathological examination revealed signs of hepatic toxicity in sub-chronic *Cc* aqueous and sub-acute *Cc*, *Cp* and *Cs* ethanol extracts-treated animals, at doses of 1000 $mgkg^{-1}$, when compared to the normal **(Figure 4.11).**

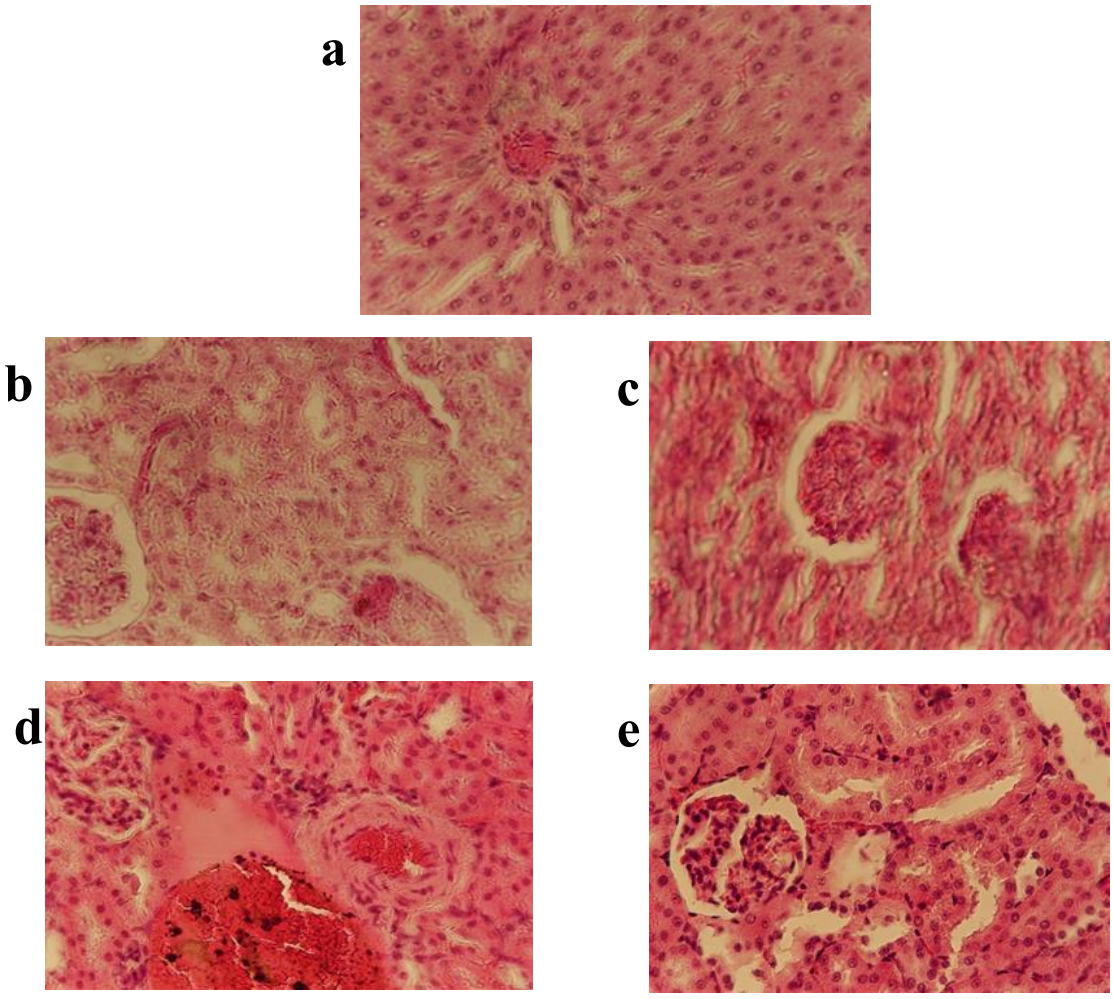

Figure 4.11. Photomicrograph of liver of experimental rats after administration of *Carica papaya*, *Cymbopogon citratus* and *Citrus sinensis* leaf extracts.

(a) Normal tubular architecture of the liver in control. **(b)** Vascular congestion in the liver of rats administered *Cc* aqueous leaf extract for 90 days at 1000 $mgkg^{-1}$ **(c)** Vascular congestion and leucocyte infiltration in the liver of rats administered *Cp* ethanol leaf extract for 28 days at 1000 $mgKg^{-1}$ **(d)** Edema and scarring of the liver of rats administered *Cc* ethanol leaf extract for 28 days at 1000 $mgkg^{-1}$ **(e)** Vascular congestion and leucocyte infiltration in the liver of rats administered *Cs* ethanol leaf extract for 28 days at 1000 $mgkg^{-1}$ (*Hematoxylin & Eosin × 40*).

4.3.2.2 Renal Toxicity/ Effects on the kidneys

Investigation of the toxic effects of *Nefang* and its constituents on the kidneys necessitated the determination of the relative organ weights of the kidney, analysis of blood urea nitrogen (BUN), plasma creatinine (CRE), uric acid and histopathological analysis.

The calculated kidney: body mass ratio of test animals after sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents did not reveal any significant changes at doses of up to 1000 $mgkg^{-1}$, when compared to the control groups as shown in **Figure 4.12**.

Administration of *Nefang* aqueous and ethanol extracts did not have any significant increase in BUN, CRE and URIC values in test animals when compared to the control. All values of test groups stayed within low to normal ranges. Upon administration of the constituent plant extracts of *Nefang*, we observed a significant ($p<0.05$) increase in BUN values in sub-acute *Pg* (ethanol), *Cp* (aqueous and ethanol) and *Cs* (ethanol) extracts-treated groups when compared to the control. Values in *MiB*, *MiL*, *Cc* and *Og* treated grouped were found to be normal **(Figure 4.13).**

Plasma CRE in all test groups were found to be normal when compared to the control **(Figure 4.14).** We also observed slight increases in URIC values after sub-acute *MiB*, *MiL* and *Cs* administration in test animals but these were not statistically significant when compared with the control **(Figure 4.15).**

Histopathological examination of the kidneys in extract treated groups revealed some morphological changes in sub-acute *Cp*, *Cc* and *Cs* ethanol extract-treated groups when compared to the control group, indicating signs of renal toxicity **(Figure 4.16).**

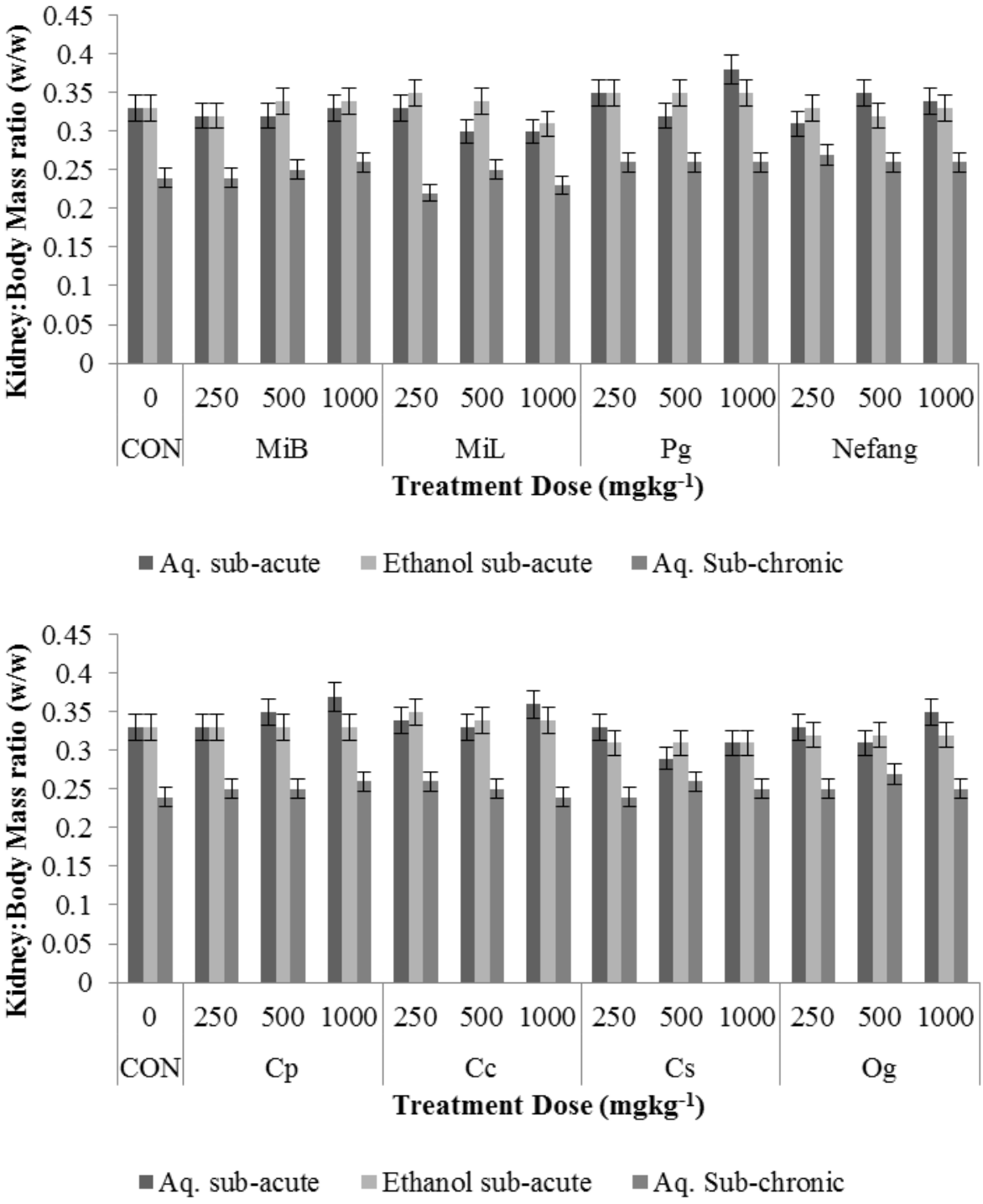

Figure 4.12. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on kidney weight of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.

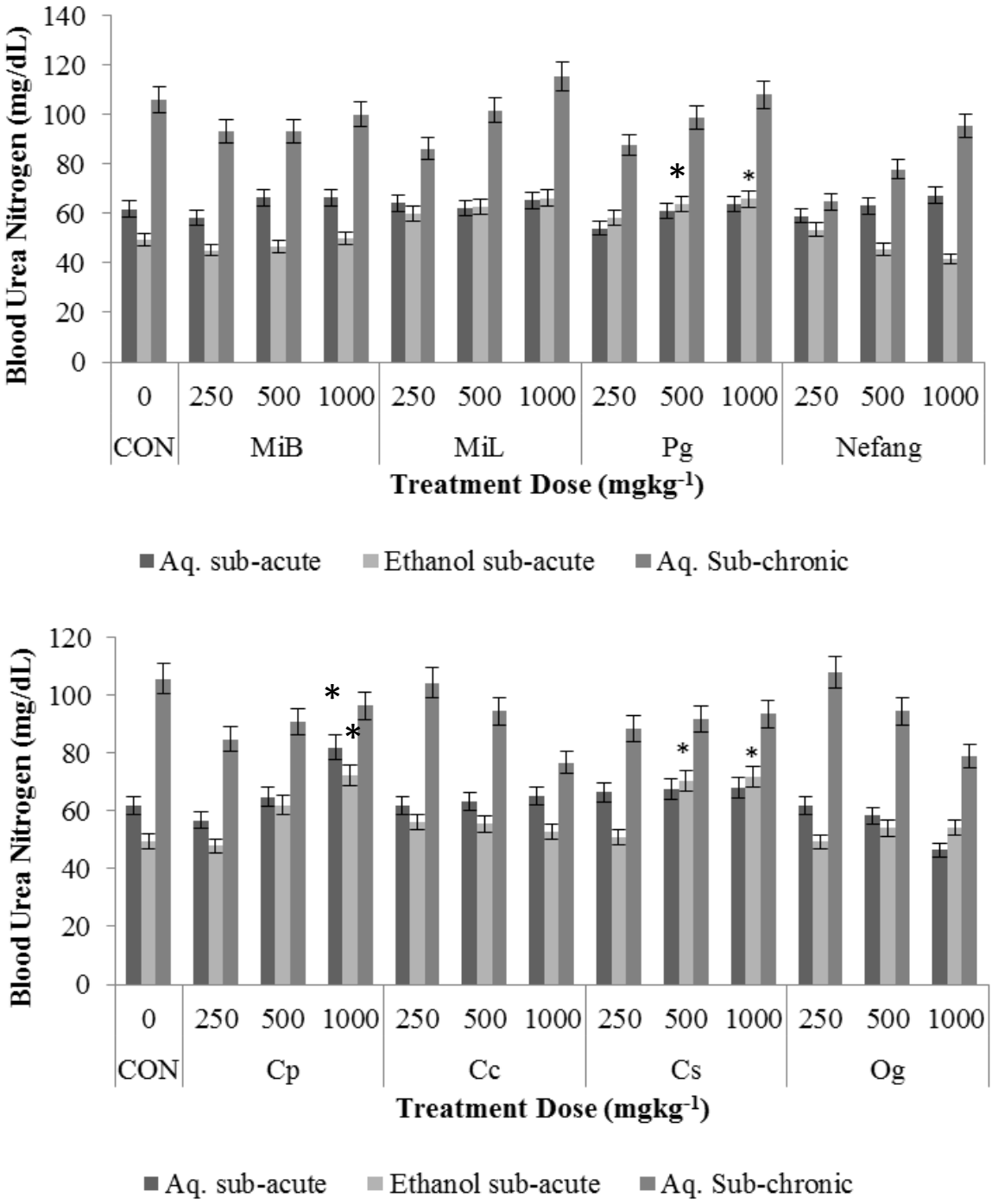

Figure 4.13. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on Blood Urea Nitrogen (BUN) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = BUN levels significantly ($p<0.05$) higher than the control*

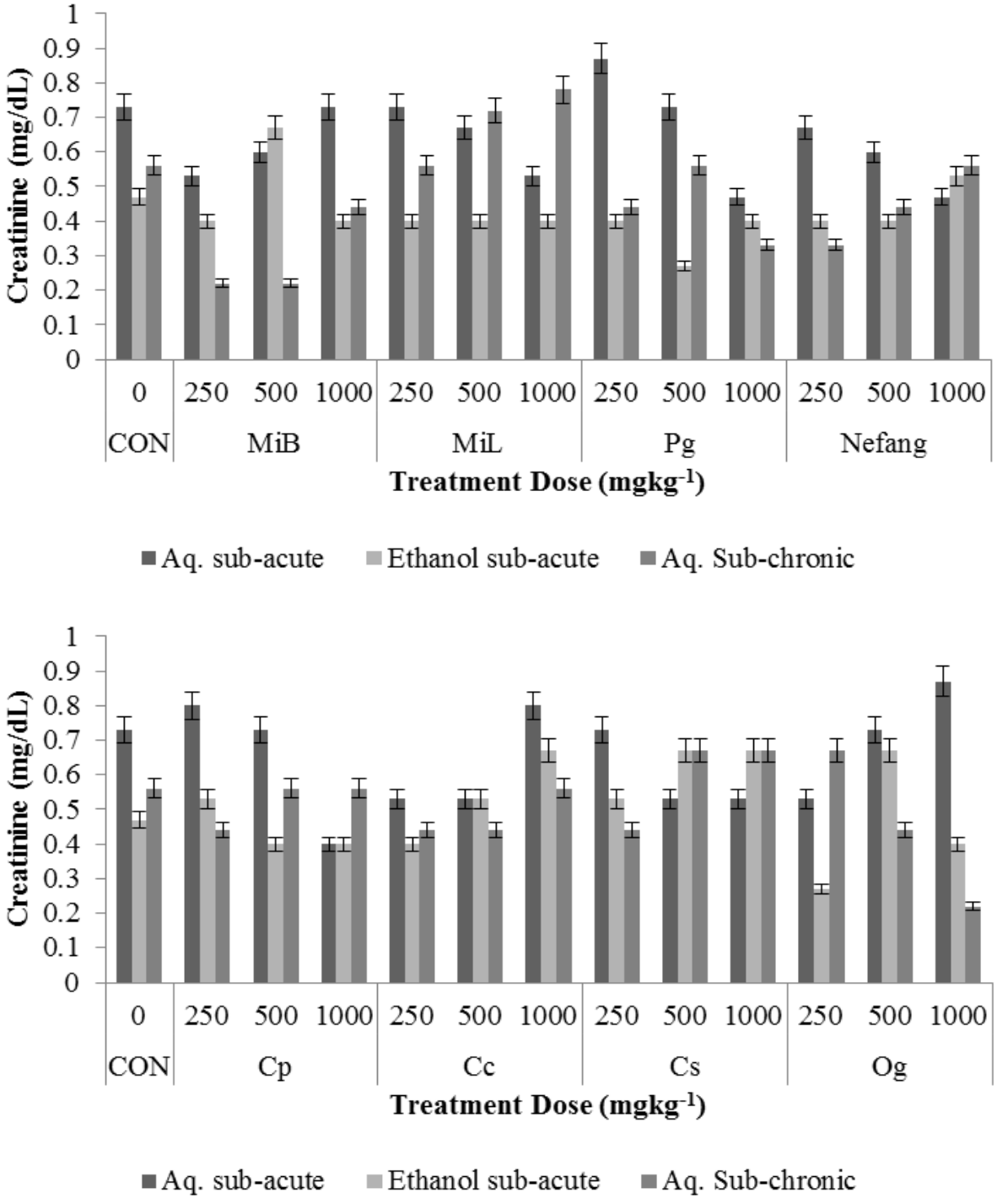

Figure 4.14. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma creatinine (CRE) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = CRE levels significantly ($p<0.05$) higher than the control*

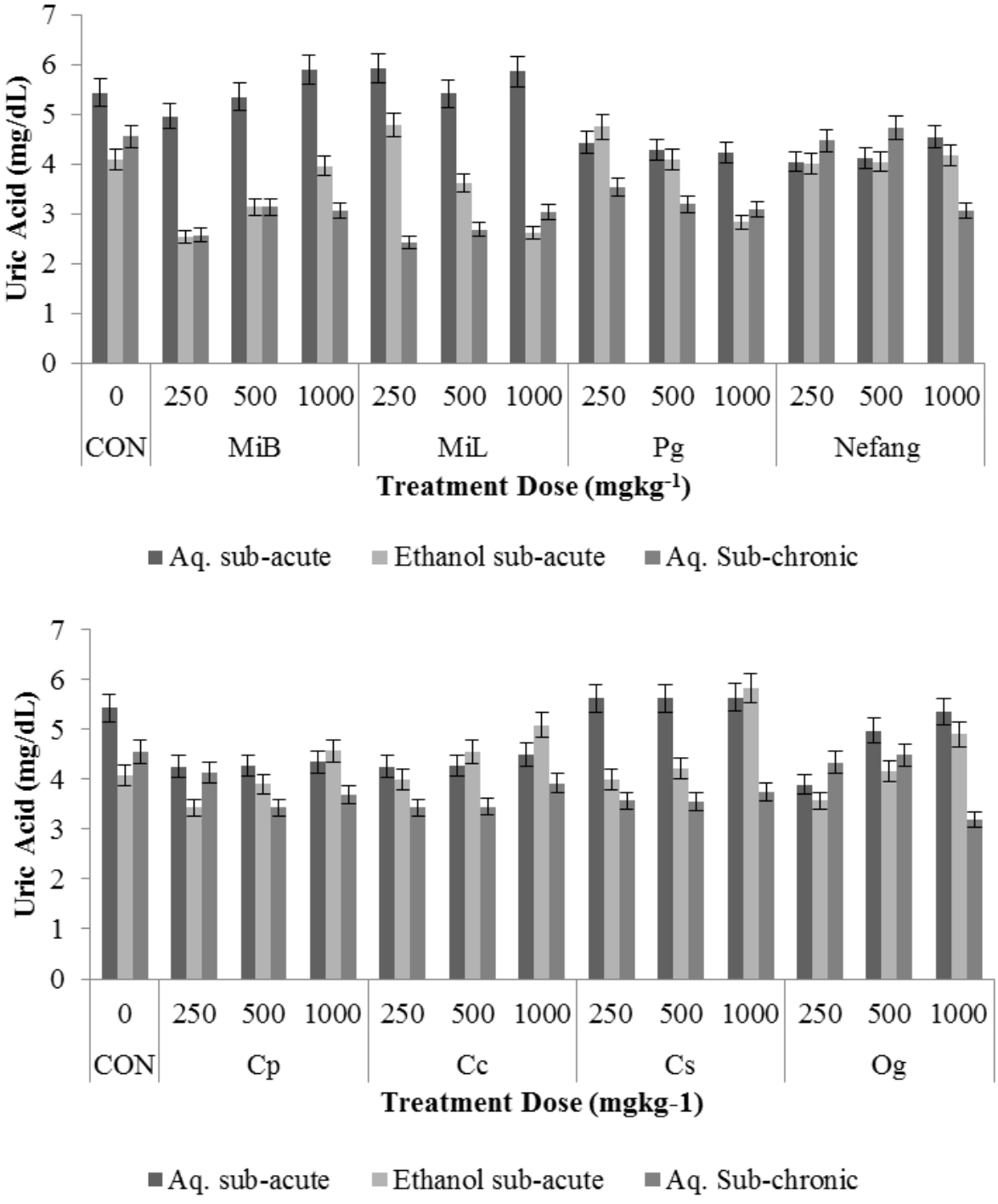

Figure 4.15. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma uric acid (URIC) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = URIC levels significantly ($p<0.05$) higher than the control*

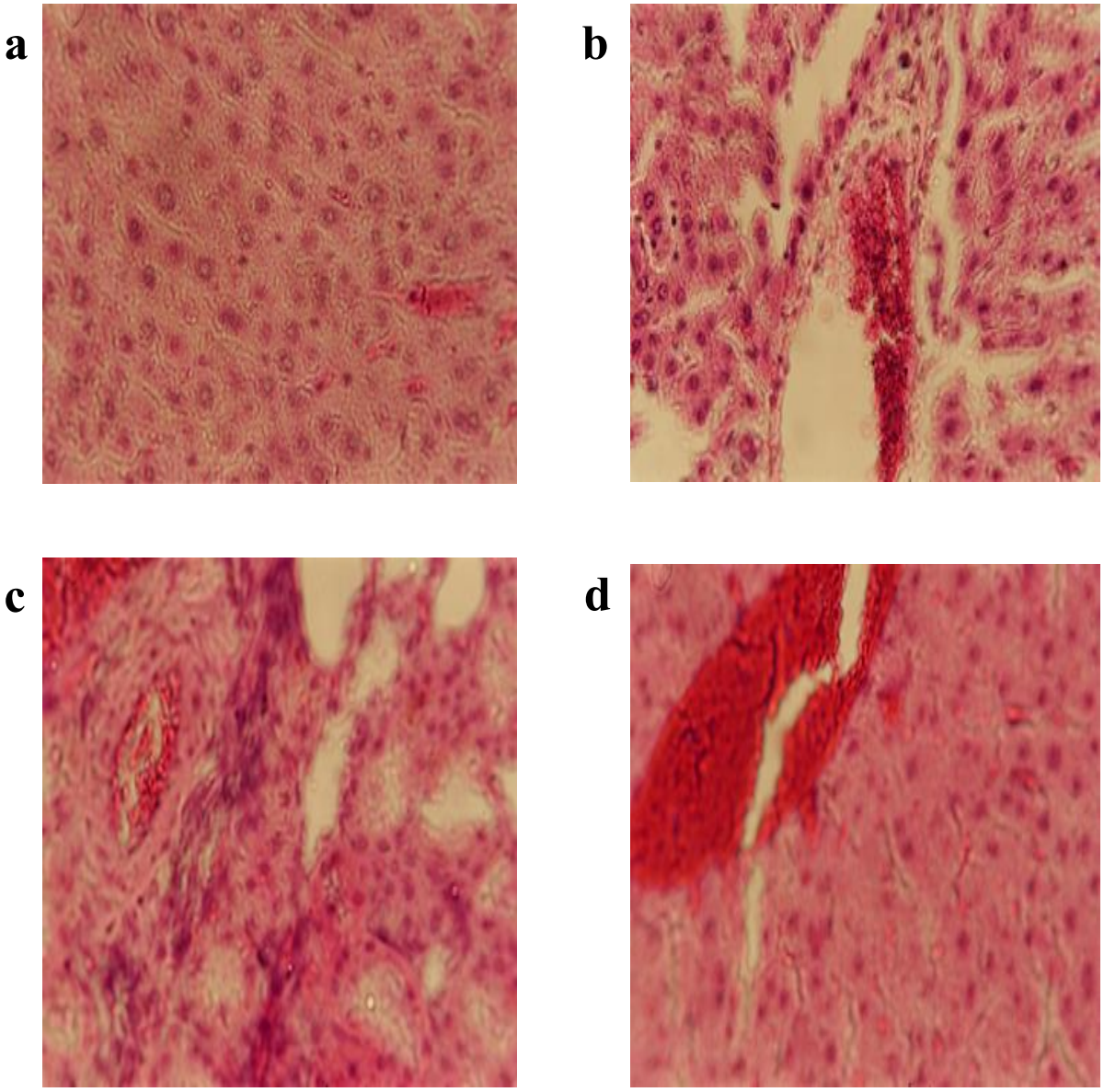

Figure 4.16. Photomicrograph of the kidney of experimental rats after administration of *Carica papaya, Cymbopogon citratus* and *Citrus sinensis* leaf extracts.

(a) Normal tubular architecture of the kidney in control. **(b)** Glumerosclerosis and tubular clarification in the kidney of rats administered *Cp* ethanol leaf extract for 28 days at 1000 $mgkg^{-1}$ **(c)** Tubular distortion in the kidney of rats administered *Cc* ethanol leaf extract for 28 days at 1000 $mgKg^{-1}$ **(d)** Mild tubular clarification and glumerulosclerosis in the kidney of rats administered *Cs* ethanol extract for 28 days at 1000 $mgkg^{-1}$. (*Hematoxylin & Eosin × 40*).

4.3.2.3 Effects on the Lipid profile

The effects of the administration of *Nefang* and its constituents on the lipid profile were evaluated by determining plasma triglycerides (**Figure 4.17**) and cholesterol (**Figure 4.18**) levels in extract-treated animals relative to the control.

Administration of *Nefang* extracts showed a significant ($p<0.05$) dose-dependent increase in TGY values in sub-acute ethanol extract-treated rats as well as CHOL levels (at 1000 $mgkg^{-1}$) when compared to the control, while sub-chronic aqueous extract-treated groups showed significantly reduced values.

However, after administration of the constituent plant extracts of *Nefang*, we observed a significant ($p<0.05$) dose-dependent increase in TGY values in sub-acute *Pg*, *Cc*, *Cs* (decreasing towards normal at 1000 $mgkg^{-1}$), *Og* ethanol and sub-chronic *Cp* aqueous extracts-treated animals relative to the control. We also observed significant ($p<0.05$) increases in sub-chronic *MIB*, sub-acute *Pg* and *Cs* aqueous extracts-treated groups at 1000 $mgkg^{-1}$. Values in all other experimental groups ranged from low to normal **(Figure 4.17).**

Analysis of plasma CHOL in experimental animals revealed a significant ($p<0.05$) increase in values in sub-chronic *Pg* and *Cc* aqueous extracts-treated groups at a dose of 1000 $mgkg^{-1}$ when compared to the control. We also observed significant decrease in values after sub-acute *MiL* and *Og* aqueous extracts-treated groups, while levels in all other experimental groups remained normal **(Figure 4.18).**

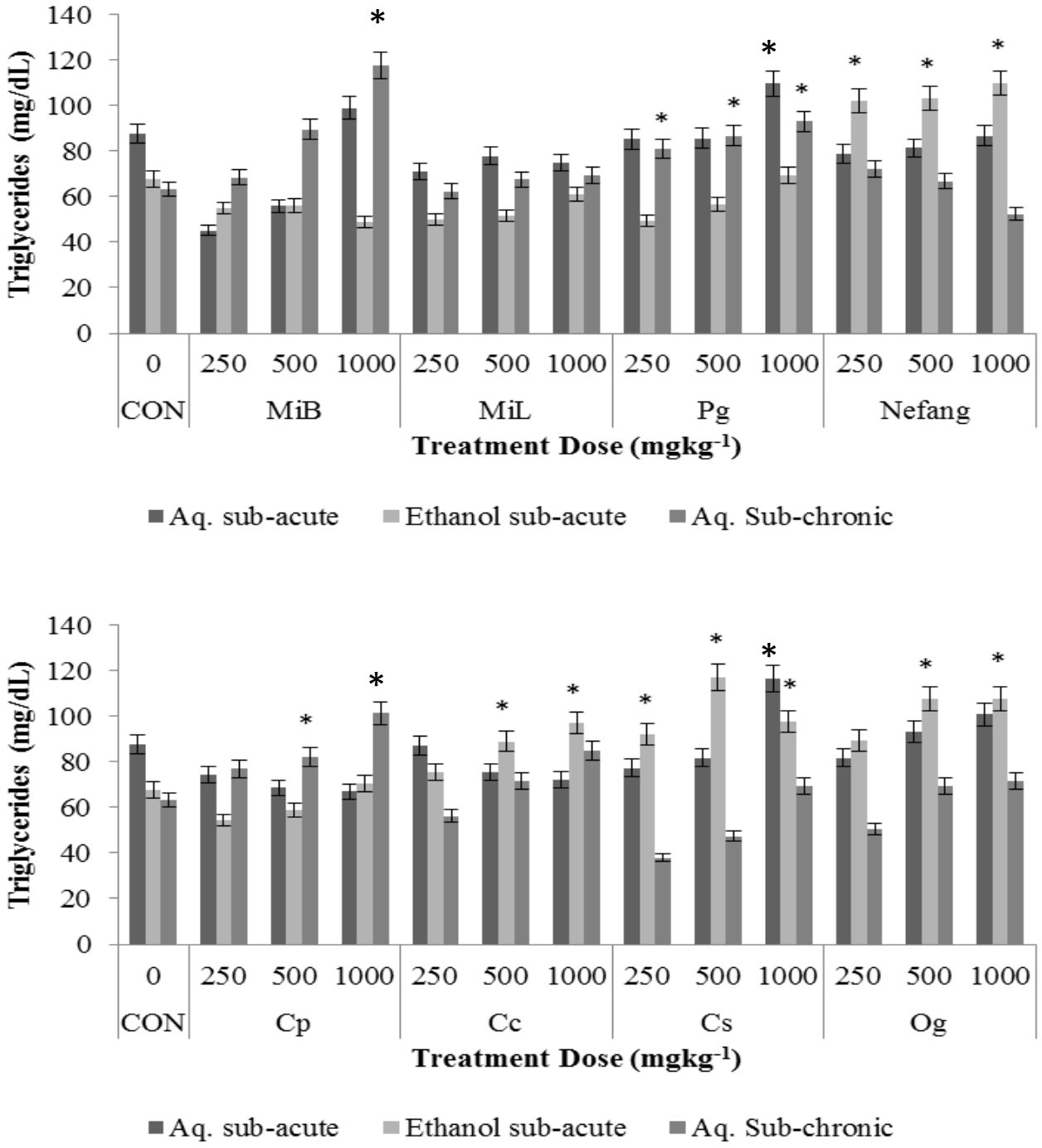

Figure 4.17. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma triglycerides (TGY) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = TGY levels significantly ($p<0.05$) higher than the control*

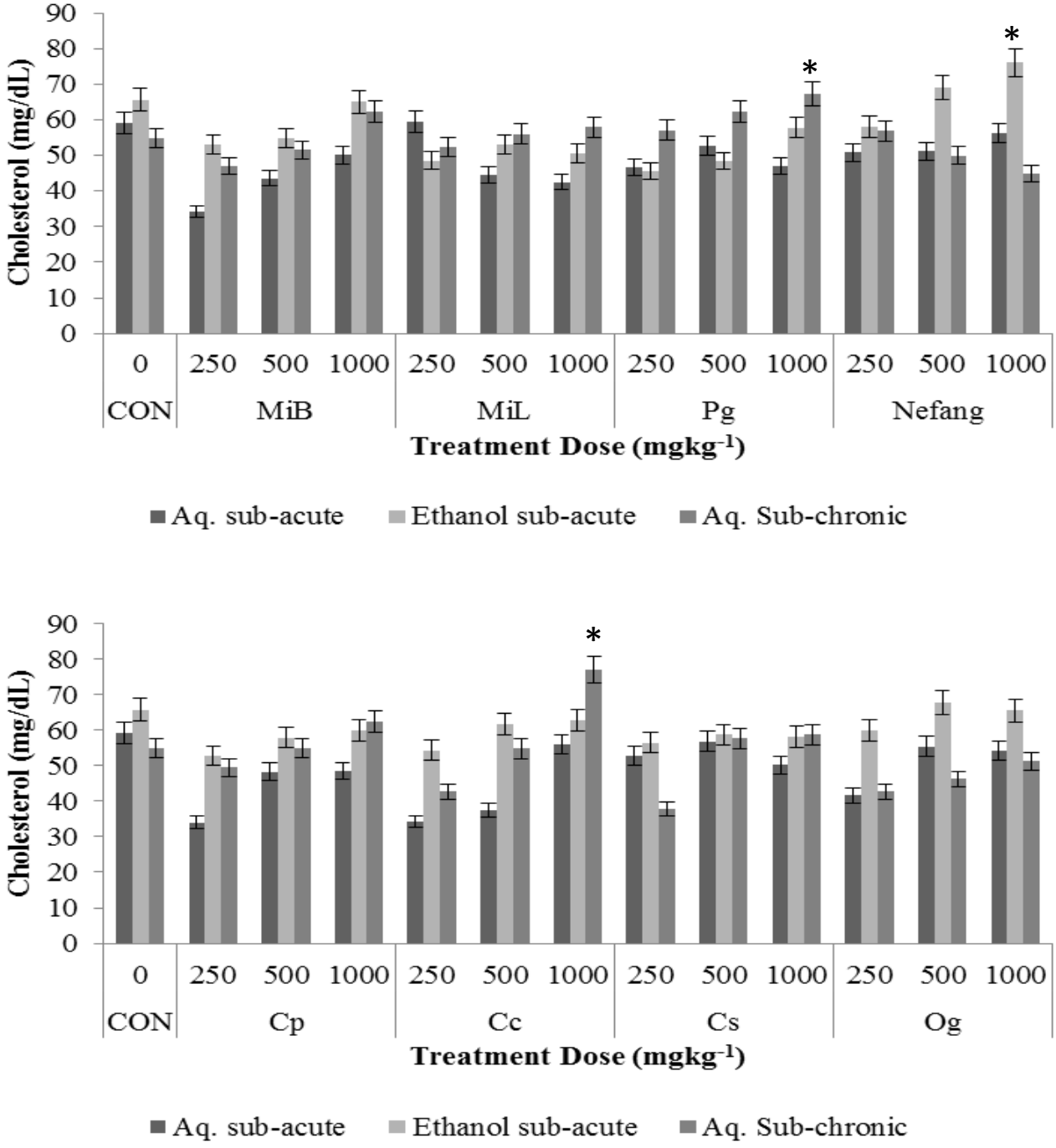

Figure 4.18. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma cholesterol (CHOL) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = CHOL levels significantly ($p<0.05$) higher than the control*

4.3.2.4 Effects on Nitrogen Balance and Glycemic levels

The effects of *Nefang* and its constituent plant extracts on the nitrogen balance and glycemic levels were determined by analyzing total proteins (TP) and glucose (GLU) in the blood plasma, as well as the overall body weight of experimental animals.

Analysis of TP revealed a significant ($p<0.05$) increase in values after sub-acute *MiB* (dose-dependent), *MiL*, *Pg* (1000 $mgkg^{-1}$), *Cp, Cc* and *Cs* aqueous extracts administration relative to the control, while levels in experimental animals treated with *Nefang* showed no significant difference **(Figure 4.19).**

Analysis of blood glucose revealed a significant ($p<0.05$) increase in GLU values in sub-acute Cs ethanol extract-treated groups. Most of the constituent ethanol plant extracts did not have significant effect on the blood glucose level in experimental animals, however, sub-acute aqueous administration of *Nefang* and its constituents significantly ($p<0.05$) lowered the GLU levels more than sub-chronic relative to the control **(Figure 4.20).**

The body weights of the experimental animals were determined at the beginning and end of the experiement and percentage body weight gained calculated. This revealed that apart from sub-acute Cc and Cs ethanol extracts administration, *Nefang* and its other constituents did not have any adverse effect on the overall body weight of experimental animals when compared to the control **(Figure 4.21).**

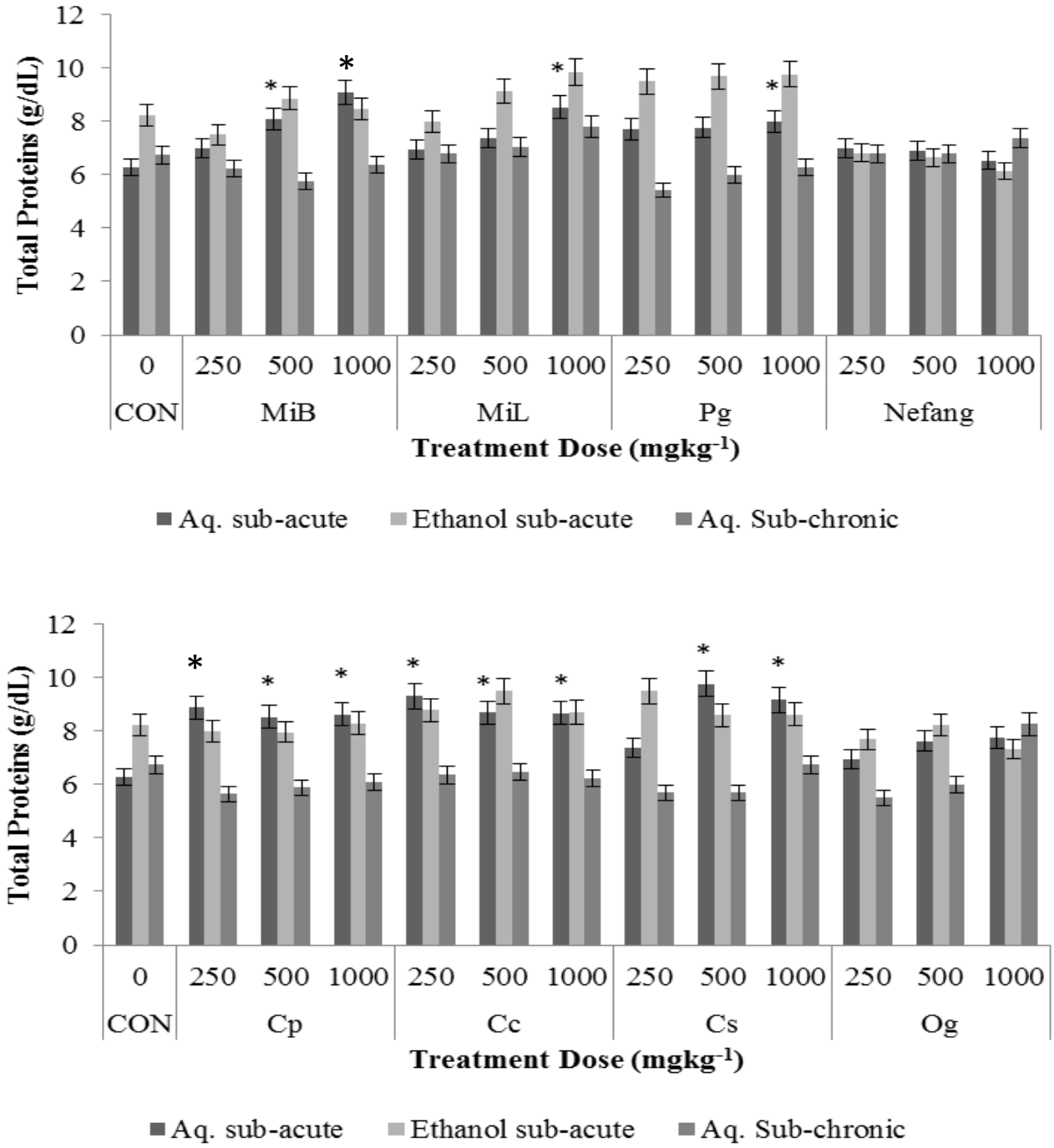

Figure 4.19. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on plasma total proteins (TP) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = TP levels significantly ($p<0.05$) higher than the control*

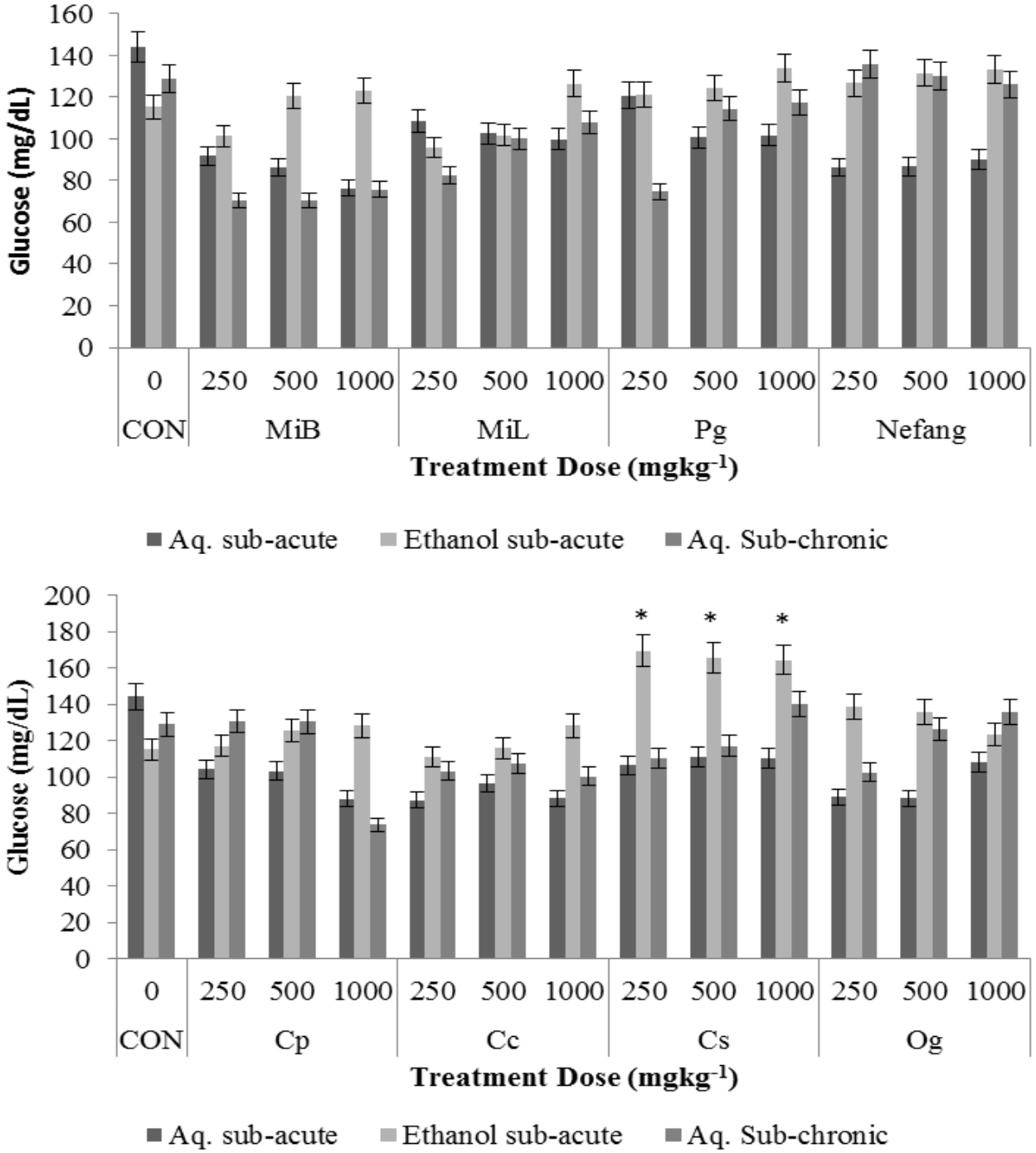

Figure 4.20. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on blood glucose (GLU) levels of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = GLU levels significantly ($p<0.05$) higher than the control*

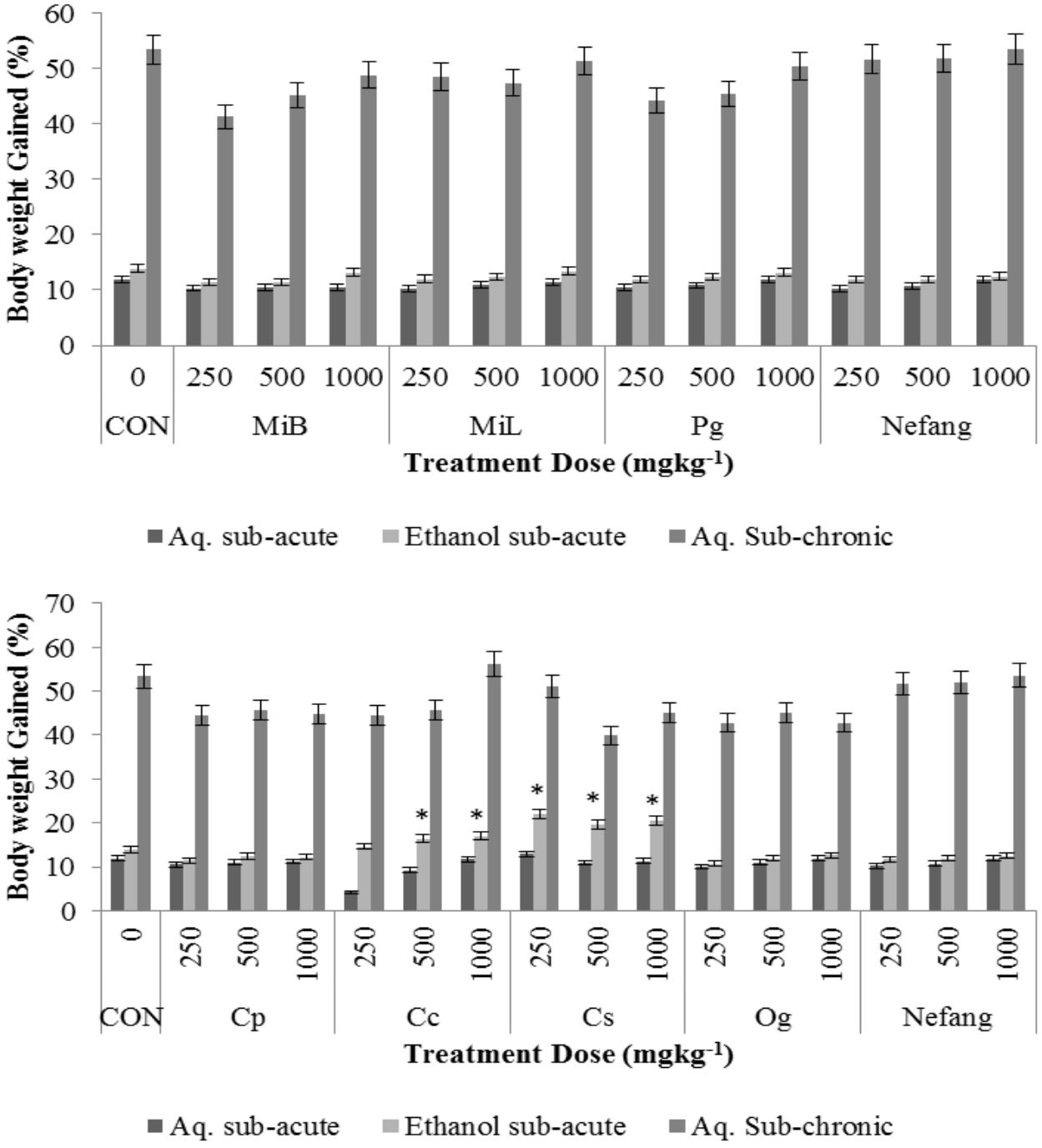

Figure 4.21. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on overall body weight of experimental rats.

Each bar represents the Mean ± SD for each group of rats, n = 6.
** = Body weight gained significantly ($p<0.05$) higher than the control*

4.3.2.5 Effects on the Hematological systems

The effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the hematological systems in experimental animals were studied by analyzing the white blood cells (WBC), red blood cells (RBC), platelet (PLT) counts and some related parameters **(Figure 4.22 – 4.27).**

White blood cells count revealed dose-dependent significant ($p<0.05$) increase in values in all sub-acute aqueous extracts-treated groups relative to the control, apart from *MiL* with normal values and *Cc* with signficiantly ($p<0.05$) high values, dose-dependently decreasing towards normal. After sub-chronic administration of the aqueous extracts, we observed dose-dependent significant increase in values in *MiL, Cp, Cs* and *Nefang* groups, whereas after sub-acute ethanol extracts administration, we noticed dose-dependent significant ($p<0.05$) increase in values in *Pg* and *Nefang* groups, which were significant at 1000 $mgkg^{-1}$, as well significant increases in *Cs* and *Og* treated groups. Apart from *MiL* and *Cp,* ethanol extract-treated groups showed significantly ($p<0.001$) higher WBC counts than aqueous extract-treated groups **(Figure 4.22)**.

Analysis of the lymphocyte number revealed dose-dependent significant ($p<0.05$) increase in values in all sub-acute aqueous extracts-treated groups relative to the control, apart from *MiL* with normal values and *MiB* which showed a dose-dependent increase which was significant at 1000 $mgkg^{-1}$. Sub-acute aqueous extract administration exhibited dose-dependent significant increase in LYM number in *MiB, MiL, Pg, Cp, Og* (1000 $mgkg^{-1}$) and *Nefang* treated groups, whereas sub-acute ethanol extract administration revealed a significant increase in the *Pg* (1000 $mgkg^{-1}$) and *Cp, Cs, Og* treated groups, which were dose-dependently decreasing towards normal **(Figure 4.23)**.

Red blood cells count analysis revealed dose-dependent significant ($p<0.05$) increase in values in all aqueous extracts-treated groups apart from *MiB* (significant only at 1000 $mgkg^{-1}$), *Cp* and *Cc*, which were initially significant but dose-dependently decreasing towards normal. The same trend was observed after aqueous sub-chronic and ethanol sub-acute administration apart from *MiB, Cp, Cs* and *MiB, Nefang* respectively. In aqueous sub-chronic administration, *MiB*-treated groups showed significantly ($p<0.001$) high RBC values dose-dependently decreasing towards normal, *Cp* showed significant values at 1000 $mgkg^{-1}$ and *Cs* showed normal values whereas in ethanol sub-acute administration, *MiB* and *Nefang* treated groups showed normal values when compared

to the control. However, RBC values after sub-chronic administration of *MiB*, MiL and Pg aqueous extracts were significantly ($p<0.05$) higher than those after sub-acute **(Figure 4.24)**. Determination of hemoglobin levels in experimental animals revealed significant increase in values after *Pg* (dose-dependent; $p<0.05$), *Cs*, *Og*, *Nefang* (dose-dependent; $p<0.001$), *MiB* and *MiL* (1000 $mgkg^{-1}$) sub-acute aqueous extracts administration when compared to the control. Sub-chronic aqueous extracts-treated groups exhibited significant increase in HGB values in *MiL*, *Pg*, *Cp*, *Og* (dose-dependent), *MiB*, *Cc*, *Cs* and *Nefang* treated groups whereas all experimental groups showed normal values after sub-acute administration of ethanol extracts, apart from *Og* (significant at 1000 $mgkg^{-1}$). *MiB* (aqueous-250 $mgkg^{-1}$ and ethanol sub-acute) and *Pg* (ethanol-250 $mgkg^{-1}$) extracts-treated groups stood out with HGB values significantly lower than that of the control **(Figure 4.25)**.

Determination of the hematocrit percentage in experimental animals revealed no significant changes in values after sub-acute and sub-chronic administration aqueous and ethanol extracts of *Nefang* and its constituents when compared the control. In as much as there were some increase and/or decrease in the values in some experimental groups, these were statistically non-significant **(Figure 4.26).**

Determination of platelets count in experimental animals revealed that values in all experiemental groups were withing the normal range apart from sub-acute *MiB*, *Cp*, *Og* and sub-chronic *Cs* aqueous extracts-treated groups; significant ($p<0.05$) increase in values were observed after sub-acute administration of *MiB* and *Og* at 1000 $mgkg^{-1}$, *Cp* at 250 $mgkg^{-1}$ which was dose-dependently decreasing to normal as well as sub-chronic administration of *Cs* at 250 $mgkg^{-1}$, dose-dependently decreasing to normal relative to the control **(Figure 4.27)**.

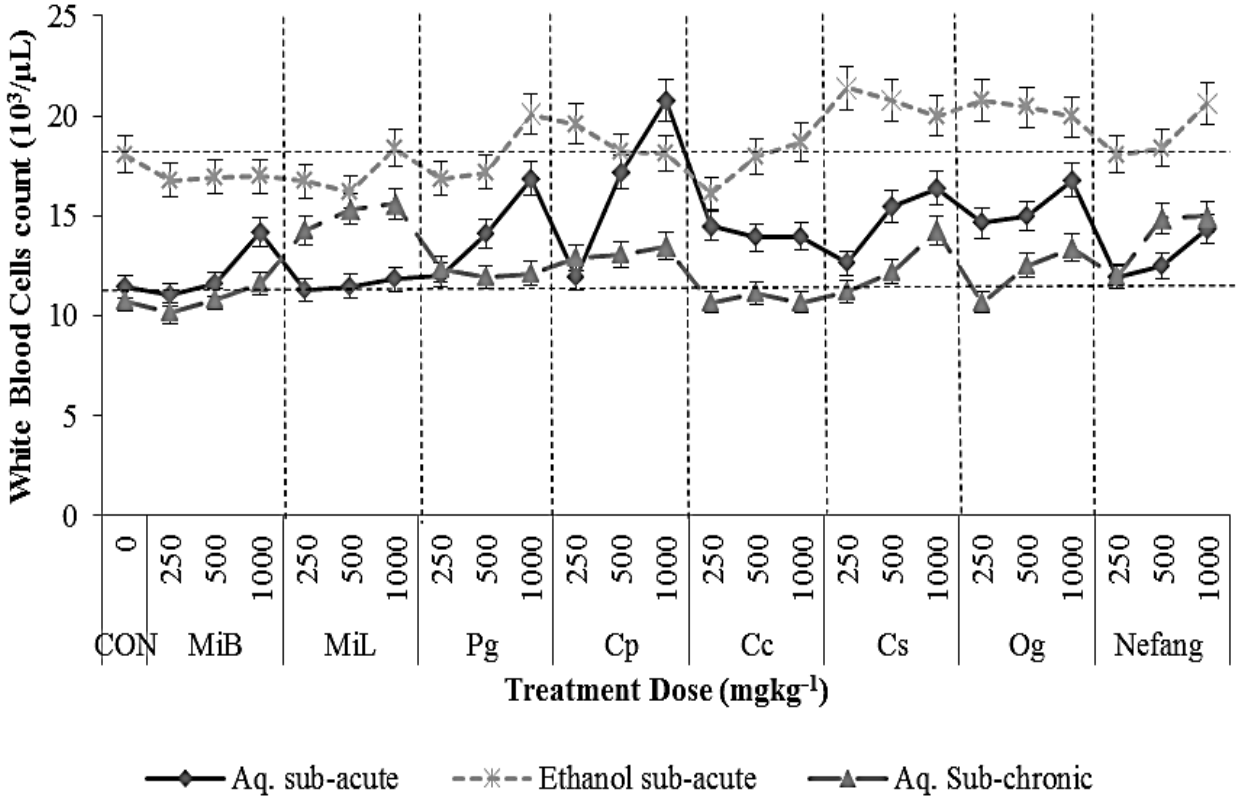

Figure 4.22. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on white blood cells (WBC) count of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6

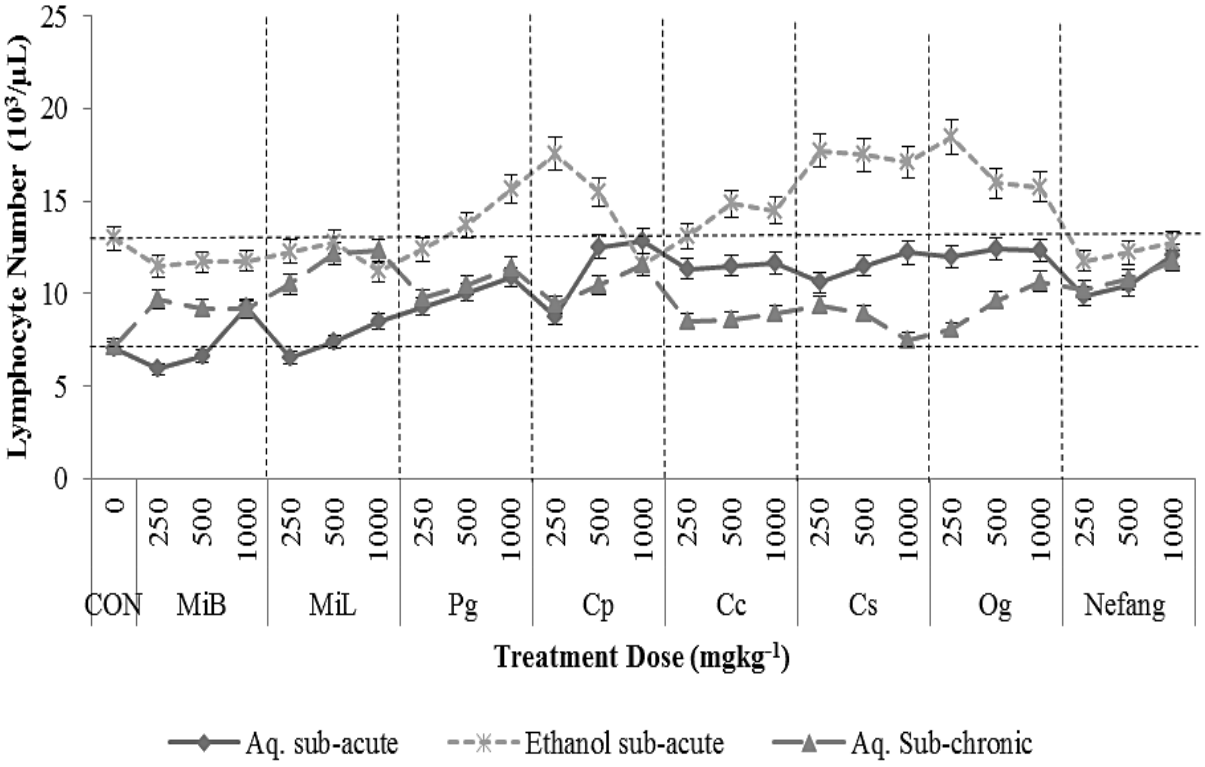

Figure 4.23. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on lymphocyte number of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6

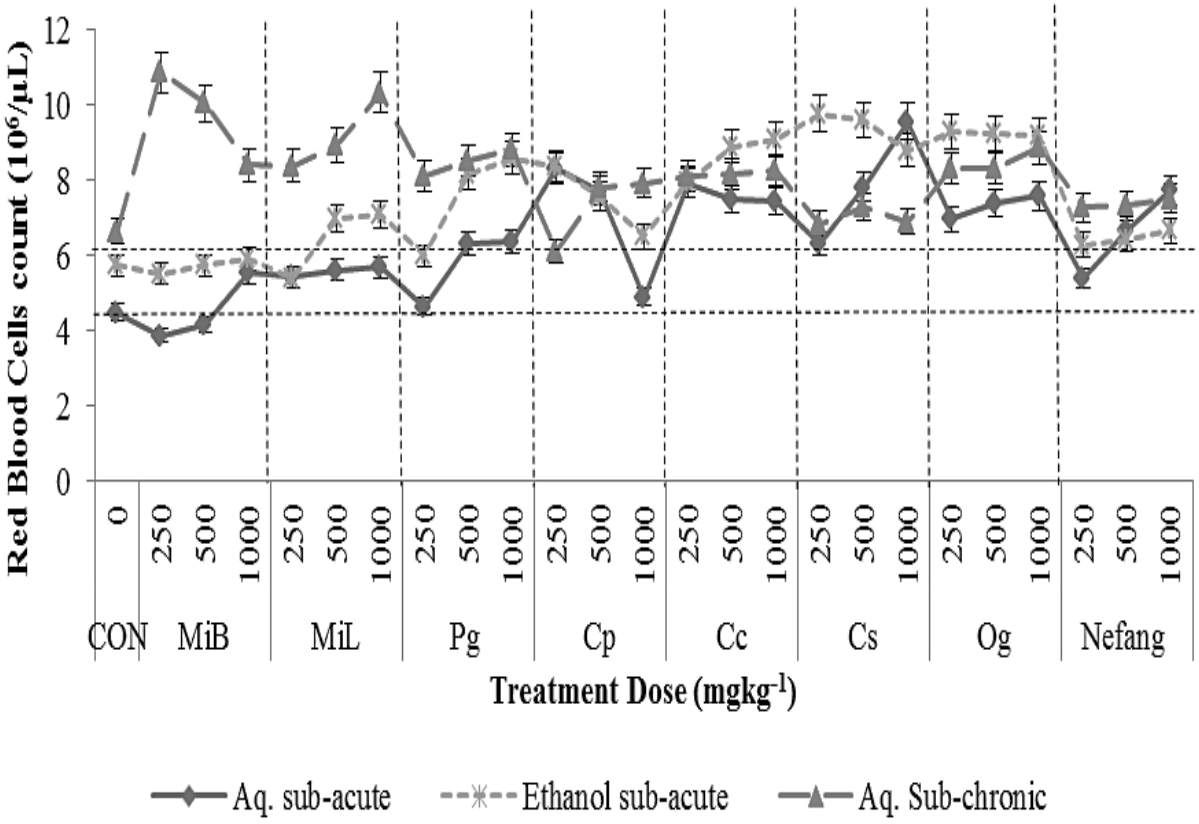

Figure 4.24. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the red blood cells (RBC) count of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6

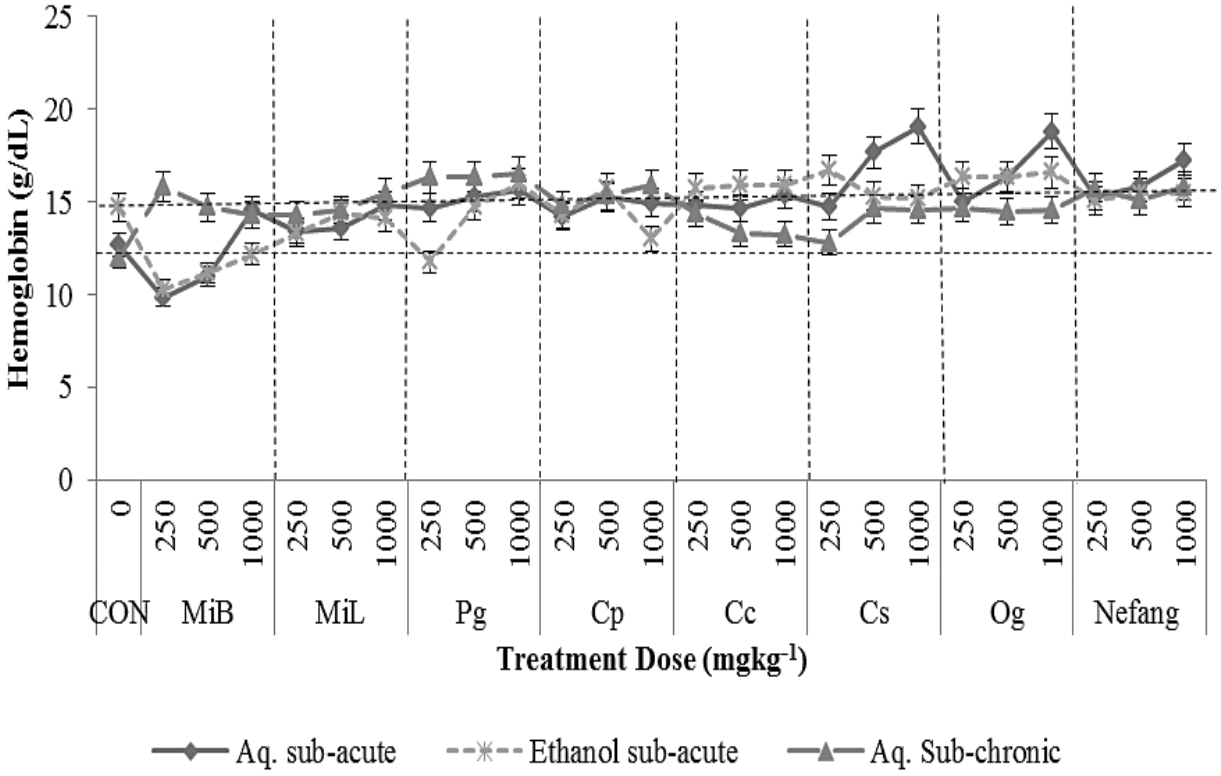

Figure 4.25. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the hemoglobin (HGB) levels of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6

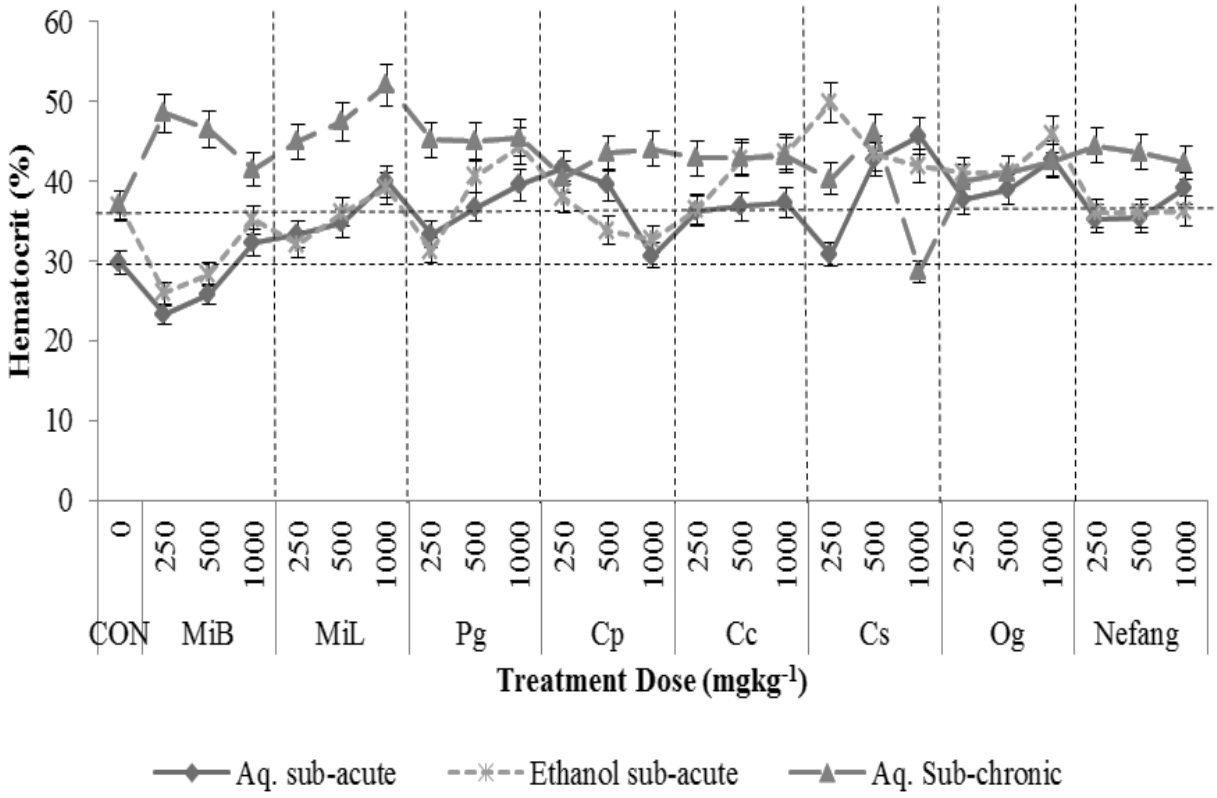

Figure 4.26. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the hematocrit (HCT) levels of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6

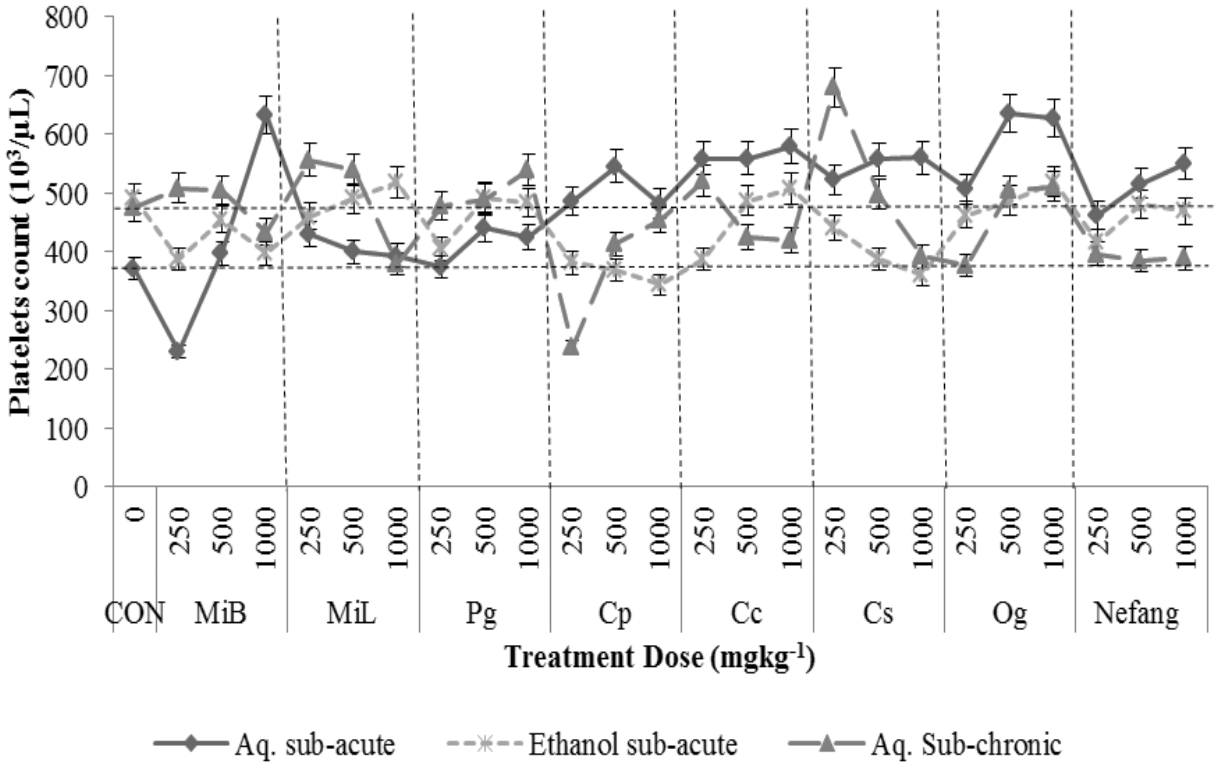

Figure 4.27. Effects of sub-acute and sub-chronic administration of the aqueous and ethanol extracts of *Nefang* and its constituents on the platelets (PLT) count of experimental rats.

Each data point represents the Mean ± SD for each group of rats, n=6.

An overview of the overall toxicity of *Nefang* and its constituents on the organ-system of experiemental rats revealed that some constituent plants showed moderate to mild toxicities but *Nefang* did not show any toxicity apart from mild toxicity exhibited by its ethanol extract on the lipid profile. No extract showed severe toxicity. This has been summarized in **Table 4.15**.

Table 4.15. Toxicological Profile of *Nefang* and its constituent plants on the organ-system of wistar rats

Extract	Hepato toxicity	Renal Toxicity	Effects on Lipid Profile	Effects on Nitrogen Balance	Effects on Glycemic Levels	Effects on WBC count	Effects on RBC count	Effects on PLT count
***Mangifera indica* bark**			**Mild** ↑TGY ↓CHOL		↓		↑ Sub-acute	↑
***Mangifera indica* leaf**	**Mild** ↑ AST		↓CHOL				↑ Sub-acute	
Psidium guajava	**Mild** ↑ AST	**Mild** (EtOH) ↑ BUN	**Moderate** (EtOH) ↑CHOL					
Carica papaya	**Mild** (EtOH) ↑ AST Liver injury	**Moderate** (EtOH) ↑ BUN Kidney injury	**Mild** ↑ TGY ↓CHOL		↓	↑		↓ Sub-acute
Cymbopogon citratus	**Moderate** (EtOH) ↑AST ALT Liver injury	**Mild** (EtOH) Kidney injury	**Moderate** (EtOH) ↑TGY ↓CHOL		↓			
Citrus sinensis	**Moderate** (EtOH) ↑ AST ALT Liver injury	**Moderate** (EtOH) ↑BUN Kidney injury	**Mild** (EtOH) ↑ TGY		(EtOH) ↑		↑	↑ Sub-acute
Ocimum gratissimum			Mild ↑ TGY		↓			↑
Nefang			**Mild** (EtOH) ↑TGY ↓ CHOL		↓			

4.4 EVALUATION FOR BIOLOGICAL ACTIVITIES OF THE CONSTITUENT PLANTS OF NEFANG THAT COMPLEMENT IN VIVO ANTIMALARIAL EFFECTS

4.4.1 In vitro Antioxidant Properties

The results of the antioxidant activity of each extract were determined using various methods presented in **Table 4.16**. No significant difference was observed between the Total phenolic content, ferric reducing antioxidant power and the radical scavenging activities of *Pg*, *MiL*, *MiB* and *Og* aqueous extracts. The analysis of the radical scavenging activity using the DPPH assay revealed that *Pg*, *MiL* and *MiB* had the greatest activity (> 90% inhibition). Their total phenolic content ranged from 61.7 to 67.2 mg catechine equivalent/g of extract. *Og* had a moderate activity (59% inhibition) and total phenolic content of 34 mg catechine equivalent/g extract. *Cc*, *Cp* and *Cs* had very weak activity and low total phenolic content.

Table 4.16. In vitro Antioxidant Activity of constituent plant extracts of *Nefang*.

Aqueous Extract (10 mg/mL)	DPPH Inhibition of Radical Activity (%)	Total Phenolic content (TPC) (mg catechine equivalent / g Extract)	FRAP (mg catechine equivalent / g Extract)
Mangifera indica bark	$90.11 \pm 0.73^{(3)}$	$66.33 \pm 1.92^{(3)}$	$587.08 \pm 8.01^{(3)}$
Mangifera indica leaf	$90.76 \pm 0.10^{(2)}$	$61.73 \pm 2.68^{(2)}$	$590.17 \pm 6.76^{(2)}$
Psidium guajava leaf	$91.65 \pm 0.38^{(1)}$	$67.15 \pm 10.57^{(1)}$	$644.17 \pm 25.69^{(1)}$
Carica papaya leaf	$13.88 \pm 3.10^{(6)}$	$6.41 \pm 0.90^{(7)}$	$117.95 \pm 31.23^{(6)}$
Cymbopogon citratus leaf	$33.74 \pm 4.01^{(5)}$	$17.03 \pm 0.39^{(5)}$	$166.72 \pm 78.37^{(5)}$
Citrus sinensis leaf	$13.81 \pm 4.42^{(7)}$	$7.59 \pm 0.98^{(6)}$	$78.09 \pm 0.76^{(7)}$
Ocimum gratissimum leaf	$59.52 \pm 7.93^{(4)}$	$34.42 \pm 1.74^{(4)}$	$353.00 \pm 9.43^{(4)}$

Data presented as Mean ± SD; n=3. In the same column, values designated different superscripts are significantly different. ()=Ranking

Evaluation of the relationship between DPPH, TPC and FRAP antioxidant activity revealed a highly significant ($p<0.01$) positive correlation between the radical scavenging activity and Total Phenol Content , the radical scavenging activity and antioxidant activity, the Total Phenol Content and antioxidant activity , with correlation indices of 0.985, 0.980 and 0.993 respectively.

4.4.2 In vivo Antioxidant Activity: Effect of *Nefang* in carbon tetrachloride-treated rats.

4.4.2.1 Effect on Physiological Parameters.

We observed a significant decrease in weight gain ($p<0.01$) in all test groups (B, C and D) compared to the untreated control group. The group treated with carbon tetrachloride alone (B) showed the least gain in weight as summarized in **Figure 4.28.** The low rate of increase in body weight was probably due the decreased water and food intake ($p<0.05$).

The relative mass of the liver in mice treated with carbon tetrachloride (group B) increased significantly. There was also a comparable increase in the mass of the liver of mice treated with a low dose of *Nefang* (100 $mgkg^{-1}$; group C) when compared to the control. These effects are summarized in **Table 4.17**. During the experiment we also observed reduced mobility and change in colour of the fur in CCl_4-treated rats which lasted the length of the experiment contrary to the test groups in which the fur regained its colour by the end of the experiment.

Treatment with *Nefang* and carbon tetrachloride seemed to have no significant effect on the relative mass of the kidney and heart.

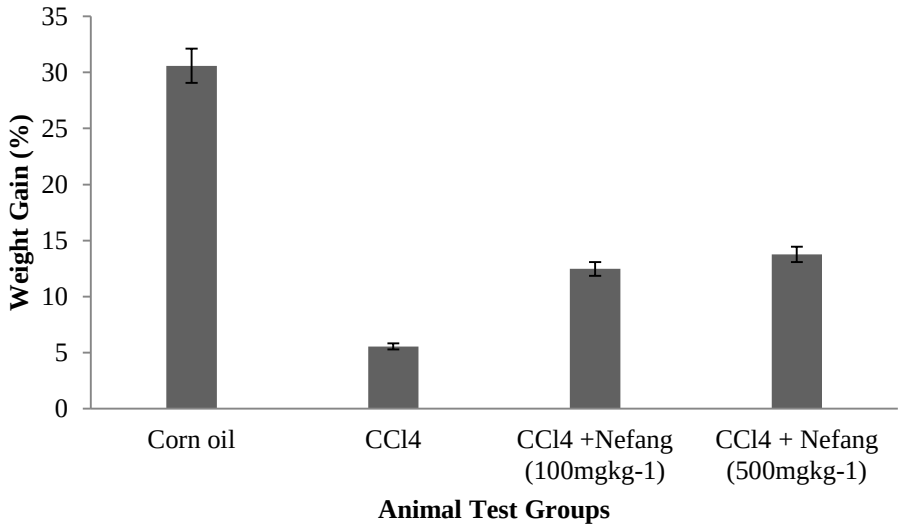

Figure 4.28. Percentage weight gain in carbon tetrachloride-treated rats compared to non-treated rats.

Bars represent means ± SD; n=5

Table 4.17. Effect of *Nefang* on selected Physiologic parameters.

Physiologic Parameters	Group A (Corn oil)	Group B (CCl_4)	Group C (CCl_4+100 mg/kg *Nefang*)	Group D (CCl_4+500 mg/kg *Nefang*)
Food intake(g)	125.92 ±16.64	85.69 ± 30.04*	93.53 ± 33.39*	94.30 ± 29.11*
H_2O intake (mL)	88.76 ± 20.1	47.69 ± 27.43*	49.61 ± 24.95*	51.46 ± 26.81*
Relative weight of Heart (g)	0.33 ± 0.19	0.33 ± 0.19	0.29 ± 0.02	0.32 ± 0.03
Relative weight of liver (g)	3.36 ± 0.39	4.78 ± 0.49*	4.37 ± 0.25*	4.02 ± 0.3
Relative weight of kidneys (g)	0.34 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.39 ± 0.01

*Data presented as Mean ± SD; n=5; Significant difference: *-p<0.05,**-p<0.01.*

4.4.2.2 Effect on Biochemical Parameters and Oxidative Stress Markers

The effects of *Nefang* aqueous extract on biochemical parameters and oxidative stress markers are presented in **Table 4.18** and **Table 4.19** respectively. These analyses revealed a significant decrease in CHOL ($p<0.05$) and TGY level ($p<0.01$) and a significant increase in BUN, CRE ($p<0.05$), ALT and AST ($p<0.01$) in group B (CCl_4-induced oxidative stressed) rats compared to the normal control group. In rats treated with *Nefang* (group C and D), we observed that at a dose of 100mgkg^{-1}, CHOL and ALT levels were abnormally high when compared to the untreated control group whereas at 500 mgkg^{-1}, CHOL was restored to normal while ALT level decreased towards normal. However, *Nefang* prevented the increase in AST, BUN, and CRE while it increased TGY levels towards normal in a dose-dependent manner when compared to the untreated group. *Nefang* had no effect on the GLU level relative to the untreated control group.

We also observed a significant decrease in SOD and CAT levels ($p<0.05$), increase in TP and MDA ($p<0.05$) in untreated control group (CCl_4-induced oxidative stressed) compared to the normal control group. At a low *Nefang* dose of 100 mgkg^{-1}, levels of SOD, CAT and MDA remained comparable to that of the untreated control groups. However, at a higher dose of 500 mgkg^{-1}, we observed a significant increase in SOD ($p<0.05$) and a decrease in MDA ($p<0.05$) levels compared to the untreated control group, showing that this activity was dose-dependent.

Table 4.18. Effect of *Nefang* on Biochemical parameters of carbon tetrachloride-treated wistar rats.

Biochemical Parameters	Group A (Corn oil)	Group B (CCl_4)	Group C (CCl_4+100 mg/kg *Nefang*)	Group D (CCl_4+500 mg/kg *Nefang*)
GLU (mg/dL)	124.07 ± 5.69	151.87 ±5.03*	160.04 ± 4.31	154.30±10.69
CHOL (mg/dL)	86.98 ± 7.60	74.92 ± 7.69*	135.71± 23.80$^{\#\#}$	91.42 ± 5.03
TGY (mg/dL)	227.47 ± 5.89	142.28±25.27**	154.89± 18.60$^{\#}$	166.01 ±3.35$^{\#}$
ALT (UI/L)	16.97 ± 2.05	97.38 ± 5.26**	126.67±12.08$^{\#\#}$	54.78 ± 2.85$^{\#\#}$
AST (UI/L)	35.52 ± 3.94	69.52 ± 1.29**	63.77 ± 7.01$^{\#}$	54.84 ± 7.75$^{\#}$
BUN (mg/dL)	35.63 ± 3.01	40.73 ± 1.50*	39.08 ± 3.32	33.12 ± 0.38$^{\#}$
CRE (mg/dL)	0.77 ± 0.09	0.88 ± 0.83*	0.88 ± 0.04	0.76± 0.05$^{\#}$

GLU-Glucose**, CHOL**-Cholesterol**, TGY**-Triglycerides**, ALT**-Alanine Aminotransferase, **AST**-Aspartate Aminotransferase, **BUN**-Blood Urea Nitrogen**, CRE**-Creatinine ($\bar{x}$ ± SD, n=5)
*Significant difference: compared to normal control-(*)-p<0.05,(**)-p<0.01, compared to untreated control-($^{\#}$)-p<0.05, ($^{\#\#}$)-p<0.01.*

Table 4.19. Effect of *Nefang* on Oxidative stress markers of carbon tetrachloride-treated wistar rats

Biochemical Parameters	Group A (Corn oil)	Group B (CCl_4)	Group C (CCl_4+100 mg/kg *Nefang*)	Group D (CCl_4+500 mg/kg *Nefang*)
SOD (UI/mg)	8.15 ± 0.35	6.77 ± 0.19*	8.54 ± 0.53	9.56 ± 0.43$^{\#}$
CAT (UI/mg)	0.1 ± 0.20	0.04 ± 0.00**	0.14 ± 0.04$^{\#}$	0.12 ± 0.30$^{\#}$
MDA (µMol/L)	1.15 ± 0.30	1.18 ± 0.19*	1.17 ± 0.07	1.07 ± 0.05$^{\#}$
TP (mg/mL)	24,05 ± 1,06	27,72 ± 1,06*	20,51 ± 1,53$^{\#}$	24,15 ± 2,8

SOD-Superoxide Dismutase, **CAT**-Catalase, **MDA**-Malondialdehyde, **TP**-Total Proteins.
$\bar{x}$ ± SD, n=4; *Significant difference: compared to normal control; (*)-p<0.05,(**)-p<0.01compared to untreated control; ($^{\#}$)-p<0.05, ($^{\#\#}$)-p<0.01.*

4.4.3 Antipyretic activity

4.4.3.1 Effect on D-Amphetamine-induced pyrexia method

Antipyretic activities of the constituent plant extracts of *Nefang* were evaluated using D-amphetamine induced pyrexia in experimental animals. In rats treated with the standard drug, paracetamol (150 $mgkg^{-1}$), we observed an initial rise in body temperature of approximately 1^oC after 30 minutes. The highest rise in body temperature observed after 60 minutes was $<2^oC$. However, after 120 minutes we observed a significant ($p<0.05$) decrease in body temperature when compared to the control. This declined to normal body temperature after 240 minutes, which was significantly ($p<0.001$) lower when compared to that of the control (**Table 4.20**).

Upon administration of the constituent plant extracts of *Nefang*, we observed the same rise in body temperature after 30 minutes in all groups, with highest body temperatures attained after about 60 minutes. After 120 minutes, we observed significant ($p<0.05$) decrease in body temperatures in animals treated with *Cc*, *Og*, *MiL*, *MiB* and *Cs*. We observed that *Cc* exhibited the best activity. After 240 minutes, *Cc*, *Og* and *Cs* exhibited significantly ($p<0.001$) better activities than the standard drug, paracetamol. *Pg* and *Cp* exhibited very weak antipyretic activities in experimental animals **(Figure 4.29).**

4.4.3.2 Brewer's Yeast-induced hyperpyrexia method

Antipyretic activities of the constituent plant extracts of *Nefang* were further evaluated using brewer's yeast-induced hyperpyrexia in experiemental animals. In rats treated with the standard drug, paracetamol (150 $mgkg^{-1}$), we observed the same trend in temperature rise like the previous exercise with D-amphetamine, with body temperatures of experimental animals coming back to normal after 240 minutes and significantly ($p<0.001$) lower when compared to that of the control (**Table 4.21**).

Cc, *Og*, *MiL*, *MiB* and *Cs* exhibited significant ($p<0.05$) better antipyretic activities while *Pg* and *Cp* exhibited lower activities when compared to the control. At the end of 240 minutes, *Cc* and *Og* exhibited comparable activities to the standard drug paracetamol. We observed once more that *Cc* exhibited the best activity amongst all the extracts tested **(Figure 4.30).**

The antipyretic effects of the constituents of *Nefang* were similar in both assays.

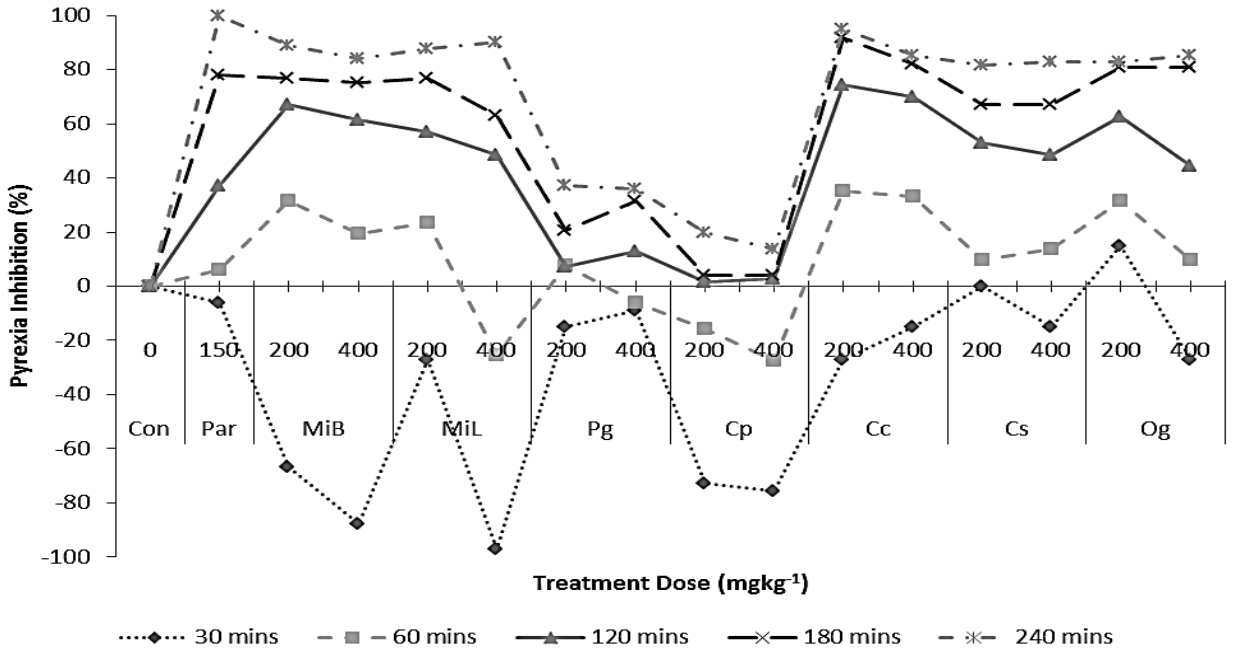

Figure 4.29. Inhibition of D-Amphetamine-induced pyrexia in wistar rats by the constituent plant extracts of *Nefang*.

Each data point represents the mean ± SD for each group of rats; n=4;
***Con** – control; **Par** – Paracetamol.*

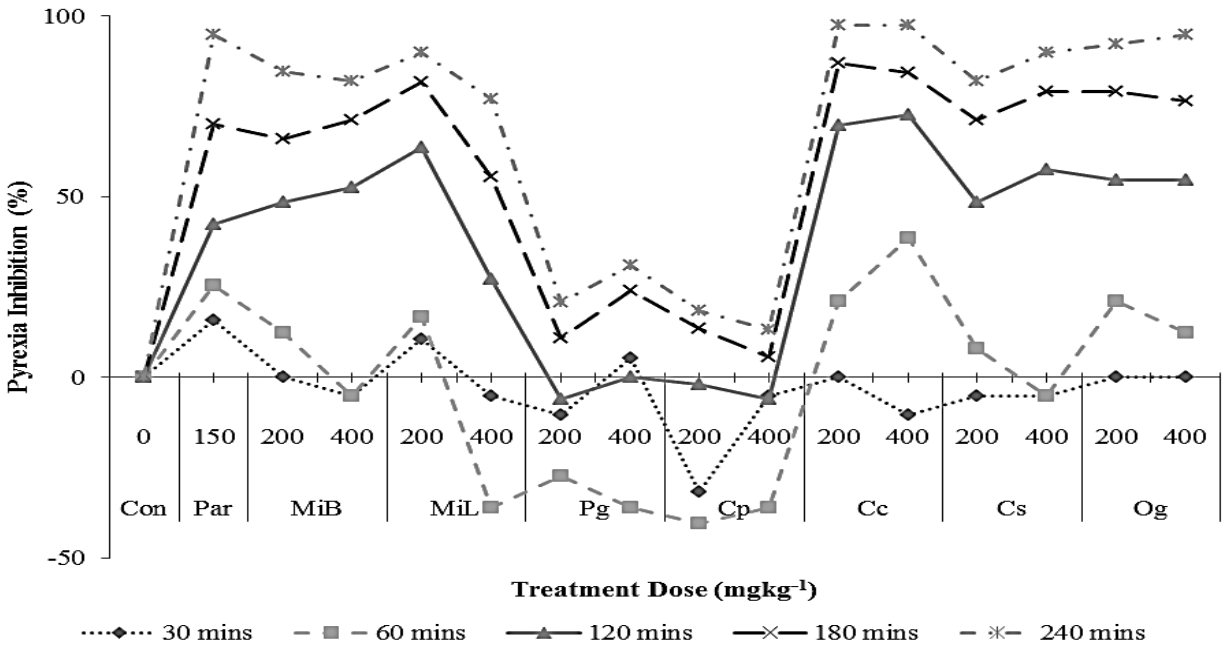

Figure 4.30. Inhibition of Brewer's Yeast-induced pyrexia in wistar rats by the constituent plant extracts of *Nefang*.

Each data point represents the mean ± SD for each group of rats; n=4;
***Con** – control; **Par** – Paracetamol.*

Table 4.20. Effect of the constituent aqueous plant extracts of *Nefang* on D-amphetamine-induced pyrexia in wistar rats.

Treatment (Drug/ Aqueous Extract)	Dose (mg/ kg)	Body Temperature (^{o}C) over Time interval (minutes) ($\bar{x}$ ± SD, n=4)					
		Basal Temperature	30	60	120	180	240
Normal Saline	-	34.95 ± 0.33	35.78 ± 0.19	36.23 ± 0.32	36.70 ± 0.38	36.78 ± 0.39	36.98 ± 0.33
Paracetamol	150	34.98 ± 0.42	35.85 ± 0.24	36.18 ± 0.28	36.08 ± 0.19*	35.38 ± 0.29*	34.98 ± 0.17**
***Mangifera indica* bark**	200	35.03 ± 0.10	36.40 ± 0.14	35.90 ± 0.22	35.60 ± 0.16*	35.45 ± 0.13*	35.25 ± 0.33*
	400	35.00 ± 0.08	36.55 ± 0.21	36.03 ± 0.15	35.68 ± 0.22*	35.45 ± 0.17*	35.33 ± 0.24*
***Mangifera indica* leaf**	200	35.00 ± 0.18	36.05 ± 0.13	35.98 ± 0.19	35.75 ± 0.29*	35.43 ± 0.17*	35.25 ± 0.26*
	400	35.18 ± 0.34	36.80 ± 0.24	36.78 ± 0.10	36.08 ± 0.10*	35.85 ± 0.45*	35.38 ± 0.37*
***Psidium guajava* leaf**	200	35.18 ± 0.39	36.13 ± 0.26	36.35 ± 0.37	36.80 ± 0.14	36.63 ± 0.19	36.45 ± 0.06
	400	35.15 ± 0.21	36.05 ± 0.19	36.50 ± 0.54	36.68 ± 0.13	36.40 ± 0.14	36.45 ± 0.33
Carica papaya leaf	200	35.05 ± 0.48	36.48 ± 0.61	36.53 ± 0.43	36.78 ± 0.49	36.80 ± 0.48	36.68 ± 0.39
	400	35.00 ± 0.35	36.45 ± 0.52	36.63 ± 0.93	36.70 ± 0.22	36.75 ± 0.52	36.75 ± 0.37
Cymbopogon citratus leaf	200	35.05 ± 0.17	36.10 ± 0.08	35.88 ± 0.22	35.50 ± 0.38*	35.20 ± 0.41*	35.15 ± 0.17**
	400	34.90 ± 0.29	35.85 ± 0.06	35.75 ± 0.25	35.43 ± 0.33*	35.23 ± 0.42*	35.20 ± 0.18**
Citrus sinensis leaf	200	34.90 ± 0.08	35.73 ± 0.13	36.05 ± 0.13	35.73 ± 0.15*	35.50 ± 0.12*	35.28 ± 0.22**
	400	34.98 ± 0.15	35.93 ± 0.10	36.08 ± 0.28	35.88 ± 0.15*	35.58 ± 0.32*	35.33 ± 0.22**
Ocimum gratissimum leaf	200	34.93 ± 0.13	35.63 ± 0.36	35.80 ± 0.36	35.58 ± 0.36*	35.28 ± 0.66*	35.28 ± 0.71**
	400	34.90 ± 0.16	35.95 ± 0.17	36.05 ± 0.17	35.88 ± 0.13*	35.25 ± 0.32*	35.20 ± 0.22**

*Significant difference * - $p<0.05$, ** - $p<0.001$ when compared to the control.*

Table 4.21. Effect of the constituent aqueous plant extracts of *Nefang* on Brewer's Yeast-induced hyperpyrexia in wistar rats.

Treatment (Drug/ Aqueous Extract)	Dose (mg/ kg)	Body Temperature (^{o}C) over Time interval (minutes) ($\bar{x}$ ± SD, n=4)					
		Basal Temperature	30	60	120	180	240
Normal Saline	-	37.00 ± 0.29	37.95 ± 0.21	38.14 ± 0.38	38.65 ± 0.32	38.91 ± 0.11	38.96 ± 0.54
Paracetamol	150	36.90 ± 0.38	37.70 ± 0.45	37.75 ± 0.33	37.85 ± 0.28*	37.47 ± 0.26*	37.00 ± 0.39**
***Mangifera indica* bark**	200	36.90 ± 0.31	37.85 ± 0.39	37.90 ± 0.41	37.75 ± 0.27*	37.55 ± 0.25*	37.20 ± 0.41*
	400	36.95 ± 0.92	37.95 ± 0.27	38.15 ± 0.54	37.73 ± 0.31*	37.50 ± 0.19*	37.30 ± 0.38*
***Mangifera indica* leaf**	200	37.00 ± 0.74	37.85 ± 0.16	37.95 ± 0.37	37.60 ± 0.18*	37.35 ± 0.11*	37.20 ± 0.28*
	400	37.05 ± 0.67	38.45 ± 0.43	38.60 ± 0.29	38.25 ± 0.17*	37.90 ± 0.28*	37.50 ± 0.19*
***Psidium guajava* leaf**	200	36.95 ± 0.51	38.00 ± 0.54	38.40 ± 0.61	38.70 ± 0.19	38.65 ± 0.38	38.50 ± 0.17
	400	37.10 ± 0.33	38.00 ± 0.15	38.65 ± 0.72	38.75 ± 0.24	38.55 ± 0.36	38.45 ± 0.11
Carica papaya leaf	200	37.00 ± 0.61	38.25 ± 0.27	38.60 ± 0.36	38.68 ± 0.22	38.65 ± 0.31	38.60 ± 0.26
	400	36.90 ± 0.68	37.90 ± 0.51	38.45 ± 0.29	38.65 ± 0.31	38.70 ± 0.29	38.60 ± 0.19
Cymbopogon citratus leaf	200	36.95 ± 0.28	37.90 ± 0.89	37.85 ± 0.18	37.45 ± 0.35*	37.20 ± 0.52*	37.00 ± 0.09**
	400	37.00 ± 0.41	38.05 ± 0.62	37.70 ± 0.09	37.45 ± 0.18*	37.30 ± 0.56*	37.05 ± 0.22**
Citrus sinensis leaf	200	37.00 ± 0.22	38.00 ± 0.20	38.05 ± 0.25	37.85 ± 0.26*	37.55 ± 0.19*	37.35 ± 0.35*
	400	37.05 ± 0.15	38.05 ± 0.31	38.25 ± 0.18	37.75 ± 0.11*	37.45 ± 0.55*	37.25 ± 0.28*
Ocimum gratissimum leaf	200	37.00 ± 0.33	37.95 ± 0.46	37.90 ± 0.42	37.75 ± 0.19*	37.40 ± 0.38*	37.15 ± 0.52**
	400	37.00 ± 0.81	37.95 ± 0.19	38.00 ± 0.19	37.75 ± 0.09*	37.45 ± 0.61*	37.10 ± 0.33**

*Significant difference * - $p<0.05$, ** - $p<0.001$ when compared to the control.*

4.4.4 Test for Anti-inflammatory Activity

The anti-inflammatory activities of the constituent plant extracts of *Nefang* at different concentrations (100, 200 and 400 $mgkg^{-1}$) were determined using Indomethacin (10 $mgkg^{-1}$) as the standard. They showed significant inhibition of carrageenan-induced inflammation in paws of experimental rats when compared to the control (**Table 4.22**). Inflammation of the paw of experimental animals was observed 60 minutes after carrageenan administration. Upon administration of the standard drug to experimental rats, the paw volumes were significantly ($p<0.001$) reduced when compared to the control after 240 minutes, giving a paw eodema inhibition of 43.73% when compared to the control (**Figure 4.31**). After administration of the constituent plant extracts of *Nefang*, only *MiL* exhibited a comparable anti-inflammatory activity to that of the standard drug by significantly ($p<0.001$) reducing the inflamed rat paw volumes by 40% at all doses. *Cp* and *MiB* also exhibited significant ($p<0.05$) anti-inflammatory activities at lower doses in experimental rats when compare to the control, with approximately 28 – 30% paw inhibition when compared to the control. *Og*, *Pg*, *Cc* and *Cs* all showed moderate but significant ($p<0.05$) anti-inflammatory activities at lower doses, with paw inhibition of approximately 20% when compared to the control.

Table 4.22. Anti-inflammatory effect of the constituent aqueous plant extracts of *Nefang* on carrageenan-induced paw edema in wistar rats.

Treatment (Drug/Aqueous Extract)	Dose (mg/kg)	Carrageenan-induced edema: Increase in Paw volumes (mL) ($\bar{x} \pm SD$, n=5)				
		0 min	60 min	120 min	180 min	240 min
Normal Saline	-	0	1.18 ± 0.08	1.28 ± 0.14	1.46 ± 0.13	1.56 ± 0.09
Indomethacin	10	0	1.17 ± 0.04	1.08 ± 0.03*	1.00 ± 0.10**	0.88 ± 0.03**
***Mangifera indica* bark**	100	0	1.07 ± 0.11	1.13 ± 0.09*	1.19 ± 0.03*	1.12 ± 0.01**
	200	0	1.15 ± 0.12	1.21 ± 0.02	1.32 ± 0.06*	1.24 ± 0.10*
	400	0	1.18 ± 0.05	1.27 ± 0.04	1.36 ± 0.03	1.26 ± 0.06*
***Mangifera indica* leaf**	100	0	1.04 ± 0.13	1.01 ± 0.08*	0.92 ± 0.08**	0.93 ± 0.06**
	200	0	1.05 ± 0.03	1.11 ± 0.08*	0.91 ± 0.14**	0.95 ± 0.11**
	400	0	1.05 ± 0.03	0.93 ± 0.03*	0.89 ± 0.06**	0.88 ± 0.08**
***Psidium guajava* leaf**	100	0	1.04 ± 0.04	1.12 ± 0.10*	1.12 ± 0.06*	1.21 ± 0.04*
	200	0	1.04 ± 0.07	1.14 ± 0.11*	1.13 ± 0.13*	1.23 ± 0.13*
	400	0	1.08 ± 0.07	1.17 ± 0.10*	1.27 ± 0.15*	1.28 ± 0.05*
Carica papaya leaf	100	0	0.97 ± 0.03	1.04 ± 0.10*	1.15 ± 0.09**	1.10 ± 0.03**
	200	0	1.10 ± 0.09	1.12 ± 0.17*	1.17 ± 0.13**	1.11 ± 0.05**
	400	0	1.11 ± 0.07	1.19 ± 0.05	1.36 ± 0.11	1.32 ± 0.09*
Cymbopogon citratus leaf	100	0	1.08 ± 0.12	1.16 ± 0.04*	1.24 ± 0.11*	1.27 ± 0.03*
	200	0	1.12 ± 0.04	1.17 ± 0.08*	1.20 ± 0.02*	1.34 ± 0.04*
	400	0	1.15 ± 0.04	1.24 ± 0.01	1.42 ± 0.06	1.48 ± 0.09
Citrus sinensis leaf	100	0	1.15 ± 0.12	1.22 ± 0.03	1.24 ± 0.05*	1.27 ± 0.13*
	200	0	1.17 ± 0.03	1.19 ± 0.04	1.35 ± 0.20	1.46 ± 0.16
	400	0	1.17 ± 0.04	1.20 ± 0.03	1.45 ± 0.04	1.56 ± 0.13
Ocimum gratissimum leaf	100	0	1.06 ± 0.05	1.14 ± 0.07*	1.23 ± 0.04*	1.22 ± 0.05*
	200	0	1.07 ± 0.06	1.16 ± 0.08*	1.23 ± 0.04*	1.24 ± 0.02*
	400	0	1.17 ± 0.03	1.26 ± 0.04	1.31 ± 0.12	1.33 ± 0.08

*Significant difference: compared to control; (*)-$p<0.05$,(**)-$p<0.01$*

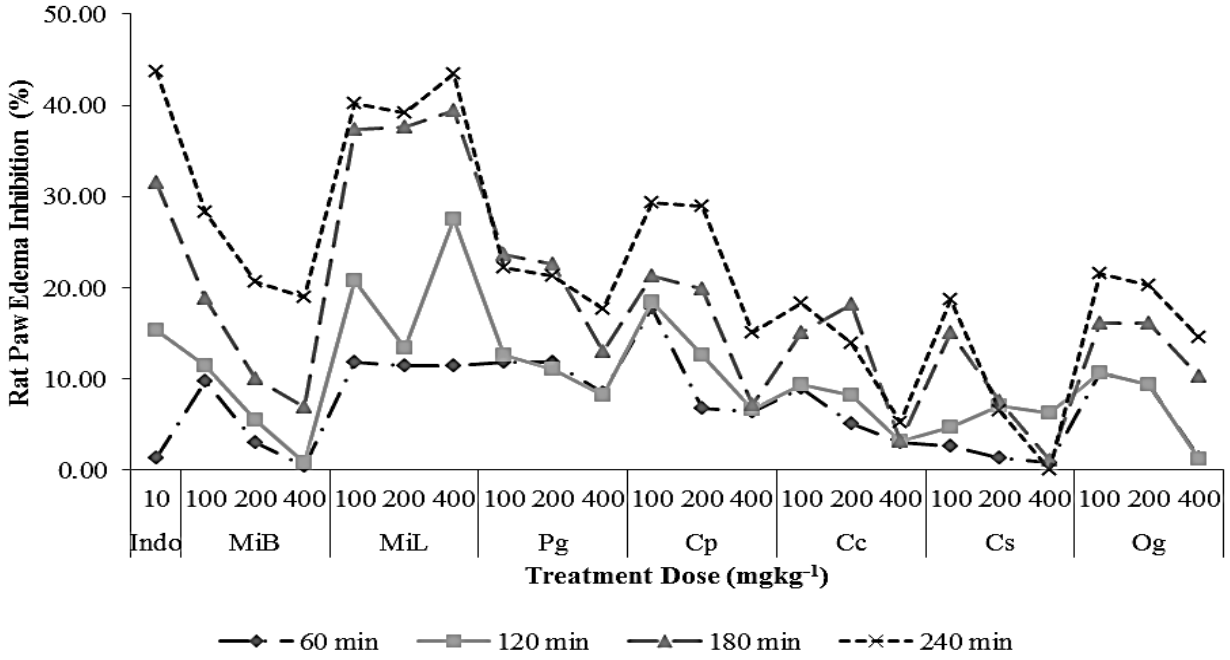

Figure 4.31. Inhibition of carrageenan-induced rat paw edema by the constituent plant extracts of *Nefang*.

Each data point represent means ± SD; n=5;
Indo *- Indomethacin*

4.4.5 Test for Antinociceptive activity

4.4.5.1 Tail Pressure method

The antinociceptive activity of the constituent plant extracts of *Nefang* on tail-induced pressure in mice was determined using an analgesy meter. The mean reaction times of mice against the gradual increase in tail pressure are presented in **Table 4.23**. The mean reaction times of the experimental mice suggested that *MiL*, *Og* and *Pg* produced significant ($p<0.001$) percentage inhibition of the threshold pain by 177.27, 167.35 and 139.58 respectively, when compared to the normal control 30 minutes after extract administration. This activity was comparable to that of the standard drug, morphine (146.67%). *MiB* and *Cp* had moderate percentage pain inhibition of 107.66 and 106.25 respectively while *Cs* and *Cc* had weak activities. Two hours post treatment, only *MiL* and *Og* still exhibited good pain inhibition levels of 129.55% and 112.24% respectively, comparable to 146.67% for morphine. *Pg* and *MiB* still exhibited moderate

activities at 83.33% and 77.42%, while *Cc*, *Cp* and *Cs* exhibited very weak activities **(Figure 4.32).** Since all the extracts showed activity, they were all subjected to further dose-response testing.

Table 4.23. Antinociceptive effect of the constituent plant extracts of *Nefang* on tail-induced pressure in mice.

Treatment (Drug/Aqueous Extract)	Dose ($mgkg^{-1}$)	Tail Pressure (units) at Designated Time Intervals ($\bar{x}$ ± SD, n=4)				
		0 min	**30 min**	**60 min**	**90 min**	**120 min**
PBS	-	6.50 ± 0.41	6.25 ± 1.03	5.13 ± 0.85	5.50 ± 0.41	5.88 ± 0.48
Morphine	5	5.63 ± 0.48	13.88 ± 0.48**	14.13 ± 1.11**	13.75 ± 0.65**	13.88 ± 1.25**
***Mangifera indica* bark**	1000	6.20 ± 0.57	12.88 ± 1.49**	11.88 ± 0.95**	11.75 ± 0.65**	11.00 ± 0.91**
***Mangifera indica* leaf**	1000	5.50 ± 0.41	15.25 ± 1.55**	13.50 ± 0.82**	13.25 ± 0.65**	12.63 ± 1.11**
***Psidium guajava* leaf**	1000	6.00 ± 0.71	14.38 ± 0.85**	14.75 ± 2.06**	13.75 ± 0.65**	11.00 ± 0.71**
***Carica papaya* leaf**	1000	6.00 ± 0.41	12.38 ± 1.49**	8.88 ± 0.85*#	8.75 ± 0.65*#	8.63 ± 0.63*#
***Cymbopogon citratus* leaf**	1000	6.50 ± 0.71	11.63 ±0.75**#	10.88 ±0.48**#	10.00 ±0.71**#	10.63 ± 0.63**#
***Citrus sinensis* leaf**	1000	6.20 ± 0.36	11.25 ±2.08**#	8.63 ± 0.85*#	8.50 ± 0.75*#	8.25 ± 1.29*#
***Ocimum gratissimum* leaf**	1000	6.13 ± 0.63	16.38 ±0.95**#	13.63 ± 1.38**	13.50 ± 0.41**	13.00 ± 1.83**

*Significant difference: * – p<0.05, ** - p<0.001 when compared to normal control; # - p<0.05, ## - p<0.001 when compared to the standard.*

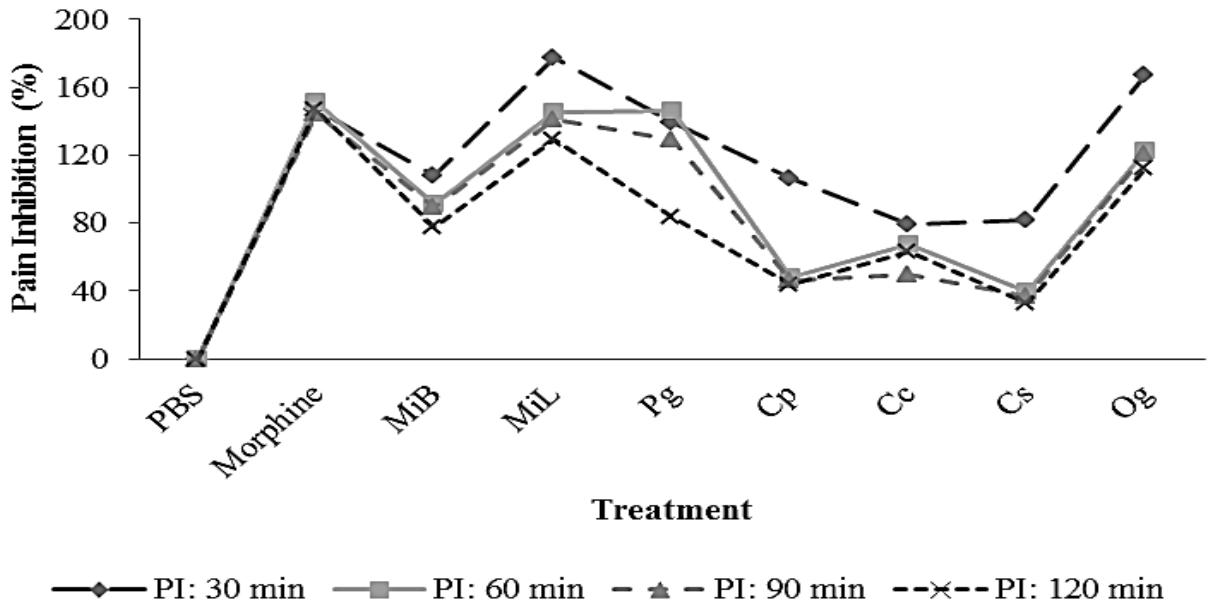

Figure 4.32. Pain inhibition of tail-induced pressure in mice by the constituent plant extracts of *Nefang*.

Each data point represents the Mean ± SD for each group of mice, n = 4.
PI – Pain Inhibition.

4.4.5.2 Tail Flick response method

In experimental animals administered standard drug, paracetamol, the mean initial tail flicking time was 4.27 seconds. The reaction time increased gradually after administration of the standard drug and a mean reaction time of 8.39 seconds was attained at the end of 60 minutes. This was maintained up to the end of 120 minutes. Analysis revealed that *Og*, *MiL*, *Pg* and *MiB* exhibited dose-dependent increases in reaction time. The reaction times after administration of *MiL* and *Pg* at doses of 600 and 1200 $mgkg^{-1}$ bwt, were comparable to that of the standard group after 60 minutes and up to 120 minutes, whereas animals treated with *Og* and *MiB* exhibited the same activity only at the highest dose of 1200 $mgkg^{-1}$ **(Table 4.24).** The mean initial tail flicking time resulted in a pain inhibition of 59.96% after 30 minutes to over 90% after 60 minutes, which was maintained to the end of the experiment (120 minutes) when compared to the reaction before drug administration **(Figure 4.33)**.

Pain inhibition (%) at the highest doses for *Og* (113), *MiL* (108), *Pg* (84) and *MiB* (88) were comparable to that of paracetamol (97) **(Figure 4.31).** At lower doses, we observed significantly ($p<0.05$) lower activities of these extracts when compared to the standard. In animals administered *Cp* and *Cc*, we observed moderate reaction times at doses up to 600 $mgkg^{-1}$ bwt, though significantly ($p<0.05$) low when compared to the standard. At higher doses as well as in animals treated with *Cs* (all doses), reaction times were significantly ($p<0.001$) low, suggesting that they show lower activities.

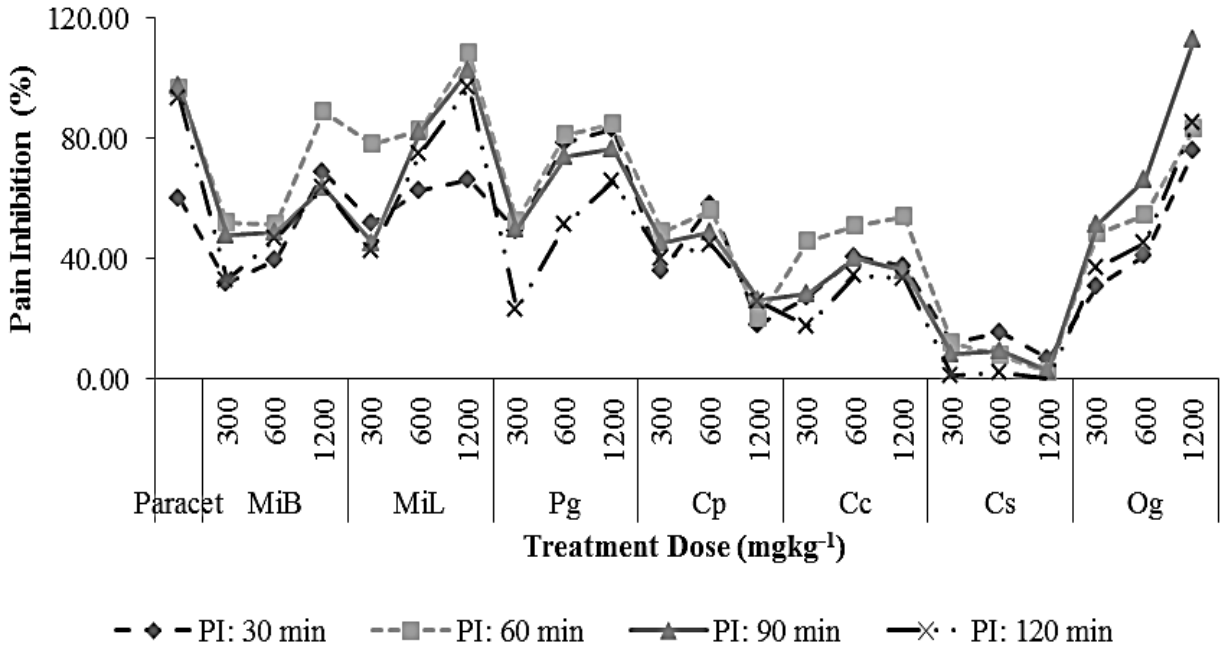

Figure 4.33. Inhibition of electric heat-induced pain on the tails of mice by the constituent plant extracts of *Nefang*

Each data point represents the Mean ± SD for each group of mice, n = 5.
***Paracet** – Paracetamol; **PI** – Pain Inhibition.*

Table 4.24. Antinociceptive effect of the constituent plant extracts of *Nefang* on electric heat-induced pain on the tails of mice.

Treatment (Drug/ Aqueous Extract)	Dose (mg/ kg)	Tail Flick Reaction Time (sec) at Designated Intervals ($\bar{x} \pm SD$, n=5)				
		0 min	30 min	60 min	90 min	120 min
Paracetamol	150	4.27 ± 0.38	6.82 ± 0.28	8.39 ± 0.20	8.43 ± 0.36	8.26 ± 0.23
***Mangifera indica* bark**	300	4.30 ± 0.38	5.62 ± 0.24	6.47 ± 0.37	6.30 ± 0.31*	5.66 ± 0.24*
	600	4.30 ± 0.38	5.96 ± 0.49	6.46 ± 0.37*	6.35 ± 0.25*	6.25 ± 0.20*
	1200	4.30 ± 0.38	7.20 ± 0.27	8.05 ± 0.41	6.99 ± 0.29	6.98 ± 0.06
***Mangifera indica* leaf**	300	4.32 ± 0.38	6.47 ± 0.25	7.59 ± 0.43	6.19 ± 0.37*	6.08 ± 0.20*
	600	4.32 ± 0.38	6.95 ± 0.14	7.80 ± 0.21	7.78 ± 0.76	7.47 ± 0.82
	1200	4.32 ± 0.38	7.09 ± 0.48	8.89 ± 0.41	8.64 ± 0.44	8.42 ± 0.36
***Psidium guajava* leaf**	300	4.25 ± 0.38	6.32 ± 0.35	6.49 ± 0.29	6.38 ± 0.17*	5.26 ± 0.17*
	600	4.25 ± 0.38	7.63 ± 0.38	7.73 ± 0.27	7.42 ± 0.46	6.45 ± 0.31*
	1200	4.25 ± 0.38	7.82 ± 0.35	7.88 ± 0.19	7.54 ± 0.37	7.08 ± 0.05
Carica papaya leaf	300	4.29 ± 0.38	5.80 ± 0.70*	6.35 ± 0.31*	6.19 ± 0.53*	5.98 ± 0.17*
	600	4.29 ± 0.38	6.73 ± 0.87	6.65 ± 0.52	6.34 ± 0.48*	6.16 ± 0.27*
	1200	4.29 ± 0.38	5.04 ± 0.62*	5.12 ± 0.39*	5.39 ± 0.63*	5.36 ± 0.29*
Cymbopogon citratus leaf	300	4.31 ± 0.38	5.42 ± 0.50*	6.22 ± 0.28*	5.48 ± 0.09*	5.00 ± 0.38*
	600	4.31 ± 0.38	6.00 ± 0.90	6.43 ± 0.33	5.98 ± 0.30*	5.74 ± 0.38*
	1200	4.31 ± 0.38	5.87 ± 0.32	6.56 ± 0.41	5.80 ± 0.25*	5.69 ± 0.32*
Citrus sinensis leaf	300	4.27 ± 0.38	4.76 ± 0.17*	4.77 ± 0.31**	4.61 ± 0.32**	4.30 ± 0.22**
	600	4.27 ± 0.38	4.92 ± 0.28*	4.59 ± 0.33**	4.65 ± 0.42**	4.35 ± 0.15**
	1200	4.27 ± 0.38	4.55 ± 0.30*	4.35 ± 0.24**	4.38 ± 0.15**	4.26 ± 0.22**
Ocimum gratissimum leaf	300	4.33 ± 0.38	5.58 ± 0.38	6.33 ± 0.50*	6.45 ± 0.26*	5.85 ± 0.14*
	600	4.33 ± 0.38	6.03 ± 0.76	6.60 ± 0.54	7.09 ± 0.68	6.19 ± 0.30*
	1200	4.33 ± 0.38	7.50 ± 0.55	7.81 ± 0.29	9.09 ± 0.69	7.91 ± 0.38

*Significant difference * - $p<0.05$, ** - $p<0.001$ when compared to the standard control.*

4.4.5.3 Hot Plate method

The effect of the constituent plants of *Nefang* in heat-induced nociceptive pain in mice was determined using the hot plate method. Piroxicam (20 $mgkg^{-1}$) and pethidine (50 $mgkg^{-1}$) were used as standards. After experimental times of 30, 60 and 120 minutes, experiemental animals treated with the standard drugs gave reaction times of 19.31, 47.49, 47.41 and 18.90, 19.15, 20.02 seconds respectively **(Table 4.25).** These produced significant ($p<0.01$; $p<0.05$) percentage increase in the threshold of pain by 87.65, 361.60, 360.80 and 100.16, 102.78, 112.02 respectively **(Figure 4.34)**, suggesting that piroxicam exhibited a better antinociceptive activity than pethidine, when compared to the control.

After administration of the plants extracts, we observed a dose-dependent increase in reaction times in animals treated with *Og*, *MiL*, *MiB* and *Pg* between 30 to 120 minutes, with *Og* showing the best reaction time of 28.98, 29.94 and 32.55 seconds after 30, 60 and 120 seconds respectively, at the highest dose of 1200 $mgkg^{-1}$. This corresponds to an increased percentage pain inhibition of 187.72, 197.20 and 223.11 during the experimental time; this was significantly ($p<0.001$) lower than that of piroxicam after 120 minutes and higher than that of pethidine ($p<0.001$). However, *MiL*, *MiB* and *Pg* all showed significantly ($p<0.001$) higher pain inhibition at 1200 $mgkg^{-1}$ in experimental animals when compared to animals that were treated with pethidine, though lower when compared to animals that were administered piroxicam. *Cp*, *Cc* and *Cs* exhibited weak activities ($p<0.001$).

Table 4.25. Antinociceptive effect of the constituent plant extracts of *Nefang* on heat-induced nociceptive pain in mice

Treatment (Drug/Aqueous Extract)	Dose (mg/kg)	Mice Reaction Time (sec) at Designated Intervals ($\bar{x} \pm SD$, n=4)			
		0 min	**30 min**	**60 min**	**120 min**
Normal Saline	-	9.51 ± 1.24	10.70 ± 1.07	10.56 ± 0.74	11.28 ± 0.67
Piroxicam	20	10.29 ± 0.61	19.31 ± 1.34*	47.49 ± 0.76**	47.41 ± 0.66**
Pethidine	50	9.44 ± 0.17	18.90 ± 0.92*	19.15 ± 0.77*	20.02 ± 1.14*
***Mangifera indica* bark**	300	9.93 ± 0.25	13.90 ± 1.21	21.75 ± 1.28*	20.81 ± 0.68*
	600	9.63 ± 0.69	13.65 ± 0.34	22.23 ± 0.71*	22.61 ± 0.70*
	1200	10.05 ± 0.26	22.91 ± 0.89*	27.29 ± 0.62*	23.79 ± 1.81*
***Mangifera indica* leaf**	300	11.11 ± 0.94	12.80 ± 0.58	16.71 ± 0.50*	16.11 ± 0.26
	600	11.21 ± 0.48	23.72 ± 0.88*	24.25 ± 1.17*	21.71 ± 0.68*
	1200	10.70 ± 0.53	23.41 ± 1.07*	32.75 ± 0.25**	28.56 ± 0.13*
***Psidium guajava* leaf**	300	10.09 ± 0.45	14.41 ± 0.73	14.57 ± 0.68	13.79 ± 1.14
	600	9.84 ± 0.34	14.86 ± 0.51	15.47 ± 0.21	18.33 ± 0.29
	1200	10.29 ± 0.53	21.27 ± 0.46*	26.29 ± 0.24*	24.22 ± 0.57*
Carica papaya leaf	300	9.63 ± 0.48	11.46 ± 0.94	11.51 ± 0.36	10.79 ± 0.61
	600	9.45 ± 0.40	12.28 ± 0.84	12.51 ± 0.38	11.01 ± 0.72
	1200	9.90 ± 0.77	12.45 ± 0.28	17.42 ± 0.25*	16.91 ± 0.62
Cymbopogon citratus leaf	300	10.07 ± 0.34	11.00 ± 0.36	11.71 ± 0.38	15.28 ± 0.56
	600	9.78 ± 0.34	13.34 ± 0.77	13.02 ± 0.45	16.08 ± 0.72
	1200	10.05 ± 0.33	15.24 ± 1.12	14.81 ± 1.10	18.85 ± 0.57
Citrus sinensis leaf	300	9.90 ± 0.44	13.24 ± 0.60	12.83 ± 0.24	13.27 ± 0.70
	600	9.86 ± 0.46	15.10 ± 0.61	14.99 ± 0.90	13.60 ± 0.43
	1200	9.73 ± 0.33	14.21 ± 0.67	16.34 ± 0.60*	15.03 ± 1.37
Ocimum gratissimum leaf	300	9.63 ± 0.36	13.66 ± 0.30	18.15 ± 0.89*	18.98 ± 0.92
	600	9.79 ± 0.28	13.66 ± 0.89	22.83 ± 0.20*	24.29 ± 1.17*
	1200	10.07 ± 0.70	28.98 ± 0.64*	29.94 ± 0.43**	32.55 ± 1.47**

*Significant difference * - $p<0.05$, ** - $p<0.001$ when compared to the control*

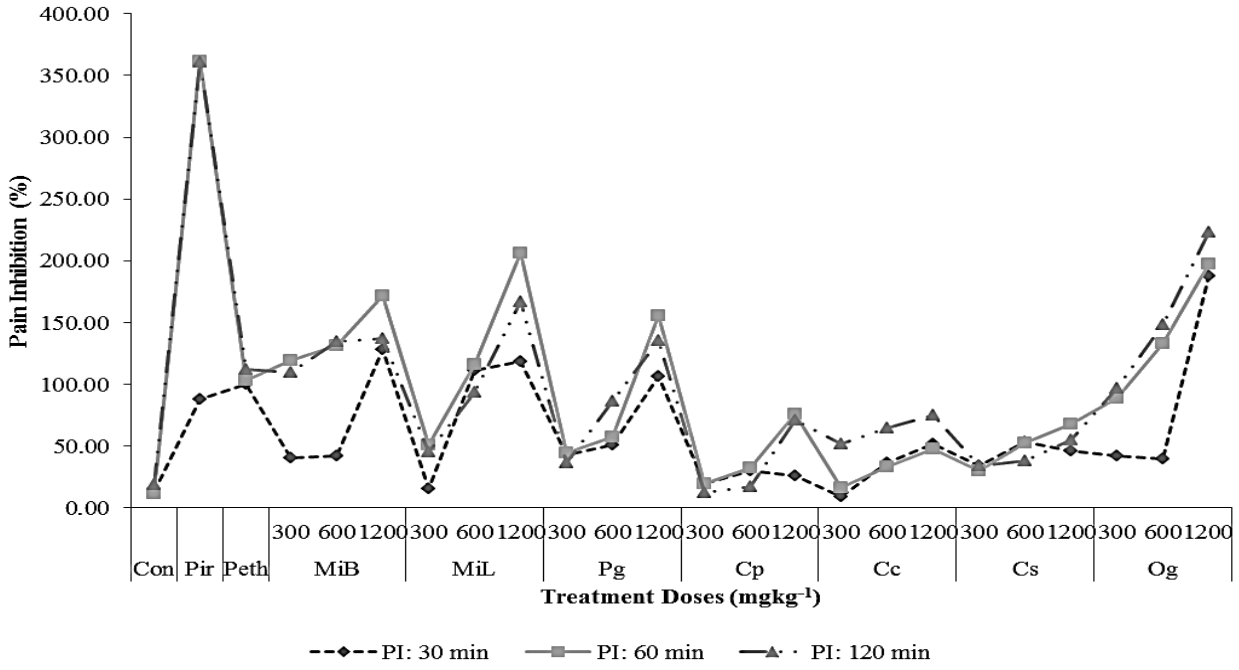

Figure 4.34. Inhibition of heat-induced nociceptive pain in mice by the constituent plant extracts of *Nefang*.

Each data point represents the Mean ± SD for each group of mice, n = 4.
***Con** – Control; **Pir** – Piroxicam; **Peth** – Pethidine; PI – Pain Inhibition.*

4.4.5 Contribution of the constituent plants of *Nefang* to overall antimalarial activity

An assessment of the biological activities of the constituent plants of *Nefang* revealed that they possess different levels of antioxidant, antipyretic, anti-inflammatory and antinociceptive activities that contribute to overall antimalarial activity. There was no constituent plant without activity. The classification of these activities has been summarized in **Table 4.26**.

Table 4.26. Classification of some biological activities of the constituent plant extracts of *Nefang*

Constituent Plant	Activity				
	Anti-plasmodial	Anti-oxidant	Anti-pyretic	Anti-inflammatory	Anti-nociceptive
***Mangifera indica* bark**	G	VG	VG	G	G
***Mangifera indica* leaf**	G	VG	VG	VG	VG
Psidium guajava	G	VG	w	m	G
Carica papaya	w	w	w	G	m
Cymbopogon citratus	w	m	VG	m	m
Citrus sinensis	w	w	VG	m	w
Ocimum gratissimum	w	G	VG	G	VG

VG (Blue) – Very Good; **G (Yellow)** – Good; **m (Orange)** – moderate; **w**- weak

5.0 DISCUSSION

The therapeutic value of synergistic interactions has been known since antiquity, and many cultural healing systems still rely on this principle with the belief that combination therapy may enhance efficacy. The study was firmly based on tradition of combining a large number of plants to create synergy, reduce toxicity and increase bioavailability and efficacy (Kokwaro, 1993; Tabuti *et al*., 2003; van Vuuren and Viljoen, 2011). Through the use of high throughput screening (HTS) and *Plasmodium* culture systems, the antiplasmodial activities of *Nefang*, its constituents and the interactions that exist between these have been characterized for the very first time. These revealed the presence of antiplasmodial and non-antiplasmodial components, as well as the presence of synergistic interactions between some of the components. We also observed that despite mild in vivo toxicity of some of the constituent plants on some organ systems, there were no observed adverse effects on the experimental animals after administration of *Nefang*. The constituent plants of *Nefang* also exhibited antioxidant, antipyretic, anti-inflammatory and antinociceptive activities. These suggest that the synergy between the biological activities of the constituent plants decreased adverse effects and increase the observed overall antimalarial activity of *Nefang*, thereby upholding phytotherapeutic tradition of polyherbals and the folk use of *Nefang*.

The formulation of *Nefang* constituted the leaves of all the six constituent plants and the bark of only one of the plants. The frequent and common use of leaves in the preparation of remedies has been reported by Muthu *et al*. (2006) and Kala (2005). These plant parts, especially the leaves were harvested either very early in the morning or in the evening when there is low irradiance. The low photosynthesis rate at these times leads to increased production of carbon-based secondary metabolites (CBSM), as well as enhancement in the production of total non-structural carbohydrates (TNC), H_2O_2 and malondialdehyde, which are related to increased secondary metabolites production (Coley *et al*., 1985; Ibrahim and Jaafar, 2012).

Eighty percent of the respondents interviewed during the study were aged above 50 years. Thus, the majority of healers from whom a great deal of ethno-medical knowledge is derived are elderly. This puts the art of herbal medicine and indigenous health care knowledge at risk because as the older people die, so does their legacy of the use of traditional medicines to manage malaria and related diseases (Kokwaro, 1993; Sofowora, 1982). There is the urgent need

for the documentation of this invaluable knowledge, since there is a persistent gap in knowledge of herbal practice between the younger and older generations. Besides, there is the believe that the older people are more competent in providing healthcare using plants and other natural substances, based on the social and cultural background of the society (UNAIDS, 2002). Most of the plants used in traditional healthcare are collected from the wild, and only a few have been domesticated. However, field observations in this study revealed that four (*M. indica, Pg, Cp* and *Cs*) of the six constituent plants are fruit plants, while two (*Cc* and *Og*) are spices. All of these are now being domesticated for consumption because over exploitation and urbanization have led to decline in resources. This practice should be extended to include other plant species, in order to ensure sustainable availability of the plants, enhance food security, improve income, health and nutritional status of the rural communities while safeguarding biodiversity and protection of the environment (Mbwambo, 2004).

Method of administration of *Nefang* is either through the conventional oral or rectal (enema) route. Enema is a rare method of rectal administration of aqueous or oily solutions or suspensions. The benefits and hazards of this rectal route have earlier been reported by Doyle (2005) and Smith *et al*. (1987). Special formulation techniques are required and the need for rectal administration to be carried out by well trained personnel.

Cytotoxicity screening is the in vitro toxicological assessment of specific adverse effects of drugs. Assessment of the cytotoxicity of *Nefang* and its constituents revealed that the CC_{50} of all the aqueous and ethanol extracts on both Hep G2 and U2OS cell lines was above 2000 µg/mL indicating the overall safety of *Nefang*, while evaluation of the in vitro antiplasmodial activities showed that *Nefang* exhibited good activity against the multidrug resistant strain of *P. falciparum* Dd2. Based on WHO and previous data, antiplasmodial activity is considered as good when EC_{50} < 50 µg/mL (Philippe *et al*., 2005; Pink *et al.*, 2005). Best antiplasmodial activities (two-fold greater activity than *Nefang*) were obtained with ethanol extracts of ***MiB*** and ***MiL***, whereas ***Cp*** and ***Og*** were least active. These findings are consistent with previous observations by Bidla *et al* (2004) for *M. indica* and ***Cc***, Ngemenya *et al* (2004) for ***Cp*** and ***Og*** and Nundkumar and Ojewole (2002) for ***Pg***. This once more confirms the antimalarial activities of some herbal extracts used in traditional medicine. In as much as plant extracts singly or in

combination have been increasingly evaluated for their in vitro antiplasmodial activities, our study is the first to demonstrate the in vitro antiplasmodial activity of *Nefang* and its component plants (singly and in paired combinations) using both CQ-sensitive and MDR *Plasmodium* parasites.

Various parameters such as localization and period of collection, plant part, drying procedure and extract preparation may modify the pharmacological response produced by a single species. Phytochemical screening of the constituent plants of *Nefang* revealed the presence of alkaloids, anthocyanins, flavonoids, phenols, saponins, tannins, triterpenes and sterols. These results are consistent with previous results from our review of the biological activity and chemical analyses of extracts of the component plants of *Nefang*. Alkaloids are one of the major antimalarial natural products and various classes have been reported to exhibit promising activities (Oliveira *et al.,* 2009). Quinine, an illustrative example, was one of the first widely used antimalarial drugs due to its parasite DNA intercalating property, possessed by many other classes. It has, however, fallen into disuse due to emerging parasite strains resistant to the drug. Consequently, it has been replaced by more effective synthetic drugs derived from the acridine and quinoline structure, such as chloroquine and mefloquine which inhibit heme polymerase and prevent the polymerization of heme to hemozoin, thereby causing oxidative-metabolic effects on the parasite, and primaquine which destroys the gametocytes of malaria parasites (Percario *et al.,* 2012). Some non-alkaloidal natural products such as terpenes, flavonoids and their related compounds have also been reported to exhibit promising antiplasmodial activities (Batista *et al.,* 2009). Triterpenoids such as Iridal extracted from *Iris germanica* L. are suspected to act against the reinvasion step rather than the maturation step of *P. falciparum*, and has cumulative inhibitory effect on the main metabolic pathways of the parasite (Benoit-Vical *et al*., 2003). In as much as the mechanism of action of flavonoids is unclear, some flavonoids have been shown to inhibit the influx of L-glutamine and myoinositol into *P. falciparum*-infected erythrocytes (Elford, 1986), while others such as a flavone glycoside from *Phlomis brunneogaleata* and iridoid from *Scrophularia lepidota* have been reported to inhibit the FabI enzyme of *P. falciparum* (Kirmizibekmez *et al*., 2004; Tasdemir *et al*., 2005).

Investigations on the efficacy of antimalarial plants usually focus on killing the parasite but rarely consider other mechanisms. Many of these herbal remedies exert their anti-infective

effects not only directly on the pathogen but by indirectly stimulating the natural and adaptive defense mechanisms of the host, thereby suppressing or eliminating the parasite (Stevenson, 2004). Therefore, some of the non-antiplasmodial secondary metabolites such as phenols could mitigate malaria parasite infection in the host by conferring a protective/ antioxidative effect against oxidative stress induced in the host parasitized red blood cells by the malaria parasite (Percario *et al.,* 2012). These results confirm that these active and non-antiplasmodial components are responsible for the overall antimalarial activity of *Nefang*.

The potent antiplasmodial activities and weak cytotoxicity profiles of most of the extracts in this study suggest high selectivity for *P. falciparum*. The reasonably high SI values for the extracts indicate that smaller quantities of such products will be needed to achieve high clinical efficacy with increased tolerability and safety (Ohrt *et al*., 2002). The effectiveness of any plant extract is dependent upon a favorable therapeutic ratio; that is the drug must kill or inhibit the parasite but have little or no toxicity to the host. The selectivity of a plant to inhibit the growth of a parasite and yet be less toxic to the host depends on differences in biochemistry between the parasite and the host. Such a plant could operate on a biochemical target in the parasite that is either absent or significantly different in the host (Mojab, 2012).

Our interaction studies with various pairs of the different solvent extracts of the constituent plants revealed the presence of twenty-three synergistic, seventeen additive and five antagonistic pairs at equipotency ratios. These results are consistent with previous observations by Azas *et al.* (2002) and Gathirwa *et al.* (2008), on different solvent plant extracts. Among the synergistic pairs, only six showed promising activity. These interactions were further characterized by isobologram analysis at variable potency ratios, wherein ***MiB/Pg***-(Aq) exhibited outright synergistic interaction at all experimental concentrations, while ***Cp/Og***-(EtOH), ***MiB/Cs***-(Aq) and ***Cp/Cs***-(Aq) exhibited antagonistic and synergistic interactions as their concentrations were inversely varied respectively. In vitro sensitivity assessment of drug combinations for malaria is used to help predict clinically useful combinations. Theoretically, in vitro synergy signifies that less than 50% of each of the components should achieve 100% therapeutic rates. The greater the synergy, the less of each component required. Therefore, reduced doses of one or both components may lead to increased tolerability and safety, more practical dosing regimens, and/or decreased cost. Additionally, synergy may allow two drugs, both less than 50% efficacious, to be

combined to achieve a very high efficacy (Ohrt *et al*., 2002). On the other hand, in vitro antagonism signifies that more than 50% of each of the components will be needed to achieve maximum therapy. The greater the antagonism, the more of each component required.

Some of the component extracts were inactive against both parasite strains and showed low SI values and high CI values in combination. This suggests the presence of weakly active and/or antagonizing components whose interactions with the active constituents could mitigate paired activity and/or overall antimalarial activity of *Nefang*. These antagonizing components could be non-toxic to the host and non-toxic to the parasite as well. Thus, eliminating such undesirable components in *Nefang* or by selectively combining the active extracts might increase overall activity and tolerability as suggested elsewhere (van Vuuren and Viljoen, 2011). Therefore, understanding the modes of interaction between the individual plant components would be of immense importance for the identification of compounds and/or mixtures for downstream clinical development.

The in vivo antiplasmodial activities of the aqueous extract of *Nefang* and its active components, ***Pg*** and ***MiB/Pg***, were investigated by evaluating the chemosuppression during early infection, while *Nefang* alone was evaluated during established infection using standard animal models. In vivo models are usually employed in antimalarial studies because they take into account the possible prodrug effect and probable involvement of the immune system in eradication of the pathogen (Wacko *et al.*, 2005). During early infection, Peter's 4-Day suppressive test was used to evaluate schizontocidal activity while the repository test was used to study the prophylactic activity. Rane's test was used to study curative ability during established infection. In all methods, determination of percent inhibition of parasitemia was the most reliable parameter. A mean parastemia level $\leq$ 90% to that of the vehicle treated animals (mock-treated control) usually indicates that the test compound is active (Peter and Anatoli, 1998). In the 4 day suppressive activity, we observed that *Nefang* and ***MiB/Pg*** significantly reduced parasitemia (in both *Plasmodium spp*) in animal models in a dose-dependent manner, with *Nefang* exhibiting antimalarial activities comparable to the standard drug used. The repository test revealed the same dose-dependent chemosuppression by *Nefang*. In the curative activity, the dose-dependent activity of *Nefang* at the highest experimental dose was observed from day two of treatement. Though its activity was slower than that of CQ, it was comparable to that of ART. Furthermore,

we observed that the survival time of *Nefang*-treated animals was also prolonged in a dose-dependent manner. Measurement of body weights and temparatures of infected animals revealed a dose-dependent preventive effect of these parameters by *Nefang*. Body weight loss and temperature reduction are a hallmark of malaria infection in animal models (Langhorne *et al.*, 2002), suggesting that an ideal plant-derived antimalarial agent should prevent body weight loss in *Plasmodium* infected animals. This dose-dependent preventive activity indicates that *Nefang* does not have any adverse effect in experimental animals as earlier observed in our toxicity studies. Increase in parasitemia levels usually results in decreased metabolic rates and a consequent decrease in body temperatures of experimental animal models (Chinchilla *et al.*, 1998), which might result in death. An ideal antimalarial agent would therefore prevent this occurrence, an effect observed from *Nefang*. Taken together, these results confirm that *Nefang* has therapeutic activity against established infection and further confirm the in vitro antiplasmodial activities reported earlier. These suggest that *Nefang* is endowed with antiplasmodial activity. Although *Nefang* exhibited a moderate in vitro antiplasmodial activity, the in vivo activity was good. In vivo antiplasmodial activity can be classified as moderate, good, and very good if an extract displayed percentage parasitemia suppression equal to or greater than 50% at a dose of 500, 250 and 100 $mgkg^{-1}$ per day, respectively (Deharo *et al.*, 2001). This therefore suggests that the overall antimalarial activity of the synergistic and additive components identified during our interaction studies, over-shadowed the antagonistic interactions. Synergy between different constituents of extracts has been documented not only for anti-malarial activity, but also for other pharmacological activities (Williamson, 2001; Houghton, 2009). Synergy, otherwise known as potentiation actually means that the effect of the combination is greater than the sum of the individual effects. The antimalarial activity of *Nefang* cannot be accounted for only by the active antiplasmodial content in the constituent plant extracts. As earlier mentioned, many anti-malarial herbal remedies may exert their anti-infective effects not only by directly affecting the pathogen, but also by indirectly stimulating natural and adaptive defense mechanisms of the host by other mechanisms. Therefore, extracts that can stimulate innate and/or adaptive immunity may be able to contribute to prophylaxis and treatment for malaria and other diseases (Masihi, 2000; Muniz-Junquiera, 2007). This suggests that a combination of the biological activities of the constituent extracts of *Nefang* could account

for the enhanced overall antimalarial activity of *Nefang*. An understanding of the pharmacodynamic or pharmacokinetic mechanisms of this action would be very important.

Toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potential of existing molecules and herbal drugs. Evaluation of the safety of *Nefang* and its constituents revealed overall decrease in adverse effects in experimental animals upon exposure to *Nefang*. In acute oral toxicity testing, the no observed advere effect level (NOAEL) for *Nefang* and its constituent was greater than 5000 $mgkg^{-1}$ bwt. Doses higher than this are generally non-classified, which is in accordance with the Organization for Economic Corporation and Development (OECD) Guidance Document for Acute Oral Toxicity Testing (Hayes, 1987; OECD, 2001). Compounds with LD_{50} values lower than 2000 $mgkg^{-1}$ bwt are generally considered to be relatively safe, since values above this are non-classified. This signifies that the aqueous and ethanol extracts of *Nefang* and its constituent plants can be considered to be non toxic at acute oral administration since the extracts were well tolerated and there were no observed adverse effects.

Comparison of body and organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic or adverse effects (Peters and Boyd, 1966; Pfeiffer, 1968) and as an assessment of therapeutic response to test articles or drugs (Winder *et al.*, 1969). In this study, the observed dose-dependent increase in bwt and increase in organ weights of the experimental animals corresponded to a decrease in percentage bwt gained when compared to the controls. Hence the extracts did not have any adverse effects on experimental animals that would cause them to loose appetite (El-Sanusi and El-Adam, 2007). The relationship between the organ: body weight ratio (Relative Orga Weight) of the experimental animals and the percentage bwt gained, with that of the controls suggests an adaptive response of the organs to the accumulation of the extracts (Jimoh *et al*., 2008). This signifies that the organ weights did not indicate any toxic or adverse effects from extract administration.

Assay of plasma biochemical parameters was performed in order to evaluate the effects of *Nefang* and its constituents on hepatic, renal, lipid, nitrogen balance and glycemic profiles of experimental animals and to give insight into pathological changes and nature of disease or other effects upon exposure to drugs. Lipid peroxidation is induced when drugs are taken resulting in the release of cytosolic enzymes into the blood stream, such as ALT, AST, ALP which when

observed in increased quantities in the plasma are indicative of liver and cellular damage (Agbor *et al*., 2005). Serum transaminase (AST, ALT) and phosphatase (ALP) are indicators of hepatic function (Konan *et al.*, 2007), URIC is the end product of purine metabolism (Cavanaugh, 2003) and it is an indicator of cardiovascular and renal diseases (Kutzing and Firestein, 2007), BUN and CRE are indicators of glomerular filtration rate (GFR), which is an indicator of the renal function (Eaton and Pooler, 2009) while blood lipids such as TGY and CHOL are some of the factors associated with artherosclerotis and cardiovascular diseases (Shaila *et al.*, 1997). The significantly increased values observed in the values of ALT, AST, ALP, URIC and BUN after the administration of the ethanol extracts of ***Cp***, ***Cc*** and ***Cs*** as well as prolonged administration of the aqueous extract of ***Cc*** at high doses, were probably indicative of liver or cellular damage of myocardial or kidney tissue. These toxicities were confirmed by the histopathological examination of the kidney and liver of these groups of experimental animals, which revealed some tissue damage. However, the normal creatinine levels indicated that these were of non-renal cause (Cavanaugh, 2003). Most plant extracts are known to produce degenerative changes to renal architecture (Khorshid, 2008) due to the presence of certain secondary metabolites which exhibit adverse effects upon. This was certainly the the reason for the observed adverse hepatic effect of these extracts at high doses. However, *Nefang* did not present any of these adverse effects, suggesting that the interaction between the plant extracts reduced the adverse effects and maintained the hepatic protection in both the aqueous and ethanol extracts.

Assessment of the effects of *Nefang* and its constituents on the lipid profile revealed that apart from *MiB*, *Cp* and *Pg* at high doses of 1000 $mgkg^{-1}$ bwt, all the extracts that elevated the TGY levels were ethanol extracts. Elevated CHOL levels were also observed only in *Pg* and *Nefang* ethanol extract treated groups. All aqueous extracts exhibited hypocholesterolomic activities and significantly reduced TGY levels at low doses. In this study, corn oil was used as the vehicle for the ethanol extracts. Previous studies reported significantly increased values in TGY and CHOL levels upon administration of corn oil diet in Swiss mice (Ahmed *et al.,* 2012) suggesting that observed hyperlipidemia might be as a result of the corn oil used as the vehicle. This suggests that *Nefang* aqueous extract and its constituents do not present any risk of hypercholesterolemia or artherosclerosis. This hypolipidemic activity might be as a result of the antioxidant property of the extracts, which lowers the level of cholesterol in the blood by increasing LDL catabolism.

The extracts might also inhibit cholesterol synthesis and delay its absorption (Rajendran and Krishnakumar, 2010) at low doses.

Blood glucose levels in experimental animals suggest that *Nefang* aqueous extract and its constituents exhibited great hypoglycemic tendencies. Only *C. sinensis* ethanol extract exhibited hyperglycemic effects in experimental rats. The antihypoglycemic activity exhibited by this extract might be due to impaired insulin action or inadequate insulin secretion (Bailey, 2000). The hypoglycemic effects may be due to the presence of hypoglycemic substances in the plant extracts (Collier *et al*., 1987; Gray and Flatt, 1999) stimulation of ß cells to produce more insulin (Khan *et al*., 1990), increasing glucose metabolism or regenerative effect of the extracts on pancreatic tissue (Shanmugasundaram *et al*., 1990) Hence, *Nefang* or some of its constituents could be used in the regulation of blood sugar levels and management of artherosclerotic diseases.

Hematological parameters analyzed included the complete blood count of experimental and control group animals. In order to understand the risk alterations in the human hematopoietic system upon exposure to drugs, analysis in toxicity studies must be carried out using animal models and then extrapolated to humans (Olson *et al*., 2000). Hematopoiesis is the process of blood cell formation. All blood cells are believed to be derived from the pluripotential stem cell, an immature cell with the capability of becoming an erythrocyte (RBC), a leukocyte (WBC), or a thrombocyte (platelet). In healthy adults, stem cells in hematopoietic sites undergo a series of divisions and maturational changes to form the mature cells found in the blood. The hematological parameters are mediators of immunity and play a vital role in immuneprotection and tissue repair (Cavanaugh, 2003; Tripathy *et al*., 2010). The WBCs protect the body from infection by foreign organisms, the RBC boost the immune system and the platelets protect blood vessels from endothelial damage as well as initiate repair of these vessels. The mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) give the volume and weight of the HGB in each RBC while the mean corpuscular hemoglobin concentration (MCHC) gives a valuable indicator of HGB deficiency. The general increase and/or normal values of these parameters and their indices when compared to the control indicate that there was no observed adverse effect of these extracts on the hematological system, which serves as an important index of the physiological and pathological status (Adeneye *et al.*, 2006). These results suggest a

stimulation of the hematopoietic system, leading to the production of these blood cells and hence a strong immuno-modulatory, antioxidant and endothelial protection and repair activity of these extracts (Guyton and Hall, 2006). These results are consistent with earlier studies on the membrane-protective activity, protection against hemolysis of the RBC (Imaga *et al*., 2009) and wound healing potential of ***C. papaya*** (Kasarla *et al.,* 2012), anti-inflammatory, free radical scavenging and antioxidant activity of ***C. citratus*** (Carbajal *et al*., 1989; Cheel *et al*., 2005), antioxidant activities of the extracts and some substances isolated from ***C. sinensis*** (Okwu *et al*., 2007). However, this is the first assessment of the effect of *Nefang* of the hematological system.

Plasmodium infection usually triggers a cascade of unpleasant physiological changes in the host such as oxidative stress, pyrexia, inflammation and nociception. Biological agents that can act against some of these may contribute towards antimalarial activities. Activities evaluated in the constituent plants included antioxidant, antipyretic, anti-inflammatory and antinociceptive.

Antioxidants are chemically diverse making their separation and quantification with regards to their source very demanding. Due to the increasing interest in antioxidants, there has been an increase in in vitro biological assays for the estimation of total antioxidant activity from different sources. The antioxidant capacity of whole blood is contained predominantly in circulating RBC whose effective and powerful antioxidant system has been highlighted as a potential major advantage for using RBCs to study ROS generation (Kinoshita et al., 2007).

Among the constituent plants of *Nefang*, the leaf extracts of ***Pg***, ***MiL***, ***MiB*** and ***Og*** exhibited potent in vitro antioxidant activity in DPPH radical scavenging and FRAP assays, which correlated with their high Total Phenolic Content. The correlation coefficients between the assays were very strong and were close to 1.

2, 2 – Diphenyl-1-Picryl-Hydrazyl (DPPH) is a commonly employed assay in antioxidant studies of plant extracts or specific compounds over a short time period, since it provides information on the reactivity of extracts with a stable free radical. The efficacies of antioxidants are usually associated with their ability to inhibit oxidative damage by scavenging free radicals (Wang *et al*., 1999). Total phenolic content estimation is a simple reproducible assay to estimate the total phenolic antioxidants in an extract (Naskar *et al*., 2010). Phenolic compounds are considered to be the most important anti-oxidative plant components (Leong and Shui, 2002) and the antioxidant activity of plant extracts correlate with the content of their phenolic compounds (Hu

and Skibsted, 2002). FRAP is a simple inexpensive method to measure the total antioxidant reducing power of plant extracts in the RBC (Prior *et al*., 2005). The fact that all the constituent plants of *Nefang* possess radical scavenging activities, TPC and FRAP (with exception of ***Cs***, ***Cc*** and ***Cp*** leaves with comparatively weak activity) suggest that *Nefang* may possess the antioxidant potential to combat oxidative stress induced during malaria infection or treatment.

In the present study, carbon tetrachloride (CCl_4) was used to induce oxidative stress so as to evaluate the potential of *Nefang* as in vivo antioxidant. Carbon tetrachloride is widely used as a toxicant for experimental induction of liver toxicity in laboratory animals, because it induces lipid peroxidation in experimental animals within few minutes of administration. Accumulation of carbon tetrachloride in the parenchyma cells results into hemolytic cleavage producing trichloromethyl (CCl_3*) and the chloride (Cl*) free radicals due to the activating effect of cytochrome P_{450} enzymes. The CCl_3* alkylates cellular proteins and other molecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen, forming lipid peroxides, leading to severe damage to some visceral organs (Tsukamoto *et al*., 1990; Terblanche and Hickman, 1991; Mizuguchi *et al.,* 2006; Preethi and Kuttan, 2009) and the massive generation of free radicals. Thus administration of carbon tetrachloride at specific doses produces marked organ damage, as evidenced by a significant ($p<0.05$) increase in renal (ALT, AST), hepatic (BUN, CRE) enzymes and lipid peroxidation. It causes hepatotoxicity, characterized by an increase in organ mass. The relative liver weight of all the groups treated with carbon tetrachloride increased as compared to the normal control group animals. However, this increase in liver weight was reduced in *Nefang*-treated groups and the effect was dose dependent, showing protective nature of *Nefang*. The rats treated with carbon tetrachloride recorded the least gain in body weight. This correlated with the reduced appetite characterized by decreased food and water intake.

Nefang seemed to offer dose-dependent protective effect because the levels of the renal and hepatic enzymes were lower in extract-treated rats as compared to the untreated control group. This was achieved by inhibiting the effect of carbon tetrachloride in the plasma.

Oral administration of CCl_4 decreased CHOL and TGY levels as earlier reported by Stern *et al.* (1965). Cholesterol has been reported to modulate the lipid bilayer membrane fluidity and other physiological processes (McConnell and Hubbell, 1971). Carbon tetrachloride causes

peroxidative degradation of the cellular membrane leading to functional morphological changes and loss of functional integrity of the membranes (Naskar *et al.*, 2010). By restoring the cholesterol level to normal, it is evident that *Nefang* may modulate the role of cholesterol in this and other physiological processes.

The human body has an effective defense system against free radical induced damage, which consists of endogenous antioxidant enzymes (e.g. CAT and SOD) and non-enzymatic enzymes (e.g. GSH). SOD and CAT form part of the enzymatic oxidative system of RBCs while MDA is an end product of lipid peroxidation in the liver and other tissues. The level of lipid peroxide is a measure of membrane damage alterations in its structure function and is measured by the level of MDA. Erythrocytes are more vulnerable to lipid peroxidation (Anandan *et al*., 1999). SOD is a metalloprotein enzyme involved in antioxidant defense, which acts by lowering the steady state level of $O_2^{\cdot-}$ while catalase protects cells against radical toxicity by catalyzing the decomposition of hydrogen peroxide to water and molecular oxygen. Decreased levels of SOD and CAT in carbon tetrachloride-treated rats may be due to the overuse of these enzymatic antioxidants or to the cross linking of these enzymes with MDA, causing an increase in the level of superoxide anion which in turn increases lipid peroxidation (Eritsland, 2000). In the present study, levels of SOD and CAT decreased and MDA levels increased in untreated control group when compared to the normal control group. This provided evidence of oxidative stress leading to enhanced lipid peroxidation, tissue damage and collapse of the antioxidant defense mechanism against free radicals. However, upon administration of *Nefang* at a high dose, these parameters did not show any stress to the antioxidative defense system, though at a low dose, levels of these parameters were comparable to that in untreated rats. This could be explained by the potent antioxidant activity or increased enzyme expression. *Nefang* therefore provided antioxidant effects.

The TP estimation gives the protein content and reflects the antioxidant capacity. The increased TP level in CCl_4-induced oxidative stress rats (group B) may be due to dehydration (Cavanaugh, 2003). TP was dose-dependently restored to normal in rats treated with the extract, revealing that *Nefang* may help boost the potential of the antioxidant system through its high phenolic content as evidenced in the in vitro studies.

Given that there is laboratory evidence that antioxidants ameliorate symptoms of malaria (Reis *et al.*, 2010), it is highly likely that the potent antioxidants found in *Nefang* contribute to

effectiveness in the management of malaria when used traditionally. This may explain why multiple herbs are included in the formulation because each definitely serves a different purpose as we might have noticed.

Pyrexia is a result of secondary impact of infection, tissue damage, inflammation, graft rejection, malignancy or other diseased states including malaria. Brewer's yeast and D-amphetamine are commonly used to induce pyrexia in rats and mice (Gupta *et al.,* 2005; Okokon *et al*., 2012). The pyrexia results from their activity such as their ability to cause infections, tissue damage and inflammation. Brewer's yeast induces pyrexia by increasing the synthesis of prostaglandins (PGE2). The infections or damaged tissue serves as a pyrogenic stimulus and the pyrogens are phagocytized by the Kupffer cells, monocytes, macrophages etc. leading to the release of pro-inflammatory mediators (cytokines), which increase the synthesis of prostaglandins near pre-optic hypothalamus area thereby triggering the hypothalamus to elevate the set point of normal body temperature (Al-Ghamdi, 2001). Amphetamine on the other hand, acts on the brain causing the release of biogenic amines from their storage sites in nerve terminals resulting in increased level of cyclic-Adenosine monophosphate (cAMP) and subsequent synthesis of prostaglandins from arachidonic acids produced in neurons receptor-mediated hydrolysis of phospholipids, leading to hyperthermia (Westfall and Westfall, 2006). Most of the anti-pyretic drugs inhibit cyclooxygenase (COX-2) expression thereby inhibiting prostaglandin biosynthesis to reduce elevated body temperature. They are however toxic to the hepatic cells, glomeruli, cortex of the brain and heart muscle. A natural prostaglandin inhibitory antipyretic remedy with minimal toxicity is therefore essential.

The results from this study shows that ***Cc***, ***Og***, ***MiL***, ***MiB*** and ***Cs*** aqueous extracts are safe and significantly reduced amphetamine and brewer's yeast induced hyperthermia in the rats at 120, 180 and 240 min post administration. This hypothermic activity might have been achieved by their action on COX-2 thereby reducing the concentration of prostaglandin in the brain or by enhancing the inherent production of the body's own antipyretic substances such as arginine and vasopressin (Chandrasekharan, 2002). An alternative could have been the mediation of their phytochemical content activity by vasodilation of superficial blood vessels leading to increased dissipation of heat as a result of a reset of the hythalamic temperature control centre (Rang *et al*.,

2007). Therefore, the antipyretic activity of these extracts could be as a result one or more of the above mentioned mechanisms, thereby contributing to the in vivo antimalarial activity of *Nefang*.

Inflammation is a common phenomenom in malaria infection and it is the reaction of living tissues towards injury. The carrageenan-induced paw eodema is a prototype for the exudative phase of acute inflammatory effects.

The development of oedema in the rat paw after the injection of carrageenan has been described as a biphasic event (Vinegar *et al.*, 1969). The initial phase which starts immediately after injection and reduces within 1 h, is attributed to the release of histamine and serotonin, while the second phase of swelling which begins at 1 h and remains through 3 h, is due to the release of prostaglandin-like substances (Crunkhon and Meacock, 1971; Georgewill and Georgewill, 2010). The second phase of oedema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents but generally NSAIDs strongly inhibit the second phase of carrageenan-induced oedema. The significant inhibition of paw oedema in rats by ***MiL***, ***Cp*** and ***MiB*** aqueous extracts suggest that they may contain biologically active substances with anti-inflammatory property. The significant anti-inflammatory activity exhibited may be due to the inhibition of any inflammatory mediators by non steroidal anti-inflammatory agents present in the extracts, which might contribute greatly in the antimalarial activity of *Nefang*. The co-existence of antinociceptive and anti-inflammatory activities observed in *Nefang*, are properties shared by most non-steroidal anti-inflammatory drugs (NSAIDs), particularly the salicylates and their derivatives. The therapeutic benefits of traditional remedies are often attributed to a combination of active constituents (Chindo *et al*., 2003). These findings lend pharmacological support to the reported folkloric uses of these plants in the treatment of inflammatory conditions.

Nociception is a common phenomenom during *Plasmodium* infection. Measuring antinociceptive activity is based on the principle that inflammation increases the sensitivity to pain and that this sensitivity is susceptible to modification by analgesics. Inflammation decreases the pain reaction threshold and this low pain reaction threshold is readily elevated by different types of analgesics (Randall and Selitto, 1957). The source of pain has been proven to detect central as well as peripheral analgesics. Analgesic properties were studied using models that could provide

different grades of noxious stimuli *i.e.* mechanical pressure on the tail using the analgesymeter and thermal stimulus using the radiant heat-induced nociceptive pain on the tail, evaluated by the tail flick method and hot plate test evaluating heat-induced nociceptive pain on the paws of mice. Mechanical pain induced by the analgesy meter provides a model for the study of non-inflammatory pain. The analgesy meter antinociceptive test is useful in elucidating centrally mediated antinociceptive responses, which focuses mainly on changes above the spinal cord level (Vongtau *et al*., 2004). The significant increase in pain threshold produced by the extracts in the analgesy meter test suggests involvement of central pain pathways. Thermal tests have several advantages including the sensitivity to strong analgesics and limited tissue damage (Ramesh, 2010). Furthermore, they utilize phasic stimulus of high intensities mimicking responses in conditions that involve high threshold pain of short duration. However, a disadvantage of this model is that since it is short lasting, it does not assess modulatory mechanisms that may be triggered by the stimulus itself (Tjolsen *et al.,* 1992) and is a less valid model for clinical pain (Eaton, 2003). The results indicate that ***Og***, ***MiL***, ***Pg*** and ***MiB*** can significantly inhibit responses to mechanical and thermal stimuli. The inhibition of thermal stimuli was dose dependent, thus showing that these extracts, at the doses administered, had strong antinociceptive activities and can contribute significantly to the antimalarial effect of *Nefang*.

6.0 CONCLUSION

This study confirms the traditional use of *Nefang* for the treatment of malaria and gives information on its formulation, folk use, efficacy and safety. We can therefore conclude as follows:

i. *Nefang* can be considered as an antimalarial agent.
ii. Combining the constituent plants of *Nefang* increased efficacy and reduced adverse side effects.
iii. The potential antimalarial activity of *Nefang* is as a result of the overall biological (antiplasmodial, antioxidant, antipyretic, anti-inflammatory, antinociceptive) activities of the constituent plants.
iv. Antagonistic interactions should not be ignored during interaction studies of polyherbals, since they could play a positive role in overall potentiation of efficacy.
v. Plants remain a dependable source of therapeutic agents for exploitation towards a rational antimalarial phytotherapeutic drug discovery.
vi. Collaboration between herbal practitioners and phytotherapy research scientists is essential.

6.0 PERSPECTIVES

In perspectives, the following are envisaged:

i. Re-formulation of *Nefang* for optimum efficacy based on in vitro activities of its constituent plants obtained in this study.
ii. Standardization and quantitative HPLC fingerprinting of *Nefang* to ensure quality and reproducibility.
iii. Clinical studies on the efficacy and safety of *Nefang* with the view to developing efficient and affordable improved traditional medicines (ITM).
iv. Interaction studies of *Nefang* and/or its constituents in combination with drugs in currents use as well as those that have fallen into disuse due to the development of parasite resistance.

REFERENCES

1 Abbas AK. Cellular and Molecular Immunology. *W.B. Saunders Company*. Philadelphia. **2000**.

2 Adeneye AA, Ajagbonna OP, Adeleke TI and Bello SO. Preliminary Toxicity and Phytochemical Studies of the stem bark aqueous extract of *Musanga cecropioides* in rats. *J. Ethnopharmacol.* **2006**, 105(3), 374-379.

3 Adjanahoun JE and Aké Assi. Plantes Pharmaceutiques de Côte d'Ivoire. **1973**; 82 pp.

4 Adjanohoun JE, Aboubakar N, Dramane K, Ebot ME, Ekpere JA, Enow-Orock EG,Focho D, Gbile ZO, Kamanyi L, Kamsu Kom J, Keita A, MbenkumT, Mbi CN, Mbiele AL, Mbome IL, Mubiru NK, Nancy WL, Nkongmeneck B, Satabie B, Sofowora A, Tamze V and Wirmum CK. Contribution to Ethnobotanical and Floristic Studies in Cameroon. Addis Ababa: *Scientific, Technical and Research Commission, Organization of African Unity.***1996**; 641 pp.

5 Afzan A, Abdullah NR, Halim SZ, Rashid BA, Semail RHR, Abdullah N, Jantan I, Muhammad H and Ismail Z. Repeated Dose 28-Days Oral Toxicity Study of *Carica papaya* L. Leaf Extract in Sprague Dawley Rats. *Molecules*, **2012**, 17, 4326-4342.

6 Afonso A, Hunt P, Cheesman S, Alves AC, Cunha CV, Do Rosario V and Cravo P. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca2+ ATPase), tctp, mdr1, and cg10. *Antimicrob. Agents Chemotherap.,* **2006**, 50(2): 480-9.

7 Agbor AG, Oben JE, Nkegoum B, Takala JP and Ngongang JY. Hepatoprotective activity of hibiscus cannabinus (Linn) against carbon tetrachloride and paracetamol liver damage in rats. *Pak. J. Biol. Sci.,* **2005**, 8, 1397-1401.

8 Ahmed IT, Hussein MMA, Mohamed M. Soliman MM and Abdelrahman AA. Comparative Studies on the Effect of High Fish and Corn Oil Diet on Lipid Profiles, PPAR-α and FAS mRNA in Swiss Mice. *J. Investigational Biochem*., **2012**, 1(1):31-37.

9 Ahmed SA, Gogal RM Jr and Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H] thymidine incorporation assay. *J. Immunol. Methods*, **1994**, 170(2): 211-224.

10 Aiyelaagbe OO and Osamudiamen PM. Phytochemical screening of Active Compounds in *Mangifera indica* leaves from Ibadan, Oyo State. *Plant Sc. Res.*, **2009**, 2(1): 11-13.

11 Aiyeloja AA and Bello OA. Ethnobotanical potentials of common herbs in Nigeria: a case study of Enugu state. *Ed. Res. Rev.*, **2006**, 1, 16-22.

12 Al-Ghamdi MS. The anti-inflammatory, analgesic and antipyretic activity of *Nigella sativa*. *J. Ethnopharmacol*., **2001**, 76:45-48.

13 Amadi JE, Salami SO and Eze CS. Antifungal Properties and Phytochemical Screening of Extracts of African Basil (*Ocimum gratissimum* L.). *Agric. Biol. J. N. Am.*, **2010**, 1(2): 163-166.

14 Anandan R, Deepika R and Devaki T. Protective effects of Piccorhiza kurroa on mitochondrial glutathione antioxidant system in D- galactosamine induced hepatitis in rats. *Current Science*, **1999**, 76, 1543-1545.

15 Anibijuwon II and Udeze AO. Antimicrobial activity of *Carica Papaya* (Pawpaw Leaf) on some pathogenic organisms of clinical origin from South-Western Nigeria. *Ethnobot. Leaflets*, **2009**, 13, 850-864.

16 Annuaire Statistique du Cameroun. Department of Cameroon. Statoids, obtained from Institut National de la Statistique, Cameroun. **2004**.

17 Anon 5 Encyclopedia of Medicinal Africa, Vol. 44. Tournai. *Belgium. Africa Cambridge.* **1986**; 1106 pp.

18 Arima H and Danno GI. Isolation of antimicrobial compounds from guava (*Psidium guajava* L.) and their structural elucidation. *Biosci. Biotechnol. Biochem.*, **2002**, 66(8): 1727-1730.

19 Asase A, Otng-Yeboah AA, Odamtten GT, Simmonds MSJ. Ethnobotanical study of some Ghanaian anti-malarial plants. *J. Ethnopharmacol*., **2005**, 99, 273-279.

20 Awuah RT. In vivo use of extracts from *Ocimum gratissimum* and *Cymbopogon citratus* against Phytophthora palmivora causing blackpod disease of cocoa. *Ann. Appl. Biol.*, **1994**, 124(1): 173-178.

21 Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO. Phytochemical Screening and Antioxidant Activities of Some Selected

Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Trop. J. Pharmaceutical Res.*, **2008**, 7 (3): 1019-1024.

22 Ayoola PB and Adeyeye A. Phytochemical and Nutrient Evaluation of *Carica papaya* (Pawpaw) Leaves. *IJRRAS*, **2010**, 5(3): 325-328.

23 Azas N, Laurencin N, Delmas F, Di Giorgio C, Gasquet M, Laget M, Timon- David P. Synergistic in vitro antimalarial activity of plant extracts used as traditional herbal remedies in Mali. *Parasitol. Res.*, **2002**, 88, 165-171.

24 Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P and Picot S. Comparison of a SYBR green I-based assay with a histidinerich protein II enzyme-linked immunosorbent assay for in vitro antimalarial drug efficacy testing and application to clinical isolates. *Antimicrob. Agents Chemother.*, **2007**, 51, 1172-1178.

25 Bailey CJ. Potential new treatments for Type 2 diabetes. *Trends Pharmacol. Sci*., **2000**, 21, 259-265.

26 Basir R, Fazalul Rahiman SS, Hasballah K, Chong WC, Talib H, Yam MF, Jabbarzare M, Tie TH, Othman F, Moklas MAM, Abdullah WO, Ahmad Z. *Plasmodium berghei* ANKA Infection in ICR Mice as a Model of Cerebral Malaria**.** *Iranaian J. Parasitol.*, **2012**, 7(4): 62 – 74.

27 Bassey ME and Effiong EO. Preliminary investigation of herbs used in paediatric care among the people of Akwa Ibom State, Nigeria. *J. Nat. Prod. Plant Resour*., **2011**, 1(3): 33-42.

28 Batista R, Silva Júnior AJ and Oliveira AB. Plant-Derived Antimalarial Agents: New Leads and Efficient Phytomedicines. Part II. Non-Alkaloidal Natural Products. *Molecules*, **2009**, 14, 3037-3072. doi: 10.3390/molecules14083037.

29 Beckman H. Antimalarial Drugs: Their Nature, Action and Use. **1958**; 529-533.

30 Beckstrom-Sternberg SM *et al*. The phytochemical database." (ACEDB version 4.3-Data version July 1994.) *National Germplasm Resources Laboratory (NGRL*), *Agricultural Research Service* (*ARS*), U.S. Department of Agriculture. **1994**.

31 Belec L, Authier FJ, Chazaud B, Piedouillet C, Barlovatz-Meimon G, and Gherardi RK. Interleukin (IL)-1 beta and IL-1 beta mRNA expression in normal and diseased skeletal

muscle assessed by immunocytochemistry, immunoblotting and reverse transcriptase-nested polymerase chain reaction. *J. Neuropathol. Exp. Neurol.*, **1997**, 56(6): 651-63.

32 Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E and Roepe PD. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob. Agents Chemother.*, **2004**, 48, 1807-1810.

33 Benoit-Vical F, Valentin A, Mallie M, Bastide JM, Bessiere JM. In vitro Antimalarial Activity and Cytotoxicity of *Cochlospermum tinctorium* and *C. planchonii* leaf Extracts and Essential oils. *Planta Medica*, **1999**, 65, 378-381.

34 Benoit-Vical F, Imbert C, Bonfils JP, Sauvaire Y: Antiplasmodial and antifungal activities of iridal, a plant triterpenoid. *Phytochemistry*, **2003**, 62(5): 747-751.

35 Benzie IFF and Strain JJ. The ferric reducing Ability of Plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Anal. Biochem.,* **1996**, 239, 70-76.

36 Berenbaum MC. A method for testing for synergy with any number of agents. *J. Infect. Dis.* **1978**, 137, 122-130.

37 Betti JL and Lejoly J. Contribution to the knowledge of medicinal plants of the Dja Biosphere Reserve, Cameroon: plants used for treating jaundice. *J. Med. Plants Res.,* **2009**, 1056-1065.

38 Bharel S, Gulati A, Abdin MZ, Srivastava PS and Jain SK. Structure, Biosynthesis and Function of artemisinin. *Fitoterapia.* **1996**, 67, 387-399.

39 Bhat GP, Surolia N. In vitro antimalarial activity of extracts of three plants used in the traditional medicine of India. *Am. J. Trop. Med. Hyg.*, **2001**, 64, 304-308.

40 Bidla G, Titanji VPK, Jako B, Ghazali GE, Bolad A and Berzins K. Antiplamodial activity of seven plants used in African folk medicine. *Indian J. Pharmacol.*, **2004**, 36, 245-246.

41 Biswas T, Pal JK, Nashar K, Ghosh DK, Ghosal. Lipid peroxydation of erythrocytes during anaemia of the hamster with *Leishmania donovani. Mol. Cellular Biochemistry*, **1995**, 146(2): 99-105.

42 Bloland P. Drug Resistance in Malaria *WHO/CDS/DRS.4.* WHO Geneva, Switzerland. **2001**.

43 Brandao MGL, Krettli AV, Soares LSR, Nery CGC and Marinuzzi HC. Antimalarial activity on extracts and fractions from *Bidens pilosa* and other *Bidens* species (Asteraceae). *J. Ethnopharmacol.,* **1997**, 57, 131-138.

44 Brasseur P, Kouamouo J, Moyou-Somo R and Druilhe P. Multi-drug resistant falciparum malaria in Cameroon in 1987-1988. II. Mefloquine resistance confirmed in vivo and in vitro and its correlation with quinine resistance. *Am. J. Trop. Med. Hyg.*, **1992**, 46(1): 8-14.

45 Bray RS and Sinden RE. The sequestration of *Plasmodium falciparum* infected erythrocytes in the placenta. *Trans. R. Soc. Trop. Med. Hyg.*, **1979**, 73(6): 716-9.

46 Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am. J. Trop. Med. Hyg.,* **2001**, 64 (1-2 Suppl): 1-11.

47 Bronner U, Divis PC, Farnert A and Singh B. "Swedish traveller with *Plasmodium knowlesi* malaria after visiting Malaysian Borneo". *Malaria J.* **2009**, 8, 15.

48 Burkill HM. The Useful Plants of West Tropical Africa. *Royal Botanic Gardens Kew*.**1977**; 969 pp.

49 Burkill HM. The useful plants of west tropical Africa, Vol 1, *Royal Botanic Gardens, Kew*, UK. **1985;** 960 pp.

50 Burkill HM. The useful plants of west tropical Africa, Vol 3, *Royal Botanic Gardens, Kew*, *UK*. **1995;** 857 pp.

51 Butterweck V and Nahrstedt A. What is the Best Strategy for Pre-Clinical Testing of Botanicals: A Critical Perspective. *Planta Med.*, **2012**, 78(8): 747-754.

52 Caraballo A, Caraballo B and Rodriguez-Acostca A. Evaluations also preliminarde Conn antimalarial medicinal plants used in Southeast amazãnico Venezuela. *J. Braz. Soc., Trop. Med.*, **2004**, 37(2): 186-188.

53 Carbajal D, Casaco A, Arruzazabala L, Gonzalez R, Tolon Z.Pharmacological Study of Cymbopogon citratus leaves. *J. Ethnopharmacol.*, **1989**, 25(1): 103-107.

54 Castellanos JRG, Prieto JM and Heinrich M. Red Lapacho (*Tabebuia impetiginosa*)– a global ethnopharmacological commodity? *J. Ethnopharmacol.*, **2009**, 121, 1-13.

55 Cavanaugh BM. Nurse's Manual of Laboratory and Diagnostics Tests. 4th Ed. *F. A. Davis Company*, Philadelphia. **2003**; 688 pp.

56 CDC-Centre for Disease Control. Malaria Biology. **2008**. http://www.cdc.gov/malaria/biology/index.htm

57 Chandrasekharan NV. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure and expression. *Proceed National Acad. Sci.*, **2002**, 99, 13926-13931.

58 Chawira AN, Warhurst DC and Peters W. Qinghaosu resistance in rodent malaria. *Trans. R. Soc. Trop. Med. Hyg.*, **1986**, 80(3): 477-80.

59 Cheel J, Theoduloz C, Rodriguez J and Schmeda-Hirschmann G. Free radical scavengers and antioxidants from lemongrass (Cymbopogon citratus (DC) Stapf). *J. Agric. Food Chem*., **2005**, 53(7): 2511-2517.

60 Chinchilla M, Guerrero OM, Abarca G, Barrios M and Castro O. An in vivo model to study the anti-malaria capacity of plant extracts. *Rev. Biol. Trop.*, **1998**, 46, 1-7.

61 Chindo BA, Amos S, Odutola AA, Vongtau HO, Abah J, Wambebe C, Gamaniel KS. Central nervous system activity of the methanolic extract of *Ficus platyphylla* stem bark. *J. Ethnopharmacol.*, **2003**, 85, 131-137.

62 Clark IA and Cowden WB. The pathophysiology of falciparum malaria. *Pharmacology and Therapeutics*, **2003**, 99, 221-260.

63 Co EMA, Johnson SM, Murthy T, Hickman MR and Johnson JD. Recent Methods in Antimalarial Susceptibility Testing. *Anti-inf. Ag. Med. Chem*., **2010**, 9(3): 148-160.

64 Coley PD, Bryant JP and Chapin FS. Resource availability and Plant Antiherbivore Defense. *Science*, **1985**, 230, 895-899.

65 Colfer CJP, Sheil D, Kaimowitz D and Kishi M. Forests and human health in the tropics: some important connections. *Unasylva*, **2006**, 224(57): 3-10.

66 Collier E, Watkinson A, Cleland CF, Roth J. Partial purification and characterization of an insulin-like material from spinach and *Lemna gibba* G3. *J. Biol. Chem.*, **1987**, 262, 6238-47.

67 Conde-Garcia EA, Nascimento VT and Santos ABS. Inotropic effects of extracts of *Psidium guajava* L. (Guava) leaves on the guinea pig atrium. *Braz. J. Med. Biol. Res.*, **2003**, 36, 661-668.

68 Cowman AF, Karcz S, Galatis D and Culvenor JG. A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. *J. Cell. Biol.* **1991**, 113(5): 1033-42.

69 Crunkhon P and Meacock SER. Mediators of the inflammation induced in the rat paw by carrageenan. *Brit. J. Pharmacol.,* **1971**, 42, 392-402.

70 Dalziel JM. The Useful Plants of West Tropical Africa. *The Crown Agents for the colonies,* London. **1995**; 612 pp.

71 Daneshvar C, Davis TME, Cox-Singh J, Rafa'ee MZ, Zakaria SK, Divis PCS and Singh B. Clinical and Laboratory Features of Human *Plasmodium knowlesi* Infection. *Clin. Infectious Diseases,* **2009**, 49: 852–60.

72 Das PC, Das A, Mandal S, Islam CN, Dutta MK and Patra B. Antiinflammatory and antimicrobial activities of the seed kernel *Mangifera indica*. *Phytotherapy,* **1989**, 6(3): 235-241.

73 Deharo E, Bourdy G, Quenevo C, Munoz V, Ruiz G and Sauvain M. A search for national bioactive compounds in Bolivia through a multidisciplinary approach. Part V. Evaluation of the antimalarial activity of plants used by the Tecana Indians. *J. Ethnopharmacol.,* **2001**, 77, 91-98.

74 de Paula RC, Dolabela MF and de Oliveira AB. Aspidosperma Species as Sources of Antimalarials. Part III. A Review of Traditional Use and Antimalarial Activity. *Planta Med.,* **2014**, 80: 378–386.

75 Desjardins RE. *In vitro* techniques for antimalarial development and evaluation. In: Peters W and Richards WHG, editors. *Handbook of Experimental Pharmacology. Germany: Springer-Verlag*. **1984**; 179-200.

76 Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, SU XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV and Coulibaly D. A molecular marker for chloroquine-resistant *falciparum* malaria. *N. Engl. J. Med*., **2001**, 344(4): 257-63.

77 Doyle D. Per rectum: a history of enemata. *J R Coll Physicians Edinb.,* **2005**, 35, 367-370.

78 Duke JA. Handbook of Phytochemical Constituents of Grass Herbs and Other Economic Plants. *CRC Press, Boca Raton*, FL, USA. **1992**; 26 pp.

79 Eaton DC and Pooler JP. Vander's Physiology.7th Ed. *McGraw-Hill Lange*, USA. **2009**; 230 pp.

80 Eaton M. Common animal models for spasticity and pain. *J. Rehab. Res. Dev.*, **2003**, 40, 41-54.

81 Egharevba HO, Iliya I, Ibekwe N, Abdullahi MS, Okwute SK and Okogun JI. Broad Spectrum Antimicrobial Activity of *Psidium guajava* Linn.Leaf. *Nature Sc*., **2010**, 8(12).

82 Elekwa I, Okereke SC and Ekpo BO. Preliminary Phytochemical Screening of the Stem Bark and Leaves of *Psidium guajava*. *J. Med. Plants Res.*, **2009**, 3(1): 045-048.

83 Elford BC. L-Glutamine influx in malaria-infected erythrocytes: a target for antimalarials? *Parasitology Today*, **1986**, 2(11): 309-312.

84 El-Sanusi NI and El-Adam S. The Effect of low levels of Dietary *Ruta graveolens* and *Solenostemma argel* or their mixture in Bovans Chicks. *Asian J. Anim Vet. Adv*., **2007**, 2: 27-31.

85 Emanuel EJ, Wendler D, Killen J and Grady C. What makes clinical research in developing countries ethical? The benchmarks of ethical research. *J. Infect. Dis.*, **2004**, 189, 930-937.

86 *Encyclopædia Britannica Online*, s. v. "Mamfe", accessed August 20, **2014**, http://www.britannica.com/EBchecked/topic/1482559/Mamfe.

87 Eritsland J. Safety Considerations of Polyunsaturated Fatty Acids. *American J. Clin. Nutr*., **2000**, 71(1): 197S-201S.

88 Fan Y, Ding Z, Yang L, Xu L and Li K. A Preliminary Study on Bioactivity of Orange and Tangerine Peel Extracts against Aphis and Mites. *Zhongguo ZhongYao ZaZhi*, **1995**, 20, 397-398.

89 Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD and Wellems TE. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell*, **2000**, 6(4): 861-71.

90 Fidock DA, Rosenthal PJ, Croft SL, Brun R And Nwaka S. Antimalarial Drug Discovery: Efficacy Models for Compound Screening. *Nat. Rev. Drug Disc*., **2004**, 3, 509-520.

91 Gabe M. Techniques Histologiques. *Mason, 120, Boulevard Saint Germain*, Paris. **1968**; 128-243.

92 Ganesan A. The impact of natural products upon modern drug discovery. *Curr. Opin. Chem. Biol*., **2008**, 12, 306-317.

93 Gathirwa JW, Rukunga GM, Njagi EN, Omar SA, Mwitari PG, Guantai AN, Tolo FM, Kimani CW, Muthaura CN, Kirira PG, Ndunda TN, Amalenba G, Mungai GM and Ndiege IO. The in vitro antiplasmodial and in vivo anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Meru community in Kenya. *J. Ethnopharmacol.,* **2008**, 115(2): 223-231.

94 Georgewill OA, Georgewill UO. Evaluation of the anti-inflammatory activity of extract of *Vernonia Amygdalina*. *Asian Pac. J. Trop. Med*. **2010**, 3(2): 150-1.

95 Gil-Izquierdo A, Gil MI, Ferreres F and Tomás-Barberán FA. In vitro Availability of Flavonoids and other Phenolics in Orange. *J. Agric. Food Chem.,* **2001**, 49: 1035-1041.

96 Ginsburg H and Deharo E. A call for using natural compounds in the development of new antimalarial treatments - an introduction. *Malaria Journal*, **2011**, 10 (Suppl 1), S1.

97 Gray AM and Flatt PR. Insulin-releasing and insulin-like activity of the traditional anti-diabetic plant *Coriandrum sativum* (coriander). *Br. J. Nutr*. **1999**, 81, 203-208.

98 Gross D. *Papaya*: A tantalising taste of the Tropics. *Maricopa County Master Gardener Volunteer information*, University of Arizona Cooperative Extension. **2003**. www.papaya Maricopa-hort@ag.arizo.edu.

99 Guanasingh CBG and Nagarajan S. Flavonoids of *Cymbopogon citratus*. *Ind. J. of Pharm. Sc.*, **1981**, 43, 115.

100 Guerra F. The introduction of Cinchona in the treatment of malaria. Part I. *J. Trop. Med. Hyg.,* **1977**, 80(6): 112-8.

101 Guerrin PJ, Olliaro P, Nosten F, Druilhe P, Laxminarayan R, Binka F, Kilama W, Ford N and White NJ. Malaria: current status of control, diagnosis, treatment and a proposed agenda for research and development. *Lancet Infect. Dis*., **2002**, 2, 564-573.

102 Gupta M, Mazumder, UK, Kumar RS, Gomathi P, Rajeshwar Y., Kakoti BB, Selven VT. Antiinflammatory, analgesic and antipyretic effects of methanol extract from *Bauhinia racemosa* stem bark in animal models. *J. Ethnopharmacol.*, **2005**, 98(3): 267-73.

103 Guyton AC and Hall JE. Textbook of Medical Physiology, 11th Ed. *Elsevier Saunders*, USA.**2006**; 1152 pp.

104 Handa SS, Khanuja SPS, Longo G and Rakesh DD. Extraction Technologies for Medicinal and Aromatic Plants. *ICS-UNIDO International Centre for Science and High Technology*, Trieste, Italy. **2008**; 266 pp.

105 Harborne JB. Phytochemical Methods. 2nd Ed. London. *Chapman and Hall, Ltd.* **1973**; pp 1-7.

106 Harrell MC and Bradley MA. Training Manual: Data Collection Methods; Semi-structured Interviews and Focus Groups. *RAND Corporation Publication*, USA. **2009**; 148 pp.

107 Hayes AW. Guidelines of Acute Oral Toxicity Testing.2nd Ed. *Raven Press Ltd.*, New York, **1987**; 185 pp.

108 Hayton K and Su XZ. Genetic and biochemical aspects of drug resistance in malaria parasites. *Curr. Drug Targets Infect. Disord.*, **2004**, 4(1): 1-10.

109 Hendrickse RG, Adeniyi A, Edington GM, Glasgow EF, White RH and Houba V. Quartan malarial nephrotic syndrome: Collaborative clinicopathological study in Nigerian children. *Lancet*, **1972**, 1(7761): 1143-9.

110 Ho M, Sexton MM, Tongtawe P, Looareesuwan S, Suntharasamai P and Webster HK. Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific lymphoproliferation in acute *Plasmodium falciparum* malaria. *J. Infect. Dis*., **1995**, 172(3): 838-44.

111 Houghton PJ: Synergy and polyvalence: paradigms to explain the activity of herbal products. In Evaluation of Herbal Medicinal Products. *London: Pharmaceutical Press*; Houghton PJ, Mukherjee PK **2009**; 85-94.

112 Hu M and Skibsted LH. Antioxidant Capacity of Rhizome extract and Rhizome knot extract of edible lotus (*Nelumbo nuficera*). *Food Chem.,* **2002**, 76 (3): 327-333.

113 Hunt NH, Golenser J, Chan-Ling T, Parekh S, Rae C, Potter S, Medana IM, Miu J and Ball HJ. Immunopathogenesis of cerebral malaria. *Int. J. Parasitol*., **2006**, 36(5): 569-82.

114 Hutchinson J and Dalziel JM. Flora of West Tropical Africa. 2nd Ed, Vol. I, 1 and 2 rev by Keay R. W. J. (1954 and 1958), pp822; Vol. II rev by Hepper F. N. (1963), pp544; Vol. III, 1 and 2 rev by Hepper F. N. (1968 and 1972), pp574. *Crown Agents*, London. **1954-1972**.

115 Ian SEB. Species Profiles for Pacific Island Agro forestry: *Mangifera indica* (mango). **2006**. *www.traditionaltree.org*

116 Ibrahim MH and Jaafar HZ. Primary, Secondary Metabolites, H_2O_2, Malondialdehyde and Photosynthetic Responses of *Orthosiphon stimaneus* Benth. to Different Irradiance Level. *Molecules*, **2012**, 17, 1159-1176.

117 Imaga NOA, Gbenle GO, Okochi VI, Akanbi SO, Edeoghon SO, Oigbochie V, Kehinde MO and Bamiro SB. Antisickling property of *Carica papaya* leaf extract. *Afr J Biochem Res*., **2009**, 3(4): 102-106.

118 Izumiyama S, Omura M, Takasaki T, Ohmae H and Asahi H. *Plasmodium falciparum*: development and validation of a measure of intraerythrocytic growth using SYBR Green I in a flow cytometer. *Exp. Parasitol*., **2009**, 121, 144-150.

119 Janssen PAJ and Jagenea AH. A new series of potent analgesics:Dextro 2,2-diphenyl-3 methyl- Hmorholino- butryprolidine and related amides. Part 1. Chemical structure and pharmacological activity. *J. Pharm.*, **1957**, 9, 38.

120 Jimoh FO, Adedapo AA, Sofidiya MO, Masika PJ, and Afolayan AJ. Safety evaluation of the extract from the shoots of *Arctotis arctotoides* in rats and mice. *Afr. J. Biotechnol*., **2008**, 7: 3173-3177.

121 John DT and Petri WA. Markell and Voge's Medical Parasitology, 9th Edition. *Saunders Elsevier*, USA. **2006**; pp 79-106.

122 Kaira BS, Chawla S, Gupta P and Valecha N. Screening of Antimalarial Drugs: An overview. *Indian J. Pharmacol*., **2006**, 38(1): 5-12.

123 Kakudidi EKZ, Bukenya-Ziraba R, Kasenene JM. The medicinal Plants in and around Kibale National Park in Western Uganda. *Lidia*, **2000**, 5, 109-124.

124 Kala CP. *J.* Ethnomedicnal botany of the Apatani in the Eastern Himalayam region of India. *Ethnobiol. Ethnomed*., **2005**, 1, 11-18.

125 Kasarla R, Elumalai A, Eswaraiah MC, Ravi P and Naresh V. An annual review on wound-healing medicinal plants (Jan-Dec 2011) *J. Nat. Prod. Plant Resour*., **2012**, 2(1): 182-185.

126 Keay RWJ. Trees of Nigeria. *Clarendon Press,* Oxford. **1989**; 476 pp.

127 Khan A, Bryden NA, Polansky MM and Anderson RA. Insulin potentiating factor and chromium content of selected food and spices. *Biol. Trace Elem. Res*., **1990**, 24, 183-188.

128 Khorshid FA.Pre-clinical evaluation of PM 701 in experimental animals. *Int. J. Pharmacol.*, **2008**, 4, 443-451.

129 Kinoshita A, Nakayama Y, Kitayama T and Tomita M. Simulation study of methemoglobin reduction in erythrocytes. Differential contributions of two pathways to tolerance to oxidative stress. *Febs Journal*, **2007**, 274, 1449-58.

130 Kirmizibekmez H, Calis I, Perozzo R, Brun R, Donmez AA, Linden A, Ruedi P, Tasdemir D. Inhibiting activities of the secondary metabolites of *Phlomis brunneogaleata* against parasitic protozoa and plasmodial enoyl-ACP Reductase, a crucial enzyme in fatty acid biosynthesis. *Planta Medica*, **2004**, 70(8): 711-717.

131 Kirtikar KR and Basu BD. *Indian Medicinal Plants*, Lalit Mohan Basu, Allahabad, India, **1993**; 371 pp.

132 Kitchen I. Modification of an analgesy meter for pawpressure antinociceptive testing in neonatal rats. *J. Pharmacol. Meth.,* **1984**, 12, 255-258.

133 Knight DJ and Peters W. The antimalarial action of N-benzyloxy dihydrotriazines. The action of cycloguanil (BRL50216) against rodent malaria and studies on its mode of action. *Annal Trop. Med. Parasitol*., **1980**, 74, 393-404.

134 Koba K, Sanda K, Guyon C, Raynaud C, Chaumont J and Nicod L. In vitro cytotoxic activity of *Cymbopogon citratus* L. and *Cymbopogon nardus* L. essential oils from Togo. *Bangladesh J. Pharmacol*., **2009**, 4, 29-34.

135 Kokwaro JO. *Medicinal Plants of East Africa*.2nd edition.*Kenya Literature Bureau*, Nairobi, Kenya, **1993**; 401 pp.

136 Konan NA, Bacchi EM, Lincopan N, Varela SD, Varanda EA. Acute, sub-acute toxicity and genotoxic effect of a hydroethanolic extract of the cashew (*Anacardium occidentale* L.). *J. Ethnopharamcol*., **2007**, 110(1): 30-38.

137 Kong DX, Li XJ and Zhang HY. Where is the hope for drug discovery? Let history tell the future. *Drug Discov. Today*, **2009**, 14, 115-119.

138 Kretti AU, Andrade-Neto VF, Brandao MGL. and Ferrari WMS. The Search for new antimalarial drugs from plants randomly selected: a review. *Mem. Inst. Oswaldo Cruz,* **2001**, 96, 1033-1042.

139 Krishna KL, Paridhavi M and Patel JA. Review on Nutritional, Medicinal and Pharmacological properties of Papaya (*Carica papaya* Linn). *Nat. Prod. Radiance,* **2008**, 7(4): 364-373.

140 Kumar R, Krishan P, Swami G, Kaur P, Shah G and Kaur A. Pharmacognostical Investigation of *Cymbopogon citratus* (DC) Stapf. *Der Pharmacia Lettre*, **2010**, 2(2): 181-189.

141 Kutzing MK and Firestein BL. Altered Uric Acid Levels and Disease States. *J. Pharmacol. Exp. Therap*., **2007**, 324(1): 1-7.

142 Laforest J. *Safety Diagnosis Tool Kit for Local Communities. Guide to Organizing Semi-Structured Interviews With Key Informants* in The Charting a Course to Safe Living Collection, Vol 11. Québec, Institut national de santé publique du Québec. **2009**; 26 pp.

143 Lambros C and Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.*, **1979**, 65, 418-420.

144 Langhorne J, Quin SJ and Sanni LA. Mouse Models of Blood-Stage Malaria Infections: Immune Responses and Cytokines Involved in Protection and Pathology. In Malaria Immunology. 2nd edition. Edited by Perlmann P, Troye-Blomberg M. Stockholm: *Karger publisher,* **2002**, 204-228.

145 Leong LP and Shui G. An investigation of Antioxidant Capacity of Fruits in Singapore market. *Food Chem.,* **2002**, 76(1): 69-75.

146 Lindberg J. Structure-Assisted Design of Drugs towards HIV-1 and Malaria Targets. *A dissertation in Faculty of Science and Technology*-ACTA UNIVERSITATIS UPSALIENSIS-UPPSALA. **2004**.

147 Lowry OH, Rosebrouch NJ, Farr AL and Randall RJ. Protein measurement with the folin-phenol reagent. *J. Biol. Chem*., **1951**, 193, 265-278.

148 Luka CD. and Mohammed A. Evaluation of the antidiabetic property of aqueous extract of *Mangifera indica* leaf on normal and alloxan-induced diabetic rats *J. Nat. Prod. Plant Resour*., **2012**, 2(2): 239-243.

149 Lukmanul Hakkim F, Girija Arivazhagan and Boopathy R. Antioxidant Property of selected *Ocimum* Species and their secondary Metabolite content. *J. Med. Plants Res*., **2008**, 2(9): 250-257.

150 Ma XH, Zheng CJ, Han LY, Xie B, Jia J, Cao ZW, Li YX and Chen YZ. Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives. *Drug Discov. Today*, **2009**, 14, 579-588.

151 Macpherson GG, Warrell MJ, White NJ, Looareesuwan S and Warrell DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am. J. Pathol*., **1985**, 119(3): 385-401.

152 Makhija IK. and Khamar D. Anti-snake venom properties of medicinal plants *Der Pharmacia Lettre*, **2010**, 2(5): 399-411**.**

153 Makler MT and Hinrichs DJ. Measurement of the Lactate Dehydrogenase Activity of *Plasmodium falciparum* as an Assessment of Parasiteamia. *Am. J. Trop. Med. Hyg*., **1993**, 48, 205-210.

154 Maselli VM. Amphibian Neuropeptides: Isolation, Sequence Determination and Bioactivity. *A PhD Thesis, Department of Chemistry*-University of Adelaide-Australia. **2006**.

155 Masihi KN: Immunomodulatory agents for prophylaxis and therapy of infections. *Int. J. Antimicrob. Agents*, **2000**, 14, 181-191.

156 Mbagwu HO, Anene RA and Adeyemi OO. Analgesic, antipyretic and anti-inflammatory properties of *Mezoneuron benthamianum* Baill Caesalpiniaceae. *Niger Quart J. Hosp. Med.*, **2007**, 17(1): 35-41.

157 Mbwambo L. Status of Arid and Semi-arid Lands of Tanzania. Paper presented at the Drylands Agroforestry Workshop, 1st-3rd September **2004**. ICRAF Headquarters, Nairobi, Kenya, **2004**.

158 McChesney JD, Venkataraman SK and Henri JT. Plant natural products: back to the future or into extinction? *Phytochemistry,* **2007**, 68, 2015-2022.

159 McConnell HM and Hubbell WL. Molecular motion in spin-labeled phospholipids and biomembranes. *J. American Chem. Society*, **1971**, 93, 314-326.

160 Metwally AM, Omar AA, Ghazy NM, Harraz FM and El Sohafy SM. Monograph of *Psidium guajava* L. leaves. *Pharmacog. J.,* **2011**, 3, 21, 89-104.

161 Misra HP and Fridovich I. The generation of superoxide radical during the auto-oxidation of hemoglobin. *J. Biol. Chem*., **1972**, 247, 6960-6962.

162 Mizuguchi S, Takemura S, Minamiyama Y, Kodai S, Tsukioka T, Inoue K, Okada S, Suehiro S. S-allyl cysteine attenuated CCl4- induced oxidative stress and pulmonary fibrosis in rats. *Biofactors,* **2006**, 26(1): 81-92.

163 Mohamed A, Tanko Y, Okasha MA, Magaji RA and Yaro AH. Effects of Aqueous Leaves Extract of *Ocimum gratissimum* on Blood Glucose Levels of Streptozocin-induced wistar rats. *Afr. J. Biotechnol*., **2007**, 6 (18): 2087-2090.

164 Mohamed-Saleem TK, Azeem AK, Dilip C, Sankarl C, Prasanth NV and Duraisami R. Anti-inflammatory Activity of the leaf extracts of *Gendarussa vulgaris* Nees. *A. Pac. J. Trop. Biomed*., **2011**, 147-149.

165 Mojab F. Antimalarial natural products: a review. *Avicenna J. Phytomedicine,* **2012**, 2(2): 52-62.

166 Morton JF. Major Medicinal Plants: Botany, Culture and Uses. *Springfield Il*: Charles C. Thomas. **1977**.

167 Mukherjee T. Antimalarial herbal drugs. A review. *Fitoterapia,* **1991**, 62, 197-204.

168 Müller I, Genton B, Rare L, Kiniboro B, Kastens W, Zimmerman P, Kazura J, Alpers M and Smith TA. Three different *Plasmodium* species show similar patterns of clinical tolerance of malaria infection. *Malaria J*., **2009**, 8, 158.

169 Muniz-Junqueira MI. Immunomodulatory therapy associated to antiparasite drugs as a way to prevent severe forms of malaria. *Curr. Clin. Pharmacol*. **2007**, 2, 59-73.

170 Muthu C, Ayyanar M, Raja N and Ignacimuthu S. Medicinal Plants used by Traditional healers in Kancheepuram District of Tamil Nadu, India. *J. Ethnobiol. Ethnomed*., **2006**, 2, 43-53.

171 Mwaiko GL. Citrus peel oil extracts as mosquito larvae insecticides. *East Afr. Med. J.*, **1992**, 69, 223-226.

172 Nargarajun S, Jain HC and Aulaki GS. Indigenous plants used in fertility control. In: Cultivation and Utilization of Medicinal Plants. *PID Publ., CSIRL*. **1989**; 558 pp.

173 Naskar S, Islam A, Mazumder UK., Saha P, Haldar PK and Gupta M. *In Vitro* and *In Vivo* Antioxidant Potential of Hydromethanolic Extract of *Phoenix dactylifera* Fruits. *J. Scientific Res.*, **2010**, 2(1): 144-157.

174 National Academies Press. Guide for Care and Use of Laboratory Animals. 8th Ed. *Nat Res. Council of the Nat Academies.* **2010;** 248 pp.

175 Newman DJ, Cragg GM and Snader KM. Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.,* **2003**, 66: 1022-1037.

176 Ngemenya MN, Tane P, Berzins K and Titanji VPK. Antiplasmodial Activity of some Medicinal Plants used in Cameroon: preliminary toxicity studies of highly active extracts. *XIth Annual Conference of the Cameroon Bioscience Society,* 16- 18 December, **2004.**

177 Njoroge GN and Kibunga JW. Herbal medicine acceptance, sources and utilization for diarrhoea management in a cosmopolitan urban area Thika, Kenya. *Afr. J. Ecol.,* **2007**, 45, 65-70.

178 Nociari MM, Shalev A, Benias P and Russo CA. A novel one-step highly sensitive fluorimetric assay to evaluate cell mediated cytotoxicity. *J. Immunol. Methods,* **1998**, 213(2): 157-167.

179 Nosten F, Mcgready R, Simpson JA, Thwai KL, Balkan S, Cho T, Hkirijaroen L, Looareesuwan S and White NJ. Effects of *Plasmodium vivax* malaria in pregnancy. *Lancet*, **1999**, 354(9178): 546-9.

180 Nosten F and White NJ. Artemisinin-based combination treatment of *falciparum* malaria. *Am. J. Trop. Med. Hyg*., **2007**, 77, 181-192.

181 Nundkumar N and Ojewole JAO. Studies on the antiplasmodial properties of some South African medicinal plants used as antimalarial remedies in zulu folk medicine. *Meth. Find. Exp. Clin. Pharmacol*., **2002**, 24(**7**): 397-401.

182 Nwinuka NM, Monanu MO and Nwiloh BI. Effects of aqueous Extract of *Mangifera indica L.* (Mango) Stem Bark on Heamatological Parameters of Normal Albino Rats. *Pakistan J. Nutr.*, **2008**, 5, 663-666.

183 Odebiyi OO and Sofowora EA. Phyto-chemical screening of Nigerian medicinal plants – Part II. *Lloydia*, **1978**, 41(3): 234-246.

184 O.E.C.D. (Organization for Economic Cooperation Development). Guidance Document on Oral Toxicity Testing 423 and 425. Environment, Health and Safety Publications. Series on testing and Assessment N° 24. **2001**; 24 pp.

185 Ohrt C, Willingmyre CD, Lee P, Knirsch C and Milhous W. Assessment of azithromycin in combination with other antimalarial drugs against *plasmodium in vitro*. *Antimicrob. Agents Chemother*. **2002**, 46, 2518-24.

186 Ojo OO and Anibijuwon II. Studies on extracts of three medicinal plants of South-western Nigeria: *Hoslundia opposita*, *Lantana camara* and *Cymbopogon citratus*. *Adv. Nat. Appl. Sc*., **2010**, 1.

187 Okokon JE, Nwafor PA, Abia GO and Bankhede HK. Antipyretic and antimalarial activities of crude leaf extract and fractions of *Enicostema littorale*. *Asian Pac. J. Trop. Dis*., **2012**, 2(6): 442-447.

188 Okwu DE, Awurum AN and Okoronkwo JI. Phytochemical Composition and *In Vitro* Antifungal Activity Screening of Extract from Citrus Plants against *Fusarium oxysporum* of Okra Plant (*Hibiscus esculentus*). *African Crop Science Conference Proceedings*, **2007**, 8, 1755-1758.

189 Oliveira AB, Dolabela MF, Braga FC, Jacome RL, Varotti FP and Povoa MM. Plant-derived Antimalarial Agents: New leads and Efficient Phytomedicines. Part 1. Alkaloids. *Anais da Academia Brasileira de Ciencias*, **2009**, 81(4): 715-740.

190 Oliveira ED, Leite TS, Silva BA and Conde-Garcia EA. Inotropic Effect of *Citrus sinensis* (L.) Osbeck Leaf Extracts on the Guinea Pig Atrium. *Brazilian J. Med. Biol. Res.*, **2005**, 38, 111-118.

191 Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul. Toxicol.Pharmacol*. **2000**, *32*, 56-67.

192 Orwa C, Mutua A, Kindt R, Jamnadass R and Simons A. Agroforestry Database: a tree reference and selection guide version 4.0. **2009**. *http://www.worldagroforestry.org/af/treedb/*.

193 Otsuki N, Dang NH, Kumagai E, Kondo A, Iwata S and Morimoto C. Aqueous extract of *Carica papaya* leaves exhibits anti-tumor activity and immunomodulatory effects. *J. Ethnopharmacol.*, **2010**, 127, 760-767.

194 Oyedele AO, Gbolade AA, Sosan MB, Adewoyin FB, Soyelu OL and Orafidiya OO. Formulation of an effective mosquito-repellent topical product from Lemongrass oil. *Phytomedicine*, **2002**, 9, 259-262.

195 Patwardhan B. Drug discovery and development: Traditional medicine and ethnopharmacology perspectives. *SciTopics,* **2009**.

196 Percario S, Moreira DR, Gomes BAQ, Ferreira MES, Goncalves ACM, Laurindo PSO, Vilhena TC, Dolabela MF and Green MD. Oxidative stress in malaria. *International Journal of Molecular Sciences*, **2012**, 13(12): 16346-16372.

197 Pedersen BK, Ostrowski K, Rohde T and Bruunsgaard H. The cytokine response to strenuous exercise. *Can. J. Physiol. Pharmacol.*, **1998**, 76(5): 505-11.

198 Peter IT and Anatoli VK. The current global malaria situation. Malaria parasite biology, pathogenesis, and protection. *W.D.C: ASM Press*; **1998**, 11-22.

199 Peters JM and Boyd EM. Organ Weights and Water Levels of the Rat following Reduced Food Intake. *J. Nutr.*, **1966**, 90(4): 354-60.

200 Peters W and Robinson BL. The Chemotherapy of rodent Malaria. LVI. Studies on the Development of Resistance to Natural and Synthetic Endoperoxides. *Ann. Trop. Med. Parsitol*., **1999**, 93, 325-9.

201 Peters W. Drug Resistance in *Plasmodium berghei*. Vincke and lips, 1948; 1 chloroquine resistance. *Experimental Parasitol.*, **1965**, 17, 80-89.

202 Pfeiffer CJ. A mathematical evaluation of the thymic weight parameter. *Toxicol. Appl. Pharmacol.* **1968**, 13(2): 220-7.

203 Philippe G, Angenot L, De Mol P, Goffin E, Hayette MP, Tits M, Frederich M. *In vitro* screening of some *Strychnos* species for antiplasmodial activity. *J. Ethnopharmacol.*, **2005**, 97, 535-539.

204 Pink R, Hudson A, Mouries MA, Bendig M. Opportunities and challenges in antiparasitic drug discovery. *Nature Rev. Drug Disc.*, **2005**, 4, 727-740.

205 Preethi KC and Kuttan R. Hepato and reno protective action of Calendula officinalis L. flower extract. *Indian J. Exptal. Biol.*, **2009**, 47(3): 163-168.

206 Presidential Decree N° 2008/376 of 12 November 2008. Restructuring the Administrative Units of the Republic of Cameroon. *Presidency of the Republic of Cameroon*, Yaoundé; **2008**.

207 Prior RL, Wu X and Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Food and Dietary Supplements. *J. Agric. Food Chem.*, **2005**, 53(10): 4290-4302.

208 *Programme National de Lutte contre le Paludisme (PNLP). Plan stratégique de lutte contre le paludisme. Ministère de la Santé Publique, Cameroun.* **2002**.

209 Rajendran R and Krishnakumar E. Hypolipidemic Activity of Chloroform Extract of Mimosa pudica Leaves. *Avicenna J. Med. Biotech*., **2010**, 2(4): 215-221.

210 Ramesh R. Analgesic Effects of the Aqueous Extracts of Plant *Ipomea pes-tigridis* Studied in Albino Mice. *Global J. Pharmacol*., **2010**, 4(1): 31-35.

211 Randall LO and Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn.*, **1957**, 111, 409-419.

212 Rang HP, Dale MM, Ritter JM and Moore PK. Pharmacology, 6th ed. *Edinburgh: Churchill Livingstone.* **2007**, 557-587.

213 Reda MYM, EL-Tahan NR, Adel M.A. El-Hadad. Effect of Aqueous Extract *Mangifera Indica* Leaves, as Functional Foods. *J. Appl. Sc. Res*., **2010**, 6(6): 712-721.

214 Reis PA, Comim CM, Hermani F, Silva B, Barichello T, Portella AC, Gomes FC, Sab IM, Frutuoso VS, Oliveira MF, Bozza PT, Bozza FA, Dal-Pizzol F, Zimmerman GA, Quevedo J and Castro-Faria-Neto HC. Cognitive dysfunction is sustained after rescue therapy in experimental cerebral malaria, and is reduced by additive antioxidant therapy. *PLOS Pathogens,* **2010**, 6, 6, e1000963. doi: 10.1371/journal.ppat.1000963.

215 Ricke CH, Staalsoe T, Koram K, Akanmori BD, Riley EM, Theander TG and Hviid L. Plasma antibodies from malaria-exposed pregnant women recognize variant surface

antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *J. Immunol.,* **2000**, 165(6): 3309-16.

216 Ringwald P, Peyron F, Lepers JP, Rabarison P, Rakotomalala C, Razanamparany M, Rabodonirina M, Roux J and Le Bras J. Parasite virulence factors during *falciparum* malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines. *Infect. Immun.,* **1993**, 61(12): 5198-204.

217 Ryley JF and Peters W. The antimalarial activity of some quinone esters. *Annal Trop. Med. Parasitol.,* **1970**, 84, 209-222.

218 Saiman L, Mehar F, Niu WW, Neu HC, Shaw KJ, Miller G. and Prince A. Antibiotic susceptibility of multiply resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, including candidates for transplantation. *Clin. Infect. Dis*., **1996**, 23, 532-537.

219 Same-Ekobo A. Epidermiology of Malaria in Cameroon: Current Treatment and Prevention Strategies. *Workshop on Antimalaria Drugs organized by Redox Antimalarial Drug Discovery Project in April 2008,* Yaoundé, Cameroon. **2008**.

220 Santin MR, Dos Santos AO, Nakumura CV, Filho BPD, Ferreira ICP and Ueda-Nakamura T. In vitro activity of the essential oil of *Cymbopogon citratus* and its major component (citral) on Leishmania amazonensis. *Paras. Res.,* **2009**, 105, 1489-1496.

221 Saotoing P, Vroumsia T, Tchobsala, Tchuenguem FF, Njan NA and Messi J. Medicinal plants used in traditional treatment of malaria in Cameroon. *J. Ecol. Nat. Environ.,* **2011**, 3(3): 104-117.

222 Sawyer E. Traditional Medicine in Sierra Leone: a Critical Appraisal. *Nigerian J. Pharm.,* **1982**, 13:28-33.

223 Shaila HP, Udopa SL and Udopa AL. Hypolipidemic effect of Terminalia arjurna in cholesterol fed rabbits. *Fitoterapia*, **1997**, 5, 405-409.

224 Shanmugasundaram ERB, Gopinath KL, Shanmugasundaram KR, Rajendran VM. Possible regeneration of the islets of Langerhans in Streptozotocin-diabetic rats given *Gymnema sylvestre* leaf extracts. *J. Ethnopharmacol*., **1990**, 30(3): 265-269.

225 Sharma VC and Ogbeide ON. Pawpaw as a renewable energy resource for the production of alcohol fuels. *Energy*, **1982**, 7(10): 871-873.

226 Shetty Akhila J, Shyamjith, D and Alwar MC. Acute Toxicity studies and determination of median lethal dose. *Curr. Sc*., **2007**, 93, 7.

227 Shree Devi MS. Acute Toxicity and Diuretic Activity of *Mangifera indica* l. bark extracts. *Intl. J. Pharma. Bio. Sc*., **2011**, 2(3): 141-146.

228 Singh SK, Tiwari RM, Saini A and Sinha SK.Phytochemical and pharmacological investigation on stem bark of *Mangifera indica* L. *Asian Pacific J. Tropical Biomed*., **2012**, 1-5.

229 Sinha AK**.** Colorimetry of catalase. *Analytical Biochem.,* **1972**, 11, 469-474.

230 Slinkard K and Singleton VL. Total Phenol Analysis: Automation and comparism with manual Method. *Am. J. Enol. Vitic*., **1977**, 28, 49-55.

231 Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P and Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob.Agents Chemother.,* **2004**, 48, 1803-1806.

232 Smith I, Carr N, Corrado OJ and Young A. Rectal necrosis after a phosphate enema. *Age Ageing,* **1987**, 16, 328-30.

233 Smith Nigel JH, Williams JT, Plucknett DL and Talbot JP. Tropical Forests and their Crops. London: *Cornell University Press.* **1992**; 568 pp.

234 Sofowora EA. Medicinal Plants and Medicine in Africa. *John Whilley Spectrum Books,* Ibadan, Nigeria. **1993**; 289 pp.

235 Sofowora EA. Medicinal plants and Traditional Medicine in Africa.*Spectrum Books Ltd* (Ibadan). **1982**; 97-145.

236 Soladoye MO, Chukwuma EC and Owa FP. An 'Avalanche' of Plant Species for the Traditional Cure of *Diabetes mellitus* in South-Western Nigeria *J. Nat. Prod. Plant Resour*., **2012**, 2(1): 60-72.

237 Stepniewska K, Chotivanich K, Brockman A, Day NP and White NJ. Overestimating resistance in field testing of malaria parasites: simple methods for estimating high EC_{50} values using a Bayesian approach. *Mal. J.,* **2007**, 6, 4.

238 Stern PH, Furukawa T and Broody TM. Rat liver and plasma lipids after carbon tetrachloride administration. *J. Lipid Res.,* **1965**, 6, 278-286.

239 Stevenson MM and Riley EM: Innate immunity to malaria. *Nature Rev. Immunol.* **2004**, 4, 169-180.

240 Stewart I. Identification of Caffeine in Citrus Flowers and Leaves. *J. Agric. Food Chem*., **1985**, 33, 1163-1165.

241 Sudhanshu S, Neerja P, Jain DC and Bhakain RS. Antimalarial agents of plant sources. *Curr. Sc*., **2003**, 85(9): 1314-1329.

242 Sulistiarini D. *Ocimum gratissimum* L. In Oyen, L.P.A. & Nguyen Xuan Dung (Eds.): Plant Resources of South-East Asia. No. 19: Essential-oils plants. *Prosea Foundation, Bogor*, Indonesia. **1999**; 140-142.

243 Tabuti JRS, Lye KA and Dhillion SS. Traditional herbal drugs of Bulamogi, Uganda: plants, use and administration. *J. Ethnopharmacol.*, **2003**, 88(1): 19-44.

244 Talcott S and Talcott S. Phytochemical Attributes Contribute to the Health-Promoting Benefits of Mangos: Mass Spectroscopic and HPLC Characterization of Mango (*Mangifera Indica L.*) following Enzymatic Hydrolysis. *Final Research Report to the National Mango Board, Department of Nutrition and Food Sciences,* Texas A and M University. **2009**.

245 Tanyi EM. Kenyang Segmental Phonology. *Cameroon Association for Bible Translation and Literacy (CABTAL),* Yaoundé, Cameroon, **2000**; 92 pp.

246 Tasdemir D, Guner ND, Perozzo R, Brun R, Donmez AA, Calis I, Ruedi P: Anti-protozoal and plasmodial FabI enzyme inhibiting metabolites of *Scrophularia lepidota* roots. *Phytochemistry*, **2005**, 66(3): 355-362.

247 Tchoumbougnang F, Amvam Zollo PH, Dagne E and Mekonnen Y. *In Vivo* Antimalarial Activity of Essential Oils from *Cymbopogon citratus* and *Ocimum gratissimum* on Mice Infected with *Plasmodium berghei*. *Planta Medica*, **2005**, 71, 20-23.

248 Terblanche J and Hickman R. Animal models of fulminant hepatic failure. *Digestive Diseases and Sciences,* **1991**, 36, 770-774.

249 Thanh NV, Toan TQ, Cowman AF, Casey GJ, Phuc BQ, Tien NT, Hung NM and Biggs B. Monitoring for *Plasmodium falciparum* drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998-2009. *Malaria J.,* **2010**, 9: 181.

250 Titanji VPK, Zofou D, Ngemeneya MN. The Antimalarial potential of medicinal plants used for the treatment of malaria in Cameroonian Folk Medicine. *Afr. J. Trad. CAM*. **2008**, 5(3): 302-321.

251 Tjølsen, A., Berge, O., Hunskaar, S., Rosland, J. H. and Hole, K. The formalin test; an evaluation of the method. *Pain*, **1992**, 51, 5-17.

252 Trager W and Jensen JB. Human malaria parasites in continuous culture. *Science*, **1976**, 193, 673-675.

253 Triglia T, Menting JG, Wilson C and Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U S A*, **1997**, 94 (25): 13944-9.

254 Tripathy S, Pradhan D and Anjana M. Anti-inflammatory and antiarthritic potential of *Ammania baccifera* Linn. International. *J. Pharma and Bio Sciences*, **2010**; 1(3): 1-7.

255 Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review, *Seminar in Liver Disease*, **1990**, 10(1): 56-65.

256 Turner RA. Screening method in Pharmacology, *Academic Press*, New York & London. **1965**; 268 pp.

257 UNAIDS. *Ancient Remedies, New Disease: Involving traditional healers in increasing access to AIDS care and prevention in East Africa*. United Nations Programme on HIV/AIDS, Geneva, Switzerland.
http://data.unaids.org/publications/irc-pub02/jc761ancientremedies_en.pdf; **2002**.

258 Vaghasiya YK, Shukla VJ and Chanda SV. *J. Pharmacol. Toxicol*., **2011**, 6, 113-123.

259 van Vuuren S, Viljoen A. Plant-Based Antimicrobial Studies – Methods and Approaches to Study the Interaction between Natural Products. *Planta Medica*, **2011**, 77(11): 1168-1182.

260 Vinegar R, Scheirber W, Hugo R. Biphasic development of carrageenan edema in rats. *J. Pharmacol. Exp. Ther.*, **1969**, 150, 328-334.

261 Vongtau HO, Abbah J, Mosugu O, Chindo BA, Ngazal IE, Salawu AO, Kwanashie HO, Gamaniel KS. Antinociceptive profile of the methanolic extract of Neorautanenia mitis root in rats and mice. *J. Ethnopharmacol.*, **2004**, 92(2 & 3): 317-324.

262 Waako PJ, Gumede B, Smith P and Folb PI. The in vitro and in vivo antimalarial activity of *Cardiospermum halicacabum* and *Momordica foetida*. *J. Ethnopharmacol.*, **2005**, 99, 137-143.

263 Wagner H and Ulrich-Merzenich G. Synergy Research: Approaching a new generation of Phytopharmaceuticals. *Phytomedicine,* **2009,** 16, 97-110.

264 Wang M, Jin Y and Ho CT. Evaluation of resveratrol derivatives as potential antioxidants and identification of a reaction product of resveratrol and 2, 2-diphenyl-1-picryhydrazyl radical. *J. Agric. Food Chem.*, **1999**, 47, 3974-3977.

265 Wasswa P and Olila D. The in vitro Ascaricidal Activity of Selected Indigenous Medicinal Plants used in Ethno Veterinary Practices in Uganda. *Afr. J. Trad.* CAM., **2006**, 3, 94-103.

266 Wauthoz N, Balde A, Balde ES, Van Damme M and Duez P. Ethnopharmacology of Mangifera indica L. Bark and Pharmacological Studies of its Main C-Glucosylxanthone, Mangiferin. *Int. J. Biomed. Pharmaceutical Sc*., **2007**, 1(2): 112-119.

267 Weekley C and Smith DS. Malaria: the clinical basics. Global Health Education Consortium (GHEC), **2013**.

268 Weiss EA. Some Indigenous Plants used domestically by East African Coastal Fishermen. *Eco. Bot*., **1979**, 33, 35-51.

269 Westfall TC and Westfall DP. Adrenergic agonists and antagonists. In: The pharmacological basis of therapeutics. 11th ed. *New York: McGraw*. **2006**.

270 White N and Olliaro P**.** Strategies for the prevention of antimalarial drug resistance: rationale for combination chemotherapy for malaria. *Parasitol Today***, 1996,** 12**:**399-401.

271 White NJ. Drug resistance in malaria. *Br. Med. Bull.*, **1998**, 54(3): 703-15.

272 White NJ. Plasmodium knowlesi: The fifth human malaria parasite. *Clin. Infect. Dis.*, **2008**, 46: 172-3.

273 WHO. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. WHO/EDM/TRM/**2000**.1.

274 WHO. Severe *falciparum* malaria. *Trans. R. Soc. Med. Hyg*. **2000**, 94 (suppl 1): S1-S90.

275 WHO. Guidelines on Good agricultural and Collection Practices (GACP) for medicinal plants. http://www.who.int/medicinedocs/en/d/Js4928e/

276 WHO. Roll back Malaria Department. c2004; [cited 2005 May 6]. **2004** http://www.who.int/malaria/resistance.

277 WHO. World Malaria Report. Geneva, World Health Organisation, **2005**. http://rbm.who.int/wmr.2005.

278 WHO. Guidelines for the treatment of Malaria/ World Health Organisation. WHO/HTM/MAL/2006.1108. **2006**.

279 WHO. World Malaria Report. Geneva, World Health Organisation. **2008**. http://rbm.who.int/wmr.2008.

280 WHO. World Malaria Report **2009**. http://whqlibdoc.who.int/publications/2009/9789241563901_eng.

281 WHO. Joint assessment of the response to artemisinin resistance in the greater Mekong sub-region. November 2011–February 2012. Summary report [cited **2013** Dec 11]. http://whothailand.healthrepository.org/bitstream/123456789/1484/3/120418-Artemisinin Resistance in GMS SummaryReport.

282 WHO. World Malaria Report **2013**. http://www.who.int/malaria/publications/world_malaria_report_2013/en/

283 Willcox M. Improved Traditional Phytomedicines in Current Use for the Clinical Treatment of Malaria. *Planta Med.,* **2011**, 77, 662-671.

284 Williamson EM. Synergy and other interactions in phytomedicines. *Phytomedicine*, **2001**, 8, 401-409.

285 Winder CV, Lembke LA and Stephens MD. Comparative bioassay of drugs in adjuvant-induced arthritis in rats: Flufenamic acid, mefenamic acid, and phenylbutazone. *Arthritis Rheum.,* **1969**, 12, 472-482.

286 Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W and Kremsner PG. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. *Infect. Immun.,* **1998**, 66(12): 6040-4.

287 Yanez DM, Manning DD, Cooley AJ, Weidanz WP and Van Der Heyde HC. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J. Immunol.,* **1996**, 157(4): 1620-4.

288 Yen GC and Duh PD. Scavenging effects of methanolic extracts of peanut hulls on free radical and active oxygen species. *J. Agric. Food Chem.*, **1994**, 42, 629-632.

289 Zapfack L, Ayeni JSO, Besong S and Mdaihli M. Ethnobotanical Survey of the Takamanda Forest Reserve. Consultancy Report Submitted to PROFA (MINEF-GTZ) Mamfe S. W. Province Cameroon. **2001**; 90 pp.

ANNEX I: ETHICAL CLEARANCE

1.1 Ethical Clearance from Kenyatta National Hospital/University of Nairobi Ethics Review Committee (KNH/UON-ERC)

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KENYATTA NATIONAL HOSPITAL APPROVED 05 DEC 2012 ETHICS & RESEARCH COMMITTEE

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke
Link:www.uonbi.ac.ke/activities/KNHUoN

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/324

5th December 2012

Mr. Protus Arrey Tarkang
Dept. of Pharmacology and Pharmacognosy
School of Pharmacy
University of Nairobi

Dear Sir

ETHICAL APPROVAL – PHD – RESEARCH PROPOSAL "EVALUATION OF MEDICINAL PROPERTIES AND SAFETY OF A POLYHERBAL PRODUCT (NEFANG)" (P652/12/2012)

Reference is made to your application for ethics approval.

This is to inform you that the KNH/UoN-Ethics and Research Committee has reviewed and approved your application for research approval.

The approval dates are 5th December 2012 to 4th December 2013.

This approval is subject to compliance with the following requirements.

a) Only approved documents will be used.
b) The project implementation will fully adhere to the three ethical principles of research.
c) All changes must be submitted for review and approval by KNH/UoN-ERC before implementation.
d) Submission of a request for renewal of approval must be received at least 30 days prior to expiry of the current approval.
e) Clearance for export of biological specimens must be obtained from KNH/UoN-ERC for each batch of shipment.
f) Submission of **Executive summary** report within 90 days upon completion of the study.

For more details consult the KNH/UoN ERC website www.uonbi.ac.ke/activities/KNHUoN

Yours sincerely

PROF. A.N. GUANTAI
SECRETARY, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN
The Dean, School of Pharmacy, UoN
The Chairman, Dept. of Pharmacology and Pharmacognosy, UoN
The HOD, Records, KNH
Supervisors: Prof. A.N. Guantai
Dr. F.A. Okalebo
Dr. W. Bulimo
Prof. G.A. Agbor

1.2 Ethical Clearance from Kenyatta National Hospital/University of Nairobi Ethics Review Committee (KNH/UON-ERC) – Renewed

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke
Link:uonbi.ac.ke/activities/KNHUoN

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref. No.KNH/ERC/R/25

25th March 2014

Protus Arrey Tarkang
Dept.of Pharmacology and Pharmacognosy
School of Pharmacy
University of Nairobi

Dear Protus

Re: Approval of annual renewal - Evaluation of the Medicinal Properties and Safety of a Polyherbal Product (Nefang) P652/12/2012)

Refer your communication of March 5, 2014.

This is to acknowledge receipt of the study progress report and hereby grant you annual extension of approval for ethical research Protocol **P652/12/2012.**

The renewal periods are 5th December 2013 – 4th December 2014.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN- ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.

g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

Protect to Discover

For more details consult the KNH/UoN -ERC website **www.uonbi.ac.ke/activities/KNHUoN**

Kindly forward the informed consent documents for endorsement with updated stamp.

Yours sincerely

PROF. A.N. GUANTAI
CHAIRPERSON, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN
The Dean, School of Pharmacy, UoN
The Chairman, Dept. of Pharmacology and Pharmacognosy, UoN

1.3 Ethical Clearance from the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaounde-Cameroon

REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie

MINISTERE DE LA RECHERCHE
SCIENTIFIQUE ET DE L'INNOVATION

INSTITUT DE RECHERCHES MEDICALES
ET D'ETUDES DES PLANTES MEDICINALES

REPUBLIC OF CAMEROON
Peace – Work – Fatherland

MINISTRY OF SCIENTIFIC
RESEARCH AND INNOVATION

INSTITUTE OF MEDICAL RESEARCH
AND MEDICINAL PLANT STUDIES

ef. : 076 /82 – 62/MINRESI/M000
Ref. :
ef. :
r Ref. :

THE DIRECTOR

Yaoundé, 1st June 2011

INSTITUTIONAL REVIEW BOARD (IRB)

PROTUS ARREY TARKANG
LABORATORY OF PHARMACOLOGY,
CENTRE FOR THE RESEARCH ON MEDICINAL
PLANTS AND TRADITIONAL MEDICINE,
INSTITUTE OF MEDICAL RESEARCH AND
MEDICINAL PLANTS STUDIES (IMPM)
YAOUNDE, CAMEROON.

RE: APPROVAL FOR ETHICAL CLEARANCE FOR A PhD RESEARCH TITLED "EVALUATION OF THE MEDICINAL PROPERTIES AND SAFETY OF A POLYHERBAL PRODUCT (*NEFANG*)"

Reference is made to the above mentioned subject

I am pleased to inform you that our Institutional Review Board (IRB), has approved ethical clearance of the above mentioned study based on the recommendations of its ethical review committee held on the 29th May 2011. This ethical approval for the part of your PhD research to be carried out within Cameroon will entail an exploratory survey involving humans and preclinical studies involving laboratory animals.

The validity of this ethical clearance is one year from 1st June 2011 to the 31st May 2012. You will be required to apply for a renewal of the ethical clearance upon this deadline if the study is not yet completed. You will also be expected to submit bi-annually progress reports to the Institutional Review Board and a final report upon completion of your PhD research at the University of Nairobi, Kenya,.

With the committee's best wishes for the success of your research.

Sincerely,

1.4 Ethical Clearance for animal use from Institut Pasteur Korea-Institutional Animal Care and Use Committee (IPK-IACUC)

<FORM NO. 4> REPORT OF REVIEW OF ANIMAL PROPOSAL

Result of Review	X Approval ▫ Conditional Approval ▫ Review after Renewal of Protocol ▫ Rejection
Primary Investigator	Lawrence Ayong
Title of Application	"Animal model for antimalarial drug efficacy testing"
IACUC No.	IPK-12009
Length of Project	11/ 15/2012 ~ 11 /14 /2013

Date of Approval	10/29/2012
President of IPK-IACUC	Regis Grailhe (Sign) 

ANNEX II: PRIOR INFORMED CONSENT (PIC) FORM

UNIVERSITY OF NAIROBI

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY. P. O. Box 19676, NAIROBI, 00202 TEL. 0202 725099

INTERVIEW WITH HERBS SELLERS, MOTHERS, COMMUNITY LEADERS AND ELDERS

PROJECT TITLE: EVALUATION OF THE MEDICINAL PROPERTIES AND SAFETY OF A POLYHERBAL PRODUCT (*NEFANG*)

OBJECTIVES OF THE INTERVIEW: Standardization of a polyherbal concoction composed of the leaves and bark of *Mangifera indica* and leaves of *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis* and *Ocimum gratissimum*.

BENEFITS: In accordance with the provisions of the United Nations Framework Convention on Biodiversity at the Earth Summit in Rio de Janeiro, Brazil in 1992, the issues relating to environmental law and policy are conservation of biological diversity, sustainable use of the components, fair and equitable sharing of benefits, access to genetic resources, transfer of relevant technologies, consideration of all rights over these resources and technologies, availability of appropriate funding to develop these issues.

Therefore, all signatories are required to respect, preserve, and maintain the knowledge, innovations, and practices of the indigenous communities.

CONTACTS OF ALL THE RESEARCHERS

1. Professor A. N. Guantai
   Department of Pharmacology and Pharmacognosy

University of Nairobi, Kenya
Tel: +254202725099
E-mail: anguantai@yahoo.com

2. Dr. F. A. Okalebo
Department of Pharmacology and Pharmacognosy
University of Nairobi, Kenya
Tel: +254737434204
E-mail: okalebof@yahoo.com

3. Dr. G. A. Agbor
Institute of Medical Research and Medicinal Plants Studies (IMPM)
Yaoundé, Cameroon
Tel: +23777223674
E-mail: agogae@yahoo.fr

4. Protus Arrey Tarkang
Institute of Medical Research and Medicinal Plants Studies (IMPM)
Yaoundé, Cameroon
Tel: +23799928611
E-mail: ptarkang@yahoo.co.uk

ASSURANCE OF ANONYMITY AND CONFIDENTIALITY

I do hereby assure you that ALL the information you have provided will be kept strictly anonymous and confidential and will be made available ONLY to the researchers named above and used only for research purposes. Any other use of the traditional knowledge and associated materials including but not limited to commercial development, may only proceed after concluding a co-operative research development agreement. Results of the work will be communicated to the community.

Any unauthorized commercial use of the above without the said agreement shall be subject to an automatic 20 – 50% royalty rate of gross revenue from such sales together with the damages.

Signature and/or Fingerprint: _____ Date: _____
NAME:

Signature: _____ Date: _____
PROTUS ARREY TARKANG

ANNEX III: QUESTIONNAIRE

UNIVERSITY OF NAIROBI

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY. P. O. Box 19676, NAIROBI, 00202 TEL. 0202 725099

INTERVIEW WITH HERBS SELLERS, MOTHERS, COMMUNITY LEADERS AND ELDERS

NAME OF PERSON/COMMUNITY GIVING INFORMATION AND/OR MATERIAL

_____

VILLAGE OF RESIDENCE: _____

POSITION: _____

PROJECT TITLE: EVALUATION OF THE MEDICINAL PROPERTIES AND SAFETY OF A POLYHERBAL PRODUCT (*NEFANG*)

OBJECTIVES OF THE INTERVIEW: Standardization of a polyherbal concoction composed of the leaves and bark of *Mangifera indica* and leaves of *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis* and *Ocimum gratissimum*.

1. How do you harvest the various plants included in Nefang? _____

   _____

2. How do you dry the plant parts? _____

3. What precautions do you take when harvesting and drying? _____
   _____
4. How do you mix the various components?_____
   _____
5. In what proportions do you mix the various components? _____
   _____
6. Does changing the proportions affect the effectiveness of the preparation? _____
7. What's the role of each of the plants? _____
   _____
   _____
8. Are there plants which you can omit from the formulation without affecting the effectiveness? _____
9. For how long can the formulation be stored without losing its activity? _____
10. What instructions do you give patients on how to use the formulation? _____
    _____
    _____
11. For how long is the formulation used by patients? _____
    _____
12. What side effects do patients complain about? _____

_____

_____