NEUROBEHAVIOURAL AND HISTOMORPHOLOGICAL CHANGES IN THE HIPPOCAMPUS AND CEREBELLUM OF WISTAR RATS TREATED WITH ARTESUNATE AND AMODIAQUINE

BY

ABDULGAFAR NIYI POPOOLA
B.Sc. Human Anatomy (Ilorin), M.Sc. Human Anatomy (Ilorin)
181076

A Thesis in the Department of Anatomy,
Submitted to the Faculty of Basic Medical Sciences
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

DECEMBER, 2021

Certification Page

I certify that this work was carried out by Popoola Niyi AbdulGafar, matriculation number 181076 in the Department of Anatomy, University of Ibadan.

……

Supervisor

Dr I. O. Imosemi

B.Sc. (Calabar); M.Sc. (Ibadan); Ph.D. (Lagos)

DEDICATION

I dedicate this achievement to the glory of Almighty God and to the service of humanity.

ACKNOWLEDGEMENTS

I thank Almighty God for bringing this work to a reasonable completion. My sincere appreciation goes to my supervisor, Dr I. O. Imosemi for his painstaking commitment, constant encouragement and goodwill mentorship towards supervising this work to a meaningful conclusion. I also appreciate the Acting Head of Department, Dr. R. S. Ajani. I wish to thank Professor A. O. Malomo and Dr O. Owoeye of the Neurotrauma and Neuroregeneration unit of the Department for their immense contribution to the successful completion of this work. My thanks also goes to other members of the unit. My heartfelt gratitude also goes to the teaching and non-teaching staff of the Departments of Anatomy, Physiology, Pharmacology, Biochemistry and Veterinary Anatomy of the University of Ibadan for their cooperation and support throughout this period.

I am sincerely grateful to my parents: Alhaji R. S. and Alhaja K. A. Popoola, my dear wife: Mrs S. T. Mejiosu, my children, my brothers and sisters for their prayers and support both morally and financially. I also appreciate the effort of all other stakeholders on the success of this work, including Mrs Bobola Farinloye, Dr Adesoji Adetona, Mr Abdul Lateef Olawumi Akeem and Mr Ifeoluwa Temitope Ajayi of the Histopathology unit in the University College Hospital (UCH); Mr Jide Okedere of Institute for Advanced Medical Research and Training (IMRAT); Mr Madukwe Jonathan of National Hospital, Abuja; my boss and mentor, Dr A. A. Abubakar; my fellow students in the Department and other good people that have contributed one way or the other towards the success of this work. Lastly, I really appreciate Tertiary Education Thrust Fund (TETFUND) for the grant awarded to me. God bless you all.

ABSTRACT

Artemisinin-based Combination Therapy (ACT) is among the best chemotherapeutic options for malaria. Artesunate (AS) + Amodiaquine (AQ) is a commonly used ACT in Nigeria, having good efficacy but with side effects involving memory loss and impaired motor coordination ranging from acute to chronic. Reports on acute effect of AS+AQ on the cerebellum and hippocampus are controversial and there is dearth of information on its delayed effect on these brain areas. This study was designed to evaluate the effects of AS+AQ administration on neurobehaviour and morphology of the hippocampus and cerebellum in Wistar rat after three-day acute treatment.

Eighty adult male Wistar rats (120±5g) were divided into 4 groups (n=20): Group A: Control (CT: Distilled water), Group B: AS (4mg/kg), Group C: AQ (10mg/kg) and Group D: AS (4mg/kg) + AQ (10mg/kg). Drugs were administered orally, once daily for 3 days and animals monitored for 14 days. Weights of the animals were recorded. Spatial memory and motor function were evaluated using Morris water-maze and fore-limb grip tests, respectively. Animals were sacrificed by cervical dislocation on the 4^{th} and 15^{th} day. Collected blood samples were analysed for full blood count. Excised cerebelli and hippocampi were processed for lipid peroxidation, biochemical markers of oxidative stress including nitric oxide (NO). Histological (Haematoxylin & Eosin) and immunohistochemical techniques including Glial Fibrillary Acid Protein (GFAP) and Cyclo-oxygenase II (COX-2) were also carried out. Data were analysed by ANOVA at $\alpha_{0.05}$.

There was no significant variation in weight, neurobehavioural, haematological, biochemical, morphological and immunohistochemical parameters between all groups for the 3 days. At 14 days, AQ group had significant weight loss (30.40%) compared with the CT group and significantly increased swimming time (22.86±2.70s) compared with the CT group (4.14±0.74s). The AS (8.00±1.11s) and AQ (7.43±0.92s) had significantly decreased grip time compared with CT (17.86±1.22s); AQ significantly increased RBC (8.30±0.13/µL) and PCV (57.00±1.30%) compared with CT (RBC: 6.90±0.31/µL, PCV: 45.80±1.36%). Lipid peroxidation (per nmmol/g) increased significantly in AS (233.07±2.26), AQ (257.14±2.06) and AS+AQ (223.32±1.99) compared with CT (180.33±0.47). Ditto for NO (/µm/mg) (CT: 455.49±3.93, AS: 472.32±0.91, AQ: 525.74±10.11, AS+AQ: 480.95±2.917). The cerebellar Purkinje cell population was significantly reduced in the AS (2.50±0.29) and AQ (1.25±0.25) groups compared with CT (5.75±0.48), indicating pyknosis. Hippocampal Cornus Ammonis 1 cells were significantly depopulated in the AS (10.75 ±0.48) and AQ (7.25±0.48) groups compared to the CT (13.25±0.25) group, also indicating pyknosis. The GFAP expressions (per %area) significantly increased in the cerebelli of AS (55.95±1.85) and AQ (57.79±1.85) groups compared with CT (23.60±3.02) and in AS (76.81±7.19) and AQ (83.71±3.78) groups of hippocampus compared with CT (55.19±3.86), indicating astrogliosis. Compared with CT (59.36±5.61), AS (83.99±5.18) and AQ (88.32±0.88) had significantly increased hippocampal COX-2 expression per %area, indicating inflammation.

Neurobehavioural and morphological changes due to Artesunate and Amodiaquine administration in Wistar rats were significant only after 14 days.

Keywords: *Artemisinin Combination Therapy*, Artesunate, Amodiaquine, Hippocampus, Cerebellar cortex.

Word Count: 466

TABLE OF CONTENTS

CHAPTER FIVE: DISCUSSION

CHAPTER SIX: SUMMARY AND CONCLUSION

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ACT	=	Artemisinin Combination Therapy
AQ	=	Amodiaquine
AS	=	Artesunate
AS+AQ	=	Artesunate Amodiaquine combination
BAS	=	Basophil
CA	=	Cornu Ammonis
CAT	=	Catalase
CB	=	Cerebellum
COX-2	=	Cyclooxygenase-2
CT	=	Control
DG	=	Dentate gyrus
DHA	=	Dihydroartemisinin
DEAQ	=	Desethylamodiaquine
EOS	=	Eosinophil
GFAP	=	Glial Fibrillary Acidic Protein
GL	=	Granular layer
GSH	=	Glutathione
H & E	=	Hematoxylin and Eosin
Hb	=	Haemoglobin
HP	=	Hippocampus
iNOS	=	Inducible Nitric Oxide Synthase
LPO	=	Lipid peroxidation
LYM	=	Lymphocyte
MCH	=	Mean Corpuscular Haemoglobin
MCHC	=	Mean Corpuscular Haemoglobin Concentration
MCV	=	Mean Cell Volume
MDA	=	Malondialdehyde
MF	=	Mossy fibres
ML	=	Molecular layer
NEU	=	Neutrophil
NO	=	Nitric oxide
OS	=	Oxidative stress
PC	=	Pyramidal cell layer

PCV = Packed Cell Volume

PL = Purkinje cell layer

PLAT = Platelet

PM = Polymorphic layer

RBC = Red Blood Cell

SC = Schaffer Collateral

SOD = Superoxide dismutase

WBC = White Blood Cell

CHAPTER ONE

1.0 INTRODUCTION

1.1 General background

The rise in resistance of malaria parasites to conventional drugs and the move to avoid treatment failures and peculiar side effects of some monotherapies have given birth to new therapeutic approaches. Prominent among these new therapeutic approaches are artemisinin-based combination therapy (ACT). Artemisinin-based combination therapy has been approved by the World Health Organization (WHO) as one of the standard drugs for the treatment of malaria infections (WHO, 2015). It involves concurrent consumption of artemisinin product and a different drug that has schizonticidal action in the blood. These two drugs are expected to target different biochemical parts of the parasite in order to exploit their synergistic and additive potentials. The advantage of this combination lies in the improvement in the treatment efficiency coupled with prolonged resistance to the individual drug component of the ACT (Oreagba, 2010; WHO, 2015). Artemisinin-based combination therapies have been documented to increase cure performance rate, decrease resistance ability of parasites and reduce transmission of parasites that are drug-resistant. The advent of ACTs has been very successful because it has curtailed the spread of malaria parasites and lowered the chance of parasite recrudescence (WHO, 2015).

At the onset of the treatment of malaria, artemisinin eradicates larger percentage of the plasmodium parasites, while the few left over of the parasites are cleared by the slow-acting partner drug. Artemisinin posseses a unique mode of action that differs from other conventional antimalarial drugs and this determines its advantage over other drugs in terms of preventing the parasites resistance. Attempt to further avoid resistance to artemisinin and avoid treatment failure as well as peculiar side effects lies in its combination with another non-artemisinin-based therapy (Lyda *et al.*, 2007). Artemisinin-based combination therapy could be fixed combination therapy where

components are co-formulated in the same tablet or a free combination therapy where components are co-administered separately. This therapy has become very popular via its effectiveness against multidrug resistance *falciparum* strains. Artesunate-amodiaquine combination (AS+AQ) is one of the most effectively used ACTs available. It is prepared in diverse formulations, such as in single fixed-dose or separate co-package. Its effectiveness in the combating malaria is adequately reported in the literature (Adjuik *et al*., 2002).

Artesunate (AS) is a product of artemisinin (qinghaosu), an active constituent of Artemisia annua plant (a Chinese herb). In spite of the effectiveness of artemisinin derivatives treatment therapy for malaria; it has a number of side effects. Clinical researches opined that AS is relatively safe for consumption whilst other researchers ascribed a number of neurological symptoms such as coma to auditory impairments and motor effects to its use (Panossian *et al*., 2006). Animal studies reported that artemisinin and its derivatives induced neurotoxic and deadly effects on different ages of experimental animals irrespective of the specie used (Schmuck *et al*., 2009). Arteether derivative was however found to be the culprit in most cases (Li and Wu, 2002).

Amodiaquine (AQ), a 4-aminoquinoline is employed for curing malaria. It is efficacious for strains of *Plasmodium falciparum* (*P. falciparum*) that are resistant against chloroquine. Following oral ingestion of AQ, it is substantially metabolised to desethylamodiaquine, the form that elicits the bulk of antimalarial activity of the drug. Amodiaquine has been reported to possess some complications, including life-severing effects, examples of which include neutropaenia, hepatitis and fulminant hepatic failure, possibly causing hepatic encephalopathy; this implies that the encephalon may be severed in different ways by treatment with AQ (Adjuik *et al*., 2002).

The hippocampus (HP) extends antero-posteriorly on the medial side of each temporal lobe. It is a gray matter that projects off the base of the lateral ventricle in the temporal horn. It folds inwards as an archicortex. Its cortex becomes three or four layers from the initial six layers (Purves, 2011). Hippocampal formation refers to the HP proper, dentate gyrus (DG) and the subiculum (Sb) and sometimes the presubiculum, parasubiculum, and entorhinal cortex are considered as part of the hippocampal formation (Purves, 2011). The HP proper is curved, resembling a seahorse, and a ram's horn (*Cornu Ammonis*). *Cornu Ammonis* (CA) is the nomenclature ascribed to the hippocampal subfields one to four as CA (1-4) (Purves, 2011). The HP helps to keep short-term memory, spatial information, cognitive memory and in processing of emotions. These functions have been identified in animal experiments showing that hippocampal neurons fire depending on the

position of the animal within its environment, and in human experiments showing that hippocampal neurons fired to particular pictures and faces. Bilateral ablation or inactivation of the human hippocampus is associated with profound memory deficits (Purves, 2011).

The cerebellum (CB) on the other hand, is a part of the brain, situated directly posterior to the pons Varolii and just below the occipital lobe. It is located in the posterior cranial fossa. It is demarcated by the tentorium cerebelli, a derivative of dura mater, from other lobes. The fourth ventricle also separates it from the pons Varolii. The cerebellum forms a key part of the brain because of its function in coordinating motor movement and maintaining balance and equilibrium. It also controls muscle tone and voluntary muscle activity, coordinates gait and maintains posture (Jimsheleishvili and Dididze, 2019).

Reports have shown that the use of ACTs distress diverse parts of the encephalon (Ekanem *et al*., 2009). Examples of the brain parts affected are hypothalamus (Purves *et al*., 2004) and the cerebellum, as in the case of dystonia (Faloju and Lesi, 2010).

Effects of ACTs in different parts of the body have been subjected to investigations by some researchers around the world. For instance, artequin (artesunate + mefloquine) was reported by Ekanem *et al*. (2009) to increase the population of reactive astrocytes in the hippocampus proportionately with dosage. By implication, this may alter neuronal environment and impair uptake of neurotransmitters, and possibly affecting the hippocampal roles. On the contrary, few other researchers opined that artemisinin derivatives have little or no harmful effect in human nervous system; however, there is paucity of data on their histological activities using experimental animals (WHO, 2000). Therefore, it would be necessary to conduct scientific experiments with clinically applicable regimen of artemisinin and its derivatives using experimental animals. In addition, drug abuse associated with antimalarials especially, is a common act among victims of malaria, hence possible toxicity of the drugs on some body organs cannot be ruled out (Izunya *et al*., 2010).

1.2 Statement of the problem

Although the effectiveness of artesunate and amodiaquine in their separate and combined (ACT) forms against malaria parasites is not questionable, the drugs have history of toxicity and side effects such as dizziness, body aches, vertigo, ataxia, slurred speech, etc. (Schmuck *et al*., 2009, Nontprasert *et al*., 2002). High doses of AS reportedly caused loss of brainstem and eye reflexes and gait disturbances in

experimental animals (Genovese *et al*., 2000; Nontprasert *et al*., 1998, 2002). Studies also revealed that artesunate and amodiaquine may reduce cognitive activity in mouse (Onaolapo *et al*., 2013). Ubulom *et al*. (2015) also reported that adult Wistar rats administered with acute oral doses of Artesunate/Mefloquine combination (Artequin) and Mefloquine alone caused proliferation of astrocytes. Artesunate co-administered with mefloquine caused astrogliosis around the HP and consequently retarding the learning and memory functions of the HP (Ekanem *et al*. 2009). Furthermore, AS+AQ combined administra-tion also posed some level of toxicity on prostatic/testicular structures (Obianime and Aprioku, 2009). Artesunate and AS+AQ also increased atherogenic indices, such as low and high lipid level cholesterol and therefore increased the possibility of coronary heart disease (Adebayo *et al*., 2011).

However, in spite of all these findings, there are still much grey areas about the nature of morphological, biochemical and behavioural alterations, extent and mechanism of toxicity that artesunate and amodiaquine in their separate and combined forms, posed on the hippocampus and cerebellum of experimental animals especially over delayed post-acute period.

1.3 Justification of the study

A careful and thorough search through the literatures on the consequence of AS, AQ and AS+AQ on the brain revealed many symptoms-based assessments coupled with few neurobehavioural and haematological studies. Few histomorphological studies of artesunate and amodiaquine and some other ACTs on the hippocampus and cerebellum reported in literatures are also acute based. Reports of the delayed post-acute effect of artesunate and amodiaquine on the hippocampus and cerebellum are very scanty in the literature. Immunohistochemical evaluations of the neurological effects of these drugs are rather too scarce. Therefore, in Nigeria and Africa by extension, where malaria is prevalent and indiscriminate use of ACT are rampant (Ekong *et al*., 2009); it is pertinent to investigate the neurobehavioural, biochemical, histomorphological and immunohistochemical effects of AS and AQ seperately and combined, on the hippocampus and cerebellum of experimental animals.

1.4 Research questions

1. Can AS and AQ separately or combined affect the cellular morphology of the CB and HP of the treated animals?

2. Can AS and AQ separately or combined, affect the neuronal and glial cells reactivity and neuroinflammation in the cerebellum and hippocampus of the treated animals?

3. Can AS and AQ separately or combined, affect the body and brain weights of the animals?

4. Can AS and AQ separately or combined, affect the spatial memory, cognition, and motor coordination of the animals?

5. Can AS and AQ separately or combined, induce oxidative stress (OS) in the brain tissues of the animals?

6. Can AS and AQ separately or combined, affect the blood profiles of the treated animals?

1.5 Research hypotheses

Hypothesis 1:

H_0: oral administration of AS and AQ and their combination will not have significant acute and delayed post-acute effects on the cellular morphology of the hippocampus and cerebellum of the animals.

H_1: oral administration of AS and AQ and their combination will have significant acute and delayed post-acute effects on the cellular morphology of the hippocampus and cerebellum of the animals.

Hypothesis 2:

H_0: oral administration of AS and AQ and their combination will not have significant acute and delayed post-acute immunohistochemical effects on the neuronal inflammation and glial cells reactivity in the HP and CB of the Wistar rats.

H_1: oral administration of AS and AQ and their combination will have significant acute and delayed post-acute immunohistochemical effects on the neuronal inflammation and glial cells reactivity in the HP and CB of the Wistar rats.

Hypothesis 3:

H_0: oral administration of AS and AQ and their combination will not have significant acute and delayed post-acute effects on the body and brain weights of the animals.

H_1: oral administration of AS and AQ and their combination will have significant acute and delayed post-acute effects on the body and brain weights of the animals.

Hypothesis 4:

H_0: oral administration of AS and AQ and their combination will not have significant

acute and delayed post-acute effects on the spatial and cognitive memories and motor coordination of the animals.

H_1: oral administration of AS and AQ and their combination will have significant acute and delayed post-acute effects on the spatial and cognitive memories and motor coordination of the animals.

Hypothesis 5:

H_0: oral administration of AS and AQ and their combination will not have significant acute and delayed post-acute effects on lipid peroxidation (LPO) and OS indices in the rat brain.

H_1: oral administration of artesunate and amodiaquine and their combination will have significant acute and delayed post-acute effects on LPO and OS indices in the rat brain.

Hypothesis 6:

H_0: oral administration of AS and AQ and their combination will not have significant acute and delayed post-acute effects on the animals' blood profiles.

H_1: oral administration of AS and AQ and their combination will have significant acute and delayed post-acute effects on the animals' blood profiles.

1.6 Objectives of the study

1.6.1 Broad objective

This study broadly investigated the acute and delayed post-acute effects (deleterious or otherwise) of the separate and combined administrations of artesunate and amodiaquine on the hippocampus and cerebellum of experimental animals.

1.6.2 Specific objectives

This study was designed to:

1. Investigate the acute and delayed post-acute effects of the separate and combined administrations of AS and AQ on the histomorphology of the HP and CB of the experimental animals.
2. Evaluate the acute and delayed post-acute immunohistochemical activities of the separate and combined administrations of AS and AQ on the neuronal inflammation and glial cells reactivity in the HP and CB of the Wistar rats.
3. Investigate the acute and delayed post-acute effects of the separate and combined administrations of AS and AQ on the body and brain weights of adult Wistar rats.
4. Evaluate the acute and delayed post-acute neurobehavioural effects of the separate and

combined administrations of AS and AQ on the spatial learning memory, cognition and muscular strength of the rats.

5. Find out the acute and delayed post-acute biochemical effects of separate and combined administrations of AS and AQ on LPO and OS indices in the brain of the animals.
6. Determine the acute and delayed post-acute effects of the separate and combined administrations of AS and AQ on blood profiles and differential white blood cell profiles of the animals.

1.7 Significance of the study

In addition to the data available in the literature as provided by various scientists, on the possible side effects and damages that could result from the administration of artesunate and amodiaquine on experimental animals, this study has helped to provide relevant information on the delayed post-acute effects of artesunate and amodiaquine on Wistar rats. The data provided by this study comprises of body and brain weights, neurobehavioral parameters of spatial memory and motor coordination, haematological profiles, biochemical indices of oxidative stress, histomorphology and inmmunohistochemistry of the CB and HP of adult Wistar rats.

1.8 Scope of the study

This study was limited to the evaluation of the standard dosages of AS and AQ on the morphology and histochemistry of Adult Wistar rats' brain, including their weights, neurobehavioral and haematological profiles within 3-day acute and 14-day delayed post-acute period.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Brief history of artemisinin

Artemisinin is a product of extraction from the herbal tree, *Artemisia annua* (Oreagba, 2010). *Artemisia annua* is in turn originated from northern part of China (Van *et al.*, 1999). The tree is popularly called 'annual worm wood', 'sweet annie' and or 'sweet worm wood' in English and 'qinghao' or 'huaghuahao' in Chinese. It is a member of the family Asteraceae (Compositae) (Ferreira and Janick, 2002).

The antimalarial property of artemisinin was first described in the hand book of prescription for emergencies by Zhouhou Beinjefang, in which 43 malaria treatment methods were described (Van *et al.*, 1999). Breakthrough of artemisinin was prompted by the reaction to the North Vietnamese leaders' request hinged on the elimination of their soldiers by malaria pandemic at the Vietnam War (Liwang and Xin-Zhaun, 2009). A research committee was then inaugurated headed by a chairman together with the then Vietnam Premier, which summoned an urgent effort to find solution to the prevailing menace. A discussion on the action plan was then held on 23rd May, 1967, thus named "project 532" (Liwang and Xin-Zhaun, 2009). The goal of the research team so inaugurated then was to find prevention and treatment for *falciparum* malaria, the type that was prevalent in the tropical areas. The team then embarked on the screening of the traditional Chinese pharmacopaedia, bringing about discovery of more than ten plants with good antimalaria activities of which *Artemisia annua* was one of them. The leaf of *Artemisia annua* was reported to eradicate parasites from the victims' bodies more than the existing drugs in history (Liwang and Xin-Zhaun, 2009). In the study, artemisinin was discovered in 1972 from the leaf of *Artemisia annua* (annual worm wood) and the medicine was named Qinghaosu in Chinese (Miller and Panosian, 1997).

Artemisinin was highly unknown to the world for about seven years until the result of 5,000 traditional Chinese medicines tested by the Chinese scientists for curing malaria was circulated through the Chinese medical journal in 1979 (Van *et al.*, 1999). Other

scientists not within China afterwards got aware of the discovery. Following the worldwide resistance emergence of plasmodium species to current antimalaria medications (e.g chloroquine, quinine and mefloquine in 1960s), it became imperative to quest for more effective antimalarial drugs. With the acceptance of artemisinin in 2006 as the choice of drug against malaria treatment, the WHO then called for instantaneous cessation to artemisinin monotherapy in favour of combining artemisinin with another antimalaria medication in what is called artemisinin-based combination therapy, so as to avert the risk of parasite developing resistance (WHO, 2015).

2.1.1 Derivatives of artemisinin

Artemisinin has been described as a sesquiterpene lactone possessing endoperoxide bridge, which is crucial in combating malaria-causing plasmodia. It does not dissolve readily in water or oil, but its carbonyl group can be broken down to dihydroartemisinin (DHA) and other derivatives which are soluble in water (AS) and oil (artemether and arteether), also with high antimalarial activities (Fig. 2.1). Several ways of complete chemosynthesis of artemisinin and numerous efforts to manufacture the medication using genetically modified microorganisms are documented (Li and Wu, 2002), but the large-scale origin of artemisinin remains *Artemisia annua* plant (Zeng *et al*., 2008).

2.2 Artesunate

Artesunate (AS) is a hemisuccinate derivative of hydroartemisinin. It is hydrophilic and unstable in neutral solution. Its oral formulation undergoes total hydrolysis prior entering the body transport system. After parenteral administration of this drug, it is swiftly hydrolysed to the active form dihydroartemisinin, and after 16-25 hours of this route of administration, artesunate was confirmed to have cleared fever in victims with severe falciparum malaria (CDC- USA, 2016).

In patients with severe malaria, artesunate has been advocated as the *sequanon* choice of management. It is now a preferred choice to quinine in severe malaria management (CDC- USA, 2016). Artesunate was found better in terms of life saving compared to other antimalarials. Artesunate had also been reported to manage less severe categories of malaria by oral administration. It was reported to be active against almost all *Plasmodium spp* (Sinclair *et al*., 2012).

2.2.1 Chemical structure of artesunate

Artesunate has a tetracyclic structure having triaoxane and lactone rings. The triaoxane ring contains endoperoxide bridges which are suspected to play the major role in its antimalaria activity. This compound has a molecular weight of 282g/mol (see Figures 2.1 and 2.2) (Oreagba, 2010).

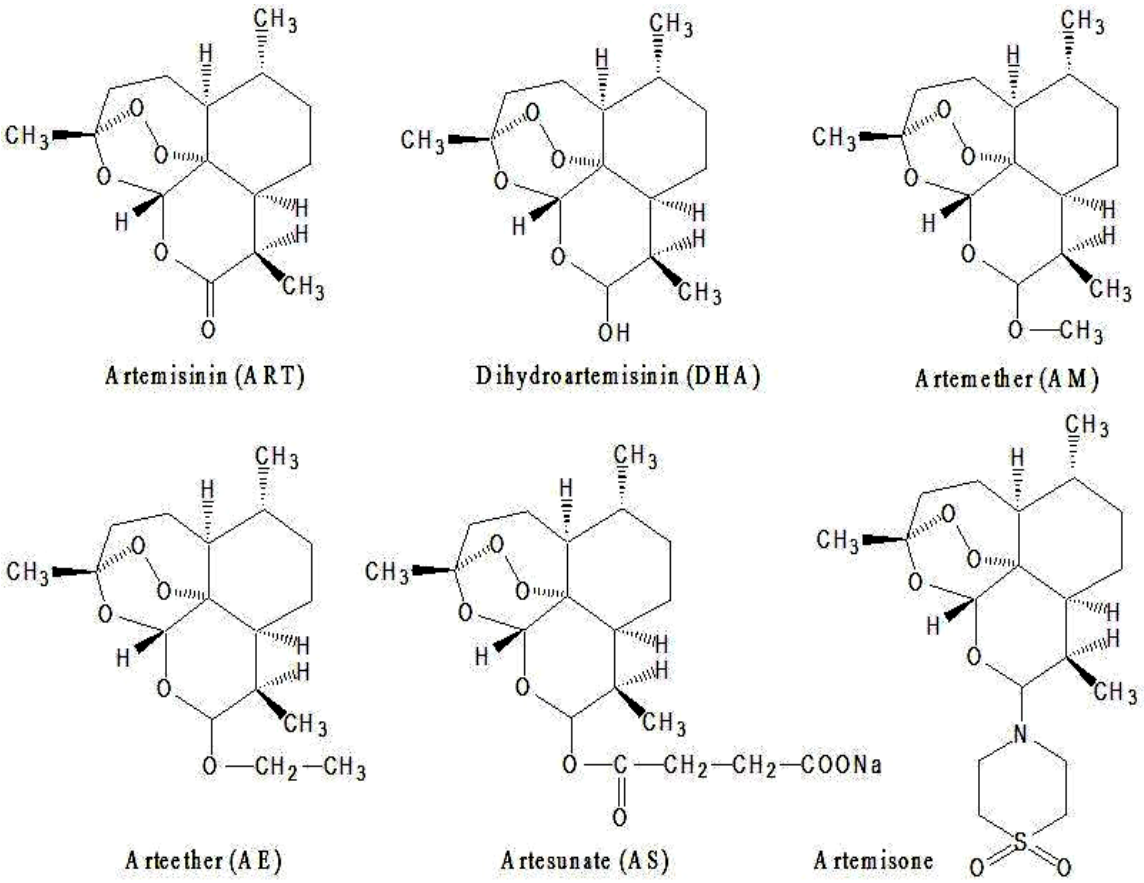


Figure 2.1. Artemisinin and its derivatives (Liwang and Xin-zhuan, 2009)

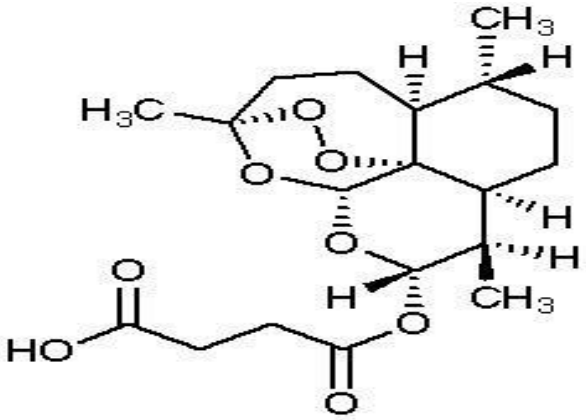


Figure 2.2. Chemical Structure of Artesunate
Formula: $C_{15}H_{22}O_5$ (Liwang and Xin-zhuan, 2009)

2.2.2 Metabolism of artesunate

Metabolism of the AS is reported to be achieved predominantly by the liver cytochrome P450 enzyme CYP2B6 following oral administration. Following oral ingestion, artesunate is quickly metabolised within minutes into DHA. The mean absorption time of DHA when taken orally slightly extends beyond one hour (1hr). The plasma half-life of artesunate is between 3 and 29 minutes while DHA has a plasma half-life ranging from 40 to 95 minutes. Artemisinin also auto-induce P-450 metabolising catalyst, leading to its reduced density in the serum in succeeding administrations (Asimus *et al*., 2007). Zhao and Song (1989) reported that shortly after artesunate administration intravenously, its concentration in the intestine markedly increased compared to the other visceral organs; brain level was second, followed by the levels in the kidneys and liver. Artemisinin derivatives are the quickest medicines to attack all malaria parasites in their different erythrocytic stages. However, one of the limitations is that their elimination half-life is very snappy (approximately 1 hour), which limits their use as a monotherapy for malaria treatment. In humans, these products are swiftly converted into their bioactive metabolite, DHA, which is subsequently get rid of by glucuronidation. The swift excretion of artemisinins in humans is beneficial in avoiding the development of plasmodium that would be resistant to the drugs (Nosten and White, 2007).

2.2.3 Mechanism of action of artesunate

All artemisinin derived drugs currently in vogue are products of biologically active dihydroartemisinin, which becomes highly potent once the parasite is present in the erythrocyte (Oreagba, 2010). Several studies have shown that artemisinin exhibits anti-malaria potentials by initiating redox homeostasis in plasmodium parasites; however, there is no uniform agreement by researchers on the mechanism artemisinin derivatives obliterates the parasites (Kappe *et al*., 2010). According to Ginsburg and Atamna (1994), when the malaria-causing *Plasmodium* specie taints an erythrocyte, it ingests hemoglobin into its digestive vacuole, thereby generating OS. Thereafter, heme's iron immediately breaks the peroxide bridge in artemisinin that is consumed, forming iron-oxo species which brings about series of reaction that produces toxic reactive oxygen species (free radicals) that bind with parasite protein through alkylation. This harm and eventually kills the plasmodium parasite (Meshnick, 2002; Oreagba, 2010). The discharge of the generated reactive oxygen species from endoperoxide bond obliterates

the parasites that are found in the red blood cells (RBC) (Meshnick, 2002). Cleavage of Artemisinin endoperoxide bridge by heme's iron was initially initiated by isolating heme adducts from artemisinin-treated *Plasmodium falciparum* (Meshnick, 2002). The authors therefore opined that iron may play some essential parts in the action of artemisinin, which may occur by activating artemisinin to free radicals. On the other hand, artemisinin interrupts cellular redox reactions in malaria parasite. It also suppressed digestive vacuole cysteine proteases action of *plasmodium* treated with it. This assertion was proven by in-vitro experiment showing build-up of heamoglobin in the parasite treated with artemisinin and retardation of hemozin. Artemisinin damaged the digestive vacuole membrane of plasmodium parasite. This opinion is buttressed with the assertion that the digestive vacuole of plasmodium parasite has been formed by the mid-ring stage (which is artemisinin-sensitive) of the parasite blood cycle (Haynes *et al.*, 2011).

2.3 Brief history of amodiaquine

Amodiaquine is schizonticidal, that is, toxic to the *Plasmodium species* in the blood. It attacks all species of *Plasmodium falciparum* in their erythrocytic stages. It is a 4-aminoquinoline antimalarial drugs used for malaria therapy (Olliaro and Mussano, 2006). Amodiaquine is effective for Plasmodium species that are resistant to chloroquine (Olliaro and Mussano, 2006). Following oral ingestion, AQ is mostly transformed to desethylamodiaquine, the active determinant of its antimalarial action (Adjuik *et al*., 2002). Amodiaquine has proven more tolerable and acceptable for malaria treatment than chloroquine, although this is not generally applicable. Some of the brand names of amodiaquine that exists are Camoquin and Flavoquine. It is available in tablets of 150 to 600 mg and the advocated dosage is 10 mg/kg body weight once daily for 3 days usually in combined form with other antimalarial drugs (Durrani *et al*., 2005). Amodiaquine is now currently compounded with artesunate in two separate tablets containing 50 mg of AS and 153 mg base of AQ. However, tablets of AS and AQ have now been co-formulated by the Drugs for Neglected Diseases initiative (DNDi). Artesunate-amodiaquine combination has proven to be a more potent combination compared to amodiaquine single form (Durrani *et al*., 2005). Some complications of amodiaquine include itching, skin rash, nausea and diarrhea (National Library of Medicine, 2015). The use of amodiaquine has also been implicated in agranulocytosis and severe liver toxicity. The main mechanism underlying the adverse

reactions of amodiaquine is associated to the formation of quinoneimine, a high valent compound. Numerous circumstances of acute dystonic reactions and bradycardia have also been reported with the use of AQ, but mostly in adults (Akpalu and Dodoo, 2005).

2.3.1 Chemical structure of amodiaquine

Amodiaquine has a chemical formula: $C_{20}H_{22}ClN_3O$ and a molecular weight of 355.866g/mol. (see Figure 2.3) (Liwang and Xin-zhuan, 2009).

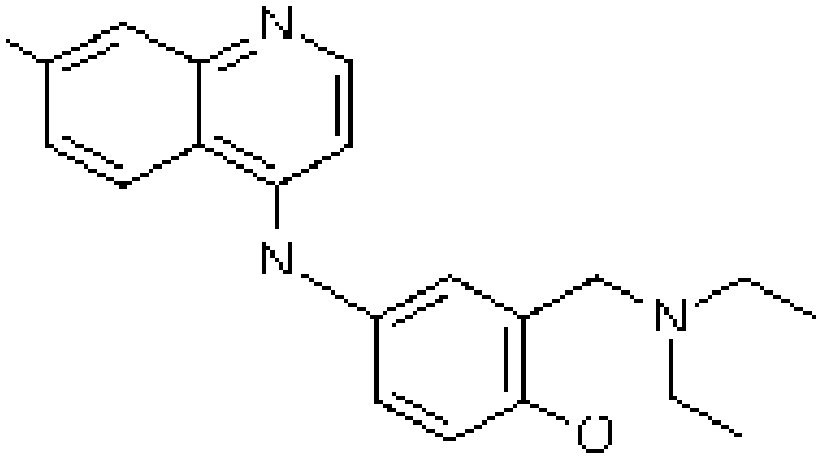


Figure 2.3. Chemical Structure of Amodiaquine

(Liwang and Xin-zhuan, 2009)

2.3.2 Metabolism of amodiaquine

Amodiaquine is assimilated through the digestive system and accumulates in organs like the liver and kidney. The main enzyme that metabolises amodiaquine in the liver is cytochrome (CYP) P2C8 which is a diverse isoform of hepatic cytochrome p450 2C8, having its gene on chromosome 10q24. This enzyme converts the drug to the primary metabolite monodesethylamodiaquine (DEAQ), then secondary metabolites; 2-hydroxyamodiaquine and bisdesethylamodiaquine (Li and Wu, 2002). Further metabolism includes oxidation and glucuronoconjugation. In healthy volunteers, the mean time to maximum concentration (T_{max}) of AQ ranges from 0.6 to 1.3 hour and the T_{max} of DEAQ ranges from 3 to 5.5 hours. Also, in healthy volunteers, the eradication of AQ follows first- order kinetics, and the mean elimination half-life of AQ ranges from 5.3 to 7.9h, while that of DEAQ ranges from 9 to18 days (Pussard *et al*., 2009).

2.3.3 Mechanism of action of amodiaquine

Amodiaquine is a derivative of aminoquinoline and usually behaves by attaching to the plasmodium deoxyribonucleic acid (DNA) thereby blocking its production and that of the ribonucleic acid (RNA). Consequently, it also blocks protein manufacture and acts against the nonsexual erythrocytic forms of Plasmodium species (National Library of Medicine, 2015). Amodiaquine stores in the lysosomes of the plasmodium parasites thereby rendering the parasites incapable to digest haemoglobin, hence preventing the parasites from deriving energy from haemoglobin digestion. AQ also binds to the parasites' nucleoproteins causing inhibition of its DNA and RNA polymerases (O'Neill *et al.*, 1998). Maggs *et al.* (1988) opined that AQ triggers production of free radicals in the form of amodiaquine-quinine-immine and semi quinine-immine which are responsible for triggering lipid peroxidation. Furthermore, lipid peroxidation had been reported to cause injury to tissue *in vivo* (Mayes, 2000). Lipid helps to replace worn-out membranes and helps in quick transmission of depolarization waves along myelinated nerve by acting as electrical insulators (Mayes, 2000). Proteins on the other hand help to preserve and restore worn-out tissues and help to form neurotransmitters and enzymes. Neurodegeneration may set in when these roles are interfered and the brain may become deficient in its tasks, and death may occur afterwards (Singh *et al*., 2013).

It was also reported that amodiaquine retards the damage of ferriprotoporphyrin IX in plasmodium, a process that is glutathione-dependent. This results to the accumulation

of this toxic peptide in the parasite. All species of plasmodium are attacked by AQ in the red blood cell (Olliaro and Musano, 2006). Report also showed there are some three distinct mechanisms by which amodiaquine produced chemically reactive species: autoxidation, peroxidase-catalyzed oxidation and N-chlorination (Maggs *et al*., 1988).

2.4 General structure of the brain (Encephalon)

The encephalon is the semisolid part of central nervous system which lies within the cranium. It weighs about 1400 g in an adult human (Afifi and Bergman, 2005). It is guarded from the external assaults by three barriers: the cranium, meninges (dura, arachnoid and pia maters) and the cerebrospinal fluid. The meninges are also involved in the Blood Brain Barrier (BBB). Ascending in sequence from the spinal cord is the principal division base on development and includes the hindbrain (rhombencephalon), the midbrain (mesencephalon), and the forebrain (prosencephalon) (see Figure 2.4) (Afifi and Bergman, 2005).

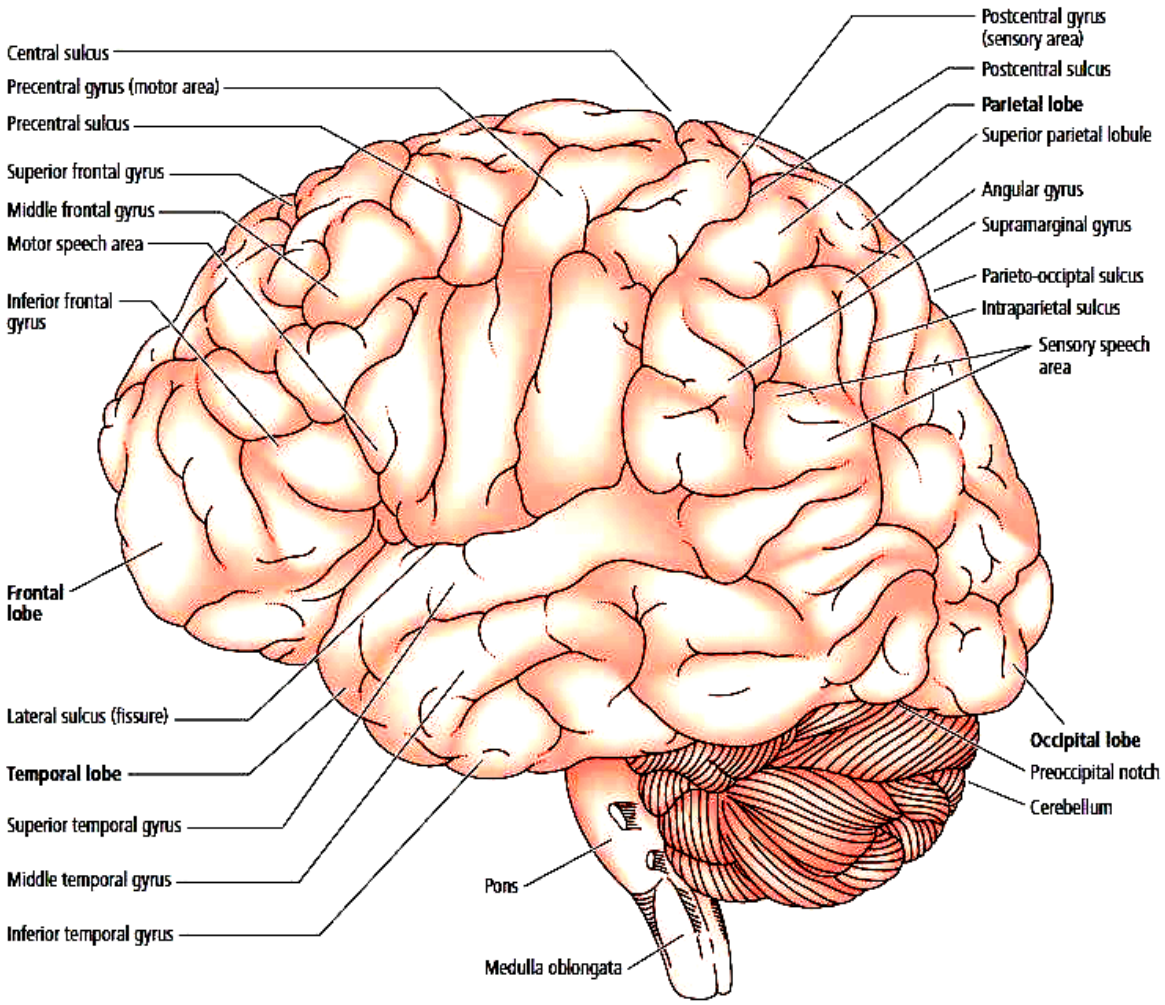


Figure 2.4. The lateral view of the brain (Afifi and Bergman, 2005)

2.4.1 Embryology of the hippocampus

Hippocampal regions start forming during the embryonic days (E15-21 in rat) and persist after birth. Neurons of the hippocampus move from the three components of its neuroepithelium (ammonic neuroepithelium, dentate neuroepithelium and glioepithelium). The products of ammonic neuroepithelium include pyramidal cells and large neurons of stratum oriens and radiatum; dentate neuroepithelium is the origin for granule cells and large neurons of stratum moleculare and hilus; while the glioepithelium generate glial cells in the fimbria. Pyramidal cells afterwards become the pyramidal cell layer; CA1 forms first and then CA3 appears. The granule cells of the dentate gyrus (DG) start to be generated at postnatal stage (Danglot *et al*., 2006).

2.4.2 Gross Anatomy of the hippocampus

The HP is a gray matter that projects off the base of the lateral ventricle in the temporal horn. It folds inwards as an archicortex. Its cortex becomes three or four layers from the initial six layers (Purves, 2011). Hippocampal formation refers to the HP proper, dentate gyrus (DG) and the subiculum (Sb) and sometimes the presubiculum, parasubiculum, and entorhinal cortex are considered as part of the hippocampal formation (Purves, 2011).

The HP proper is curved, C-shaped resembling a seahorse, and a ram's horn or *Cornu Ammonis* (literally "Ammon's horn", abbreviated CA). *Cornu Ammonis* (CA) is the nomenclature ascribed to the hippocampal subfields one to four as CA (1-4) (Purves, 2011). At the CA, the hippocampal cortex becomes compacted into a single layer with densely packed pyramidal neurons, resembling a tight U shape (see Figures 2.5 and 2.6). The lower end of this curve embeds into the dentate gyrus that is curved posteriorly. HP has similar composition in all mammals but has different neural circuits. The cross-section of the dentate gyrus has three or four layers of cells depending if the hilus is included (Purves, 2011).

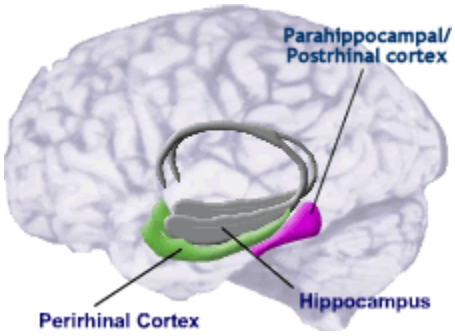


Figure 2.5. The brain showing the hippocampus (Gupta, 2012)

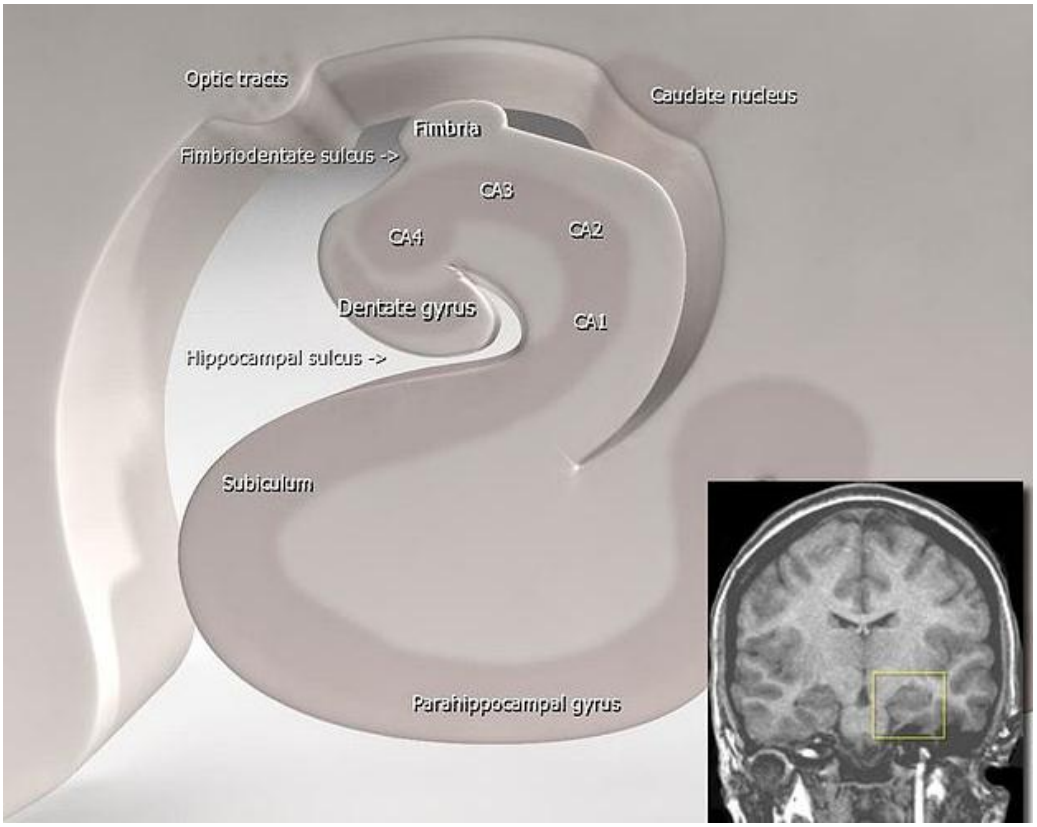


Figure 2.6. Gross Anatomy of the hippocampus (Anderson *et al*., 2007).

2.4.3 Histology of the hippocampus (Hippocampal cells and layers)

The HP has similar cellular composition in all mammals but with different neural circuits. Hippocampal formation refers to the HP proper, dentate gyrus (DG) and the subiculum (Sb).

2.4.3.1 Hippocampus proper

The most common components used to describe the HP proper are the DG and the CA. The DG contains the fascia dentata and the hilus while the term CA, *Cornu Ammonis* was derived from an Egyptian deity Amun, whose head was like that of a ram. The CA is differentiated into subfields CA1, CA2, CA3, and CA4 (Figure 2.6). However, the CA4 is actually the "deep, polymorphic layer of the DG" (Anderson *et al*., 2007).

The CA regions are also divided into strata (or layers) as follows:

- **Stratum oriens** (str. oriens): this layer is superficial to the alveus. It contains: the cell bodies of basket cells, the trilaminar cells and the basal dendrites of pyramidal neurons.

- **Stratum pyramidale** (str. pyr.): this layer contains: the cell bodies of the pyramidal neurons, synapses from the Mossy fibers that pass through stratum lucidum (in CA3 subfield), the cell bodies of many interneurons, including axo-axonic cells, bistratified cells, and radial trilaminar cells.

- **Stratum lucidum** (str. luc.): this layer is one of the thinnest strata in the HP and only found in the CA3 region. It contains Mossy fibers from the DG granule cells.

- **Stratum radiatum** (str. rad.): this layer contains septal and commissural fibers, Schaffer collateral fibers, basket cells, bistratified cells, and radial trilaminar cells.

- **Stratum lacunosum** (str. lac.): this layer is thin, containing Schaffer collateral fibers, perforant path fibers from the superficial layers of entorhinal cortex. It is often grouped together with stratum moleculare into a single stratum called stratum lacunosum-moleculare (str. l-m.) due to its small size.

- **Stratum moleculare** (str. mol.): this layer is the most superficial. It contains the synapses between the perforant path fibers and the distal, apical dendrites of pyramidal cells (Anderson et al., 2007).

2.4.3.2 Dentate gyrus

The layers of the DG are as follows (Figure 2.8):

- **The polymorphic layer** (poly. lay.) is the most superficial layer. It contains many interneurons, and the axons of the dentate granule cells pass through this stratum on the way to CA3.
- **Stratum granulosum** (str. gr.) contains the cell bodies of the dentate granule cells.
- **Stratum moleculare, inner third** (str. mol. 1/3) is where both commissural fibers from the contralateral DG run and form synapses.
- **Stratum moleculare, external two thirds** (str. mol. 2/3) is the deepest layer of DG, sitting just superficial to the hippocampal sulcus (Anderson *et al*., 2007).

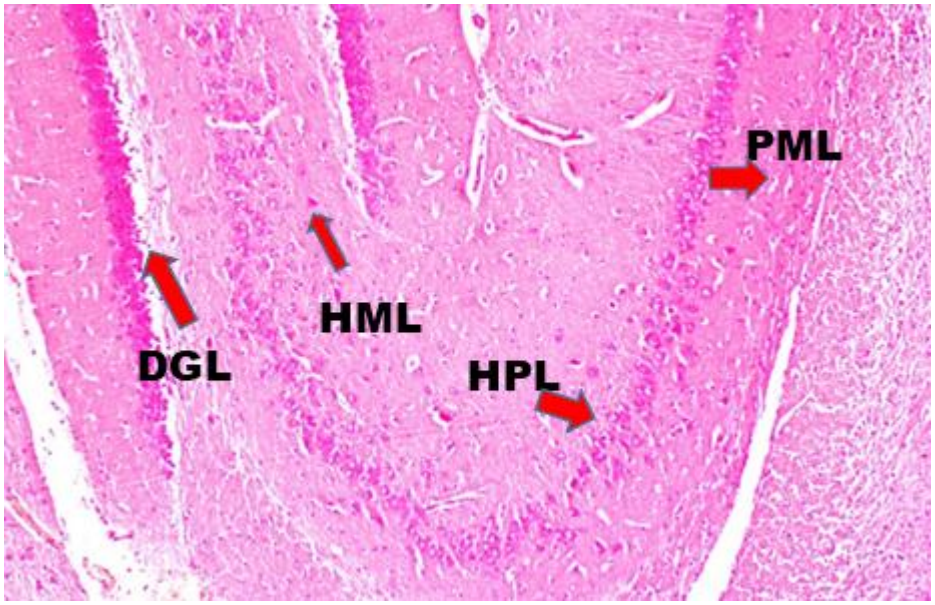


Figure 2.7. Hippocampus: Haematoxylin & Eosin stained (X100). DGL = dentate granular layer; HPL = hippocampus pyramidal layer; HML = hippocampus molecular layer; PML = polymorphic layer. (Adapted from Bench work: Popoola, 2018)

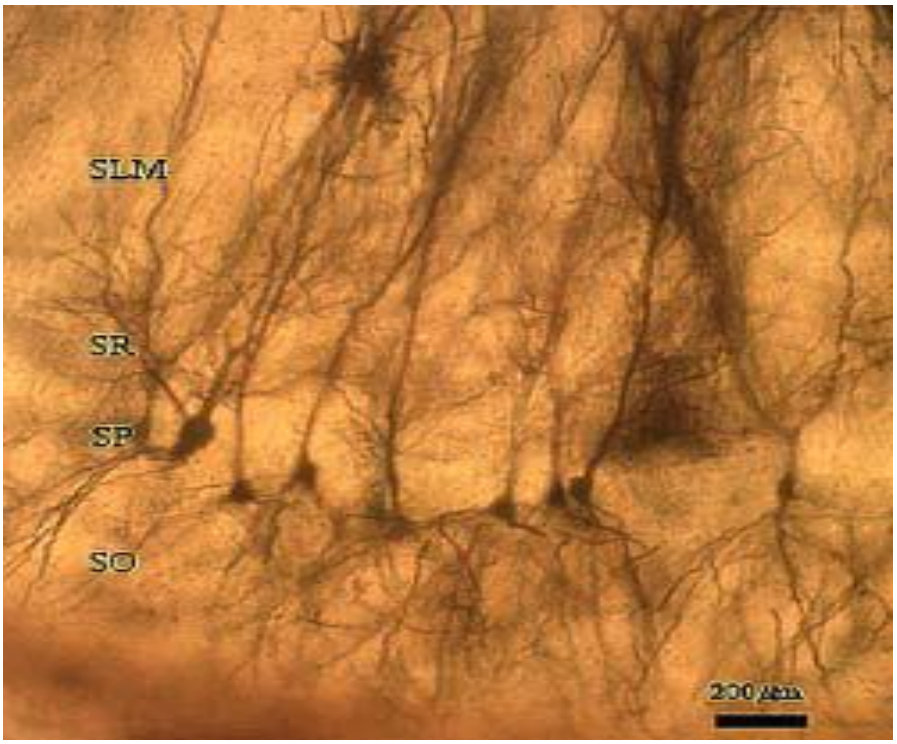


Figure 2.8. Frontal section of the rat brain illustrating parts of the hippocampus proper. SO - stratium oriens, SP - stratum pyramidale, SR - stratum radiatum, SLM - stratum lacunosum-moleculare. (Falougy, 2008).

2.4.4 Memory pathway (circuitry) in the hippocampus

The HP receives and sends projections to other brain parts through the Entorhinal cortex (EC), situated underneath the frontal part of the HP. The hippocampal formation is made up of several sub regions, such as the CA1–4, the DG, and the subiculum (Sb). These sub-regions are connected by two major neural circuits: the monosynaptic and trisynaptic circuits. The trisynaptic circuit transmits impulse from the EC to the DG through the perforanth path (PP), which penetrates through the Sb. Message, then flows from the DG to CA3 through the mossy fibres (MF) pathway. Lastly, impulse then flows from CA3 to CA1 along the schaffer collaterals (SC). The projections to the Sb and the EC make the circuit complete. The monosynaptic fibres bypass the DG and CA3 and instead send inputs directly from the EC to CA1 (see figure 2.6) (Gupta, 2012).

The entorhinal cortex (EC) constitutes the principal hippocampal input while its main output is through CA1 to the Sb (Kandel and Schwartz, 2013). Input reaches CA1 via variable pathways. Axons from EC emanating from layer III form the source of the direct perforant path (PP). These synapse with the extreme dendrites of CA1 neurons.

On the contrary, axons erupting from layer II form the indirect pathway and transmit impulse to CA1 through trisynaptic circuit. At the onset, the axons pass via the PP to the dentate gyrus' granule cells (1st synapse). Thereafter, the axons travel through the mossy fibres (MF) to CA3 (2nd synapse). After which, the CA3 axons known as SC link up with the apical dendrites and then extend to CA1 (third synapse) (Kandel and Schwartz, 2013). Axons from CA1 then project back to the EC, completing the circuit (Figures 2.9 and 2.10) (Gupta, 2012).

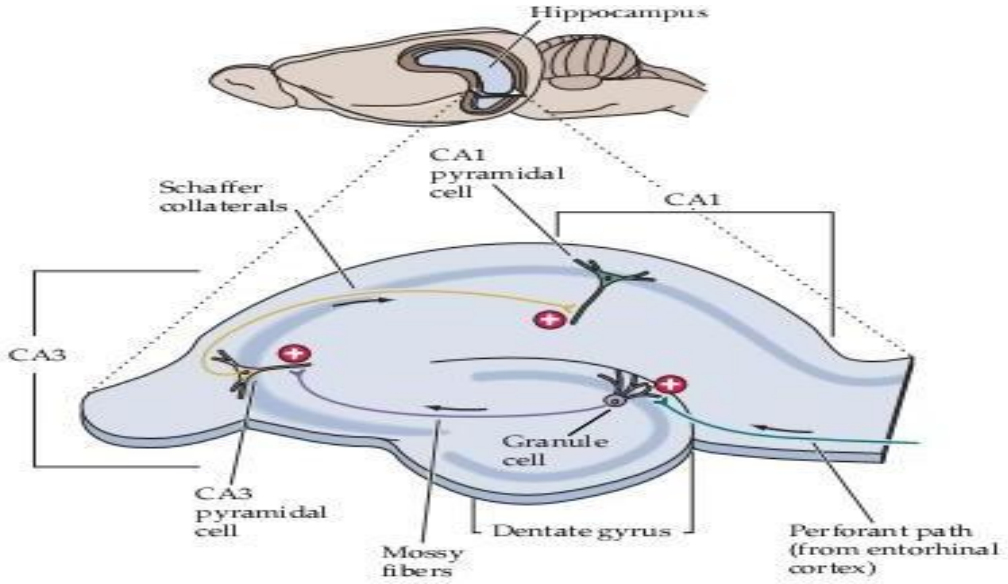


Figure 2.9. Cells of the hippocampus (Gupta, 2012)

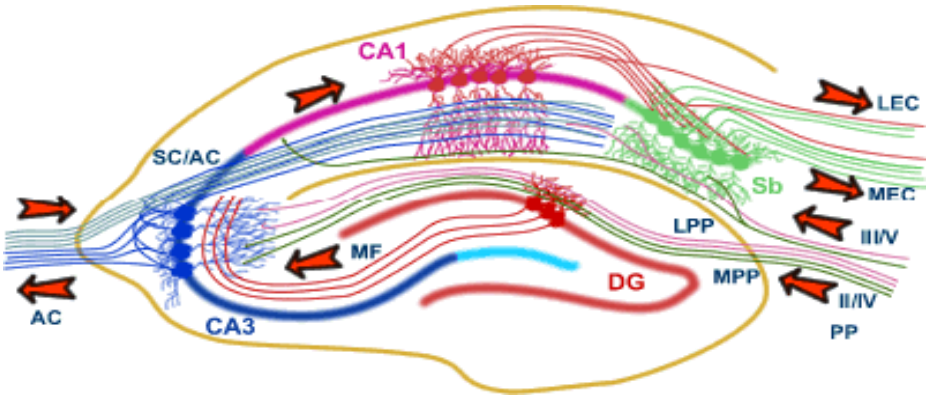


Figure 2.10a. Circuitry of the hippocampus (Gupta, 2012)

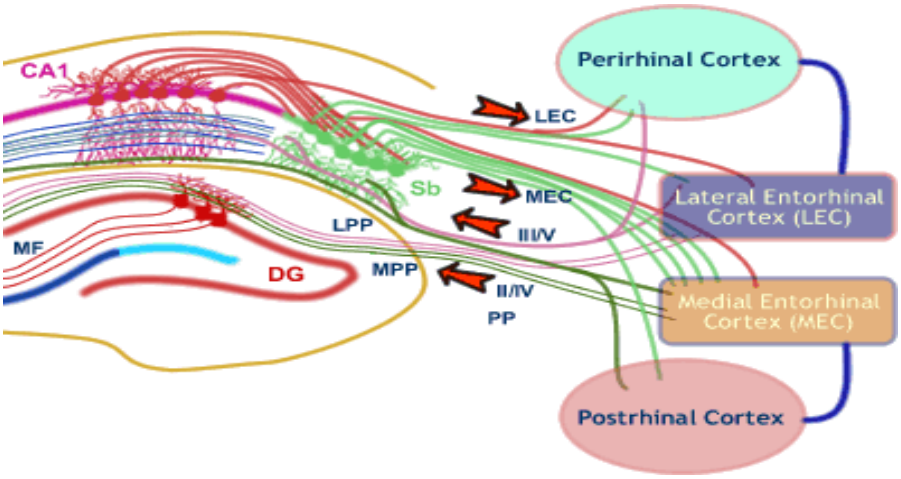


Figure 2.10b. Circuitry of the hippocampus (Gupta, 2012)

2.4.5 Blood supply to the hippocampus

The HP is vascularized by the collateral branches of the posterior cerebral artery (PCA) and the anterior choroidal artery (ACA). These arteries further give rise to network of superficial hippocampal arteries (SHA) that in turn lead to deep intrahippocampal arteries (DIA) located intraparenchymally. The peripheral hippocampal arteries may also emanate from the trunk of the choroidal posterolateral, splenial, inferior and temporal arteries (Duvernoy *et al*., 2005). Out of the SHAs, the medial hippocampal arteries are the most developed and often present. The posterior hippocampal arteries often accompany the medial hippocampal arteries via the subiculum. The posterior and the medial hippocampal arteries follow a common straight path, with many anastomoses, parallel to the hippocampal sulcus towards the Margodenticulatus. The perforating arteries arise from this anastomotic network and enter the HP between the indentations of the Margodenticulatus and supply the hippocampal body and tail (Figure 2.11) (Duvernoy *et al*., 2005).

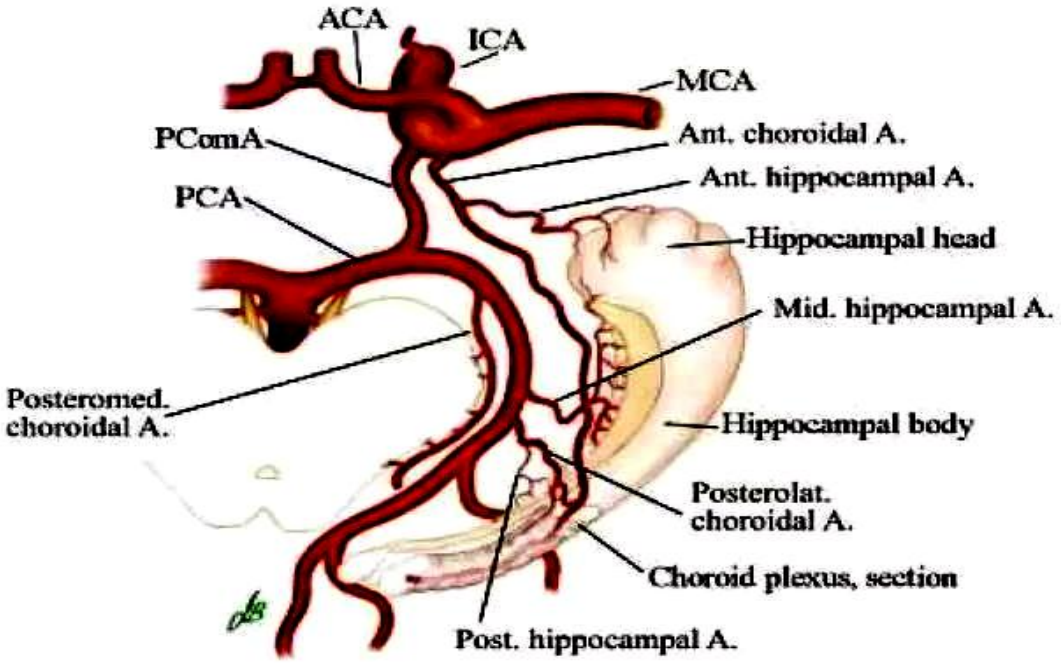


Figure 2.11. Arterial supply of the Hippocampus
(Duvernoy *et al*., 2005)

2.4.6 Hippocampus of rat

The rat's HP developed deep within the temporal lobe. Like that of human, the rat's HP is also elongated antero-posteriorly, from the dorsal to the ventral end. It is curved and possesses subfields CA4 to CA1 defined along the curve. The rat's hippocampal formation is majorly sited in the posterior and lateral walls of the cerebral cortex. The HP proper is C-shaped and is located beneath the tiny entorhinal cortex. The sulcus hippocampi form the ventral and caudal borders of the dentate gyrus (Turner *et al.*, 1998). The HP proper, dentate gyrus and Sb are epitome of simple cortex, constituted by a primary neuron and its surrounding interneurons. These major neurons are arranged in one layer of a three-layered structure, contrary to six-layered neocortex. Pyramidal cells form the main neurons in the HP proper and the Sb. They have varying size and density throughout the HP proper. This variation helps to distinguish the fields of the HP proper (Turner *et al*., 1998).

In contrast to many other encephalic parts where neurogenesis is accomplished by birth, the DG forms an outstanding exception in the mammalian brain where its granular cells that form its main neurons multiply over a long time, commencing during gestation and continues after birth. Modern findings now suggest that neurogenesis continues in the DG throughout the human life time. The segmentation of the hippocampal pyramidal cells is concluded before parturition and their structure appear elementary at parturition. The pyramidal neuron matures after birth as well as proliferation of their axonal connections and dendrites (see Figures 2.12 and 2.13) (Gahr, 2002).

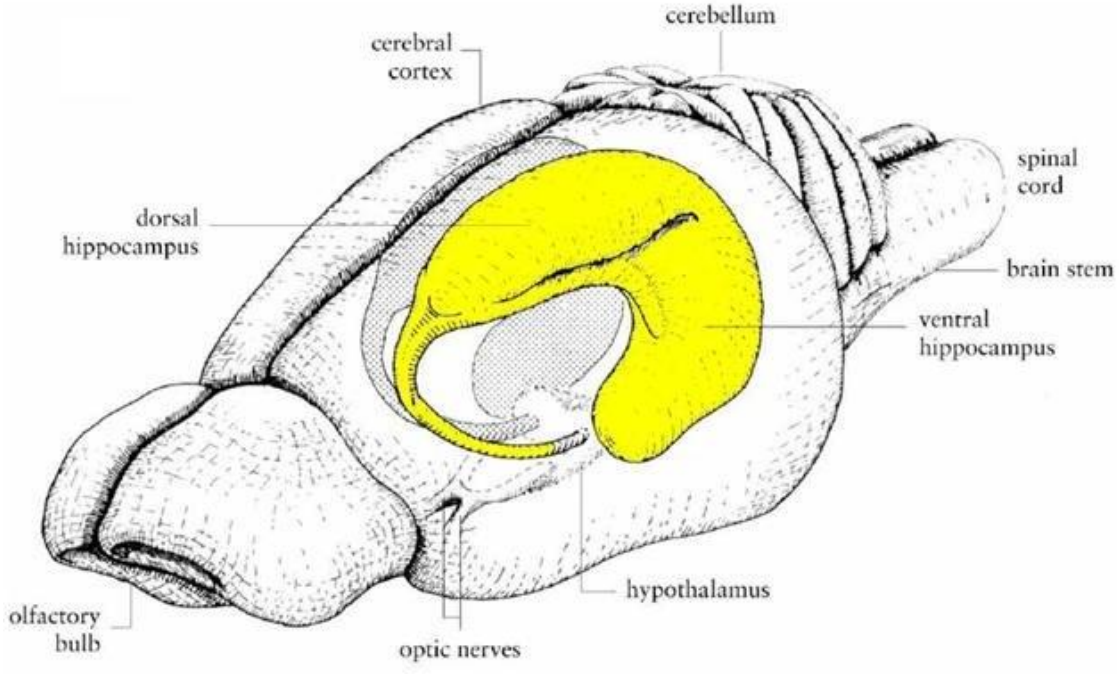


Figure 2.12. The rat brain showing the hippocampus and the cerebellum
(Kevin, 2007)

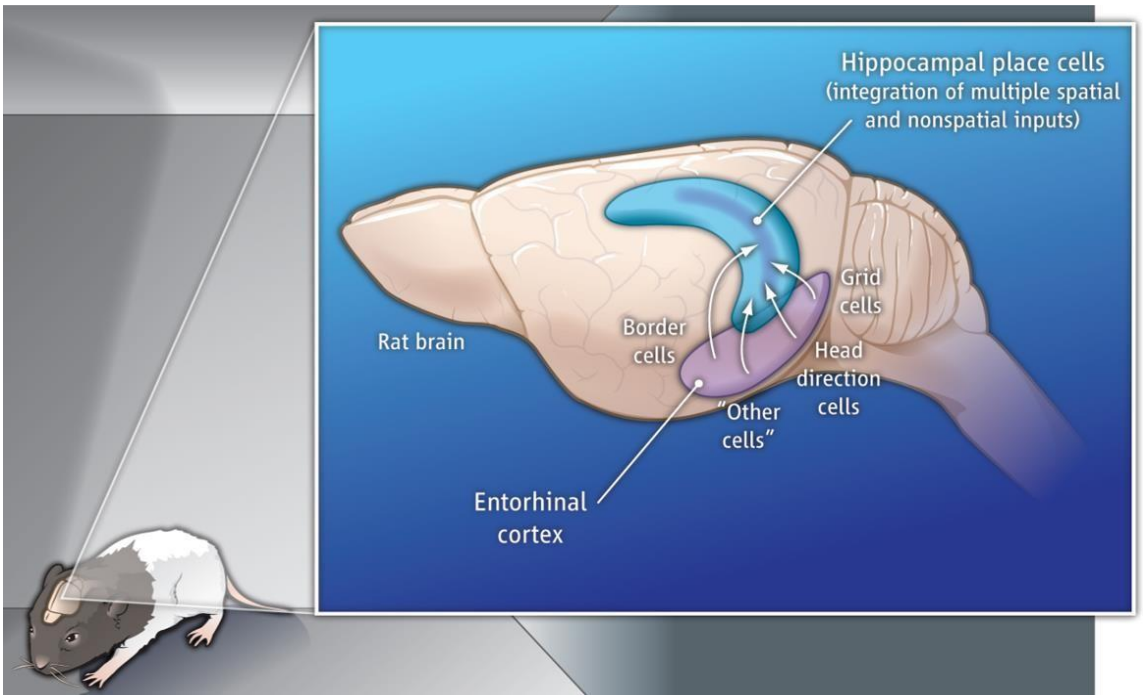


Figure 2.13. The rat hippocampus showing the hippocampal cells
(Zhang, *et al*., 2013)

2.4.7 Embryology of the CB

The primordium of the CB initially appeared as a pair of projections lateral to the alar plate facing the fourth ventricle at about 42 days (CRL 12–16 mm) of embryonic development. Between 49 to 63 days (CRL 28 mm), a pair of dorsal projections, the rhombic lip also appears on the alar plate. It bent sideways and fused with the midbrain medially. At this time, the primitive choroid plexus seemed to be part of the cerebellar hemisphere forming an eosinophilic matrix centrally. In this period, the inferior olive has evolved in the thick medulla. The cerebellar vermis grew much after the hemisphere perhaps from a central cell cluster around the aqueduct (Hill, 2016).

As at 77–84 days (CRL 70–90 mm), the cerebellar hemisphere was thick like the mid-brain. It also gives rise to an apparent laminar configuration. Evidence of vermis fissures is seen at the central part. By the 105–112 days (CRL 110–130 mm), it has already comprised the primitive dentate nucleus. The nodule and flocculus were recorgnised; vermis appear thick like the hemisphere where it followed numerous deep fissures (Hill, 2016).

2.4.8 Gross anatomy of the CB

The CB is found in the posterior cranial pit behind the brainstem. It is demarcated from the cerebrum by an annexe of the dura matter called tentorium cerebelli. It is linked to different brain parts such as the brainstem. The CB possesses about two-third of the total brain neurons. Its circuitry helps in motor control and learning. It is not involved in movement initiation, consequently, its injury is not linked with paralysis, but it is involved in synchronized, accurate, and fluent performance of voluntary movements and their adaptive adjustment. It is also involved in non-motor cognitive and affective functions (Herculano-Houzel, 2010).

It contains two hemispheres laterally separated by a cerebellar vermis. Its surface contains transverse folds called folia. The outer layer of the CB is highly folded forming a gray matter called the cerebellar cortex which encloses a body of white matter called the *arbor vitae* (tree of life) with numerous branching process. The *arbor vitae* also environ three pairs of cerebellar nuclei which are entrenched in another body of white matter called corpus medullare located at the central part of the CB (Roostaei *et al*., 2014) (see Figures 2.14 and 2.15).

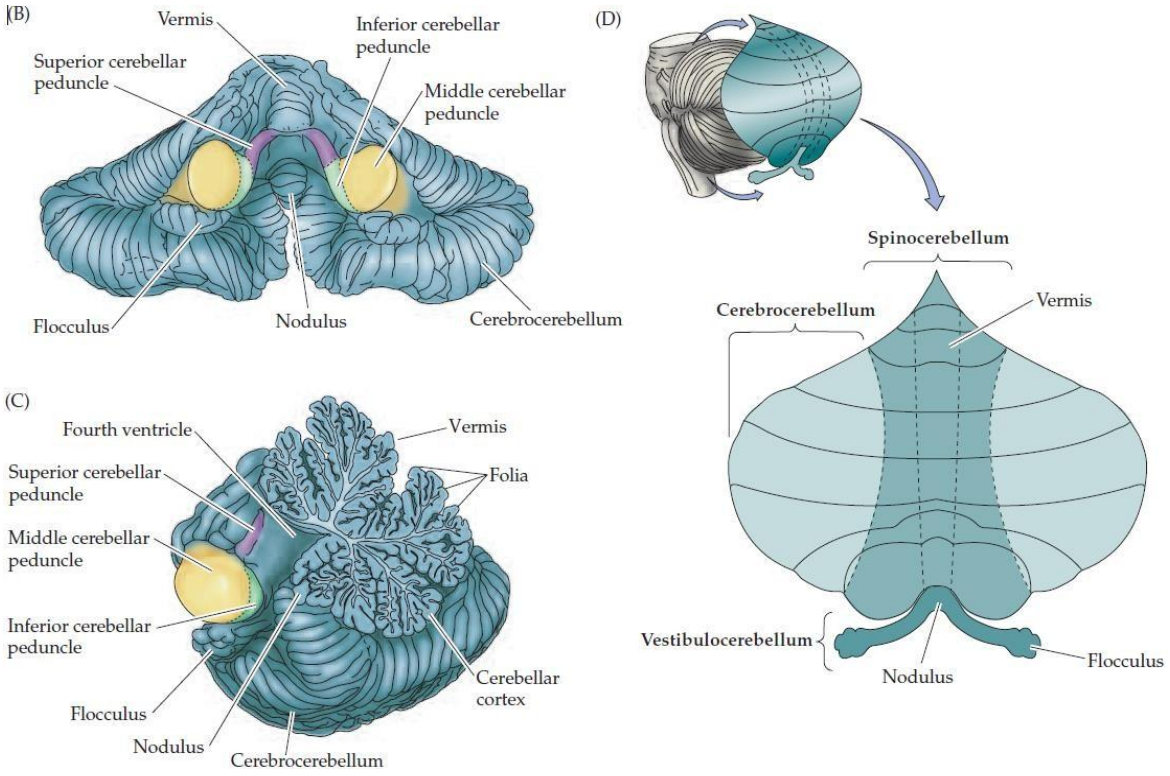


Figure 2.14. Gross structure of the Cerebellum (Purves *et al*., 2004)

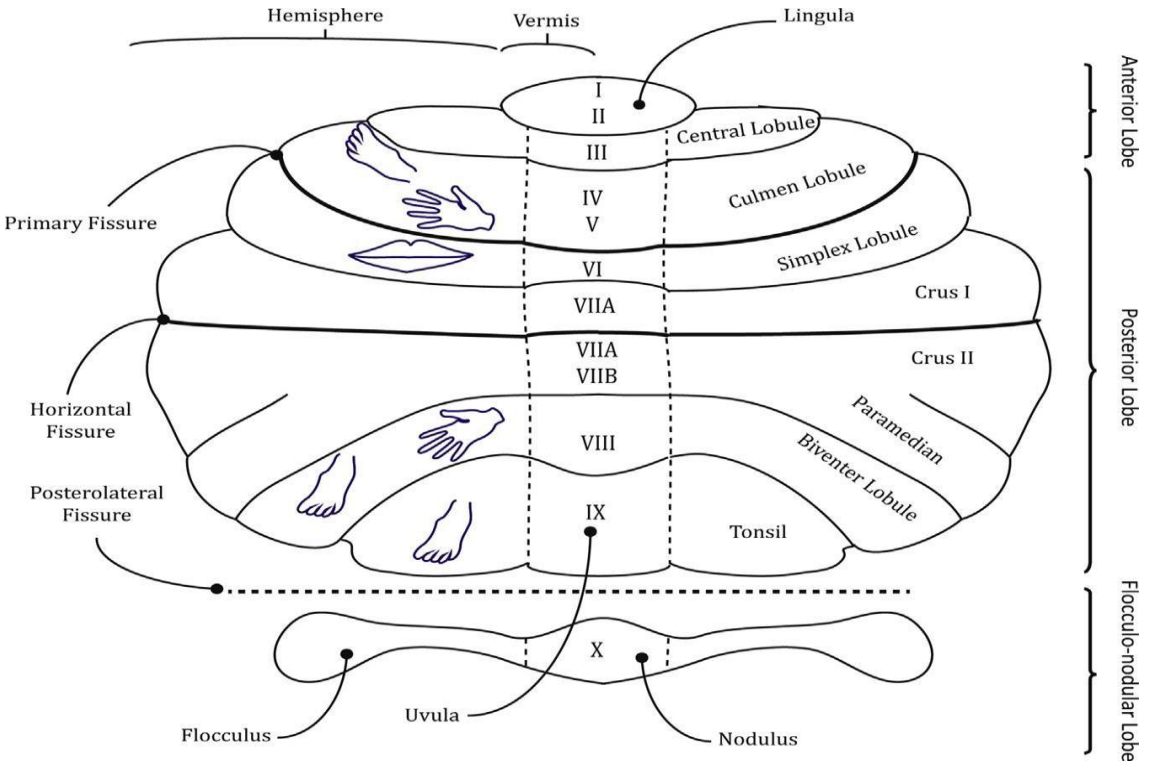


Figure 2.15. The lobes, fissures and cortex of the left cerebellum (Roostaei *et al*., 2014)

The CB is also made up of 3 lobes namely, anterior, posterior and flocculonodular lobes. Demarcating the nodes are two transverse fissures, the primary and the posterolateral fissures. The lobes of the CB are further subdivided into ten transverse lobules I–X. The superior, middle and inferior cerebellar peduncles attach it to the brainstem (Roostaei *et al*., 2014).

2.4.9 Cerebellar circuitry

The principal excitatory input pathways to the CB are the mossy and climbing fibers. The former emanate from different part like the pontine and vestibular nuclei, spinal cord and the reticular formation principally through the middle and inferior cerebellar peduncles and distribute fibres to the Purkinje (PKJ) layer indirectly. The climbing fibers on the other hand arise entirely from the contralateral inferior olivary complex and directly relate information to the PKJ cells. Both of these fibers relate with the deep cerebellar nuclei (Figure 2.13). Mossy fibers synapse with the neurons in the other layers. They also form a sequence of expansions called rosettes in the granular layer which connects with dendritic expansion of the granule cells (dendritic claw) to form cerebellar glomeruli (Roostaei *et al*., 2014). Inhibition of PKJ cells targets is achieved by g-aminobutyric acid (GABA) neurotransmission. These cellular axons constitute the only output of the cerebellar cortex (Kandel and Schwartz, 2013).

2.4.10 Histology of the cerebellum

Microscopically, the cerebellar cortex is tri-layered, the outer molecular layer, the middle PKJ cell layer, and the inner granular layer (Figures 2.16 and 2.17). The granular layer possesses nucleic acids in the nuclei of its numerous small cells hence, it is well defined. The PKJ cell layer comprises of one layer of large PKJ cell peri-karyons that are effortlessly identifiable. The molecular layer contains many axons and dendrites with capillaries. There are four pairs of nuclei within the cerebellar white matter; these are the fastigial, dentate, emboliform, and globose nuclei (Yassa, 2006).

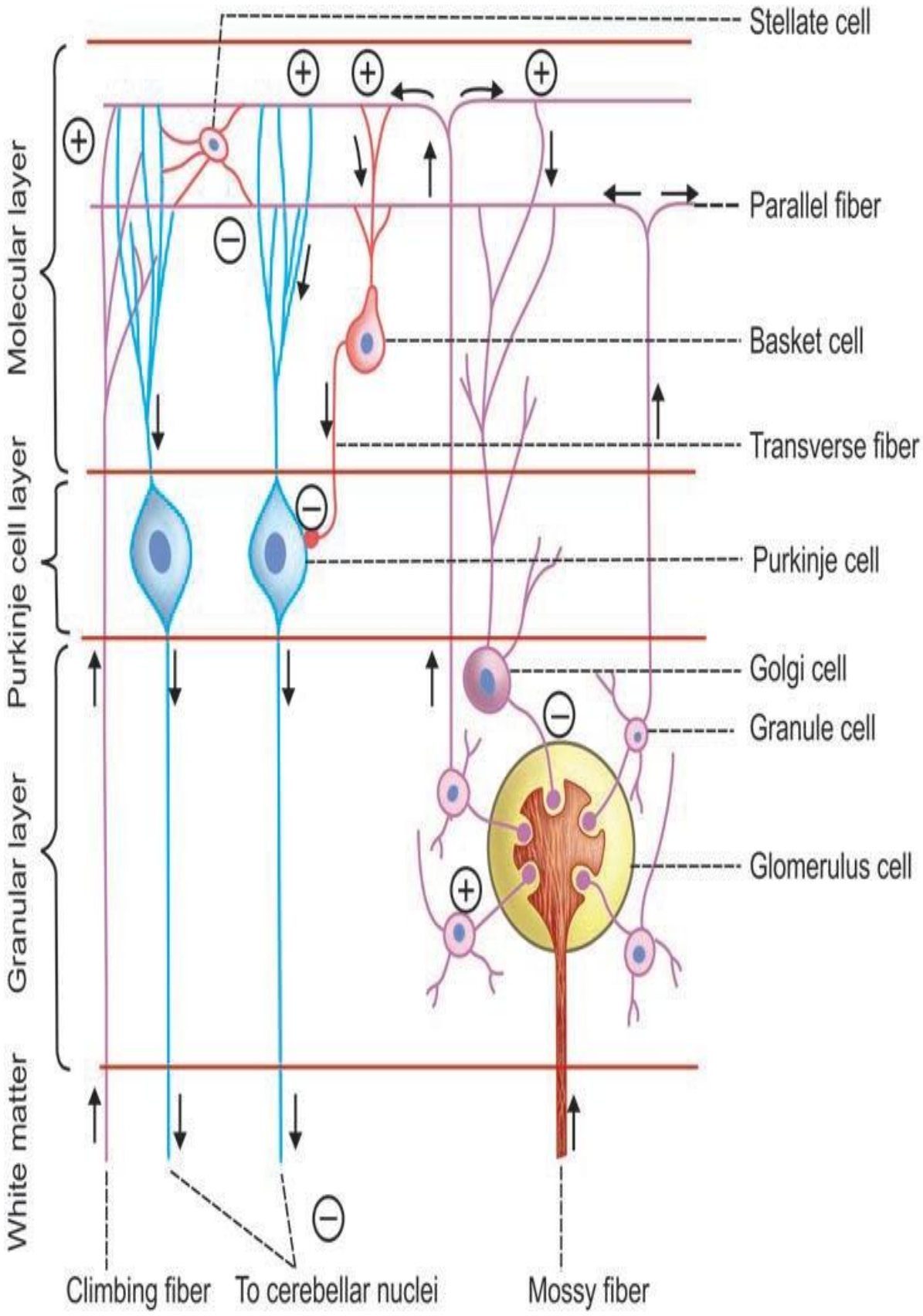


Figure 2.16. The cerebellar cortex and its circuitry (Sembulingam and Sembulingam, 2012).

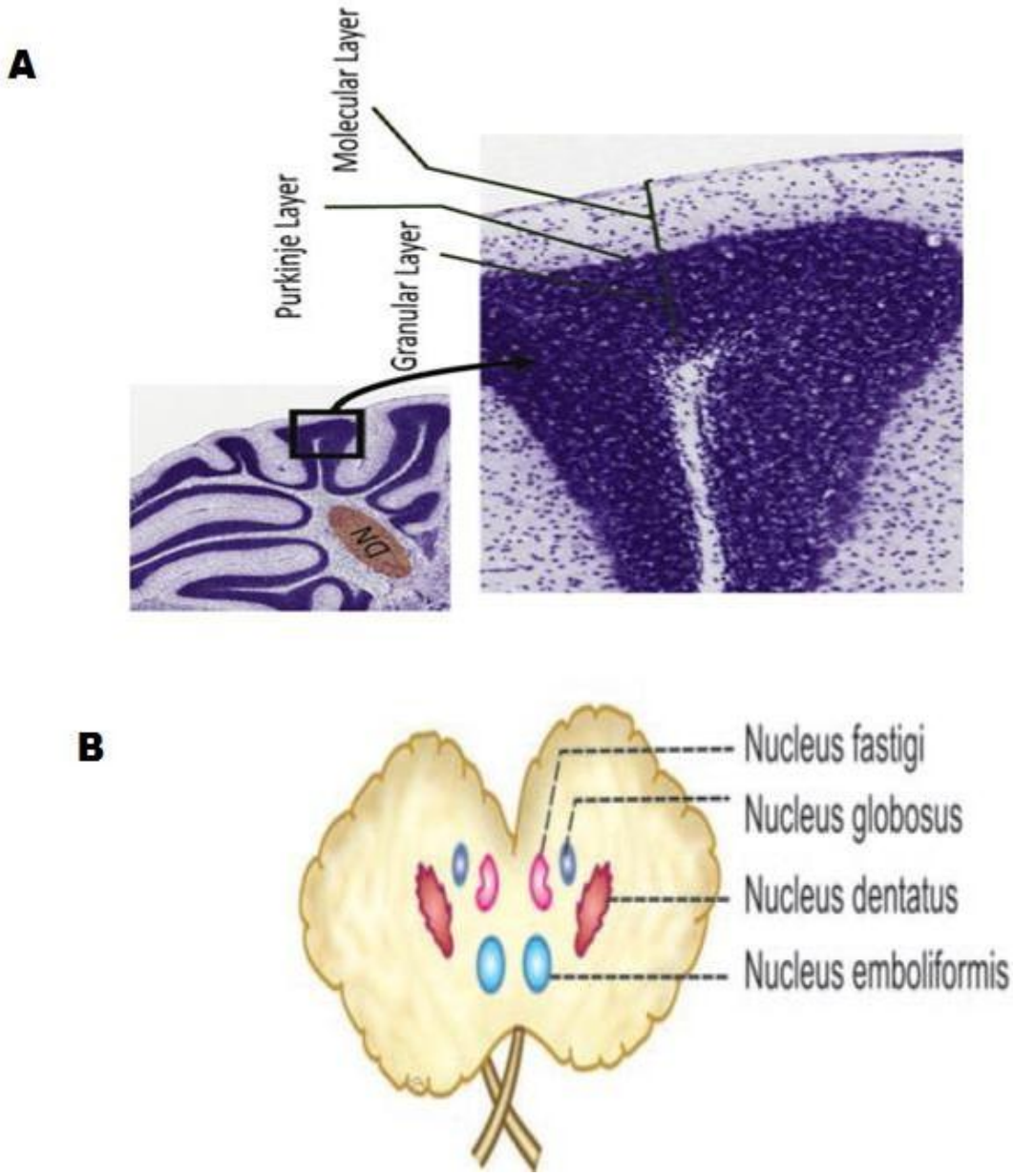


Figure 2.17. Histology of the cerebellum (Ross and Pawlina, 2010).

2.4.11 Blood supply to the cerebellum

The vessels to the CB are derived from vertebrobasilar system which in turn emanates from the early part of the subclavian arteries and proceeds superiorly via the transverse foramina of the superior six cervical vertebrae. The right and left vertebral arteries meet after entering the cranial vault via the foramen magnum at the pontomedullary junction where they form the basilar artery. Out of the three major branches that vascularise the CB, two emanate from the basilar part of the system while one emanates from each vertebral branch of the system: the superior cerebellar artery (SCA), the anterior and inferior cerebellar artery (AICA), and the posterior and inferior cerebellar artery (PICA) (Figure 2.18) (Delion *et al*., 2016).

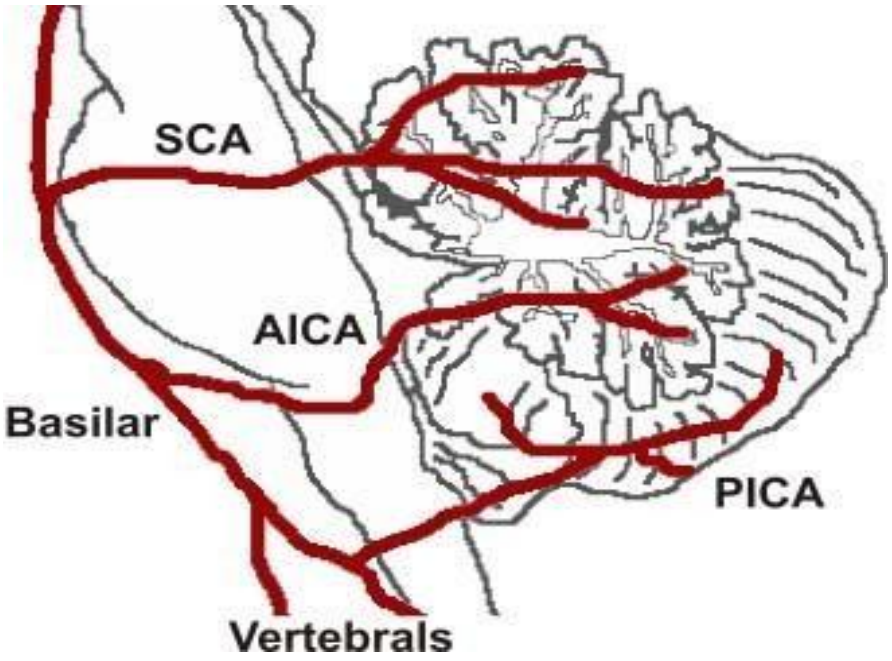


Figure 2.18. Arterial supply to the cerebellum. (Delion *et al*., 2016)

2.4.12 The cerebellum of rat

The CB of rat, like that of human is housed in the posterior cranial fossa, dorsal to the brainstem and pontine nuclei. Despite its nomenclature as the “little brain”, about half of the neuronal population is contained within it (Kandel and Schwartz, 2013). The CB has three lobes: the anterior, posterior and flocculonodular lobes. The CB is further divided into lobules by transverse (interlobular) fissures. These lobules are further partitioned by the foliations. The CB is also split by a median vermis into two hemispheres laterally. It is en-sheathed by a superficial cortex. The cerebellar output is provided by the cerebellar nuclei within the white matter (Gao *et al*., 1996).

The cerebellar function lies in three regions; the vestibular, the spinal and the cerebral parts of the CB. The vestibulocerebellum is presumably the phylogenetically oldest of these parts, which is presumably the same as flocculonodular lobe. It participates in regulating balance and eye movements. The spinocerebellum consists of the vermis and paravermis. It receives impulses from the spinal cord and it is assumed to chiefly partake in reflex motor control of the striated muscles. Lastly, the cerebrocerebellum is phylogenetically the newest part of the CB. It consists of most of the hemispheres and receives impulse majorly from the cerebral cortex. It helps in the preparation, organization and learning of complex movements and some cognitive functions (Figure 2.19) (Gao *et al*., 1996).

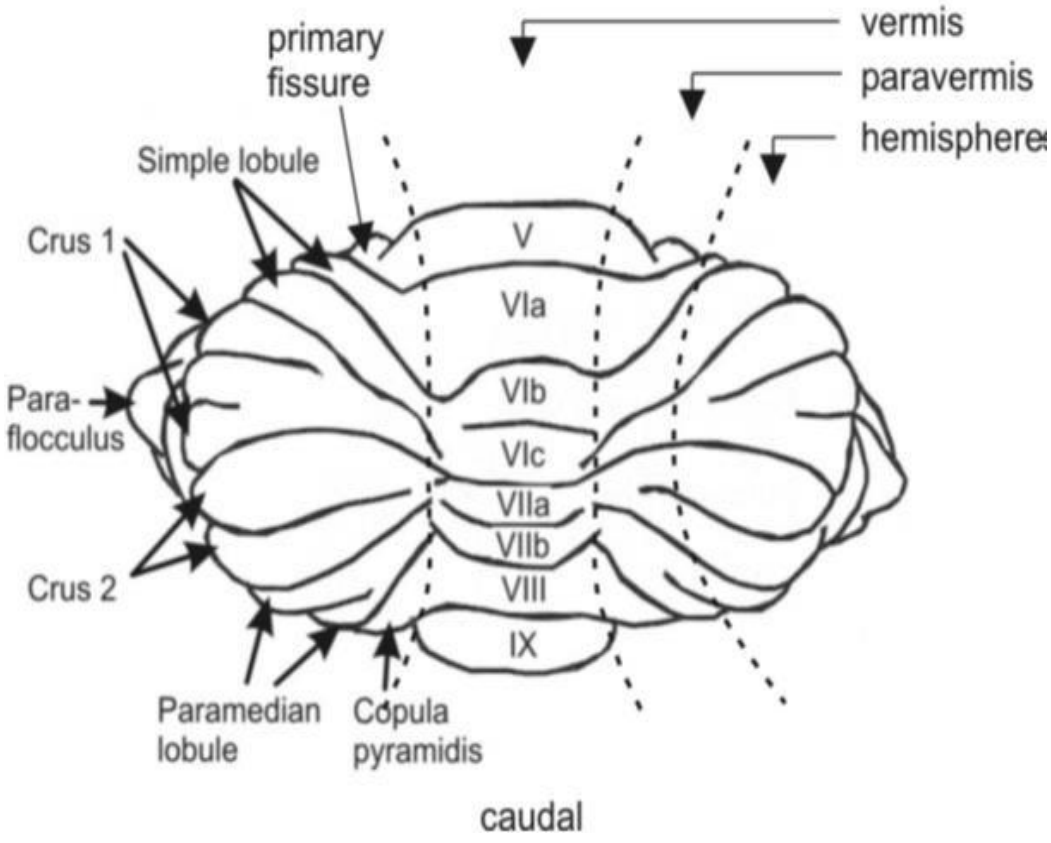


Figure 2.19. Gross anatomy of the rat cerebellum (posterior view)

(Martinez and Armengol, 2015)

2.4.13 The cellular parts of the rat cerebellum

The cerebellar cortex

The rat's cerebellar cortex has an identical appearance. It has three stata supero-inferiorly, namely, the molecular layer, the PKJ cell layer and the granular layer followed by a white matter (Martinez and Armengol, 2015). The molecular layer predominantly consists of the superficial division of climbing parts of the axons of granule cells that have their origin from the granular layer, and are known as the parallel fibers (see Figure 2.20). These parallel fibers travel with the folia long axis, and in the rat, a parallel fiber's length may be up to 4.6-5.0mm (Martinez and Armengol, 2015).

The Purkinje cell layer consists of the largest neurons and forms the sole output cells of the cerebellar cortex. The dendritic tree of the PKJ cell extends into the molecular layer lying perpendicular to the course of the parallel fibers. Each PKJ cell of the rat receives excitatory inputs from up to 175,000 parallel fibers, from one or two climbing fibres of granule cell axons (Martinez and Armengol, 2015) and from only one climbing fiber The innermost granular layer consists of a vast number of various neurons constituting the most abundant cells in the CB (Martinez and Armengol, 2015).

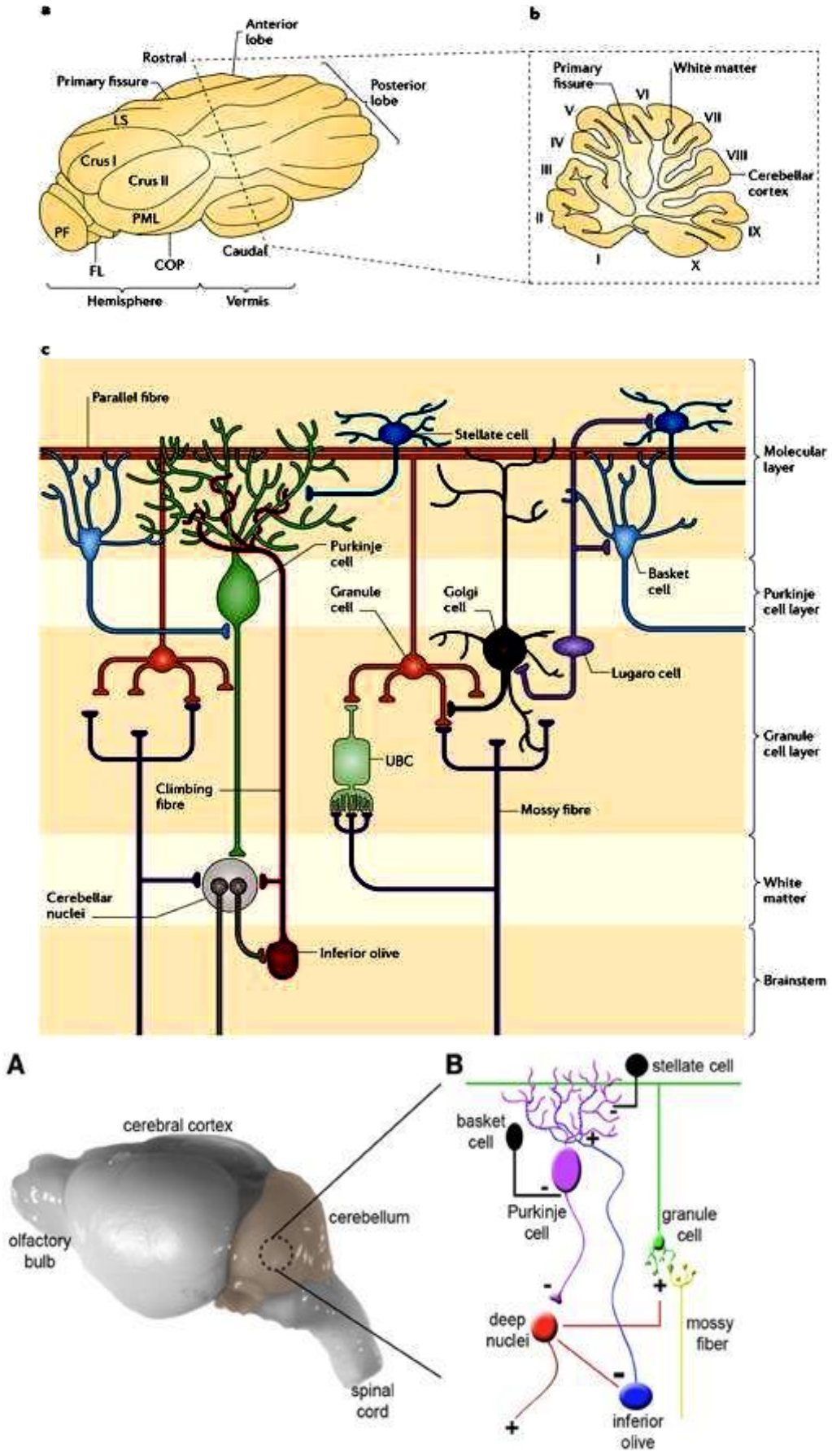
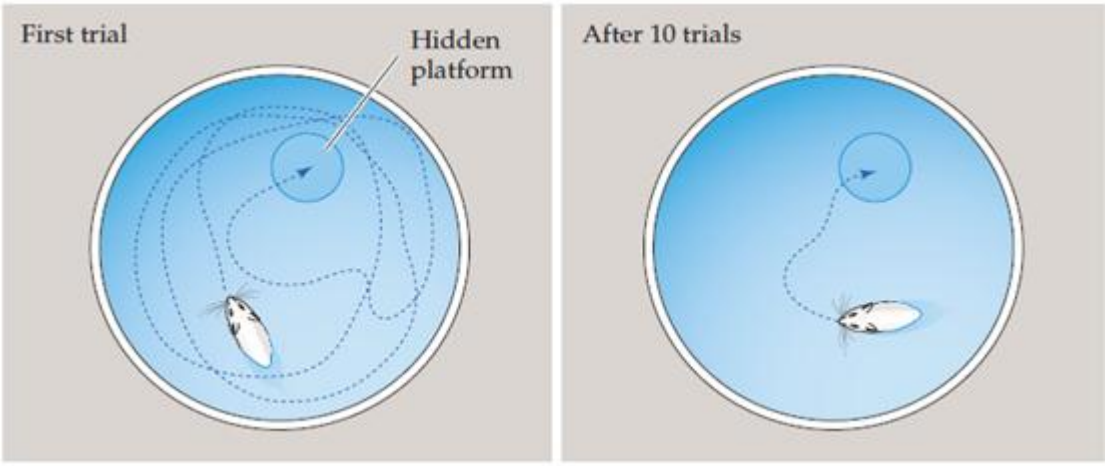


Figure 2.20. Histology of the rat cerebellum (Martinez and Armengol, 2015)

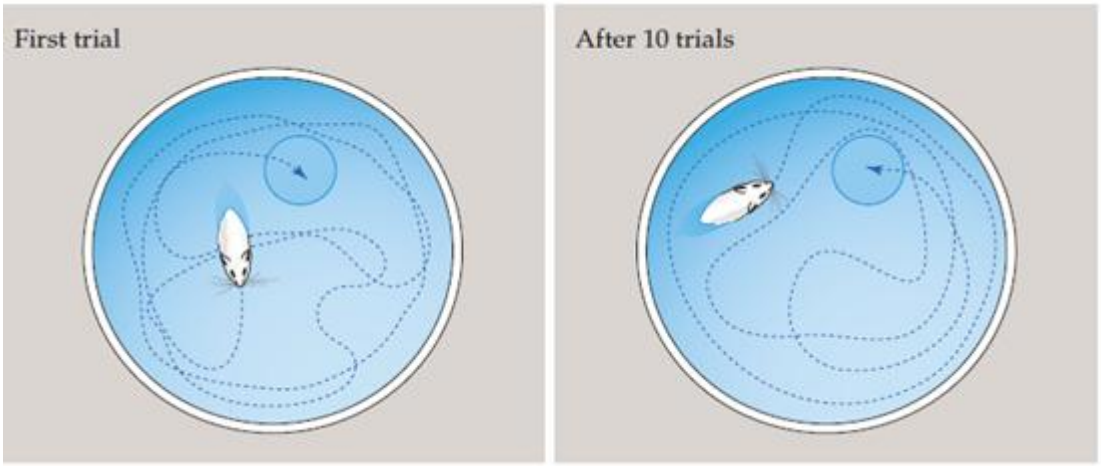
2.5 Neurobehavioural Assessments

2.5.1 Morris Water Maze (MWM) Test

In MWZ Test, the animal is allowed to swim in a pool of opaque water having a hidden platform submerged under the water surface (Figure 2.21). Apparently healthy animals are allowed to search randomly until they locate the submerged platform. The animal soon learn to locate the platform within a short time following repeated testing However, animals with hippocampal assault failed to learn to find the platform, suggesting that the HP plays a main role in spatial memory (Eichenbaum, 2000; Schenk and Morris, 1985).



A: Apparently healthy rat



B: Rat with hippocampal assault

Figure 2.21. Morris Water Maze Apparatus. (Eichenbaum, 2000; Schenk and Morris, 1985).

2.5.2 Fore-limb grip Test (Grip strength test)

Fore-limb grip test is commonly used to evaluate rats and mice with neuromuscular disorders. It also assess the effects of different substances (such as drugs, chemicals, etc.) on motor performance. In this test, the animal is allowed to grip a bar or rod connected to a monitoring device and its tail is pulled horizontally. The maximum time taken by the animal to hold on to the rod is recorded as the forelimb grip strength (Figure 2.22) (Smith *et al*., 1995).

Figure 2.22 Forelimb Grip Strength Test (Smith, *et al*., 1995).

2.6 The Haematological profiles of study

Blood is the red coloured fluid that flows through the arteries and veins. Human blood contains plasma (the liquid portion), blood cells (red and white), and cell fragments called platelets (Sembulingam and Sembulingam, 2009).

2.6.1 Main components of blood

Blood cells, including platelets constitute 45% of human blood, while plasma forms the remaining 55%. The diagram below (Figure 2.23) shows red blood cells (RBCs), white blood cells (WBCs) of different types (large, purple cells), and platelets (Sembulingam and Sembulingam, 2009).

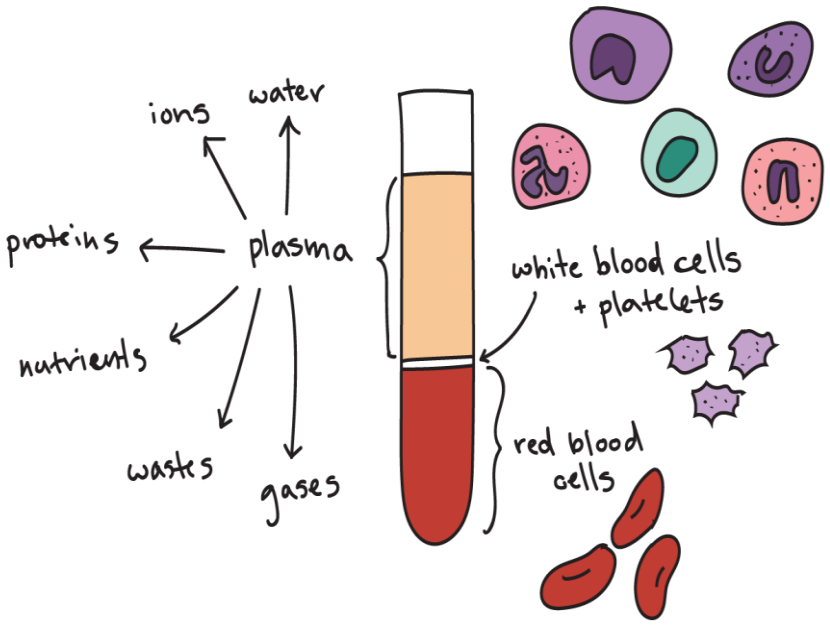


Figure 2.23. Components of blood (Sembulingam and Sembulingam, 2009).

2.6.1.1 Plasma

The plasma contains mainly water (about 90%), ions, proteins, nutrients, wastes, and dissolved gases (forming the remaining 10%). The plasma helps to maintain the blood pH and osmotic balance (Sembulingam and Sembulingam, 2009).

2.6.1.2 Red blood cells (Erythrocytes)

Red blood cells, also known as **erythrocytes**, contain heamoglobin, which has affinity for both oxygen and carbon dioxide. They help deliver oxygen to tissues and remove carbon dioxide when they circulates through the body. In the lungs, RBCs take up oxygen and release the oxygen to the body cells as they circulate through the body tissues. They also remove carbon dioxide from the tissues back to the lungs for elimination (Sembulingam and Sembulingam, 2009).

2.6.1.3 White blood cells (Leukocytes)

White blood cells, also known as **leukocytes**, constitute less than 1% of the blood cells. They help in immune responses by recognizing and removing micro-organisms. There are five major types of WBC, and these are divided into two different groups, according to their appearance under a microscope:

- the **granulocytes**, which have granules in their cytoplasm and include neutrophils, eosinophils, and basophils,.
- the **agranulocytes**, which do not have granules in their cytoplasm and include monocytes and lymphocytes (Sembulingam and Sembulingam, 2009).

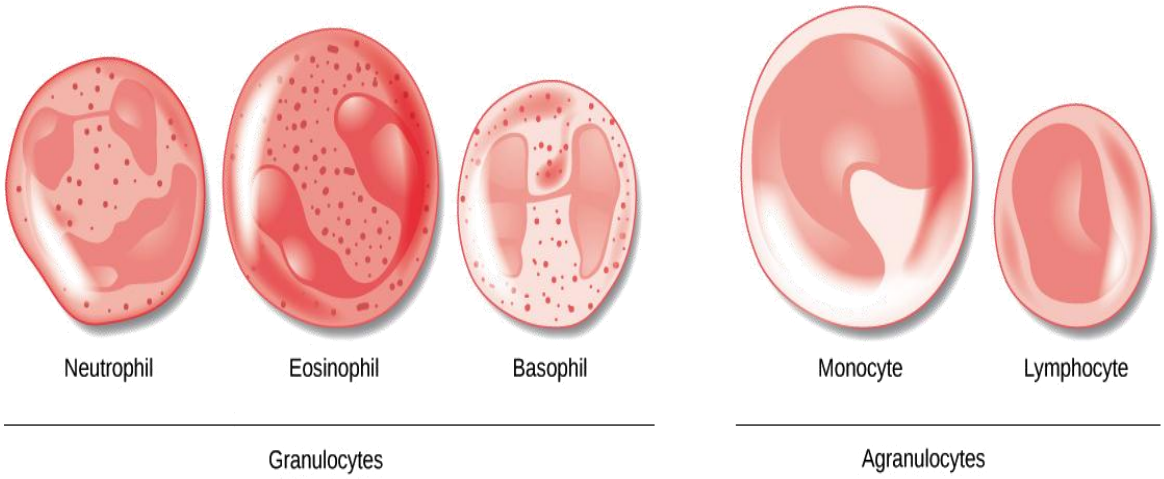


Figure 2.24: Forms of White Blood Cell (Sembulingam and Sembulingam, 2009).

2.6.1.4 Platelets (Thrombocytes)

Platelets, also called **thrombocytes**, are cell fragments involved in blood coagulation. They are formed from megakaryocytes. They help to arrest bleeding at a wound site where they form a sticky plug called clotting. They do this by releasing signals, which attract other platelets making them become sticky. Platelets also initiates clotting by conversion of fibrinogen to fibrin that forms threads which reinforce the platelet plug (Sembulingam and Sembulingam, 2009).

2.7 Biochemical markers of oxidative stress

2.7.1 Malondialdehyde (MDA)

This was employed to check for lipid peroxidation. It is a marker of OS in the tissues. The method of Beuge and Aust (1978) was used to assay for MDA.

2.7.2 Nitric oxide (NO)

Nitric oxide (NO) plays an essential role as physiological messenger and effector molecule in several biological systems, neuronal tissues inclusive. Based on its wide range of systemic applications, there are robust interests in measuring it in biological tissues and fluids as a marker of inflammation (Dawson and Dawson, 1995).

2.7.3 Superoxide dismutase (SOD)

This is an antioxidant and a marker of OS. The enzyme protects the cells against dangerous levels of superoxide which is a form of free radical reactive oxygen specie (ROS) (Nozik-Grayck *et al*., 2005).

2.7.4 Catalase (CAT)

Catalases usually catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Chelikani *et al.,* 2004). This was assayed to check for hydrogen peroxide and generation of other free radicals, it is therefore an antioxidant and a marker of OS.

2.7.5 Glutathione (GSH)

Glutathione is a peptide containing cysteine moiety. It is found in almost all living organisms. Its system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione S-transferases (Meister, 1994). This is also used as a marker of OS and inflammation.

2.8 Histological Techniques: Preparation of Tissues for Microscopic Examination

Tissue preparation for light microscope can be achieved through the following underlined procedures (Bancroft and Gamble, 2008):

2.8.1 Fixation

Fixation is the process of treating or immersing tissues in chemical solutions called fixatives in order to avoid autolysis. Tissue or organ should be quickly and sufficiently fixed in an appropriate fixative following excision from the animal or even before, in order to preserve its structure and molecular composition.

2.8.2 Dehydration

After fixation, the water content of the fixed tissue is removed by passing it through an increasing grade of ethanol (usually from 70% to 100% ethanol).

2.8.3 Clearing

Clearing helps to replace the ethanol used in dehydration with a solvent miscible with the embedding medium. The clearing agent mostly suitable for paraffin embedding is xylene. Clearing also makes the tissues become transparent.

2.8.4 Embedding/Infiltration

Embedding involves soaking the tissue in a semi-solid medium to facilitate sectioning. Embedding materials include paraffin and plastic resins. Once the tissue is impregnated with the solvent (xylene), it is placed in melted paraffin in the oven, typically at 58-60°C.

2.8.5 Sectioning

Sectioning of hard block of tissue is done with a microtome by its steel or glass blade to a thickness of 1-10 µm. The sections are then floated on water and transferred to glass slides for staining.

2.8.6 Staining

Staining of tissues is necessary to make the various tissue components conspicuous and allow distinctions to be made between them. The most commonly used out of all dyes is the combination of **hematoxylin and eosin** (**H&E**). Hematoxylin stains the cell nucleus and other acidic structures (such as RNA-rich portions of the cytoplasm and the matrix of hyaline cartilage) blue. In contrast, eosin stains the cytoplasm and collagen pink (Bancroft and Gamble, 2008).

2.9 Immunohistochemistry

Immunohistochemistry involves incubating a tissue section having a particular protein in a solution containing an antibody to that protein. Binding of this antibody to the said protein then occurs, location of which can thereafter be seen with either the light or electron microscope, depending on the type of compound used to label the antibody. In immunocytochemistry, the most important requisite is the availability of an antibody against the protein that is to be detected. This means that the protein must have been previously purified and isolated so that antibodies can be produced (Bancroft and Gamble, 2008).

2.9.1 Glial Fibrillary Acidic Protein (GFAP)

Glial Fibrillary Acidic Protein is a cell-specific marker that distinguishes astrocytes from other glial cells. In the central nervous system, astrocytes show different responses to almost every type of injury or disease. In such pathological conditions, increase in size (hypertrophy) and increase in number (hyperplasia) of astrocytes are usually noticed, and those cells form abundant glial fibrils that can be demonstrated by immunostaining. These astrocytes are thus referred to as reactive astrocytes (Lyck *et al*., 2008).

2.9.2 Cyclo-oxygenase-2 (COX-2)

This is a marker for neuro-inflammation**.** COX-2 has been consistently shown to be constitutively expressed in organs, such as the brain. It was established that non-steroidal anti-inflammatory drugs (NSAIDs) exert their anti-inflammatory properties through the inhibition of COX enzymatic activity, thus preventing prostaglandin synthesis (Vane, 1971).

2.9.3 inducible Nitric-Oxide Synthase (iNOS)

This is used to express the level of oxidative stress through the release of free radical's reactive nitric oxide species (RNOS).

2.10 Anti-malarial drugs and substances affecting the hippocampus and cerebellum

2.10.1 Toxicity of artemisinin and its derivatives

The neurotoxic dose of oral artemether and artesunate that caused death in 50 % of animals was approximately 300 mg/kg/day (Nontprasert *et al*., 2002). Some research reports suggested a favorable safety profile and a good tolerability with the use of artemisinin and its derivatives and that most reports of their deleterious activities in

clinical applications are not supported by research data (Meshnick, 2002). However, reports of toxicity of artemisinins in experimental animals have triggered worries about the safety of the drugs, but these are suggested to be hinged on high dosages intake of artemisinins for long time. Research with experimental animals has shown that over-dosages with artemisinin may cause neuronal damage, but this has not been established in man (Nontprasert *et al*., 2002). The safety of artemisinin in treating malaria during pregnancy is another area of concern. The loss of embryo in a pregnant mother has been reported with the use of artemisinin parenterally in animals and this has been associated to possible inhibition of erythropoiesis (Clark *et al*., 2008). Report of declined erythropoiesis in human after receiving artemisinin suggests that the drug has the tendency of causing embryotoxicity in women during early pregnancy (Clark, 2009).

2.10.2 Empirical data of the effects of AS, AQ and AS+AQ on body and organ weights

Utoh-Nedusa *et al*. (2009) reported a gain in body weight following administration of artemisinin in experimental rats. Olumide and Raji (2011) reported significant gain in body and relative organ weights of rats that received AS at a low dose and substantial decrease in the animal's weight treated for 6 weeks. Izunya *et al.* (2010) reported that standard and double standard doses of artesunate did not alter the body weights of rats. Ubulom *et al*. (2015) likewise documented an upsurge in the body temperature and decline in feeding rate of their experimental animals administered with amodiaquine and cifloxacine combination. Blundell *et al*. (2017) in their study opined that drug that causes reduction in weight usually act on the hypothalamus to reduce feelings of hunger.

2.10.3 Empirical data of the effects of AS, AQ and AS+AQ on animal behaviour

Ekong *et al*. (2008) documented that amodiaquine in graded doses on oral administration for three and six days caused increase in total locomotor and exploration activities, and reduced anxiety in rats. Ekong *et al.* (2008) reported that standard dose of artesunate didn't exhibit damaging effect and may not affect behaviour. Furthermore, AS-AQ combination also showed no significant change in behaviour of rats, indication that this drug may not have altered locomotion and may be neither anxiolytic nor anxiogenic at its recommended doses or time (Ekong *et al.*, 2010). Therapeutic doses of AS, AQ and AS+AQ caused reduction in spatial memory scores in mice on three (3) day administration (Onaolapo *et al.*, 2013).

2.10.4 Empirical data of the effects of AS, AQ and AS+AQ on haematological profiles

Papiya *et al*. (2015) in their subchronic study on artesunate reported no change in the level of hemoglobin (Hb), total red blood cell (RBC) count, platelet (PLT), lymphocytes (LYM), basophil (BAS), mean cell volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) while total white blood cell (WBC) count, neutrophil (NEU), eosinophil (EOS), packed cell volume (PCV) and mean cell hemoglobin (MCH) were increased significantly at a double normal dose.

Utoh-Nedusa *et al*. (2009) reported that dihydroartemisinin significantly raised the PCV, the total WBC count and NEU count in rats. Similarly, Aprioku and Obianime (2011) reported that AS, dihydroartemisinin, and artemether significantly and dose-dependently increased WBC count and lymphocyte count while neutrophil count was decreased by artemether at double therapeutic dose in rats. Agoma *et al.* (2008) reported that white blood cell counts showed slight increase especially in lymphocytes while neutrophil level decreased as treatment progressed.

2.10.5 Empirical data of the effects of AS, AQ and AS+AQ on biochemical indices

Amodiaquine triggered peroxidation of lipid and caused agranulocytosis (Farombi, 2000) and these processes were reported to cause nervousness, violence, misperception, character fluctuations, and others over a prolonged use (Tester-Dalderup, 1984) and spontaneous motion when acute dosages are consumed. Furthermore, lipid peroxidation had also been reported to cause tissue impairment *in vivo* (Mayes, 2000). Utoh-Nedusa *et al.* (2009) documented that dihydroartemisinin does not alter serum level of alanine-aminotransferase, serum alkaline phosphatase and serum aspartate amino transferase actvities. Aprioku and Obianime (2011) reported dose-dependent increase in serum prostate acid phosphatase and glutamate oxalo-acetic transaminase in artesunate and dihydroartemisinin exposed animals. Dihydroartemisinin also causes dose-dependently increase in serum glutamate pyruvate transaminase and triglyceride level.

Ekong *et al.* (2008) reported that AQ+AS combination reduced brain cholesterol level and triglyceride in experimental animals treated for six days and increased level of these bio-molecules in the group treated for three days. Aprioku and Obianime (2011) in their comparative study with artesunate, amodiaquine, and different ACTs on the biochemical indices, reported an increase in the level of basal serum prostate acid

phosphatase by amodiaquine and sulfadoxine/pyrimethamine. Artesunate/sulfadoxine/ pyrimethamine significantly increases serum urea in doses tested. Basal serum creatinine level was increased by sulfadoxine/pyrimethamine and decreased by AS+AQ combination at their subclinical doses. The level of uric acid in serum was substantially reduced by half standard dose of AS+AQ. The blood cholesterol level was found drastically diminished following intake of clinical doses of AS and AQ. The raised serum level of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were significantly reversed when folic acid was co-administered with AS (Aprioku and Obianime 2011). Literature also indicates that an imbalance between reactive oxygen metabolites and antioxidant defense results in OS (Gibananada and Hussain, 2002).

2.10.6 Empirical data of the effects of AS, AQ and AS+AQ on the nervous system

Ubulom *et al*. (2015), after administering adult Wistar rats with oral doses of Artesunate/Mefloquine combination (Artequin) and oral doses of Mefloquine for 3 days reported that their hippocampi exhibited huge and heavy number of astrocytes and astrocytes' processes. Another group administered with Mefloquine and graded doses of Artequin revealed smaller and weakly visible oligodendrocytes cum vacuolation. The same study revealed loss of pyramidal neurons in the treated groups. These findings suggested that the drugs, at the administered dosages affected supporting glia cells and that Mefloquine and Artesunate/Mefloquine induced dose-dependent reactive astrocytes and astrocytes' processes formation in the HP. This, according to the authors may alter neuronal environment and impair the uptake of neurotransmitter thus altering the hippocampal function. Ekanem *et al*. (2009) reported that, when the CNS is insulted, astrocytes, the neuro-supportive cells, usually become more populated around the damaged sites so that the injured neurons may be repaired; these astrocytes are therefore tagged reactive astrocytes. Abbas and Nelson (2004) opined that the emergence of reactive astrocytes is an indication of early signs of cell loss. Thus, the activities of Mefloquine and Artesunate/Mefloquine combination on astrocyte as seen in the study of Ubulom *et al*. (2015) indicate their harmful potential on the neurons of the HP. The significance of this lies in the fact that transformed glial cell anatomy and physiology contribute to mental disorders such as forgetfulness and seizures (Edgar and Sibille, 2012). Dystonic reaction associated the use of AQ was first reported by Akindele and Odejide (1976).

Oligodendrocyte helps in axon survival and function, injury of which may cause axon degeneration, as a result of lack of energy metabolites e.g. lactate, which it conveys solely as monocarboxylate transporter. Possible disruption of this transporter as shown in the alteration in the structure and population of oligodendrocytes in the study of Ubulom *et al*. (2015) may injure axon and depopulate neurones in the HP of rats affecting the normal functioning of the HP (Edgar and Sibille, 2012).

Artemisinin and its derivatives were reported to cause gait disturbances, prominent loss of brain stem and eye reflexes and prominent neuropathic lesions (Nontprasert *et al*., 2002). Artesunate was also reportedly implicated in ataxia and slurred speech (Miller and Panosian, 1997). Ekong *et al*. (2009) documented that a 3-day administration of AQ+AS caused histological alterations in the CB such as shrinkage of the Purkinje cells; another group administered with same dose for six days, and double dose for three days showed shrinkage and loss of the PKJ cells and shrinkage of Purkinje cell layer. The consequence of this may be insufficient emission from the cerebellar cortex to other brain and body parts, PKJ cells being the major cortical output of the CB. Cerebellar dysfunction might result, culminating in some motor problems such as vertigo (dizziness), step disturbances and tremor.

The use of amodiaquine within the therapeutic dose was said to cause peripheral neuropathy, vertigo and lethargy (weakness or inactivity characterized by lack of energy) occasionally (Pfizer, 2016). The use of amodiaquine in large doses has also been reported to cause syncope (spontaneous loss of consciousness owing to inadequate blood supply to the encephalon), spasticity (state of painful and involuntary muscular contraction), tremor and spontaneous shakings (Pfizer, 2016).

Eweka and Adjene (2008) in their study on the sub-acute effect of AS (in graded dosages) on the superior colliculus of Wistar rat reported cell clumping, swelling, and intercellular vacuolations around the stroma of the superior colliculus. This suggested that varying dosages of AS over a sub-acute period of administration (13 days) may be harmful to the neurons of the intracranial visual relay centre which mayhamper vision.

2.10.7 Empirical data of the effects of other agents on the hippocampus and cerebellum

Omotosho and Babalola (2014) reported that Wistar rats exposed to varying doses of cigarette smoke had decreased cerebellar weights, morphology of the CB and cell layers depleted. Chronic intake of nicotine triggered OS in the CB. Chronic nicotine

consumption was reportedly caused substantial loss of cerebellar white matter (Tewari *et al*., 2010). Gamma radiation caused a substantial alteration in the morphology of rats' CB, such as weight retardation, width retardation, etc. (Owoeye *et al*., 2011).

Schmitz *et al.* (2005) reported that cranial radiotherapy caused damage to rat cerebellar neurones while Malomo *et al.* (2005) documented neuronal contractions with the same agent. Rats dosed with varying dosages of tramadol reportedly had mild distortion of their Purkinje cells such as shrank nucleus with an indented membrane; the cerebellar granular layer also exhibit shrank cells, without nuclei. Tramadol treatment also revealed a tremendous reduction in the width of PKJ and granular layers and a tremendous rise in the width of the molecular layer in the animals. The PKJ cell count reduced drastically in the treated groups (El-Bermawy *et al*., 2015).

Cyanide consumption by mother Wistar rats elicited slight variations in the foetal cerebellar development postnatally (Malomo *et al*., 2004). Osuagwu *et al*. (2007) stated that the parenteral administration of phenytoin in Wistar rats caused decrease in cell density of the pyramidal layer of the CA 1 subfield of the HP cum reduction in the mean body weight. The same substance also caused OS in the CB (Imosemi *et al*., 2010). Zhang *et al*. (2016) in a review paper opined that opiate drugs in general, such as morphine and heroin depleted neural progenitors in the sub-granular zone of dentate gyrus. Consumption of alcohol is also reported to cause cognitive and memory impairments associated with the HP in both human consumers and in laboratory animal experiments (Berry and Matthews, 2004). Acute intake of cocaine reportedly improves cognition in humans and improves learning and memory in experimental animals (Garavan *et al*., 2008). Also, acute cocaine consumption alters hippocampal long-term potentiation that aid learning and memory (Bliss and Collingridge, 1993).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Procurement of animals

Eighty (80) Adult Male Wistar rats (*Rattus norvegicus*) of weight range 110g to 200g were obtained from the animal house of the Department of Veterinary Physiology, University of Ibadan and used for this experiment.

3.2 Procurement of drugs

Tablets of Artesunate and Amodiaquine were procured from Diadem pharmacy, Ojoo Area, Ibadan (manufactured by ADAMS Pharmaceutical Co., Ltd., Anhui, China).

3.3 Care and grouping of Animals

The animals were kept under standard laboratory conditions with 12 hour light/dark condition. They were made to have access to feed and water *ad- libitum* throughout the period of the experiment. Thee experiment was divided into two phases: acute and delayed post-acute phases. Each phase consisted of four groups, hence the animals were grouped into four (N=20). Each of the four groups were further sub-divided into two groups (n=10). The first sub-group represented the acute phase (4 days) while the second sub-group represented the delayed post-acute phase (15 days). All grouping was done randomly.

Group A (CT) - Distilled water (control group)

Group B (AS) - Artesunate (4 mg/kg BW)

Group C (AQ) - Amodiaquine (10 mg/kg BW)

Group D (AS+AQ) - Artesunate (4mg/kg BW) + Amodiaquine (10 mg/kg BW).

These doses are the standard therapeutic doses of the two drugs in human, thus simulating a situation in which the ACT is used, but in this case, without any malaria infection (Adebayo *et al*., 2011).

3.4 Drug preparation

Stock solution

Tablets of AS and AQ were grinded separately into powder. Sixteen (16) mg of AS (i.e.- 0.8 mg, a dose taken by the rat of highest weight- 200 g x 20 animals) was dissolved in 40mls (i.e. 2 ml, a volume taken by the rat of highest weight x 20 animals) of distilled water while twenty (40) mg of AQ [i.e. 2 mg, a dose taken by the rat of highest weight (200g) x 20 animals] was dissolved in 40mls (i.e. 2 ml, a volume taken by the rat of highest weight x 20 animals) of distilled water. Weight of drugs were measured by sensitive weighing balance while distilled water was measured by calibrated cylinder obtained from the department of Anatomy, University of Ibadan.

The following calculations were done as a guide:

For Artesunate (AS):

4 mg/kg B.W of AS means a 1kg rat will receive 4 mg of AS, therefore animal of X g weight will receive (X g x 0.004 mg) dose of AS.

By standard, animal of weight 100 g should receive 0.5 ml volume of drug preparation (OECD, 2000); therefore, animal of X g weight will receive (X g x 0.005 ml) concentration of (X g x 0.004 mg) dose of AS.

For Amodiaquine (AQ):

10 mg/kg B.W of AQ means a 1kg rat will receive 10 mg of AQ, therefore animal of X g weight will receive (X g x 0.01 mg) dose of AQ.

By standard, animal of weight 100 g should receive 0.5 ml volume of drug preparation (OECD, 2000); therefore, animal of X g weight will receive (X g x 0.005ml) concentration of (X g x 0.01 mg) dose of AQ.

For Artesunate (AS) +Amodiaquine (AQ):

Animal of X g weight will receive half (X g x 0.005 ml) concentration of (X g x 0.004 mg) dose of AS + half (X g x 0.005 ml) concentration of (X g x 0.01 mg) dose of AQ.

Drugs were administered with the use of oral gavage once per day at 09:00 hours for three (3) consecutive days.

3.5 Drug administration

The drugs were administered to the rats in mg/kg body weight as aqueous suspension using oral gavage. The drug suspension was continuously shaken to ensure homogenous delivery to the animals. The drugs were administered to the four groups A, B, C, and D as follows:

Group A received distilled water *ad-libitum* throughout the duration of the study (Control group)

Group B received 4 mg/kg body weight of Artesunate (AS group) for 3 consecutive days.

Group C received 10 mg/kg body weight of Amodiaquine (AQ group) for 3 consecutive days.

Group D received 4/10 mg/kg body weight of Artesunate/Amodiaquine (AS+AQ group) for 3 consecutive days.

All animals for the acute phase were sacrificed on the 4th day while those for the delayed post-acute phase were sacrificed on the 15th day.

This design was to mimic the dosage of administration of the drugs in human as recommended by WHO.

3.6 Ethical consideration

Approval of laboratory experimental ethics was sought and obtained from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC), with reference number UI-ACUREC/App/10/2016/002.

3.7 Gross morphometry

3.7.1 Body weight: As an index of physical status of the animals, the weight of each animal was monitored over the period of the study. The animals were weighed on day 0 and day 4 as initial and final weights respectively for the acute study; while the animals for the delayed post-acute study were weighed on days 0 and 15 as the initial and final weights, and these were used to determine weight difference. The weights were taken with Electronic Weighing balance (Model: RS 225, Range: 0-600g, Make: Citizen). The weighing scale was switched on and zeroed with the container placed on it. Each rat was placed in the container and the readings were recorded. The formula below was used to calculate the percentage weight difference:

$$\textbf{\% Weight difference} = \frac{\text{Final weight (D4/D14)} - \text{Initial weight (D1)}}{\text{Initial Weight (D1)}} \times 100$$

3.7.2 Brain Weight: After cervical dislocation and opening of the skull, the weight of the extracted brain of each animal was also taken with the aid of a sensitive weighing balance which was zeroed with a piece of paper placed on it. The whole brain of each sacrificed rat was placed on the paper and the readings were taken.

3.7.3 Brain body weight ratio: The brain body weight ratio for each animal was calculated with the formula:

$$\text{Organ to body weight index} = \frac{\text{Brain weight}}{\text{Animal weight}} \times 100$$

3.8 Neurobehavioural studies

The following neurobehavioural tests were carried out on the 1st, 2nd, 3rd (acquisition trials) and 4th days (probe trial) for acute study; and 11th, 12th, 13th (acquisition trials) and 14th days (probe trial) of the experiment for delayed post-acute study:

3.8.1 Morris water maze test: it is a neurobehavioural test of spatial memory and cognition of the rats, a function of the hippocampus.

Procedure

The rats were placed inside a mini circular swimming pool measuring 50 cm in height, 160 cm in diameter and filled with water to a 44 cm depth (appendix XV). The water was made opaque by adding milk and was periodically drained for cleaning and disinfection. The pool was divided into four quadrants by four equally spaced points around the edges of the pool designated as north (N), east (E), south (S) and west (W). A platform, made from a circular, water- resistant material of 10 cm height was placed 1 cm below the water level. This served as the hidden escape platform. The rats were allowed to locate the submerged platform within a maximum period of 2 minutes and were allowed to stay on it for 30 sec. The animals that did not locate the platform within 2 minutes were manually guided to the platform. The escape latency time to locate the platform was recorded for each trial. Each rat was given a daily session of five trials for the first 3 days while the probe trials were conducted on the 4^{th} and 14^{th} days of the acute and delayed post-acute phases respectively. Each rat was dried with a dry towel after each trial. Total swim time, swim distance, amount of time spent in each quadrant and the frequency of crossing the escape quadrant were quantified by means of hand-scored according to the method of Morris, 1984 modified by Vorhees and Williams, 2006.

3.8.2 Motor function test (Forelimb grip strength): this was used to objectively quantify the rats' muscular strength - a function of cerebellum, and to evaluate the drugs' effect and toxins on muscular and neurodegeneration (Pichon *et al*., 2014).

Procedure

The forelimb paws of the rat were placed horizontally on suspended metal rod (measuring 2 mm in diameter and 1 m in length), 1 m above a landing area filled with soft bedding. The duration of time each rat was able to grip the metal rod was recorded with a stop watch. A maximum time of 2 minutes was allotted to each rat. This was carried out on days 4 and 14 for the acute and delayed post- acute studies respectively.

3.9 Haematological Studies

3.9.1 Sample preparation for haematological studies

One milliliter of blood sample was withdrawn from each group of animals by ocular puncture 24 hours after the last drug administration (day 4) for the acute study and on day 14 for the delayed post-acute study. This was done by puncturing the medial canthus of the animal's eye with EDTA capillary bottles. Autohaematological Analyser Machine (Make: Japan Model: CNME030119) was used for the analyses, using the method of Baker *et al*. (1998).

3.9.2 Determination of Packed Cell Volume (PCV)

An EDTA capillary tube was used to draw capillary blood up to about three quarters of the tube. Sealant was used to seal the unfilled end. The filled capillary tube was then placed in one of the numbered slots of the microhaematocrit centrifuge (Make: Japan, Model: 00126824) with the sealed end against the rim. The blood was centrifuged at 12000 rpm for 10 minutes in the microhaematocrit centrifuge. Immediately after centrifuging, the PCV was read from microhaematocrit reader (Baker *et al.* 1998).

3.9.3 Red blood cell count

RBC count was determined by the method described by Baker *et al.* (1998). Hayem's solution was drawn into a standard red cell pipette up to the 101 mark as a diluent, and then transferred to a small test tube. About 0.02 ml of blood was drawn with capillary tube and emptied into the test tube containing the diluent. The tube was then shaken for proper mixture. A drop of red-cell suspension was thereafter transferred to a counting chamber and then counted under the light microscope (x 40) (Baker *et al.* 1998).

3.9.4 White blood cell count

About 0.38 ml of diluting fluid was dispensed into a small test tube. Also, 0.02ml of well mixed EDTA anticoagulated blood was added to the diluting fluid in the test tube.

The diluted sample was then mixed and loaded into the counting chamber. Using the improve Neubauer Chamber, the white cells present in the 4 corner 1mm2 areas and those in the central $1mm^2$ were counted under light microscope (x 40) (Baker *et al.* 1998).

3.9.5 Differential white blood cell count

Thin blood film was prepared, air dried and fixed in Giemsa stains for 3 minutes. One volume of Giemsa stain was diluted with nine volumes of buffer; the slide was flooded and stained, then allowed to act for 15 minutes. The slides were washed and differentiated with buffer, drained and dried in the air. The back of the slide was cleaned and examined under light microscope (x 40) (Baker *et al.* 1998).

3.9.6 Haemoglobin determination

Blood sample (0.02 ml) was emptied into 2 milliliters of ammonia diluting fluid and mixed. The solution was read immediately. The colour was stable for 6-8 hours. The blood was transferred to a clean 10 mm light-path cuvette. The cuvette was placed on the cuvette holder, until the audible signal was made by the Digital Haemoglobinometer. Then, the hemoglobin values were read from the display (Baker *et al.* 1998).

3.9.7 Calculation of red cell indices

The value of PCV, Hb and RBC count was used to calculate the MCHC, MCH and MCV using the formula below:

MCH = Hb/PCV (g/dl)
MCHC = Hb/RBC (pg)
MCV = PCV/RBC (fl)

3.10 Animal sacrifice and tissue preparation

The rats for the acute study were sacrificed on day 4 while rats for delayed post-acute study were sacrificed on day 15 by cervical dislocations. The skulls were dissected and the whole brain tissues were excised. A measured part (0.1 g) from the individual brain tissues for enzyme study was homogenized in 0.4 ml of 5 % sucrose solution, after which they were centrifuged for 10 minutes at 3000 rpm. The supernatant gotten from the brain tissues were kept frozen in the freezer at -4^{o}C. The supernatant was later used for SOD, CAT, GSH, MDA, and NO analyses.

3.10.1 Malondialdehyde

Procedure

The concentration of MDA was quantified according to the method of Beuge and Aust (1978) as outlined below;

A portion of thiobarbituric acid reagent (2ml of 0.7% and 1ml of TCA) were added to 2ml of the sample. The mixture was thoroughly heated in a water bath at 100^{o}C for 20 minutes. It was cooled and centrifuged at 4000 rpm for 10 minutes.

The absorbance of the supernatant was read at a wavelength of 540nm against the reference blank of distilled H_20 after centrifuging for another 10 minutes.

$$\text{Concentration of MDA} = \frac{\text{Absorbance}}{\text{Extinction coefficient}}$$

Extinction coefficient of MDA= $1.56\times10^{3}nm^{-1}cm^{-1}$. TBA: 0.7% i.e 0.7g in 100mls. TCA: 20% i.e 2g in 100mls (Bagchi and Puri, 1998).

3.10.2 Nitric oxide (NO)

Procedure

Aromatic diamino compound 2, 3-diaminonaphthalene (DAN) was employed as an indicator of NO formation (Miles *et al*., 1996). The relatively non-fluorescent DAN reacts rapidly with N_2O_3 generated from acidified nitrite (nitrous acid) or from the interaction of NO with oxygen to yield the highly fluorescent product 2, 3-naphthotriazole. This assay is used to quantify NO generated under biologically relevant conditions (e.g. neutral pH) with minimal interference by nitrite decomposition (Miles *et al*., 1996).

3.10.3 Superoxide dismutase

Procedure

An aliquot of 0.2 ml of each of the tissue homogenates was added to 2.5 ml of 0.05 carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. A measured volume of 0.3 ml of freshly prepared 0.3 mM epinephrine was then added to the mixture. Spectrophotometer was thereafter employed to measure the absorbance of the sample at 450 nm.

- Change in ab/ min = A5-A1/2.5
- % inhibition increase in abs of sample/increase in abs of blank x100

- I unit sod = amount that cause 50% inhibition

3.10.4 Catalase

Procedure

To 0.4ml of H_2O_2 (0.2 M) was added to 1ml of 0.0 1M phosphate buffer (pH 7) followed by the addition of 0.1ml clear supernatant of the tissue homogenate (10 %w/v) and gently swirled at room temperature. The reaction of the mixture was stopped by adding 2 ml dichromate-acetic acid reagent (5% $K_2CR_2O_7$ prepared in Glacial acetic acid). The changes in the absorbance was measured at 620nm and recorded after 3 minutes' interval (Chelikani *et al.,* 2004).

Percentage inhibition was calculated using the equation

$$\% \text{ inhibition rate} = \frac{\text{absorbance of blank} - \text{absorbance of sample}}{\text{absorbance of blank}} \times 100$$

3.10.5 Glutathione

Procedure

The general thiol reagent, 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's Reagent) was made to react with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TMB molecule and recycling the GSH; thus, amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH (Tietze, 1969).

3.11 Histological study

3.11.1 Sample preparation for histological and immunohistochemical studies

The animals were sacrificed by cervical dislocations. The skulls were dissected and the whole brain tissues were removed, thereafter, the cerebellum and the hippocampus were sectioned out and preserved in 10% neutral buffered formalin (NBF). The brain tissues were passed through the process of fixation, dehydration, clearing, infiltration, embedding, sectioning and staining.

Fixation was carried out in 10% formalin and then transferred to 50%, 70%, 90% and two changes of absolute alcohol for one hour change each. Clearing of tissue was done in xylene. Infiltration was made in four changes of molten paraffin wax at 60^{o}C for 45

minutes each. The tissues were embedded in embedding moulds. When the paraffin wax was cooled, it sets as hard block, and were trimmed with a hot knife. Serial sections of 5 microns thick were made using rotary microtome. The cut sections were then floated in warm water bath at temperature of 30^{o}C - 40^{o}C and were placed on glass slides. They were finally stained with Haematoxylin and Eosin (Bancroft and Gamble, 2008).

3.11.2 Haematoxylin and Eosin (H and E)

This was employed to study the general morphology of the HP and CB.

Procedures for haematoxylin and eosin stain as described by Bancroft and Gamble (2008) was adopted. Cut sections were dewaxed in two changes of xylene for 3 minutes each, hydrated in descending grades of alcohol (90%, 70%, 50%), rinsed in distilled water, stained in haematoxylin for 15 minutes, washed in running tap water for 3 minutes, differentiated in 1% acid in 70% alcohol for few seconds, blued in running tap water for 15 minutes, stained in 1% alcoholic Eosin for 5 minutes, dehydrated rapidly in ascending grades of alcohol, cleared in xylene, mounted in distrene plasticizer xylene (DPX) and covered using clear coverslips.

3.12 Immunohistochemical study

3.12.1 Glial Fibrillary Acid Protein (GFAP)

This staining method was employed to demonstrate the expression of astrocytic glial cells around the HP and CB.

Manual immunostain procedure

Procedure for manual immunochemistry (IHC) as described by Delcambre *et al*. (2016) was adopted. Procedures for all protocols include;

1. Sectioning from formalin fixed paraffin embedding (FFPE) tissues at 3 μm and placing them on positively charged glass slides.
2. The slides were soaked in xylene three times for 5 min to remove paraffin.
3. These sections were then rehydrated through a gradient of ethanol for 5 min in each concentration, 100%, 100%, 95%, and 70% ethanol, followed by de-ionized water.
4. Tissues sections were encircled with a hydrophobic barrier pen (in order to reduce the volume of the reagents tested and liquid loss).
5. Antigen unmasking was done using the pressure cooker method of heating slides for investigating heat induced epitope retrieval (HIER) effectiveness for 10

minutes.

6. Slides were rinsed in buffer.
7. Tissues were immersed in endogenous peroxidase blocking solution (3% H_2O_2) for 5 min followed by two rinses in PBS for 5 minutes each.
8. Protein blocking reagents were applied for 20 minutes with the exception of Novolink Protein block for only 5 min.
9. Blocking reagents were removed without rinsing before adding primary antibody.
10. Primary antibody was applied in a volume of 300µl, 1:200 made in Dako Antibody Diluent (Antibodies were applied in a 37ºC, humidified incubator/chamber) for 60 minutes and were washed in 200-250 ml PBS in a glass jar twice for 5 minutes each time.
11. Antibody enhancer was applied for 10 minutes.
12. Slide were rinsed in buffer for 1 minute.
13. Polymer was applied for 15 minutes
14. Slides were rinsed in buffer for 2 minutes
15. Signal developed was applied in approximately 300 µl DAB Plus for 1-2 min.
16. Slides were rinsed in 200-250 ml ddH2O in a glass jar for 1 min.
17. Slides were counterstained in 200-250 ml 10% Harris Hematoxylin (wt/vol) in distilled water for 3 min.
18. Slides were rinsed in 200-250 ml tap water (TH_2O) in a glass jar for 1 min.
19. Slides were blued in 200-250 ml ammonia water in a glass jar for 5 min.
20. Slides were rinsed in 200-250 ml H_2O in a glass jar for 1 min.
21. Slides were dehydrated for 1 min in 200-250 ml following series of washes in a glass jar: 70% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 100% ethanol,
22. Slides were cleared in two changes of xylene
23. Slides were mounted with 2-3 drops of mounting media

3.12.2 Cyclo-oxygenase-2 (COX-2)

It was used do demonstrate inflammation if any, in the neurons of the HP and CB.

Staining Procedure

This was achieved by the procedure for manual immunochemistry (IHC) as described by Delcambre *et al.* (2016) (as in section 3.11.1).

3.12.3 Inducible Nitric-Oxide Synthase (iNOS)

This was used to express the level of OS through the release of free radical's reactive nitric oxide species (RNOS) after administration of artesunate and amodiaquine.

Procedure:

This was achieved by the procedure for manual immunochemistry (IHC) as described by Delcambre, *et al.* (2016) (as in section 3.11.1).

3.13 Histological and Immunohistochemical Photomicrography

The slides with stained sections were viewed and photographed with an Olympus U-D03 light microscope captured with Olympus DP21 (Olympus CH, Japan) at magnification (x400). The photomicrographs obtained were also adequately reported.

3.14 Cell population count and quantification of immunoreaction

Cell population and immunoreactions from histological and immunohistochemical photomicrographs respectively were quantified using Image J software (Broken symmetry software, version 1.4.3.67).

3.15 Statistical Analysis

Data from the body and brain weights, neurobehavioral, haematological, biochemical, histological and immunohistochemical studies were analysed statistically by comparing the values obtained from the treatment groups with that of the control group by one-way analysis of variance (ANOVA) using Graphpad prism statistical package software (San Diego, California, USA) version 7.02. Tukey's post-hoc multiple comparison was also conducted in order to find the level of significance at 95% confidence interval ($\alpha = 0.05$). All values were presented as Mean ± standard error of mean (SEM) and $\alpha_{0.05}$ was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 General observations

Majority of the animals in the AS, AQ and AS+AQ groups became less active and slept off after receiving the drugs. There was however no record of mortality in any of the groups.

4.2 Weight results of the experimental animals

4.2.1 Body weights of the animals for the acute study

The result of the progressive body weight change of the animals shows that there was a percentage increase in the body weights of all the groups of animals. The CT group had the highest percentage increase, followed by the AS+AQ group, followed by the AS group while the AQ group had the lowest percentage increase. There are however no significant difference in the weight difference across groups (see Table 4.1).

Table 4.1. Body weights differences of the animals for the acute study

Groups	Initial average weight (g)	Final average weight (g)	Weight difference (g)	Percentage Weight difference (%)
CT	199.10±5.49	214.60±6.62	15.50±3.90	7.20↑
AS	196.20±6.39	206.20±6.22	10.00±3.03	4.90↑
AQ	191.00±2.16	197.40±3.43	6.40±2.52	3.20↑
AS+AQ	171.90±4.01	184.90±3.19	13.00±3.00	7.00↑

Values are presented as Mean±SEM. n = 10 at $\alpha_{0.05.}$↑ = % Weight increase. CT - Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.2.2 Body weights of the animals for the delayed post-acute study

It was observed that there was percentage increase in weight of the control (CT), combination (AS+AQ) and the artesunate (AS) groups. Statistical analysis by one-way ANOVA showed that AS group had an increase significantly lower than the CT but not significantly different from the AS+AQ. AS+AQ group had a percentage weight increase not substantially different from the CT group while only the amodiaquine (AQ) group showed significant weight decrease compared with CT and the other two experimental groups (AS and AS+AQ) (see Table 4.2).

Table 4.2. Body weights differences of the animals for the delayed post-acute study

Groups	Initial average weight (g)	Final average weight (g)	Weight difference (g)	Percentage Weight difference
CT	110.00±3.06	149.60±6.09	39.60±5.38	36.00↑
AS	168.00±2.15	176.00±11.23	8.00±9.28*	4.50↑*
AQ	180.00±3.27	125.30±8.48	-54.70±8.32**	-30.40↓**
AS+AQ	138.00±2.16	156.60±6.12	18.60±6.53	13.50↑

Values are expressed as Mean ± SEM. n= 10 at $\alpha_{0.05}$. CT-Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine, ↑= % weight gain, ↓=% weight loss, *indicates increase significantly lesser than CT, ** indicates significant decrease compared to the CT.

4.2.3 Brain weights of the animals for the acute study

The result of the brain weight of the animals showed that the AS+AQ group had the highest brain weight, followed by the AQ group, followed by the CT group while the AS group had the lowest brain weight. However, statistical analysis by one- way ANOVA showed no substantial differences in the brain weights across all the groups of animals when compared (see Table 4.3).

Table 4.3. Brain weights of the animals for the acute study

Group	Brain Weight (g)
CT	1.72±0.04
AS	1.69±0.05
AQ	1.76±0.10
AS+AQ	1.78±0.06

Values are presented as Mean±SEM. n = 10 at $\alpha_{0.05.}$

CT - Control, AS - Artesunate, AQ - Amodiaquine,

AS+AQ - Artesunate+Amodiaquine.

4.2.4 Brain weights of the animals for the delayed post-acute study

Result shows that the artesunate group (AS) had the highest average wet brain weight, followed by the control (CT), followed by the combination group (AS+AQ) while the amodiaquine (AQ) had the lowest mean wet brain weight. However, statistical analysis by one-way ANOVA showed no substantial variation between the brain weights between all the groups [P_{cal} (AS vs CT) = 0.96; $\alpha_{0.05} > 0.05$], P_{cal} (AQ vs CT) = 0.52; $\alpha_{0.05} > 0.05$], P_{cal} (AS+AQ vs CT) = 0.81; $\alpha_{0.05} > 0.05$], [P_{cal} (AS vs AS+AQ) = 0.63; $\alpha_{0.05} > 0.05$]. (see Figure 4.1).

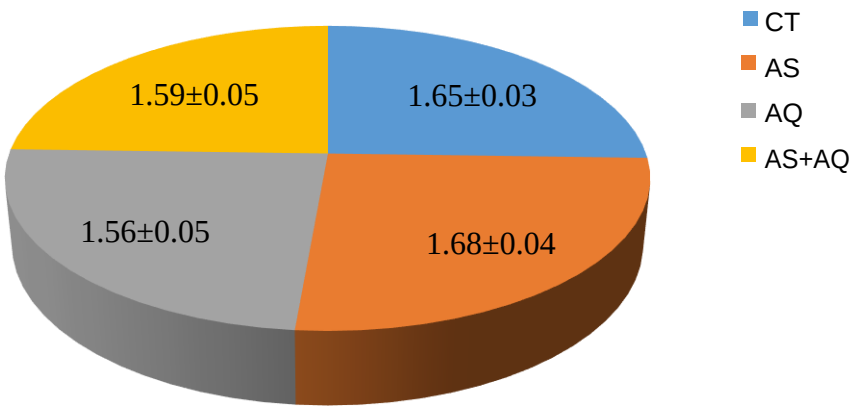


Figure 4.1: Brain Weights of the Wistar rats for the delayed post-acute study. Values are expressed as Mean ± SEM. n = 10 at $\alpha_{0.05}$. CT - Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.2.5 Organ body weight ratio of the animals

4.2.5.1 Brain to body weight index of the acute study

Table 4.4 shows that the brain-body weight ratio of the animals varied higher in the experimental groups than in the control group. The order of variation is AS>AQ>AS+AQ>CT. However, statistical analysis by one-way ANOVA revealed no substantial differences between the groups ($\alpha_{0.05}$>0.05).

Table 4.4: Brain to Body Weight Index of the acute study

Groups	Final average body weight	Average brain weight	Brain-body weight index (%)
CT	196.20±20.21	1.72±0.04	0.88
AS	171.90±12.69	1.69±0.05	0.98
AQ	191.00±6.83	1.76±0.10	0.92
AS+AQ	199.10±17.39	1.78±0.06	0.89

Values are expressed as Mean±SEM. n = 10 at $\alpha_{0.05.}$ CT- Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.2.5.2 Brain to body weight index of the delayed post-acute study

Table 4.5 revealed the brain-body weight ratio of the animals following sub-acute treatment. From the Table, AQ group had the highest brain-body weight ratio, followed by the CT group, followed by AS+AQ group while the AS group had the lowest. Only the AQ group had a brain-body weight ratio higher than the CT value. However, statistical analysis by one-way ANOVA revealed no substantial differences between the groups ($\alpha_{0.05}$>0.05).

Table 4.5. Brain to Body Weight index of the delayed post-acute study

Groups	Final mean body weight (g)	Mean brain weight (g)	Brain-body weight index (%)
CT	149.60±6.09	1.65±0.03	1.10
AS	176.00±11.23	1.68±0.04	0.95
AQ	125.30±8.48	1.56±0.05	1.25
AS+AQ	156.60±6.12	1.59±0.05	1.02

Values are expressed as Mean ± SEM. N = 10 at $\alpha_{0.05}$. CT-Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ-Artesunate+Amodiaquine.

4.3 Result of Neurobehavioural studies of the Animals

4.3.1 Neurobehavioural results of the acute study

Table 4.6 shows the result of the neurobehavioural study following acute treatment. There was increase in swimming time (escape latency) in the AQ and AS+AQ groups compared with the CT group but this was not statistically significant with Tukey's post-hoc multiple comparisons (P_{cal} = 0.99 & 0.87; $\alpha_{0.05}$ > 0.05). There was decrease in the AS group compared with CT group which was also not statistically significant (P_{cal} = 0.99; $\alpha_{0.05}$ > 0.05). However, comparison between each of AQ and AS+AQ groups with the AS group revealed statistically significant increase (P_{cal} = 0.04 & 0.01; $\alpha_{0.05}$ < 0.05). Results of the swimming distance, quadrant crossing and forearm grip showed no significant variation between all the groups.

Table 4.6. Neurobehavioural results of the acute study

	Group			
Neurobehaviour	CT	AS	AQ	AS+AQ
Swim. Time (s)	6.30±0.52	4.70±0.60	6.50±0.34[#]	6.80±0.33[#]
Swim. Dist. (m)	302.70±11.95	285.10±14.08	300.50±13.52	287.50±14.07
Quadrant Cross.	6.40±0.50	6.60±0.50	5.70±0.50	5.60±0.40
Forearm Grip (s)	7.20±4.90	6.60±0.50	6.90±0.60	6.60±0.62

Values are presented as Mean±SEM. n = 10 at $\alpha_{0.05.}$ CT - Control (Distilled water), AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine. [#] Significant increase compared with the AS group.

4.3.2 Neurobehavioural results of the delayed post-acute study

Results of post-acute study showed that the swimming time of the AQ group (22.86±2.70) was substantially higher than that of the CT (4.14±0.74) and the other two experimental groups. The AS (6.86±0.74) and the AS+AQ (6.71±1.11) groups also had a higher swimming time but not significantly different from the CT. [P_{cal}(AS vs AQ) = 0.000($\alpha_{0.05}$<0.05); P_{cal}(AS vs AS+AQ) = 1.000($\alpha_{0.05}$>0.05); P_{cal}(AQ vs AS+AQ) = 0.000($\alpha_{0.05}$<0.05); P_{cal}(AS vs CT) = 0.565($\alpha_{0.05}$>0.05); P_{cal}(AQ vs CT) = 0.000($\alpha_{0.05}$<0.05); and P_{cal}(AS+AQ vs CT) = 0.607($\alpha_{0.05}$>0.05) (see Table 4.7).

Analyses of the swimming distance revealed that the AQ group (974.3±46.16) and AS+AQ (316.0±8.97) covered a substantially higher distance compared with the CT group (175.4±31.59). Also, the AS (280.7±31.80) group had a higher swimming distance but not significantly different compared with the CT group. P_{cal} (AS vs AQ) = 0.000($\alpha_{0.05}$ < 0.05); P_{cal} (AS vs AS+AQ) = 0.868 ($\alpha_{0.05}$ > 0.05); P_{cal} (AQ vs AS+AQ) = 0.000 ($\alpha_{0.05}$ < 0.05); P_{cal} (AS vs CT) = 0.128($\alpha_{0.05}$ > 0.05); P_{cal} (AQ vs CT) = 0.000 ($\alpha_{0.05}$ < 0.05); and P_{cal} (AS+AQ vs CT) = 0.026 ($\alpha_{0.05}$ < 0.05).

The CT group had the highest frequency of quadrant entry (6.71±0.42). The order of variation is CT>AS+AQ>AQ>AS. However, there were no substantial variations between the experimental groups when compared with the control group. P_{cal} (AS vs CT) = 0.35 ($\alpha_{0.05}$ > 0.05); P_{cal} (AQ vs CT) = 0.67 ($\alpha_{0.05}$ > 0.05); P_{cal}(AS+AQ vs CT) = 0.87($\alpha_{0.05}$ > 0.05); P_{cal} (AS vs AS+AQ) = 0.87($\alpha_{0.05}$ > 0.05); P_{cal} (AQ vs AS+AQ) = 0.99($\alpha_{0.05}$ > 0.05) and [P_{cal}(AS vs AQ) = 0.94 ($\alpha_{0.05}$ > 0.05) (see Table 4.7).

Result of the fore-limb grip strength (motor function) revealed that the AS (8.00±1.11) and AQ (7.43±0.92) groups had statistical significant decrease in grip time when compared with the CT group (17.86±1.22) while the AS+AQ group (15.43±1.17) had a reduced grip time but not significantly different from the CT (17.86±1.22). [P_{cal} (AS vs CT) = 0.00; $\alpha_{0.05}$ < 0.05], [P_{cal} (AQ vs CT) = 0.00; $\alpha_{0.05}$ < 0.05], [P_{cal} (AS vs AS+AQ) = 0.00; $\alpha_{0.05}$ < 0.05], [P_{cal} (AQ vs AS+AQ) = 0.00; $\alpha_{0.05}$ < 0.05], [P_{cal} (AS+AQ vs CT) = 0.43; $\alpha_{0.05}$ > 0.05] and [P_{cal} (AS vs AQ) = 0.98; $\alpha_{0.05}$ > 0.05] (see Table 4.7).

Table 4.7. Neurobehavioural results of the delayed post-acute study

Behaviour/Group	CT	AS	AQ	AS+AQ
Swim. Time (s)	4.14±0.74	6.86±0.74	22.86±2.70***	6.71±0.42
Swim. Dist. (m)	175.0±31.80	280.70±31.80	974.30±46.16***	316.00±8.97*
Quadrant Cross.	6.71±0.42	5.43±0.53	5.86±0.51	6.00±0.65
Forearm Grip (s)	17.86±1.22	8.00±1.11###	7.43±0.92###	15.43±1.17

Values are presented as Mean±SEM. n = 10 at $\alpha_{0.05.}$ CT - Control (Distilled water), AS- Artesunate, AQ- Amodiaquine, AS+AQ-Artesunate+Amodiaquine. *** P<0.001 significant increase compared with the CT and other groups. * P<0.05 significant increase compared with the CT. ### P<0.001 significant decrease compared with the CT and other groups.

4.4 Results of Haematological Profiles of the Animals

4.4.1 Results of Haematological Profiles of the acute study

The level of RBC in the AQ group varied slightly higher compared with the CT group while that of AS and AS+AQ groups varied slightly lower compared with the CT group. There is however no statistical substantial difference between the groups ($P_{cals} > 0.05$). Also, the level of WBC in the three experimental groups (AS, AQ and AS+AQ) decreased slightly compared with the CT group but there was no statistically substantial variation between the groups ($P_{cals} > 0.05$). The level of platelets and haemoglobin concentration did not vary substantially between the control and the experimental groups. PCV level was highest in the CT group, followed by AS group, followed by AQ group while the AS+AQ group had the lowest level. Analyses of the variations however revealed no statistical significant difference ($P_{cals} > 0.05$) between all groups. (See Table 4.8).

The order of variation of neutrophil count is AS > CT > AQ > AS+AQ but there was no statistically substantial variation within and between the experimental groups and the control group. The MCV, MCH and MCHC in the experimental groups did not vary significantly from the control. Also, AS group had the highest level of lymphocytes followed by AQ, followed by AS+AQ while the CT group had the lowest. However, there was no significant difference between the groups ($P_{cals} > 0.05$) (see Table 4.8).

Table 4.8. Haematological Profiles of the acute study

B. P	CT	AS	AQ	AS+AQ
RBC (10^6/µL)	7.20±2.09	6.99±2.55	7.45±2.06	7.08±1.01
WBC (10^3/µL)	3.40±1.18	3.40±1.46	3.50±1.12	3.40±1.26
PLAT (10^3/µL)	9.52±29.34	9.37±29.52	9.13±28.28	9.29±28..36
Hb (g/dl)	14.55±1.35	14.87±1.52	14.95±1.76	14.66±1.54
PCV (%)	50.80±1.11	50.60±1.72	49.00±1.52	47.60±2.11
MCV (fl)	77.09±1.35	76.08±1.65	67.02±1.43	65.09±1.92
MCH (g/dl)	28.65±0.11	27.42±0.31	30.52±0.52	29.55±0.25
MCHC (pg)	21.65±0.36	20.87±0.58	21.95±0.62	20.76±0.55
NEU (%)	2.77±0.27	2.83±0.30	2.71±0.23	2.43±0.27
BAS (%)	0.35±0.40	0.25±0.30	0.250±0.20	0.30±0.21
EOS (%)	0.04±0.01	0.04±0.00	0.04±0.00	0.04±0.00
MON (%)	0.00±0.00	0.20±0.15	0.30±0.00	0.00±0.20
LYM (%)	6.66±0.43	7.53±0.21	7.40±0.67	6.67±0.38

Values are expressed as Mean ± SEM. n= 10 at $\alpha_{0.05}$. B.P - blood parameters, CT: Control, AS: Artesunate, AQ: Amodiaquine, AS+AQ: Artesunate+Amodiaquine. RBC: red blood cell, WBC: white blood cell, PLAT: platelet, Hb: haemoglobin, PCV: packed cell volume, MCV: Mean cell volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Heamoglobin Concentration, NEU: Neutrophil, BAS: Basophil, EOS: Eosinophil, MON: Monocyte, LYM: Lymphocyte.

4.4.2 Results of Haematological Profiles of the delayed post-acute study

For the delayed post-acute study, the RBC count increased in the three experimental groups (AS, AQ and AS+AQ) compared with the control (CT) group. However, only the AQ group had statistically substantial increase than the CT ($P_{cal} = 0.01$; $\alpha_{0.05} < 0.05$) when subjected to statistical analysis by one-way ANOVA. Similarly, the levels of WBC varied higher in AS and AQ groups but lower in AS+AQ group than the CT group. However, only the AQ group had a statistical substantial increase than the CT ($P_{cal} = 0.00$; $\alpha_{0.05} < 0.05$). Platelets levels varied higher in AS and AS+AQ groups but lower in AQ group than the CT group but these variations were not statistically significant (see Table 4.9).

Haemoglobin level varied significantly higher in the AQ group than in the CT ($P_{cal} = 0.01$; $\alpha_{0.05} < 0.05$) and AS group. It was also higher in the AS and AS+AQ groups than the CT group but the differences were not statistically significant. Packed Cell Volume level varied higher in all the experimental groups than the control but only the AQ group showed statistical significant increase ($P_{cal} = 0.00$; $\alpha_{0.05} < 0.05$) than the CT group. Mean Cell Volume varied lower in the AS but higher in AQ and AS+AQ groups than the CT group but these variations were not statistically significant. Mean Corpuscular Haemoglobin varied lesser in AS, AQ and AS+AQ groups than the CT group but these variations were statistically insignificant. The variation in Mean Corpuscular Heamoglobin Concentration between the treated groups and the control group was not substantial but the MCHC in AQ group varied significantly lower than the AS group. Neutrophil level varied substantially higher in AQ group than the CT and all other experimental groups while lymphocyte level varied substantially lesser in AQ group than the CT and other experimental groups. However, there were no statistically substantial variations in basophil, eosinophil and monocytes between each of the experimental groups and the CT group and within the experimental groups (see Table 4.9).

Table 4.9. Haematological Profiles of the delayed post-acute study

B. P	CT	AS	AQ	AS+AQ
RBC (10^6/µL)	7.90±0.31	7.00±0.33	9.30±0.13*	7.40±0.22
WBC(10^3/µL)	9.10±0.90	10.40±1.42	18.90±1.99*	8.00±0.59
PLAT(10^3/µL)	6.54±43.48	6.68±86.14	6.47±75.17	7.13±67.16
Hb (g/dl)	13.60±0.33	13.90±0.43	16.00±0.37*	14.70±0.55
PCV (%)	45.80±1.36	46.20±1.36	57.00±1.30*	50.80±1.36
MCV (fl)	66.80±1.56	65.60±1.57	67.60±0.51	67.80±1.16
MCH (g/dl)	19.20±0.37	18.80±0.58	18.80±0.37	19.20±0.20
MCHC (pg)	29.00±0.45	29.20±0.50	27.80±0.37[b]	29.00±0.32
NEU (%)	14.00±2.53	24.00±4.56	49.20±8.65*	16.60±5.76
BAS (%)	0.20±0.20	0.00±0.00	0.00±0.00	0.00±0.00
EOS (%)	0.60±0.40	1.20±0.37	1.40±.098	0.80±0.58
MON (%)	0.00±0.00	0.40±0.25	0.00±0.00	0.40±0.40
LYM (%)	85.00±2.53	74.40±4.80	55.00±9.71#	82.20±6.05

Values are expressed as Mean ± SEM. n = 10 at $\alpha_{0.05}$. B.P - Blood Parameter, CT: Control, AS: Artesunate, AQ: Amodiaquine, AS+AQ - Artesunate+Amodiaquine. * significant increase compared with the control. # significant decrease compared with the control. [b] significant decrease compared with the AS group. RBC: red blood cell, WBC: white blood cell, PLAT: platelet, Hb: haemoglobin, PCV: packed cell volume, MCV: Mean cell volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Heamoglobin Concentration, NEU: Neutrophil, BAS: Basophil, EOS: Eosinophil, MON: Monocyte, LYM: Lymphocyte.

4.5 Results of Biochemical Indices of the Animals

4.5.1 Results of Biochemical Indices of the acute study

With reference to Table 4.10, the mean level of MDA was significantly greater in the AS group than the CT ($P_{cal} = 0.02$; $\alpha_{0.05}<0.05$) and the other two experimental groups. The mean level of MDA was also higher in the AQ and AS+AQ groups than the CT group but these variations were not statistically significant. The mean level of NO was lower in the three treated groups than the CT group but not statistically significant.

Also, the mean level of SOD increased in AS group than in CT and the other two experimental groups while the level decreased in the AQ and AS+AQ groups than the CT but there were no statistically substantial changes among the groups. The same trend was also seen with CAT. The GSH mean levels were slightly higher in the AQ and AS+AQ groups compared with the CT while the level was slightly lower in the AS group compared with the CT group but these variations were not statistically substantial.

Table 4.10. Results of Biochemical Indices of the acute study

Biochemical Index	Group			
	CT	**AS**	**AQ**	**AS+AQ**
MDA (nmmol/g)	4.14±0.45	7.12±0.88*****	6.40±0.36	4.87±0.68
NO (µm/mg)	55.62±1.59	49.45±1.22	51.02±3.49	51.27±2.89
SOD (U/mg)	5.45±0.58	6.84±0.51	4.28±1.07	4.00±0.67
CAT (µm/min/mg)	2.37±0.24	2.81±0.19	2.24±0.28	2.24±0.13
GSH (U/L)	5.36±7.43	5.03±3.23	5.56±4.05	5.76±4.30

Values are expressed as Mean ± SEM. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ-Artesunate+Amodiaquine. * Significantly different from the control. MDA: malodialdehyde, NO: nitric oxide, SOD: superoxide dismutase, CAT: catalase, GSH: glutathione.

4.5.2 Results of Biochemical Indices of the delayed post-acute study

Result of delayed post-acute showed that MDA level increased significantly in all the experimental groups compared to the CT group. The MDA level also increased significantly in the AQ group compared with the AS and AS+AQ groups. Nitric oxide level was highest in AQ group, followed by AS+AQ group, followed by AS group, while the CT group had the lowest. The level varied significantly higher in the AQ and AS+AQ groups compared with the CT group. AS vs AQ [P_{cal} (AS vs AQ) = 0.00]; AQ Vs AS+AQ [P_{cal} (AQ vs AS+AQ) = 0.00]; and AQ vs CT [P_{cal} (AQ vs CT) = 0.00]; and between AS+AQ vs CT [P_{cal}(AS+AQ vs CT) = 0.03]; All P_{cal} values < 0.05] while there were no statistical substantial variations between AS vs AS+AQ groups (P_{cal} = 0.71; P > 0.05) and between AS vs CT groups (P_{cal} = 0.19; P > 0.05) (see Table 4.11).

Table 4.11 also revealed that the level of tissue SOD was lowest in the AS group followed by AQ group, followed by AS+AQ while the CT group had the highest. There was statistical substantial variation between the AS group and AQ group (P_{cal} = 0.00; P < 0.05) and AS vs AS+AQ groups (P_{cal} = 0.00; P < 0.05), while each of the three experimental groups (AS, AQ and AS+AQ) also varied significantly lower than the CT group (P_{cal} = 0.00; P<0.05). There was however no statistical substantial change between AQ and the AS+AQ groups (P_{cal} = 0.27; P > 0.05). From the same table, the tissue catalase level was observed to be substantially lower compared to the control in all the three experimental groups. There were statistical substantial variations between AS Vs AQ (P_{cal} = 0.02; P < 0.05), AS Vs CT (P_{cal} = 0.00; P < 0.05), AQ VS CT (P_{cal} = 0.00; P < 0.05) and AS+AQ Vs CT (P_{cal} = 0.00; P < 0.05). There was however no statistical substantial variation between AS and AS+AQ groups (P_{cal} = 0.15; P > 0.05).

The level of GSH varied significantly lower in the AS, AQ and AS+AQ groups compared with the CT group. The order of variation is CT > AS > AS+AQ > AQ. Analysis of variance revealed statistical substantial variation between AS Vs CT groups (P_{cal} = 0.00; P < 0.05), AQ Vs CT groups (P_{cal} = 0.00; P < 0.05) and AS+AQ Vs CT groups (P_{cal} = 0.000; P < 0.05) levels of tissue glutathione but there was no statistical substantial variation between the AS versus AS+AQ groups (P_{cal} = 0.99; P > 0.05).

Table 4.11. Results of Biochemical Indices of the delayed post-acute study

Biochemical index	Group			
	CT	**AS**	**AQ**	**AS+AQ**
MDA (nmmol/g)	18.33±0.47	23.07±2.26#	25.14±2.06#	23.32±1.99#
NO (μm/mg)	45.49±3.93	47.32±0.91	52.74±10.11#	48.95±2.917#
SOD (U/mg)	9.89±.098	5.70±0.16*	7.20±0.11*	7.54±0.13*
CAT (μm/min/mg)	10.55±1.18	6.59±1.54*	7.60±1.80*	6.01±1.07*
GSH (U/L)	11.15±0.32	7.16±0.09*	5.80±0.15*	7.08±0.25*

Values are expressed as Mean ± SEM. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * Significant decrease compared with the control. # Significant increase compared with the CT. MDA: malodialdehyde, NO: nitric oxide, SOD: superoxide dismutase, CAT: catalase, GSH: glutathione.

4.6. Results of Histological and Immunohistochemical analysis of the animals

4.6.1 H & E Result of the Cerebellum (acute)

Statistical analysis of the cerebellum photomicrograph (acute) as shown in Plate 1 and Figure 4.2 revealed that there was no statistical substantial variation in the number of viable Purkinje cells between the AS, AQ and AS+AQ experimental groups and the CT group. All cells appeared morphologically normal in both the control and the treated groups.

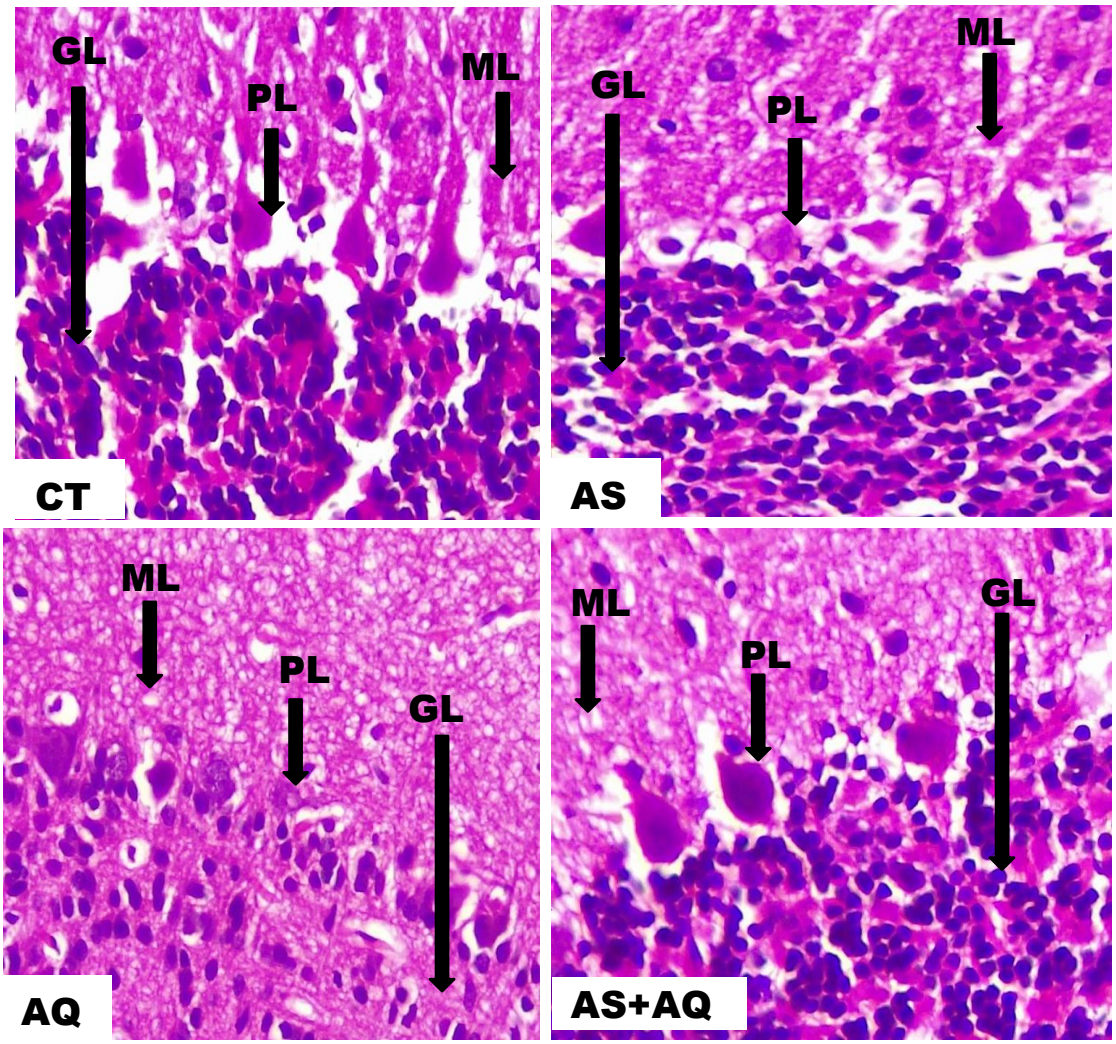


Plate 1. H & E Section of the Cerebellum of the Wistar rats (acute study). (X400). AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine, CT - Control, ML = Molecular layer; PL = Purkinje cell layer; GL = Granular layer.

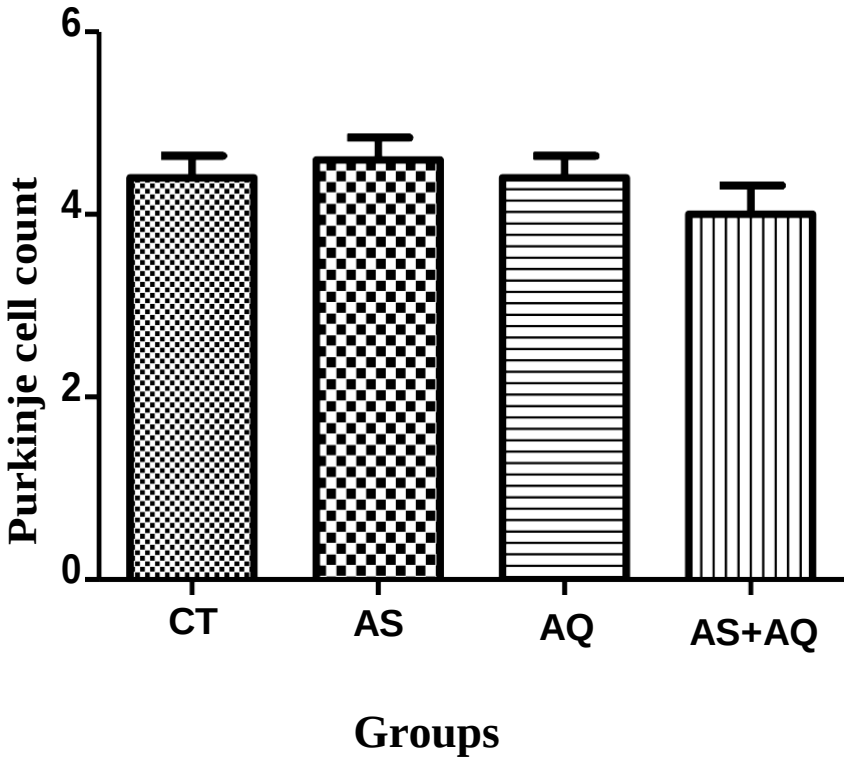


Figure 4.2. Cerebellar Purkinje Cell Count for the acute study. Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT - Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.2 H & E Result of the Cerebellum (delayed post-acute)

Statistical analysis of the cerebellum photomicrograph following delayed post-acute period revealed that there was statistically substantial decrease in the population of viable Purkinje cells in the AS (2.50±0.29) and AQ (1.25±0.25) separate groups compared to the CT group (5.75±0.48) but the AS+AQ group was not significantly different from the CT group (Plate 2 and Figure 4.3). Close observation also revealed pyknotic Purkinje cells in the AS & AQ groups compared with the CT and AS+AQ. The Granular cell layer of the AQ group also appeared denuded compared to the other groups.

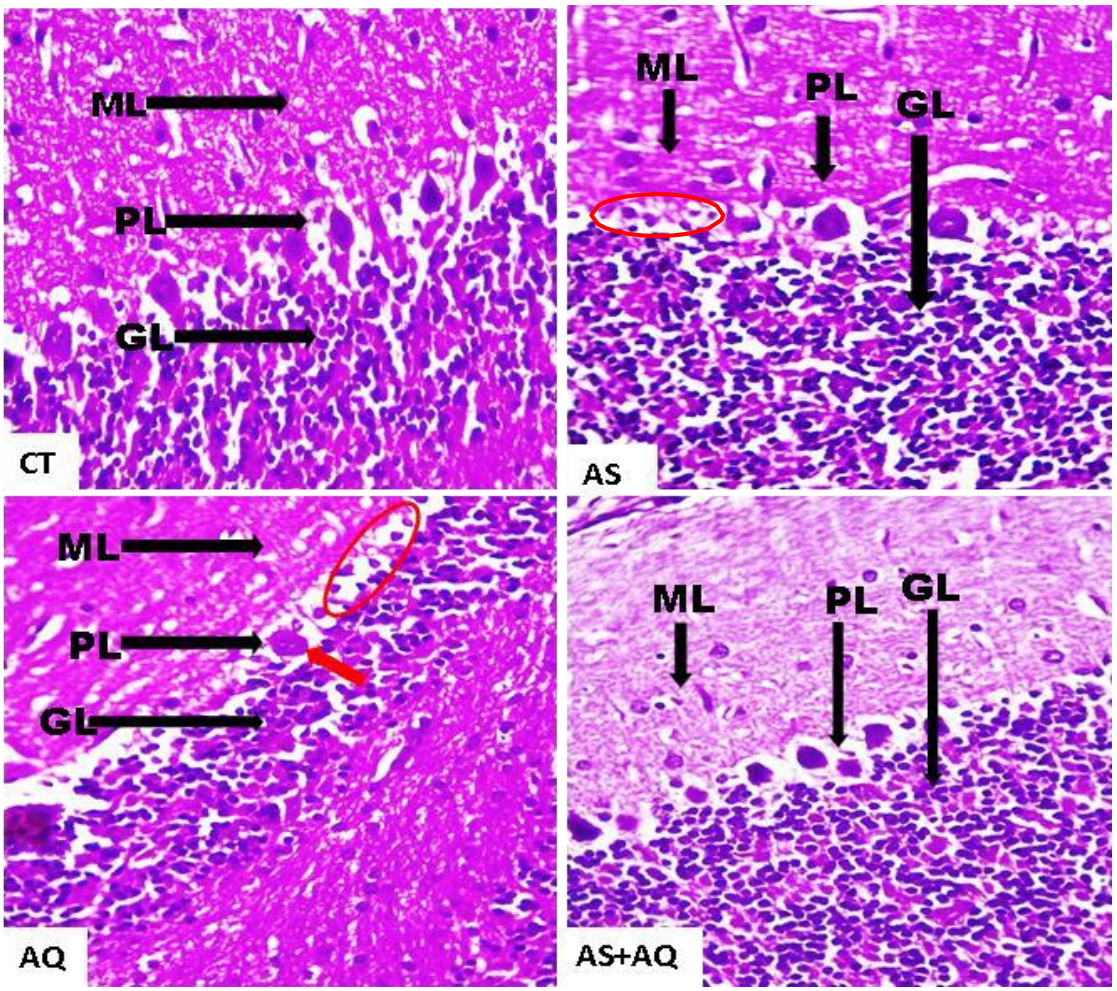


Plate 2. H&E Section of the cerebellum of the Wistar rat (delayed post-acute study). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate- amodiaquine combination group. ML = Molecular layer, PL = Purkinje cell layer and GL = Granular cell layer. The red arrow and red circles showed pyknotic (nuclear pallor and death) Purkinje cells in the AS & AQ groups. The Granular cell layer of the AQ group also appeared denuded compared to the other groups.

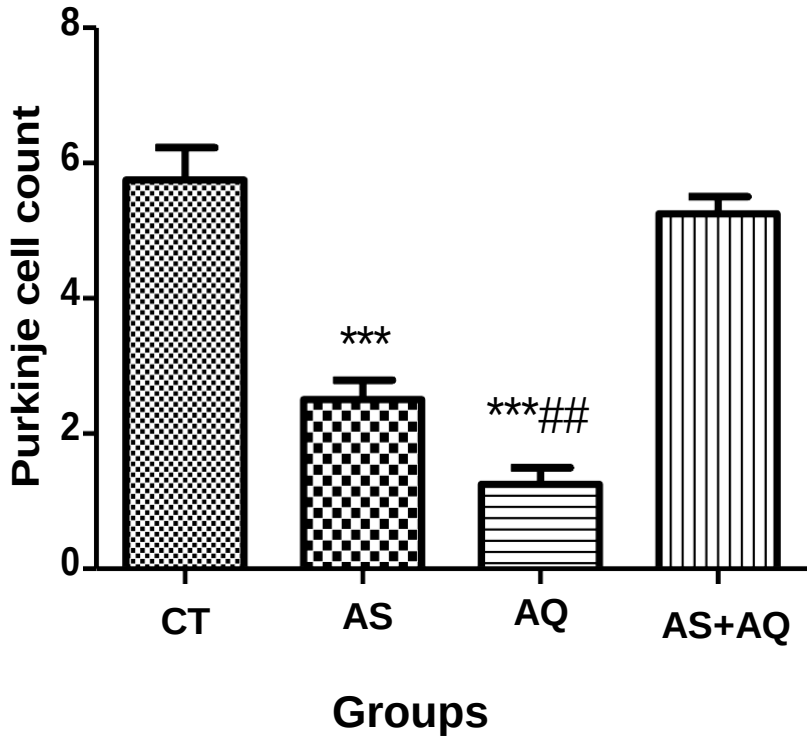


Figure 4.3. Cerebellar Purkinje Cell Count for the delayed post-acute study. Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT - Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine. *** Substantial decrease compared to the control ($P<0.001$). ## Significant decrease compared to the other groups ($P<0.01$).

4.6.3 H & E Result of the Hippocampus (acute)

Statistical analysis of the hippocampus photomicrograph (acute) as shown in Plate 3 and Figure 4.4 revealed that the population of viable Cornu Ammonis 1 cells did not vary significantly between the AS, AQ and AS+AQ groups compared to the control (CT). All cells appeared normal in both the control and the treated groups.

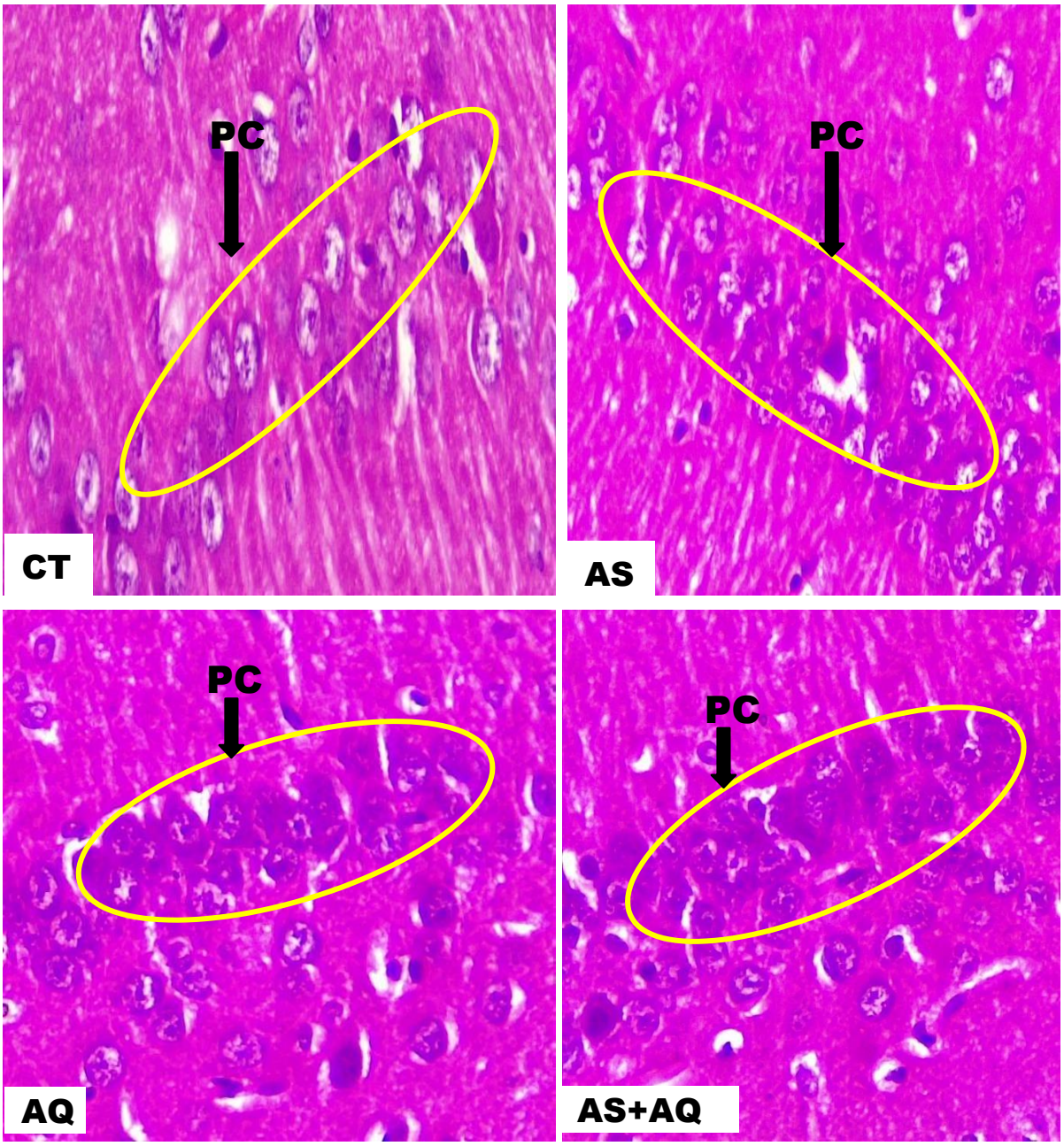


Plate 3. H&E Section of Hippocampus of the Wistar rat (acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate+amodiaquine combination group. PC = Yellow rings showing Pyramidal cells (Cornu Ammonis 1).

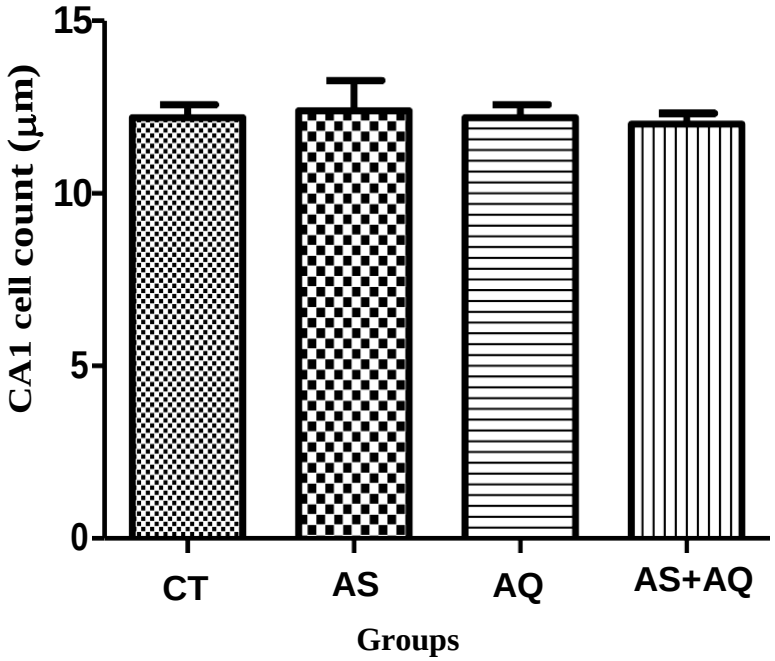


Figure 4.4. Hippocampal Cornus Ammonis 1 Cell Count for the acute study.

Means ±S.E.M. n = 5 at $\alpha_{0.05}$. CT - Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.4 H & E Result of the Hippocampus (delayed post-acute)

Statistical analysis of the hippocampus photomicrograph (delayed post-acute) as shown in Plate 4 and Figure 4.5 revealed that the population of viable Cornu Ammonis 1 cells reduced significantly in the AS and AQ groups compared to the control (CT) group. The population of viable CA1 cells also reduced in the AS+AQ group but was not statistically substantially different from the control (CT) group. There is an evidence of some element of pyknotic pyramidal cells in the AS, AQ and AS+AQ groups.

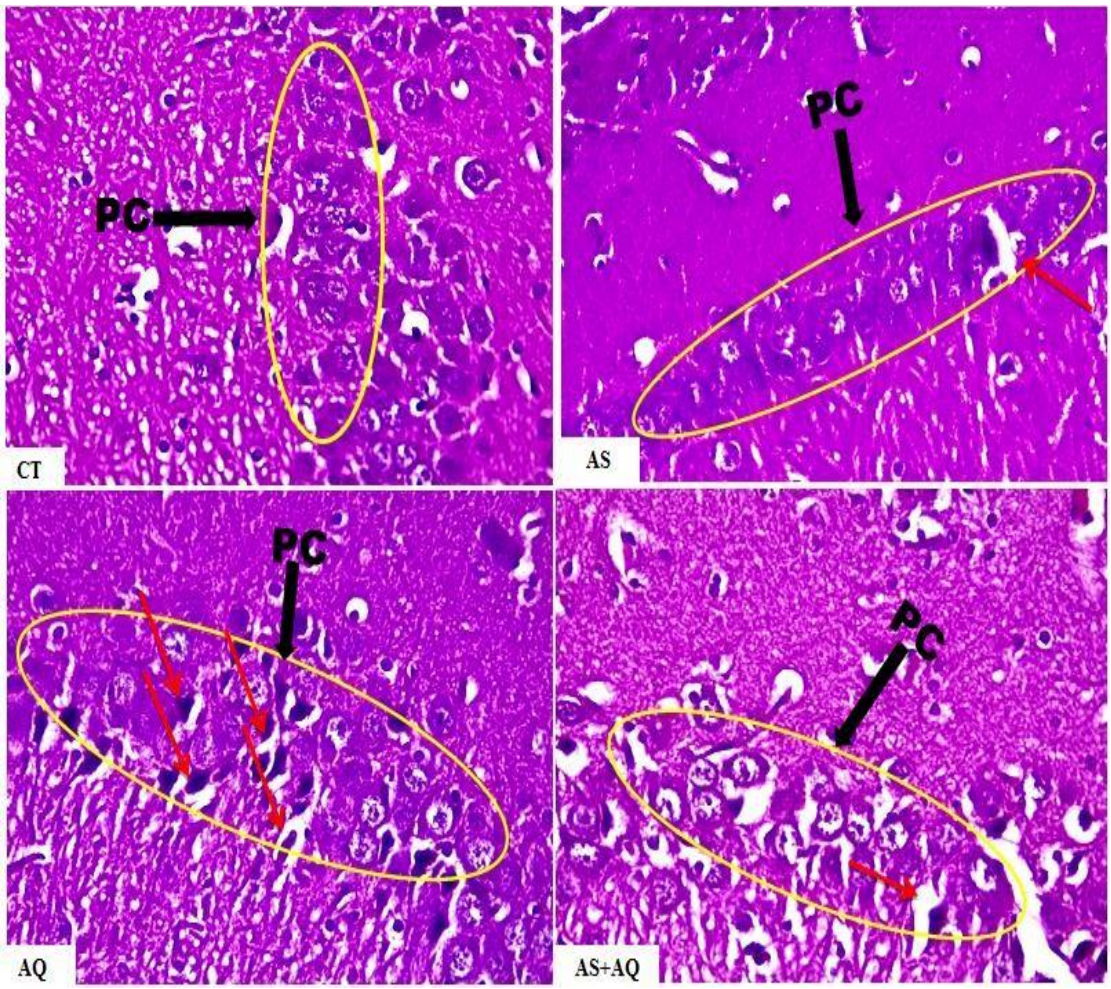


Plate 4. H&E Section of Hippocampus of the Wistar rats (delayed post-acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. PC = Pyramidal cells (Cornu Ammonis 1). Section of the AS, AQ and AS+AQ groups showed pyknotic pyramidal cells (red arrows).

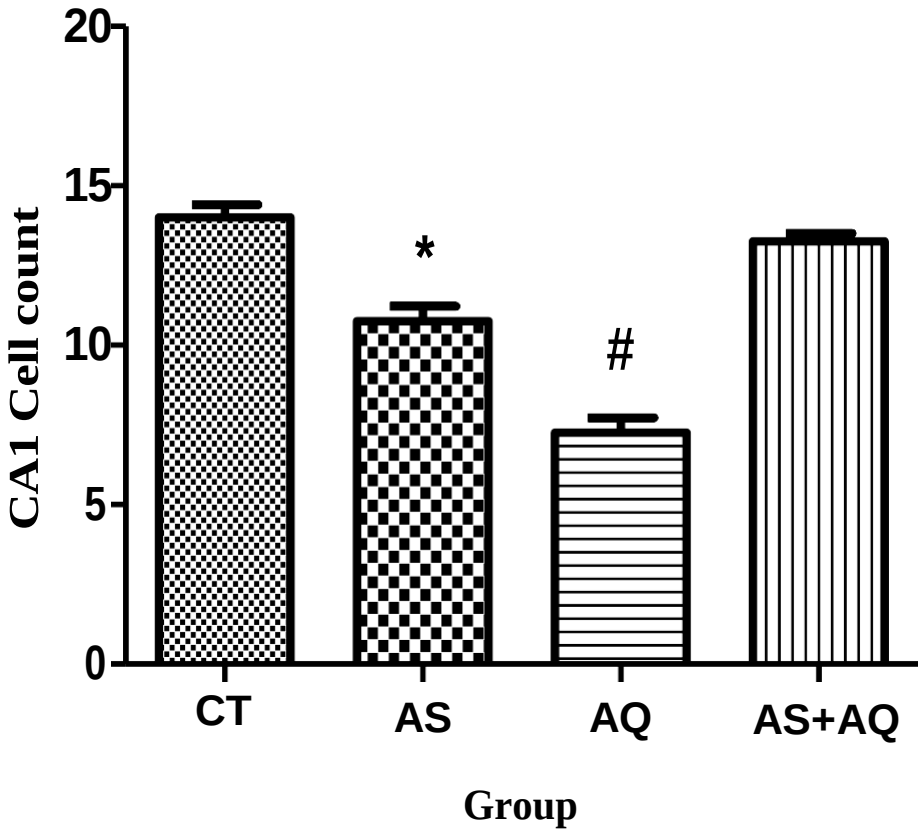


Figure 4.5. Hippocampal Cornus Ammonis 1 Cell Count (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ -Artesunate+Amodiaquine. * Significant decrease from the control. # Significant decrease compared with other groups.

4.6.5 GFAP Result of the Cerebellum (acute)

Statistical analysis of the cerebellum photomicrograph (plate 5) as shown in Figure 4.6 showed that there was a slight but insignificant increase in the level of GFAP expression by the AS+AQ group compared to the CT group but the AQ and AS groups had lower expressions of GFAP also not substantially different from the CT group.

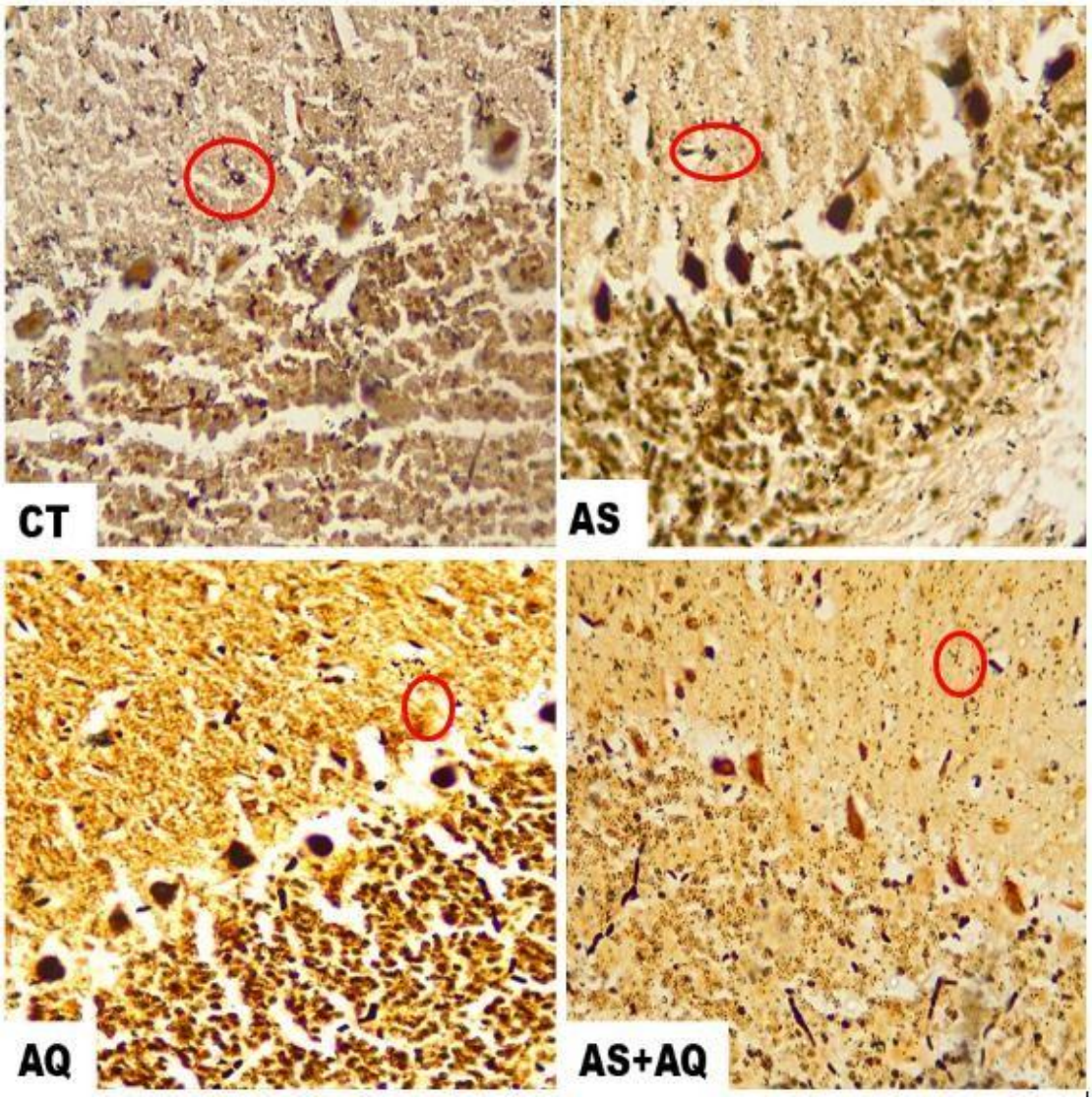


Plate 5. GFAP Section of Cerebellum of the Wistar rats (acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Red rings = astrocytes.

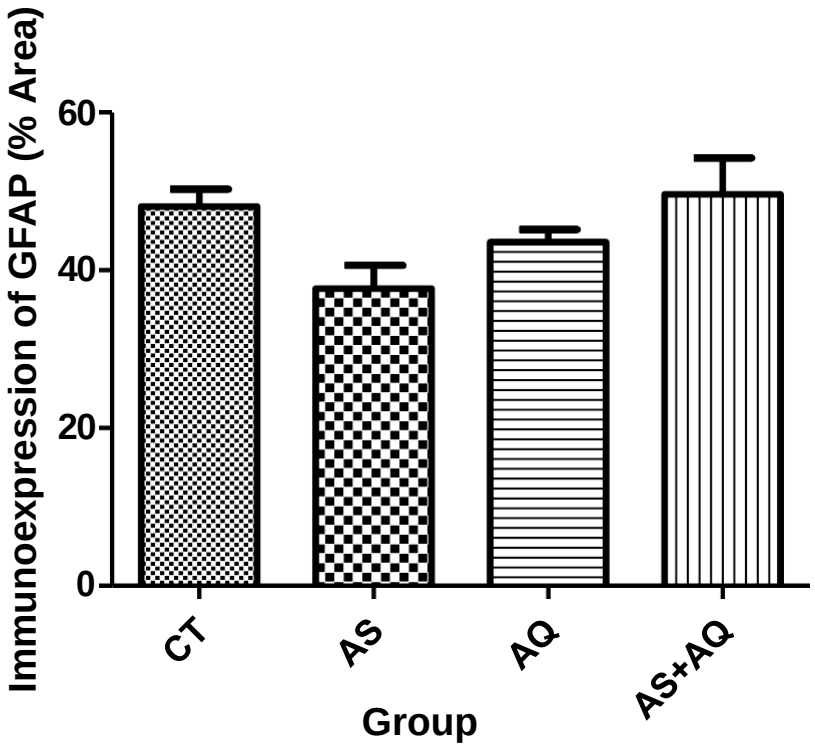


Figure 4.6. Immunoexpression of GFAP by the Cerebellum (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.6 GFAP Result of the Cerebellum (delayed post-acute)

Statistical analysis of the cerebellum photomicrograph (plate 6) as shown in Figure 4.7 revealed that there was significant increase in the level of GFAP expression in the AS and AQ groups compared with the CT and AS+AQ groups. There was however no significant difference in GFAP expression between the AS+AQ and the CT groups.

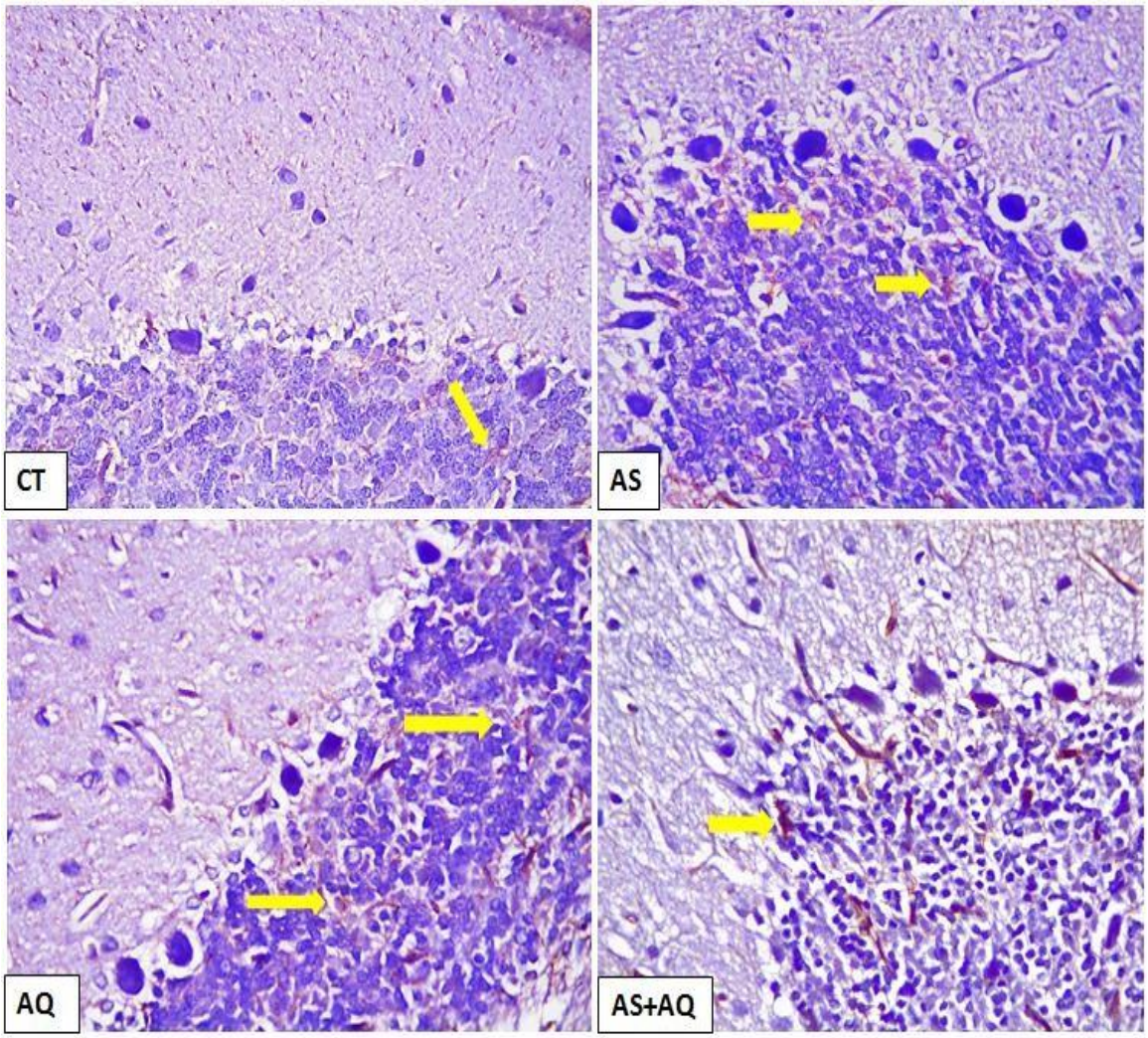


Plate 6. GFAP Section of Cerebellum of the Wistar rats (delayed post-acute). (X400). CT= control group, AS= artesunate group, AQ = amodiaquine group, AS+AQ = artesunate- amodiaquine combination group. Yellow arrows = astrocytes.

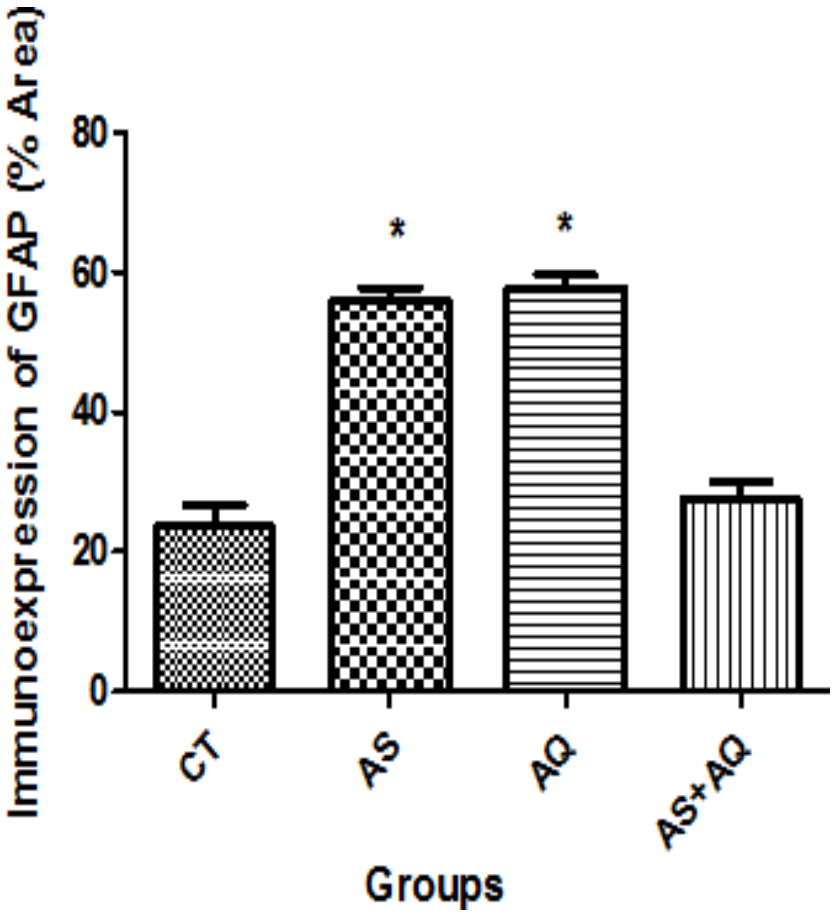


Figure 4.7. Immunoexpression of GFAP by the Cerebellum (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine. *p < 0.05 significant increase compared with the CT and AS+AQ groups.

4.6.7 GFAP Result of the Hippocampus (acute)

Statistical analysis of the hippocampus photomicrograph (plate 7) as shown in Figure 4.8 revealed that there were no substantial variations in the expressions of GFAP between the experimental groups and the CT group.

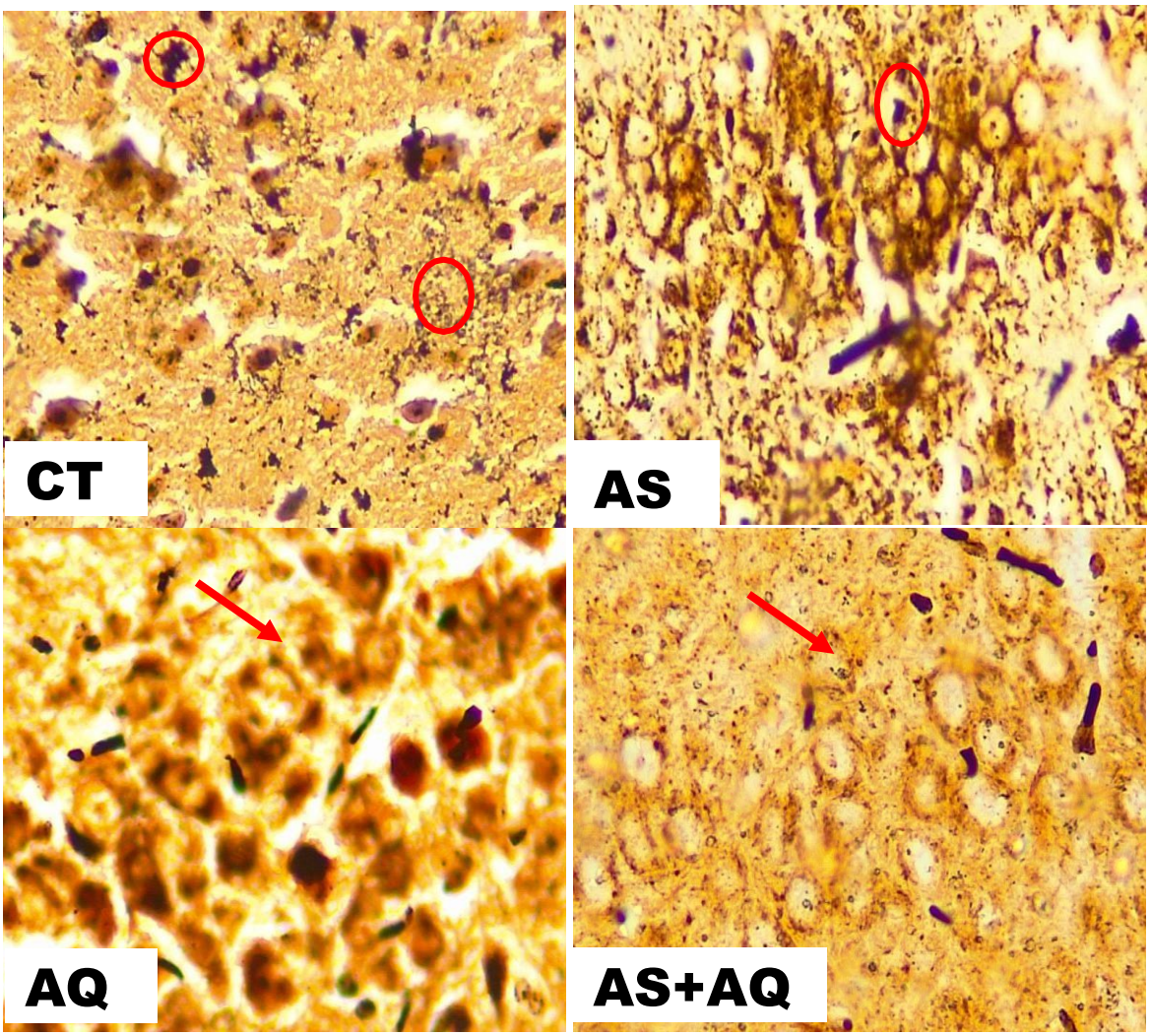


Plate 7. GFAP Section of Hippocampus of the Wistar rats (pyramidal cell (CA1) region) (acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination. Red rings and arrows = astrocytes

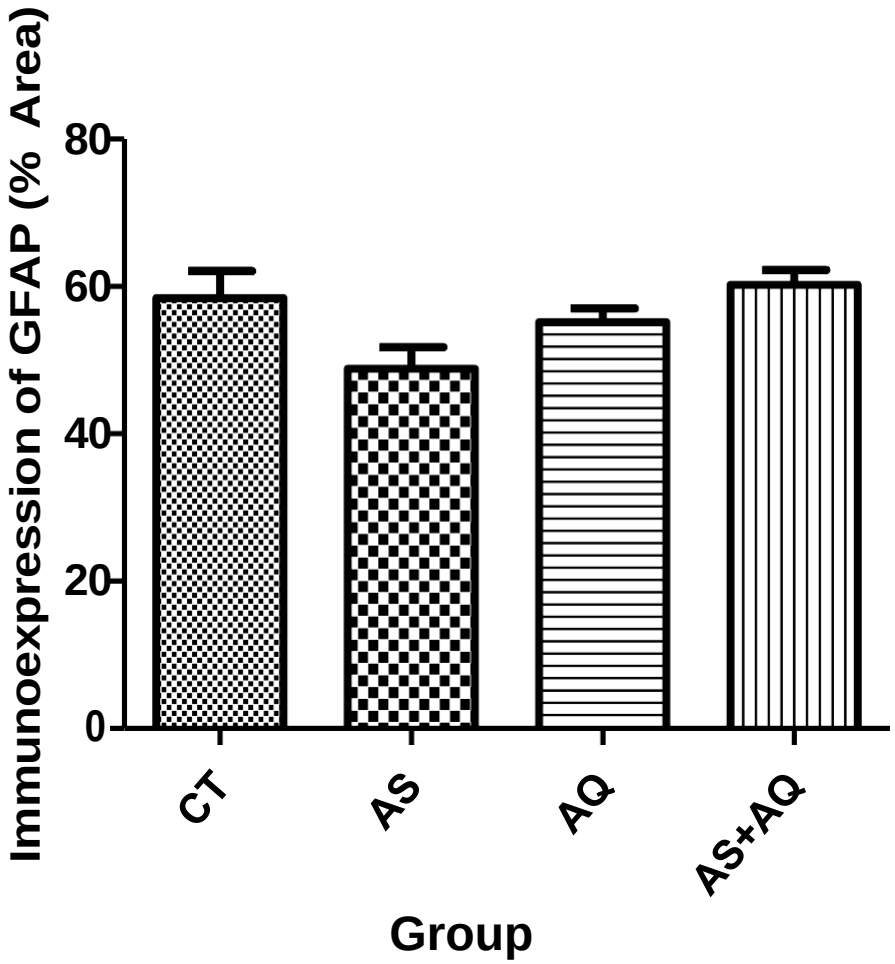


Figure 4.8. Immunoexpression of GFAP by the Hippocampus (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control (Distilled water), AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.8 GFAP Result of the Hippocampus (delayed post-acute)

Statistical analysis of the hippocampus photomicrograph (plate 8) as shown in Figure 4.9 revealed that there were significantly higher expressions of GFAP in both AS and AQ groups than the CT group while the AS+AQ group had a lower expression, although not substantial varied from the CT group.

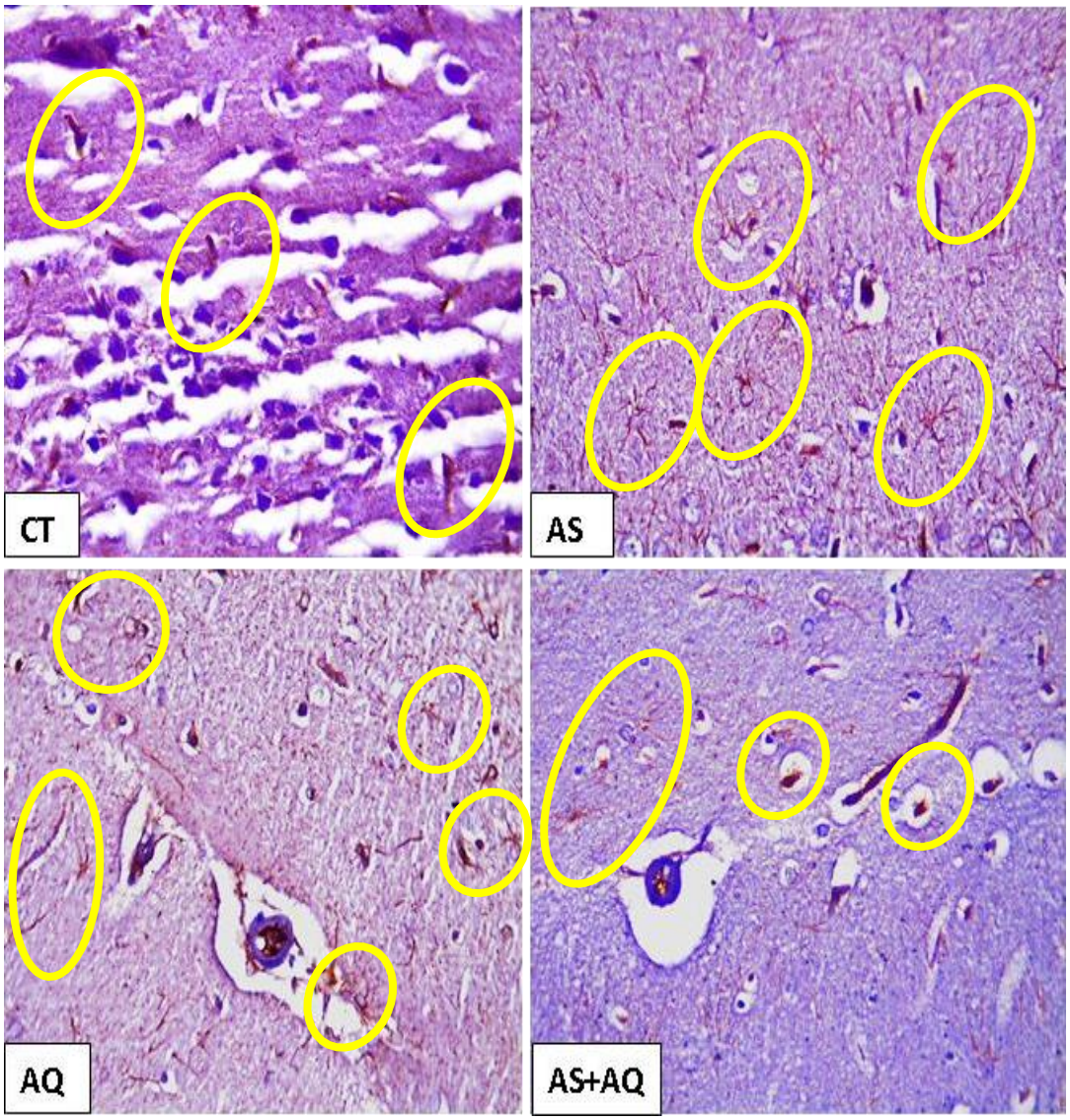


Plate 8. GFAP Section of Hippocampus of the Wistar rats (pyramidal cell (CA1) region) (delayed post-acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Yellow rings = astrocytes.

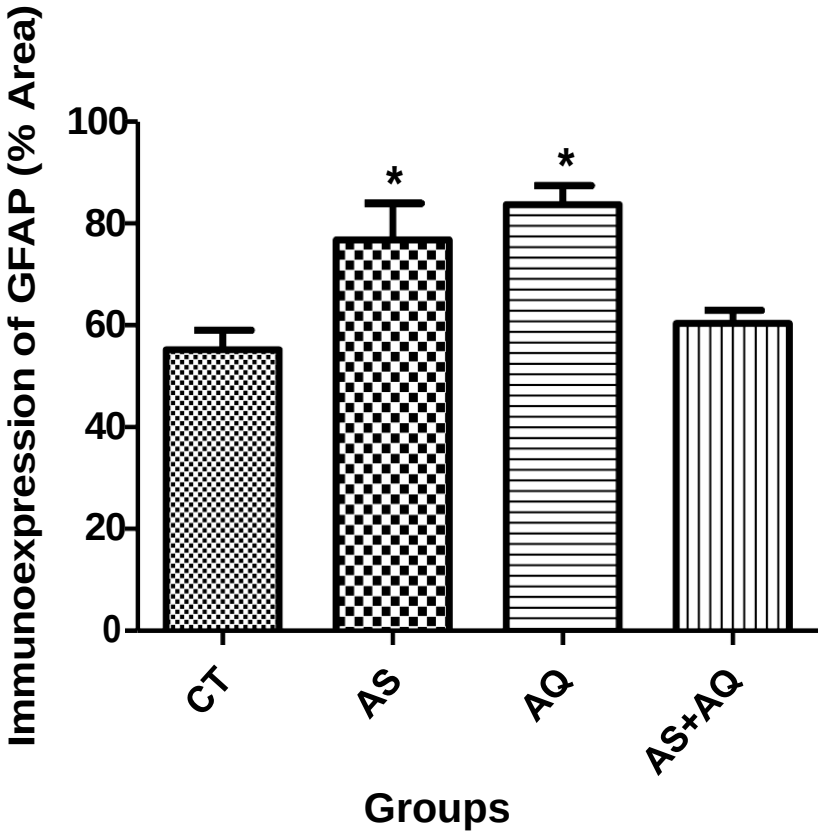


Figure 4.9. Immunoexpression of GFAP by the Hippocampus (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * Significantly higher than the control.

4.6.9 COX-2 Result of the Cerebellum (acute)

The levels of COX-2 expression by the cerebellum (plate 9) were slightly higher in the AS, AQ and AS+AQ groups compared with the CT group. Statistical analysis of the photomicrograph however revealed that there were no statistically significant differences in the expression between the three experimental groups (AS, AQ and AS+AQ) and the CT group (as shown in Figure 4.10).

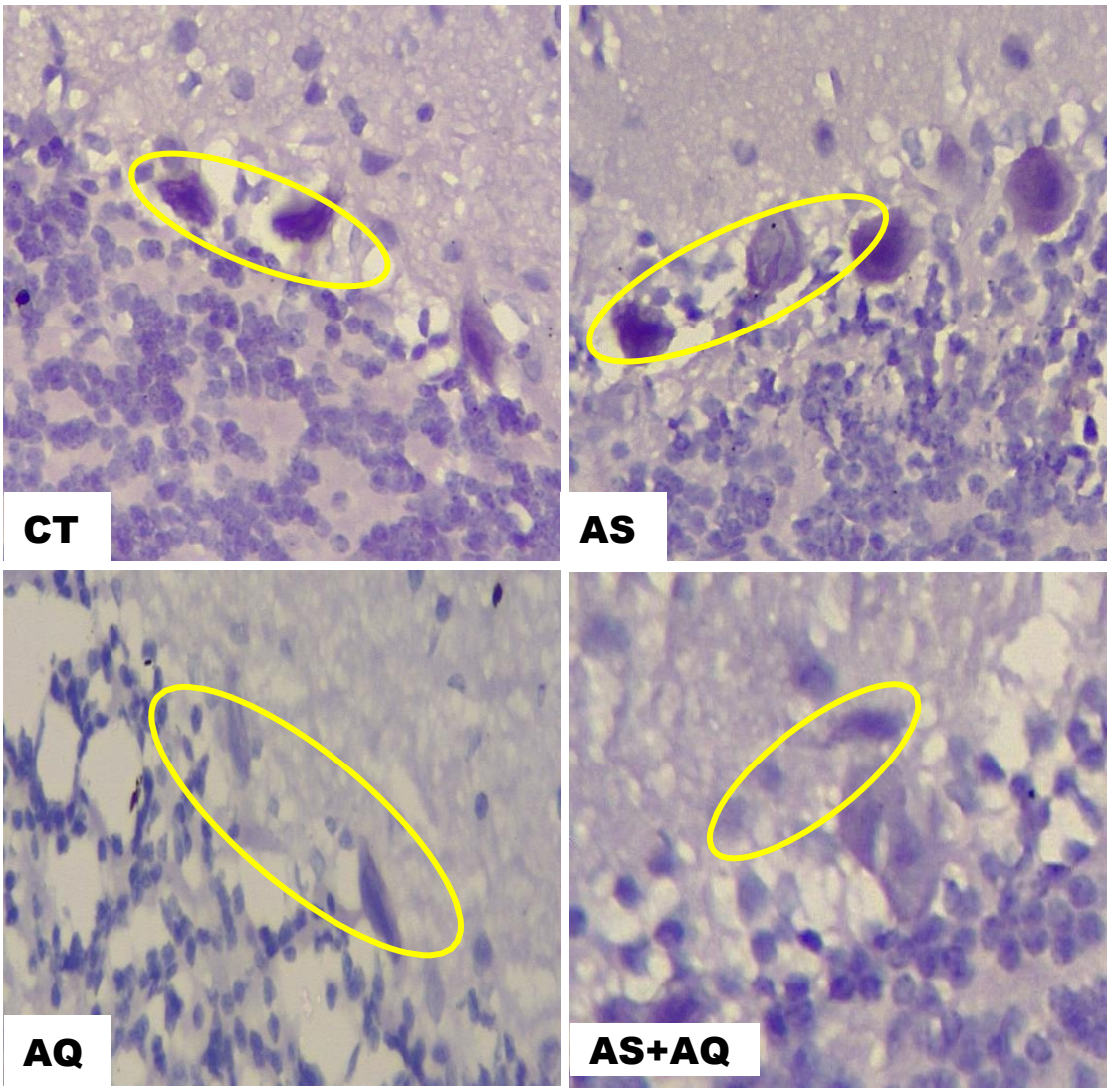


Plate 9. COX-2 Section of Cerebellum of the Wistar rats (acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Yellow rings: Purkinje cells in the Purkinje cell layer

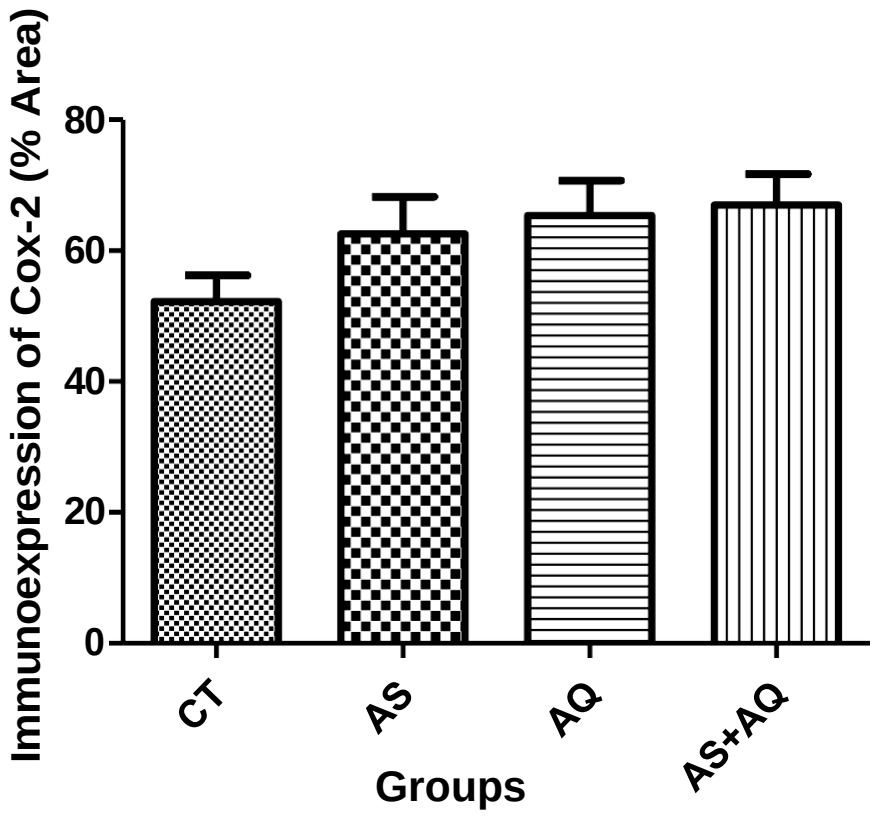


Figure 4.10. Immunoexpression of COX-2 by the Cerebellum (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control (Distilled water), AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine.

4.6.10 COX-2 Result of the Cerebellum (delayed post-acute)

Statistical analysis of the cerebellum photomicrograph (see plate 10) as shown in Figure 4.11 revealed that there were no statistically substantial variations in the level of COX-2 expression between the three experimental groups (AS, AQ and AS+AQ) and the CT group.

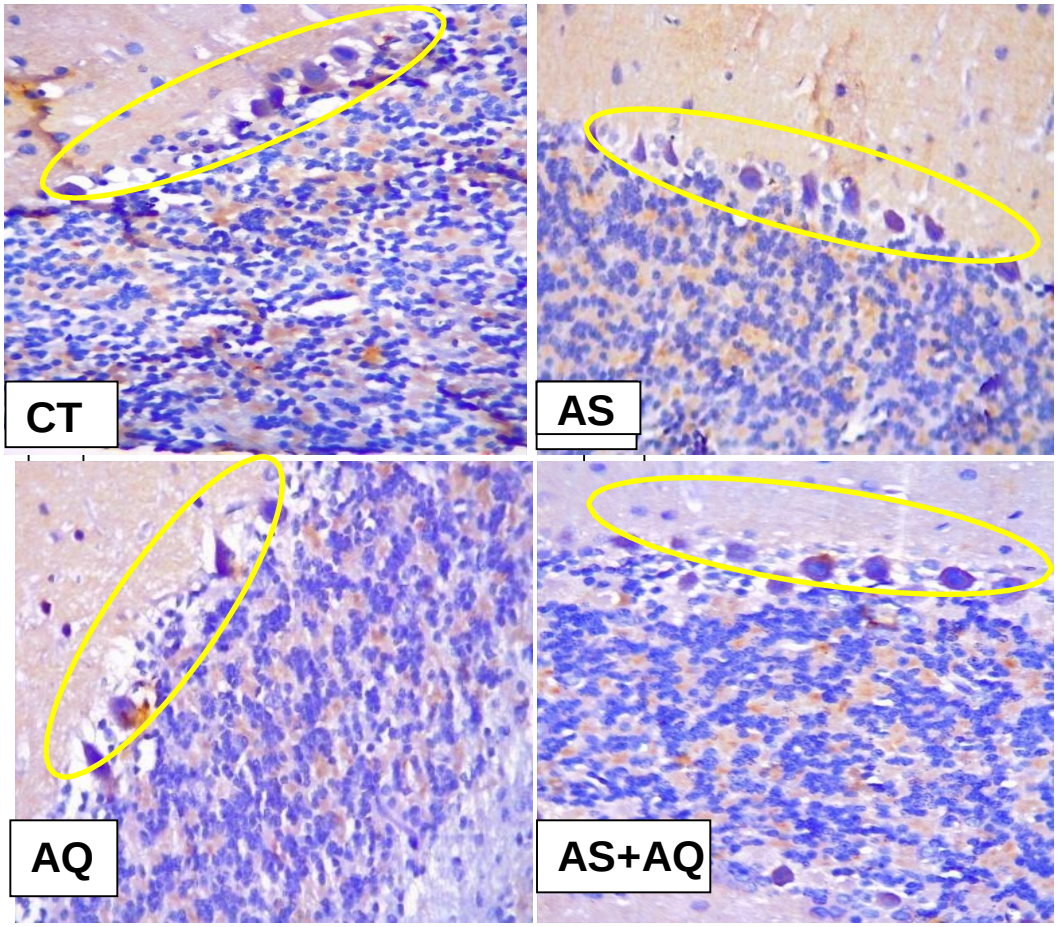


Plate 10. COX- 2 Section of Cerebellum of the Wistar rats (delayed post-acute). (X400). CT= control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Yellow rings = Purkinje cells in the Purkinje cell layer.

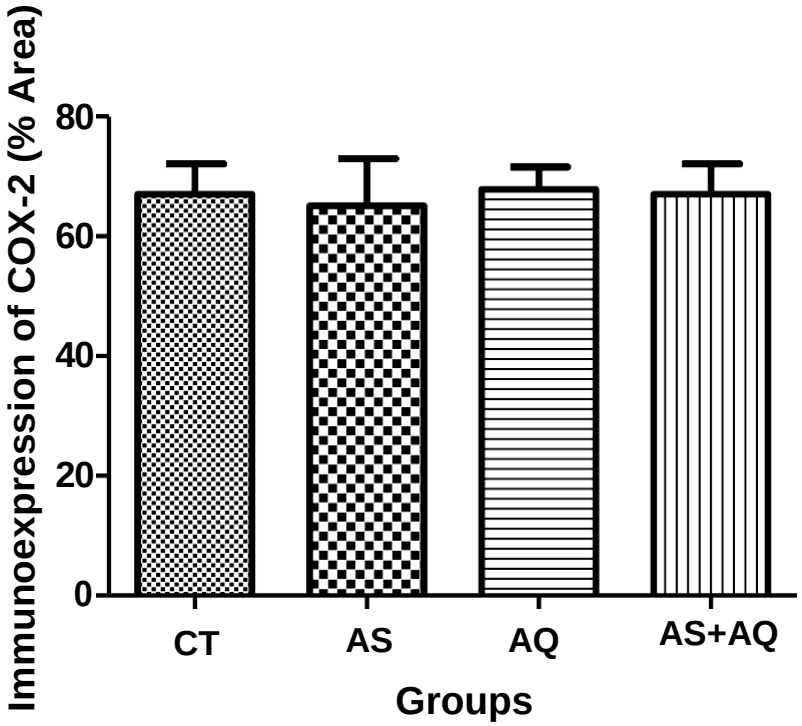


Figure 4.11. Immunoexpression of COX-2 by the Cerebellum (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine.

4.6.11 COX-2 Result of the Hippocampus (acute)

Statistical analysis of the hippocampus photomicrograph (Plate 11) as shown in Figure 4.12 revealed that COX-2 immunoexpression by the hippocampal pyramidal cells did not substantially vary between the experimental groups and the CT group.

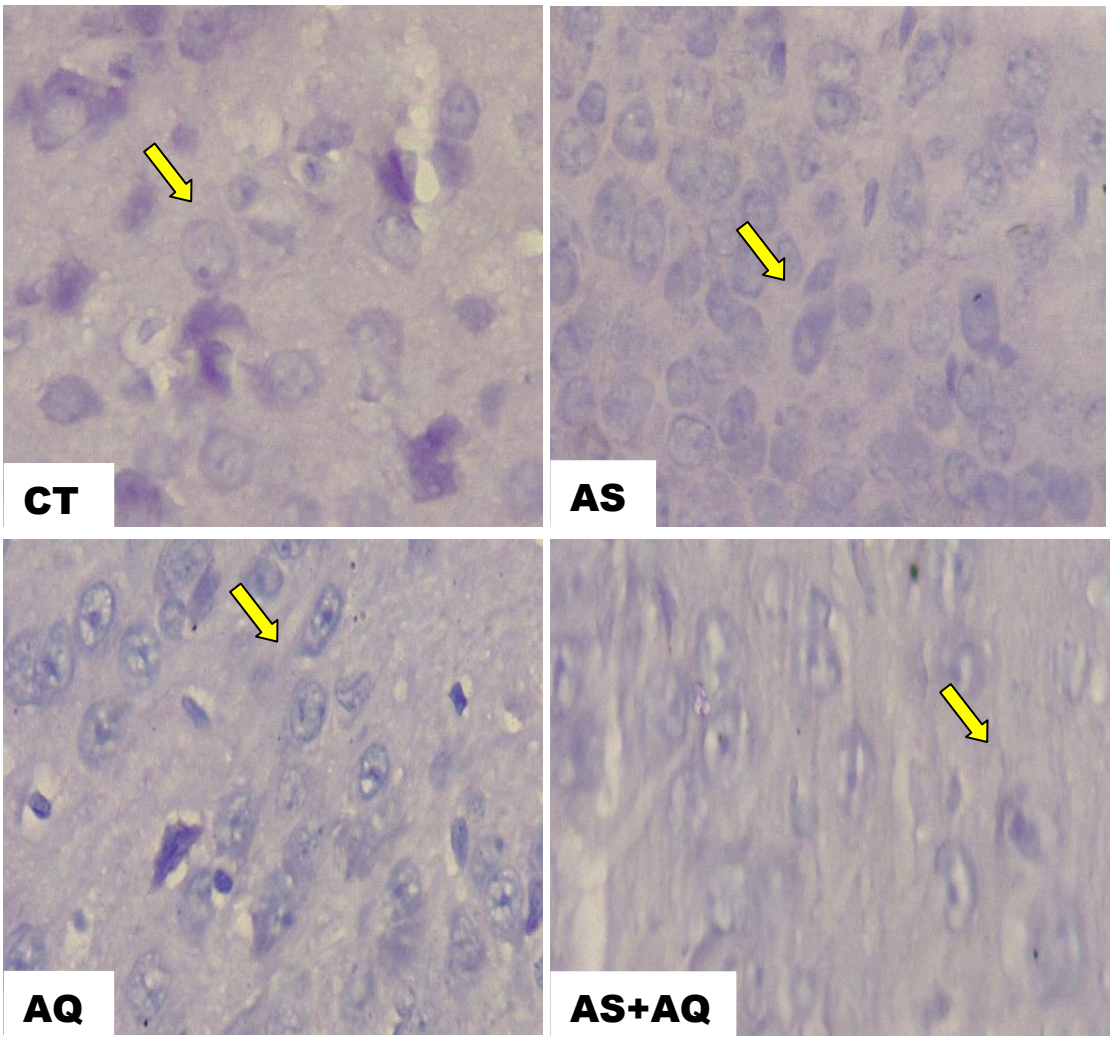


Plate 11. COX-2 Section of Hippocampus of the Wistar rats (CA1 pyramidal cell region) (acute). (X400). CT= control group, AS= artesunate group, AQ= amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. Yellow arrows: Cornu Ammonis 1 cells

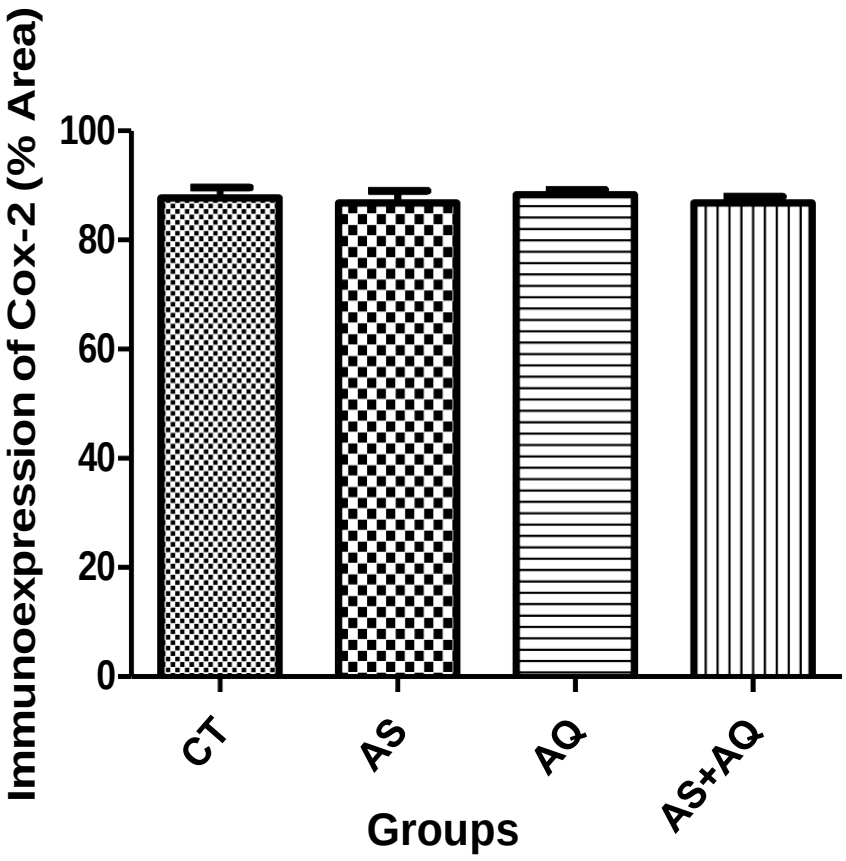


Figure 4.12. Immunoexpression of COX-2 by the Hippocampus (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine.

4.6.12 COX-2 Result of the Hippocampus (delayed post-acute)

Statistical analysis of the hippocampus (delayed post-acute) as shown in Figure 4.13 and Plate 12 revealed that COX-2 immunoexpression was substantially higher in AS and AQ groups than the CT group, while the AS+AQ group had lower expression although not substantially varied from the CT group.

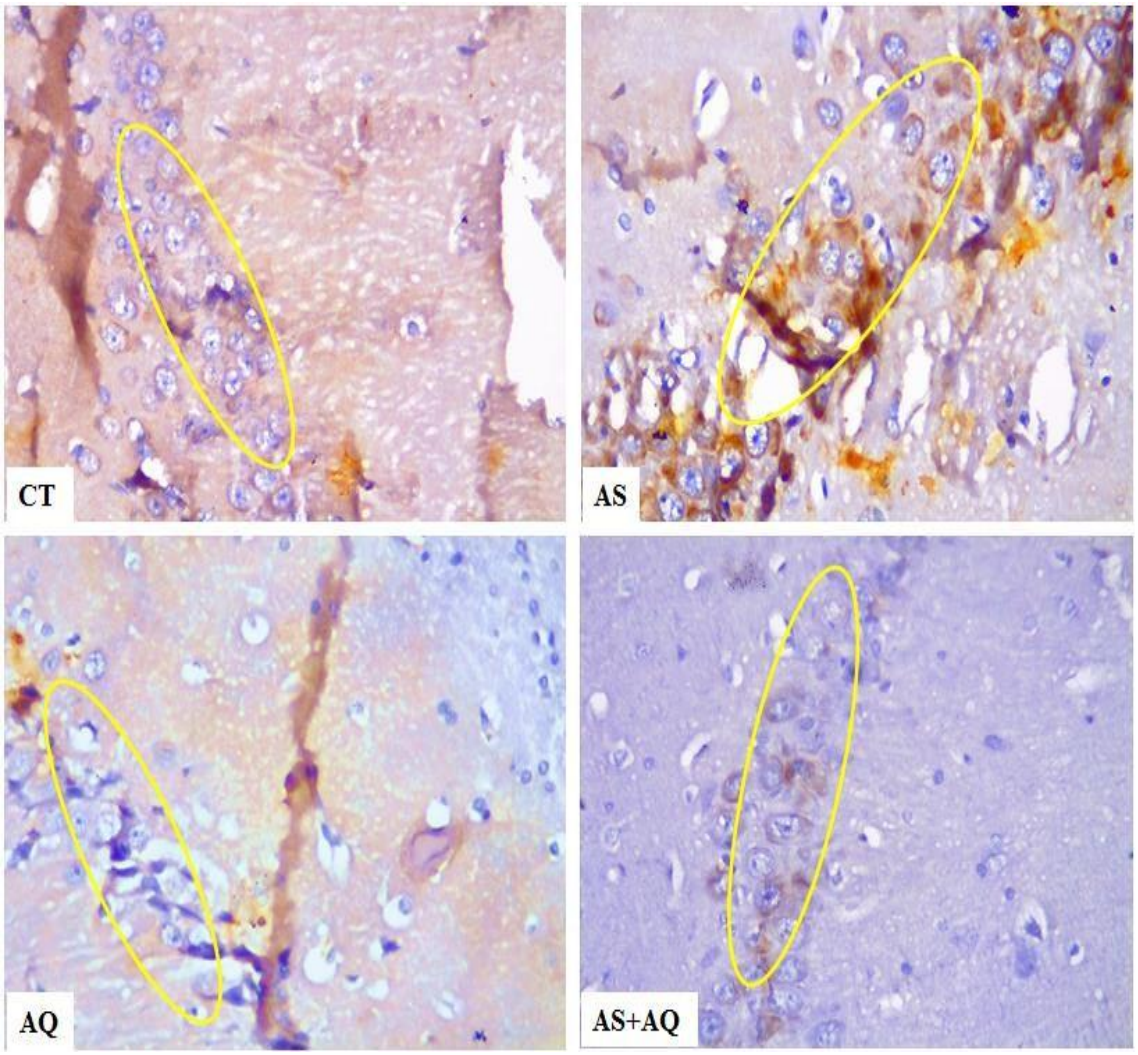


Plate 12. COX-2 Section of Hippocampus of the Wistar rats (pyramidal cell region) (delayed post-acute). (X400). CT = control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Yellow rings = Pyramidal cell layer with some Cornu Ammonis1 cells.

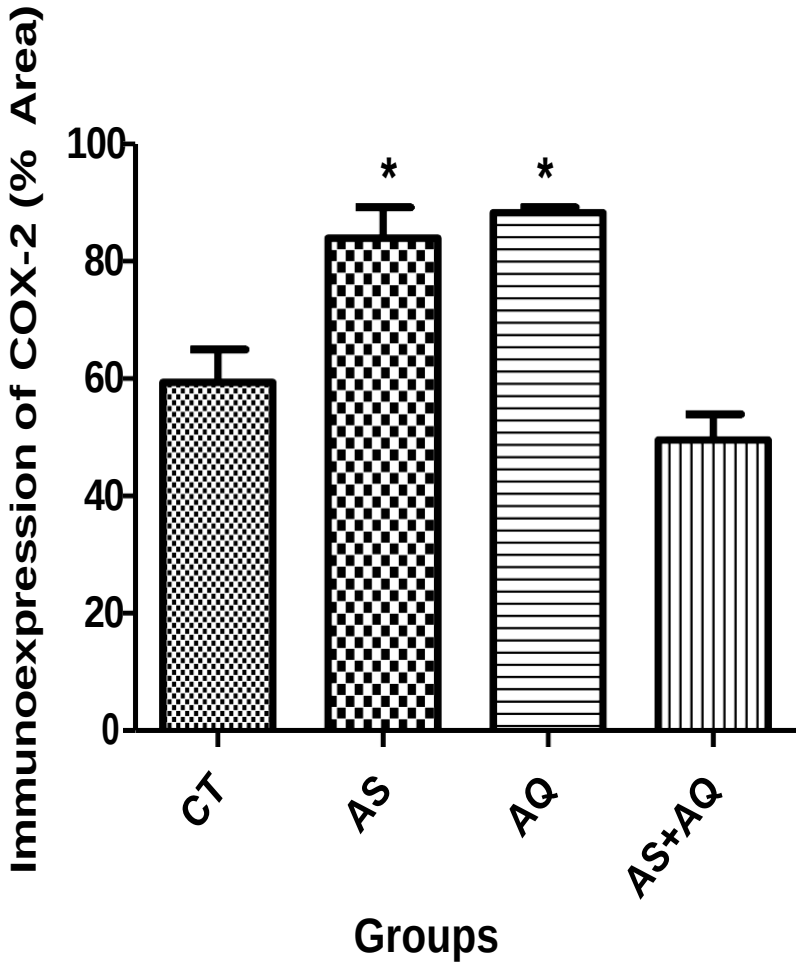


Figure 4.13. Immunoexpression of COX-2 by the Hippocampus (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * Significantly different from the control.

4.6.13 iNOS Result of the Cerebellum (acute)

Statistical analysis of the cerebellum photomicrograph (Plate 13) as shown in Figure 4.14 shows that there was no substantial variation in the immunoexpression of iNOS by the cerebellar Purkinje cells between the three experimental groups (AS, AQ, AQ+AS) and the CT.

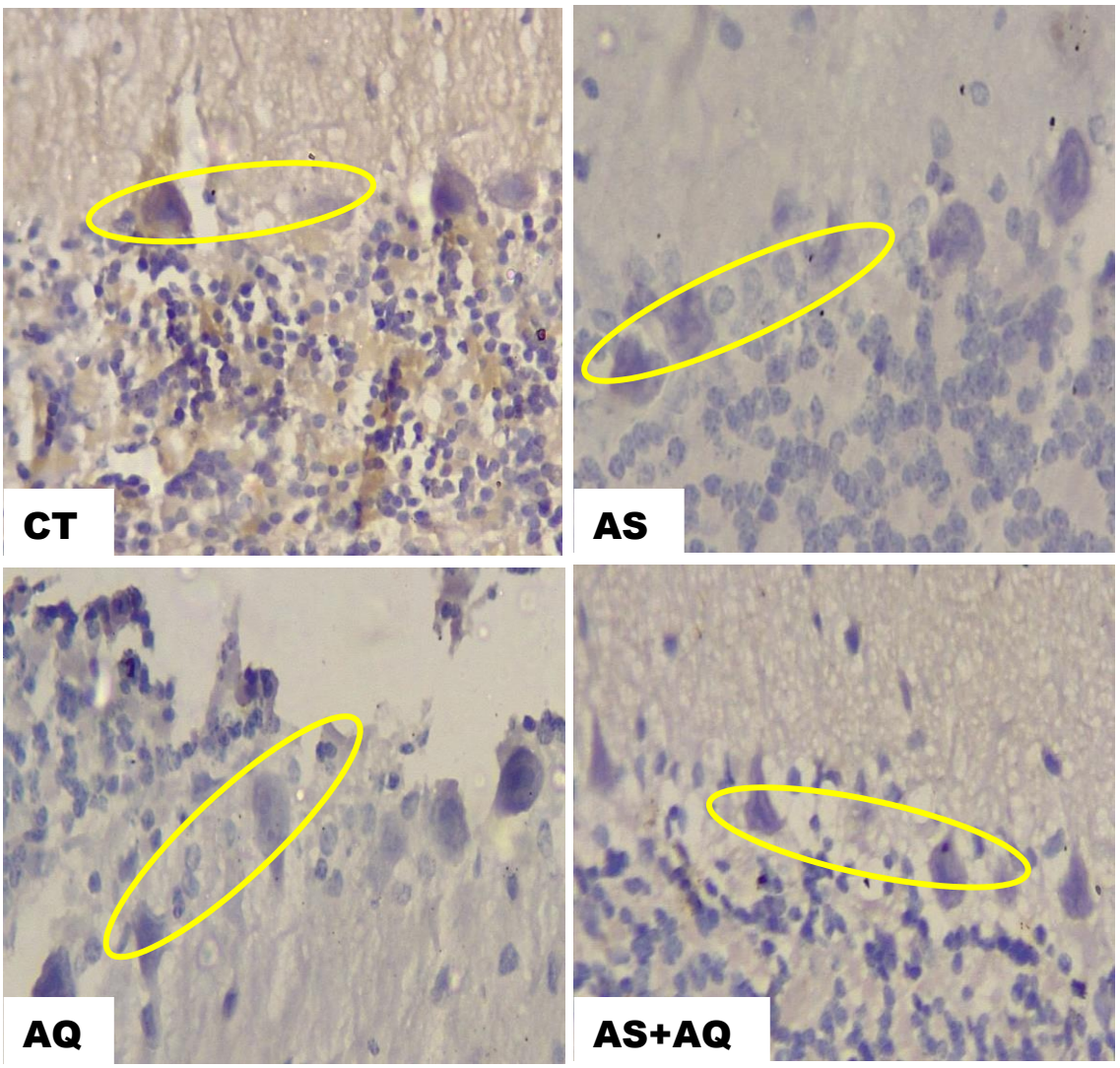


Plate 13. iNOS Section of Cerebellum of the Wistar rats (acute). (X400). CT= control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. Yellow rings show expression of NO by the Cerebellar cells.

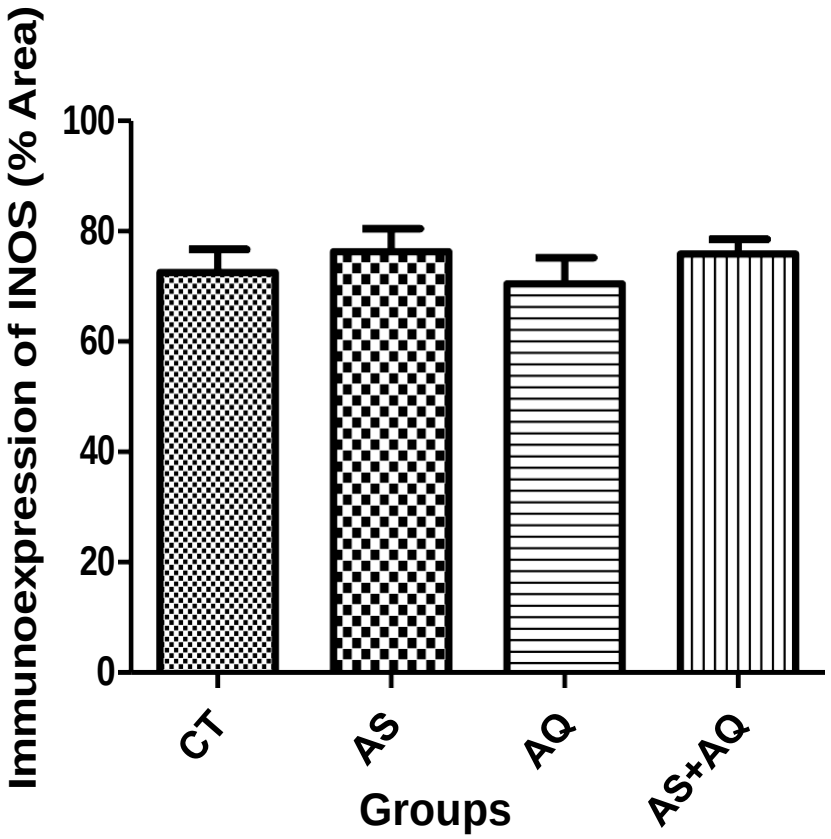


Figure 4.14. Immunoexpression of iNOS by the Cerebellum (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control (Distilled water), AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.14 iNOS Result of the Cerebellum (delayed post-acute)

Statistical analysis of the cerebellum photomicrograph (Plate 14) as shown in Figure 4.15 reveals that there was significantly higher immunoexpression of NO in the three experimental groups (AS+AQ>AQ>AS) compared to the CT.

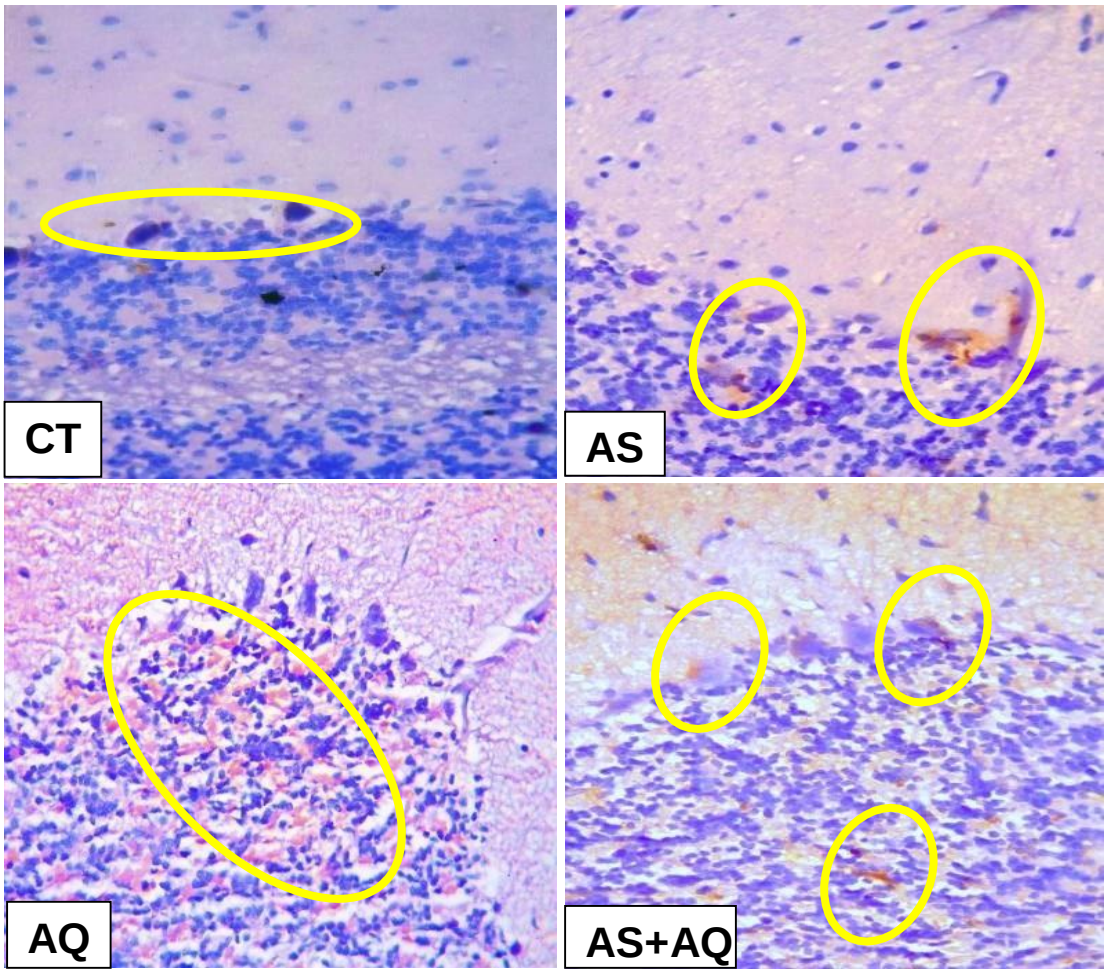


Plate 14. iNOS Section of Cerebellum of the Wistar rats (delayed post-acute). (X400). CT= control group, AS = artesunate group, AQ = amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. Yellow rings show expression of NO by the Cerebellar cells.

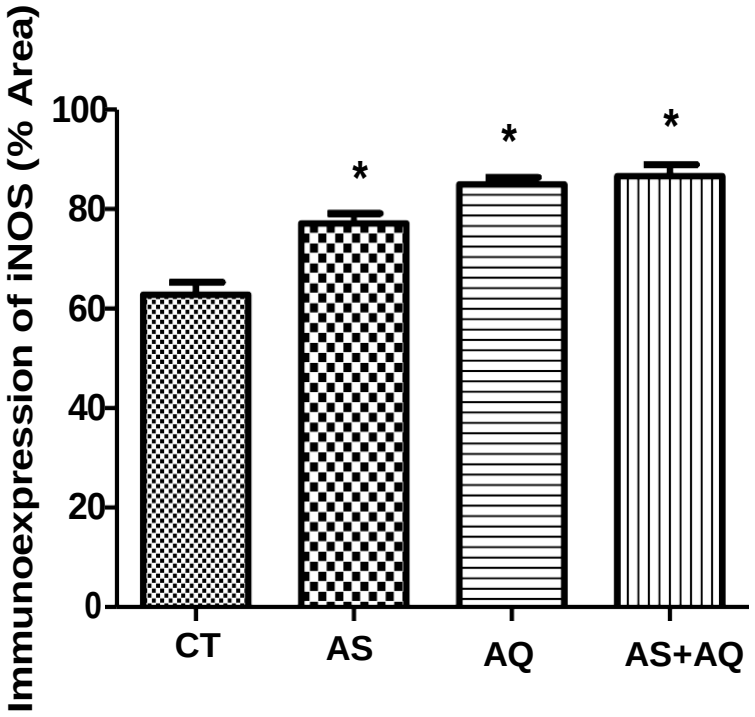


Figure 4.15. Immunoexpression of iNOS by the Cerebellum (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ - Artesunate+Amodiaquine. * Significant increase compared with the CT.

4.6.15 iNOS Result of the Hippocampus (acute)

Statistical analysis of the hippocampus photomicrograph (Plate 15) as shown in Figure 4.16 reveals that there was no substantial variation in the expression of iNOS by the three experimental groups (AS, AQ and AS+AQ) compared to the CT group.

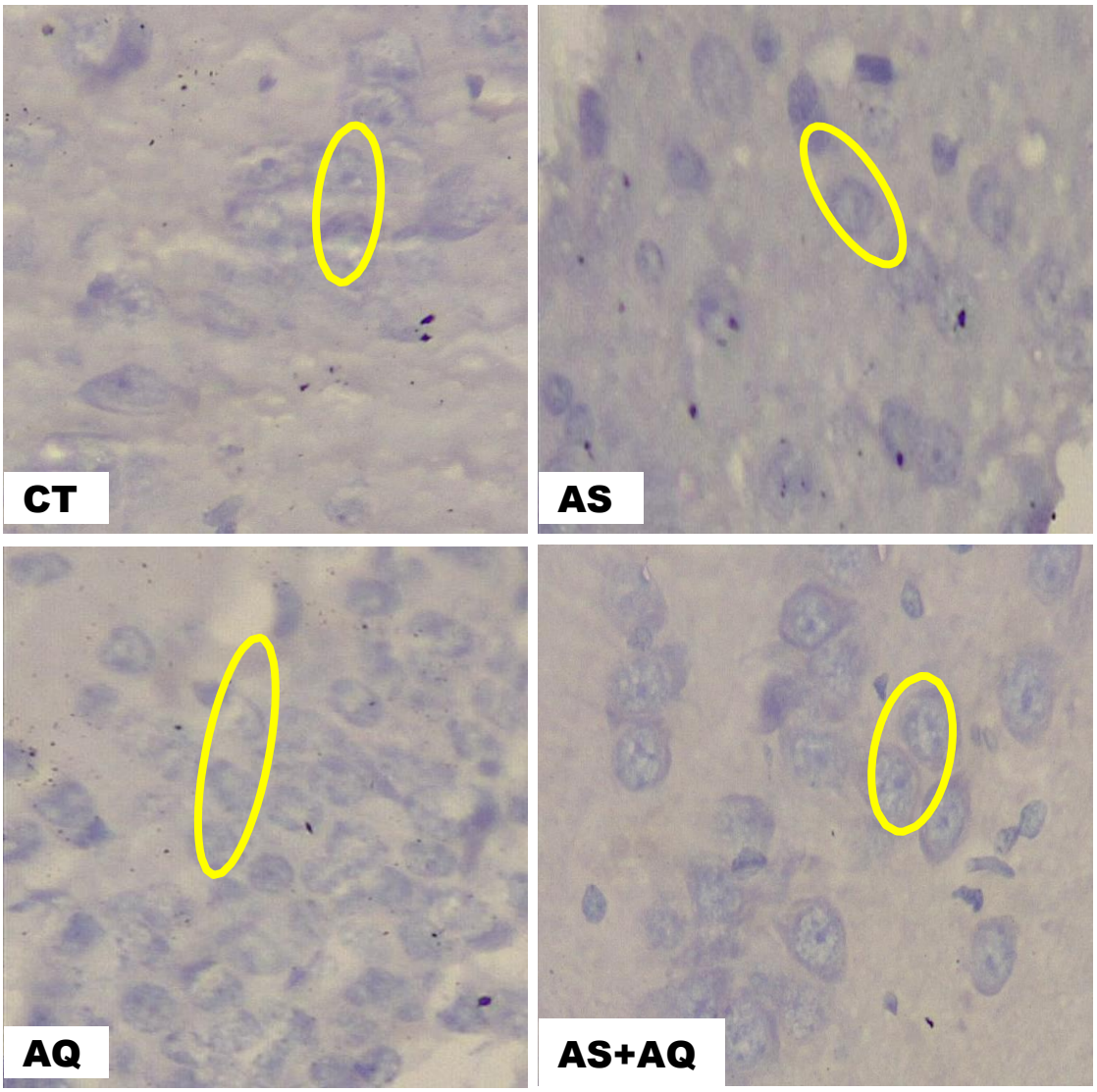


Plate 15. iNOS Section of the Hippocampus of the Wistar rats (pyramidal cell (CA1) region) (acute). (X400). CT= control group, AS= artesunate group, AQ = amodiaquine group, AS+AQ= artesunate-amodiaquine combination group. Yellow rings= CornuAmmonis 1 cells

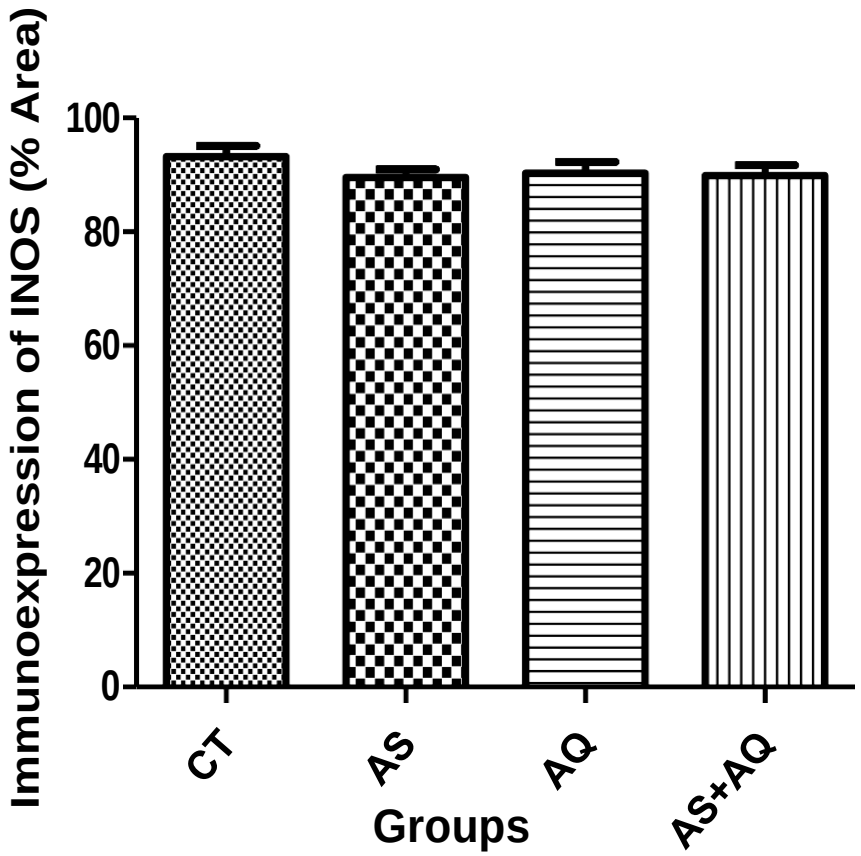


Figure 4.16. Immunoexpression of iNOS by the Hippocampus (acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT- Control, AS - Artesunate, AQ - Amodiaquine, AS+AQ - Artesunate+Amodiaquine.

4.6.16 iNOS Result of the Hippocampus (delayed post-acute)

Statistical analysis of the hippocampus photomicrograph (Plate 16) as shown in Figure 4.17 reveals that there was significantly higher expression of iNOS in the three experimental groups (AS, AQ and AS+AQ) compared to the CT group (see plate 16).

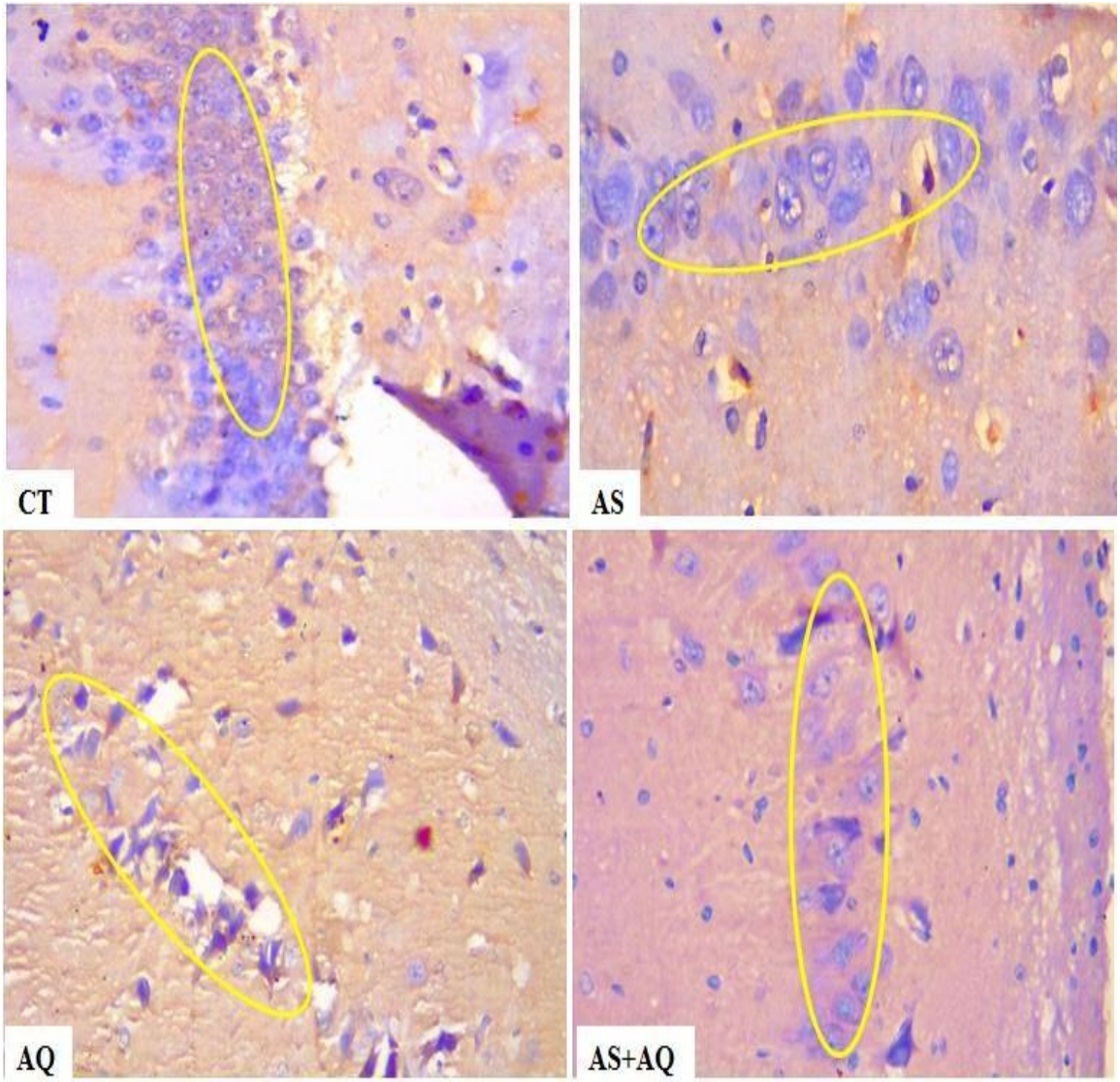


Plate 16. iNOS Section of Hippocampus of the Wistar rats (pyramidal cell (CA1) region) (delayed post-acute). (X400). CT= control group, AS= artesunate group, AQ= amodiaquine group, AS+AQ = artesunate-amodiaquine combination group. Yellow rings = Cornu Ammonis 1 cells.

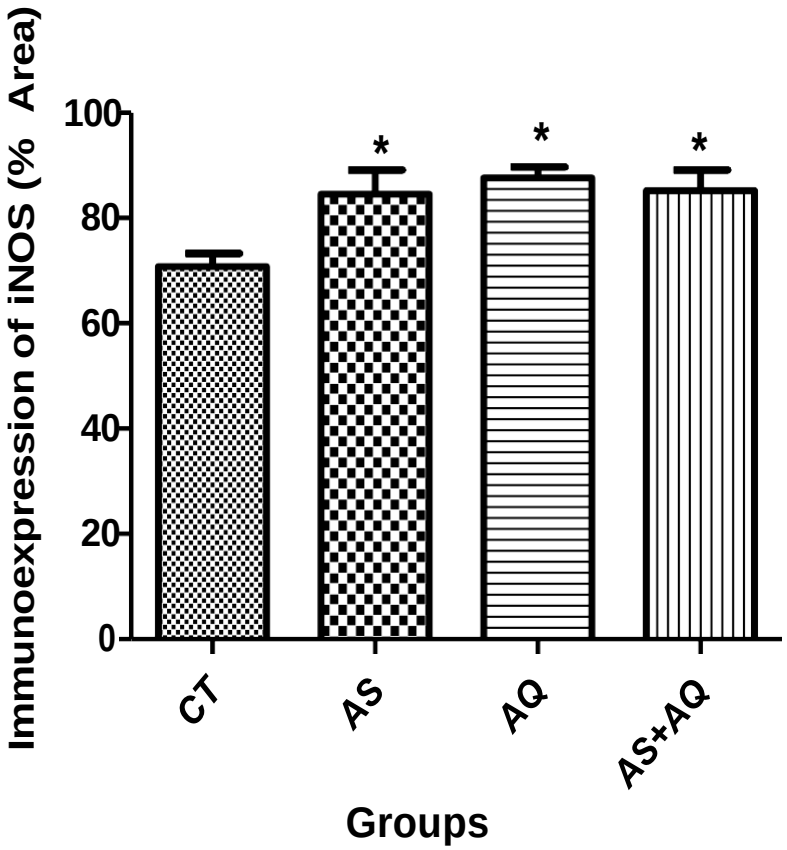


Figure 4.17. Immunoexpression of iNOS by the Hippocampus (delayed post-acute). Means ± S.E.M. n = 5 at $\alpha_{0.05}$. CT - Control, AS- Artesunate, AQ- Amodiaquine, AS+AQ- Artesunate+Amodiaquine. * Significantly increase compared with the control group.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Physical Observations

The decreased activities and dozing observed with the animals after receiving the drugs suggest that the drugs induced muscle weakness, drowsiness and unconsciousness in the animals. This finding agrees with that of Ekong *et al*. (2008) who reported dizziness, gait disturbances and other effects against the use of amodiaquine. This agreement may be associated with the similarity in the dosage of the drug administered in the two studies.

5.2 Variation in weight changes after treatment with AS, AQ and AS+AQ

5.2.1 Body weight (B.W) variations

In the acute phase of this study, the percentage increase in body weight recorded in all the experimental groups depicts that the drugs did not have any significant negative effect on food consumption and nutrient uptake. This result is in consonance with that of Izunya *et al*. (2010) who stated that AS at its standard and double standard doses had no effect on B.W of rats; and that of Utoh-Nedusa *et al.* (2009) who reported increase in mean body weight on administration of artemisinin in rats. This similarity may be based on the similarity in dosage and experimental design of the two studies. However, this finding contradicted that of Olumide and Raji (2011) who reported a substantial reduction in B.W of the rats administered with AS (2.9 mg/kg B.W) for 5 days. The contradiction may probably be due to the discrepancy in dosages between the two studies.

In the delayed post-acute phase, the weight loss recorded with the AQ group may be associated with decreased consumption of food by the animals as a result of loss of appetite and low nutrient uptake following treatment with this drug. This agreed with the report of Ubulom *et al*. (2015) who stated that there was decline in feeding rate of their experimental animals administered with amodiaquine and cifloxacine combination. Blundell *et al*. (2017) in their study opined that drug that causes reduction in weight may have acted on the appetite control centre of the hypothalamus in the

brain to reduce feelings of hunger.

On the other hand, the AS+AQ group had an increase in percentage weight change not substantially different from that of the CT group. This finding suggested that the combination form of the drugs (AS+AQ) neither reduced their appetite for food nor negatively affected the feeding pattern of the animals. Furthermore, the higher percentage weight difference recorded in the combination group may also suggest that the combination regimen of these two drugs is better than the monotherapy of the individual drug in terms of weight sustenance. This finding compared favorably with that of Utoh- Nedusa *et al*. (2009) who reported upsurge in the mean body weight of rats on administration of artemisinin. It also agreed with Olumide and Raji (2011) who reported significant increase in body and relative organ weights of rats treated with AS and that of Izunya *et al*. (2010) who also reported that AS at its standard and double standard doses had no effect on the body weights of rats. The similarities in these studies may be hinged on the similarity in dosages used.

5.2.2 Variations in brain weights and brain-body weight ratio

For the acute study, no significant variations between the brain weights of the treatment groups and the control group and between the experimental groups was recorded; this strongly suggests that the drugs had no effect on the brain weights of the animals. The brain-body weight ratio also revealed no substantial variation among all the groups depicting that the drugs may not probably have any effect on the relative brain weight. This finding is contrary to Olumide and Raji (2011) who accounted a substantial change in relative organ weight of the rats treated with AS (2.9 mg/kg B.W) for 5 days.

For the delayed post-acute phase, the result of brain weights revealed that the AS group had the highest average brain weight followed closely by that of the CT, followed by the AS+AQ group while the AQ had the lowest average brain weight, although there were no statistical substantial variations among the groups suggesting that there was no difference in the effects of the drugs on brain weights. Accordingly, the AQ group had the highest mean brain-body weight ratio while the AS group had the lowest. This conformed to the result of the body weight showing that the organ weight varies directly proportional with the body weight.

5.3 Variation in neurobehavioural parameters after treatment with AS, AQ and AS+AQ

In the acute phase of this study, result showed that there was insignificant variation in the swimming distance and time in probe trials between all the experimental groups and the CT. This was an indication that the drugs might probably have no significant modulating effects on the spatial memories of the rats within the normal therapeutic doses. Also, the insignificant variations in the frequencies of entering the escape quadrant between all the experimental groups and the CT group could probably be an indication that the drugs might have no modulating effects on the rats' cognitive memories. Further still, insignificant difference in the duration of fore-arm grip between the treatment and the CT groups and among the three experimental groups of animals strongly suggested that the drugs either separately or combined did not affect the grip strength and muscular coordination of the animals. This finding corroborated that of Ekong *et al.* (2009) who recounted that artesunate may not be harmful at its recommended dose and may not affect behaviour. However, this finding contradicted that of Onaolapo *et al*. (2013) who reported a decrease in spatial memory scores in all treatment groups that received AS and AQ compared to the CT (vehicle). The differences in these findings might be associated with the variation in the type of spatial memory tests employed in the two studies.

In the delayed post-acute phase, the significantly higher swimming time and swimming distance covered in the probe trial recorded with the AQ group compared to the CT and the other two experimental groups (AS and AS+AQ) could infer that the drug had negative effect on the spatial memory of the rats while the artesunate and the combination groups did not have significant effect on the spatial memory. However, the insignificant variations in the frequencies of entering the escape quadrant between the treatment groups and the CT group and within the experimental groups indicate that artesunate and amodiaquine separately and combined did not significantly affect the cognition of the rats. This finding was in conformity with Ekong *et al.* (2009) who opined that artesunate may not be harmful at its recommended dose and may not affect behaviour. They also reported that artesunate-amodiaquine combination showed no significant change in behaviour of rats (Ekong *et al.*, 2010). It also agreed partly with the claim of Onaolapo *et al*. (2013) who reported a reduction in spatial memory scores in all treatment groups with AS and AQ compared to the CT (vehicle) with a better performance in the artesunate-amodiaquine combination group. However, the slight

variation in the findings of the two studies may be associated with the difference in the type of animals employed.

Results of the fore-limb grip test showed that AS and AQ separate groups had significantly reduced grip strength than the CT and AS+AQ groups. This could be an indication that the two drugs might have caused muscle weakness in the animals as seen in the reduced activities observed with the animals after few minutes of receiving the drugs. This result was in consonance with the report of Pfizer (2016) that the use of amodiaquine within the therapeutic dose caused lethargy (weakness or inactivity characterized by lack of energy). However, the combination form of the two drugs showed improved grip strength in the animals similar to that of the control.

5.4 Variation in haematological profiles after treatment with AS, AQ and AS+AQ

In the acute study (Table 4.8), there were no substantial variations in the PCV, total RBC count and total WBC count within the treated groups and between the treated groups and the control. Haemoglobin concentration, platelets level, MCH, MCHC and MCV also did not vary significantly between the experimental groups and the control. This might suggest that the separate and combined administration of these drugs may not have enhancing or diminishing effects on the blood cell parameters estimated. Differential WBC count into NEU, EOS and LYMP revealed no significant variations within and between the treated groups and the control; hence the drugs may not significantly increase or decrease any of these parameters. This agreed with the results of Papiya *et al*. (2015) who in their sub-chronic study on artesunate, reported no change in the level of Hb, total RBC, PLAT, LYMP, BAS, MCV and MCHC.

In the delayed post-acute phase, the significant increased level of blood cells (RBC and WBC) as seen in the AQ group (Table 4.9) suggested that the drug stimulated the haemopoietic stem cells in the bone marrow to actively manufacture more blood cells. The significant increased level of haemoglobin in the AQ group than the CT group (Table 4.9) also strongly indicated that there was positive variation between the RBC level and the Hb level. Haemoglobin is an oxygen carrying protein in the RBC, and usually, as the quantity of RBC increases, so does the level of Hb (Sembulingam and Sembulimgam, 2012). Higher PCV level recorded in all the experimental groups, especially the AQ group (Table 4.9) than the CT group also conformed to the results of the blood cell count (RBC and PCV) indicating that the experimental drugs

boosted the blood cell count of the animals within the delayed post-acute period after oral administration of normal therapeutic dosages. The two drugs whether separately or combined however, did not have anysignificant effects on the MCV, MCH and MCHC of the animals. These findings partly agreed with Papiya *et al*. (2015) who recorded no substantial change in the level of Hb, total RBC, PLAT, LYMP, BAS, MCV and MCHC in their subchronic study on artesunate (AS) while total WBC, NEU, EOS, PCV and MCH levels amplified significantly at a higher dose of double the normal dose of artesunate per day. It also partly conforms to Utoh-Nedusa *et al*., (2009) report, that dihydroartemisinin significantly raised the PCV, the WBC and percentage NEU counts in rats. It also conforms to Aprioku and Obianime (2011) who also reported that artesunate, dihydroartemisinin, and artemether significantly and dose-dependently increased white blood cell count and that of Agoma *et al.* (2008) who reported that the mean of all the blood parameters were within the normal limits following artesunate/mefloquine treatment while there was slight increase in WBC count. The concordances of the present findings with the previous findings may be associated with the similarity in dosages of drugs administered and duration of study while the discrepancies may be due to range of factors such as the longer duration of sub-chronic to chronic and even higher dosages of administration as reported in some of the previous studies.

Furthermore, NEU level varied higher in the experimental groups (especially the AQ group) than the CT group while the level of LYMP varied lower in the experimental groups (especially the AQ group) than the CT group. This showed that the drugs (especially AQ) caused proliferation of granulocyte (NEUT) and scanty production of agranulocyte (LYM). This finding contradicted the previous reports that AQ caused agranulocytosis, also known as agranulosis or granulopenia, meaning low level of white blood cell granulocytes count especially the Neutrophils, a condition also called neutropenia (Akindele and Odejide, 1976 and Akpalu and Dodoo, 2005) as well as Agoma *et al.* (2008) who narrated that artesunate/mefloquine initiated slight increase in WBC count; which was more prominent in the lymphocyte count while the neutrophil count decreased as treatment progressed. This variation might be due to the shorter duration of this study compared to the longer duration in the previous studies.

5.5 Variation in biochemical indices after treatment with AS, AQ and AS+AQ

In the acute phase (Table 4.10), the results obtained from the biochemical analyses revealed no substantial variations in the SOD, CAT, GSH and NO levels between the experimental and the control groups indicating no toxic effect potential from the drugs within the dosage and period of treatments used. However, only the group treated with artesunate had substantial rise in MDA level than the CT. This might suggest that the drugs had very low potentials to generate free radicals within the acute duration.

In the delayed post-acute phase (Table 4.11), the result of MDA tissue level showed that it was significantly highest in AQ group, followed by AS group, followed by AS+AQ group while the CT group had the lowest level. This strongly suggested that the experimental drugs, separately or combined triggered the production of MDA. Malondialdehyde has earlier been described as the dialdehyde of malonic acid and a by-product of lipid metabolism in the body. It is reactive electrophile specie that causes OS in cells and form covalent protein adducts, known as advanced lipoxidation end products (ALE). It also forms mutagenic deoxyribonucleic acid (DNA) adducts when it reacts with deoxyadenosine and deoxyguanosine in DNA. It is a biomarker of lipid peroxidation by some drugs or toxins and oxidative damage to lipids because oxidized lipids are able to produce MDA as decomposition product tissues (Nair *et al*., 2008). The increased level of MDA in the AQ, AS and AS+AQ groups suggested that lipid peroxidation occurred in the brain tissues of the experimental animals which could be hinged on generation of free radicals such as hydrogen peroxide, nitric oxide and superoxide anions. This damaging process was highest in the AQ group than the AS group and in AS than the combination (AS+AQ) of the two drugs. This might infer that the combination therapy may be better in managing lipid peroxidation process and reducing toxic stress to the brain cells than the individual drugs. Earlier reports have shown that AQ, in its mechanism of action against malaria parasite, adheres to nucleoproteins of the parasites thereby hindering its DNA and RNA polymerases (O'Neill *et al*, 1998). It also triggers free radicals in the form of amodiaquine-quinine-immine and semi quinine-immine which triggers lipid peroxidation (Maggs *et al*, 1988, Clarke *et al*., 1990). By implication, this could be the reason MDA is significantly highest in the AQ group. Furthermore, lipid peroxidation had been reported to cause damage to tissue *in vivo* (Mayes, 2000). Lipid helps to replace worn-out membranes and serve as electrical insulators thereby allowing quick dissemination of depolarization

waves along myelinated nerve (Mayes, 2000). Disruption of this function may result in neurodegeneration, which might cause the brain to perform deficiently (Singh *et al*., 2013).

The level of nitric oxide (NO) also varied significantly higher in AQ group (Table 4.11) than the CT and other experimental groups (AS and AS+AQ). This result further shows that AQ generated free radicals including NO which have also migrated to the brain. NO is a marker for inflammatory changes in the brain. It is the main secretory product of mammalian cells that initiates host defense by either direct effect or intercellular signaling (Dawson and Dawson, 1995). The substantial upsurge in the level of NO in the brain tissue of AQ group suggests inflammatory responses in the brain tissues of the animals that received the drug.

The level of tissue SOD was significantly reduced in the AS, AQ and AS+AQ groups compared to the CT group. Superoxide dismutase is a form of enzyme antioxidants which act as radical scavenger. It catalyses the conversion of superoxide anion into O_2 and H_2O_2 hence they are markers of OS (Zelko *et al*., 2002). SODs are present in majority of aerobic cells and in extracellular fluids (Nozik-Grayck *et al*., 2005). Therefore the low level of SOD in the AS, AQ and AS+AQ groups is a strong indication that the drugs generated substantial amount of free radicals in the brain tissues of the experimental animals leading to the low level of SOD in those groups compared with the CT group which might have resulted from the overwhelming activity of the free radicals.

The result also showed that the tissue catalase level was significantly lower in the experimental groups (AS, AQ and AS+AQ) than the CT group (Table 4.11). Catalases are also antioxidant enzymes that speed up the breakdown of H_2O_2 to water and O_2 using either iron or manganese as cofactor (Chelikani *et al*., 2004) hence they are indicators of OS. Their levels are expected to be low where there are increased generations of free radicals because high amount of this antioxidant enzyme have been engaged by the free radicals causing reduction in amount present in the tissue. This might explain why the levels of CAT were low in the experimental groups than in the CT group.

The amount of glutathione (GSH) varied substantially lower in all the experimental groups than the CT group (Table 4.11). The order of variation is AQ < AS+AQ < AS < CT. Glutathione is a non-enzymatic antioxidant. It has an antioxidant property because the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized

and reduced (Meister, 1994). It is also a marker of OS due to its antioxidant properties. These three biochemicals (SOD, CAT and GSH) are agents of protection against OS. Therefore, the decreased level of these agents in the experimental groups (AS, AQ and AS+AQ) compared to the control is an indication of their extinction owing to their mopping off activities of the generated free radicals. According to Gibananada and Hussain (2002), OS results from an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and antioxidant defense of the body. This consequently caused oxidative damage such as oxidative modification of cellular macromolecules, induction of cell death by apoptosis or necrosis, as well as structural tissue damage (Nair *et al*., 2008).

5.6 Histological and Immunohistochemical changes in the rats' cerebellum and hippocampus after treatment with AS, AQ and AS+AQ

In the acute study, the general morphological analysis of the cerebellum as observed under the light microscope (Plate 1, Figure 4.2), using image J soft-ware for population count revealed that the population of viable Purkinje cells in each of the three experimental groups did not vary significantly from that of the control. Since the control group was administered with distilled water, expected to be devoid of any pathological changes and a standard of comparison, therefore the population of viable Purkinje cells observed in the experimental groups suggested moderate population, i.e neither overpopulated nor underpopulated. Furthermore, the level of immunohistochemical expressions (GFAP, COX-2 and iNOS) in the experimental groups (Figures 4.6, 4.10 and 4.14 respectively) appeared not significantly different from the control, suggesting that there were moderate level of astrocytes, no inflamed neurons and no OS. These results suggested there were no evidences of astrogliosis (a sign of neuronal degeneration), neuroinflammation, OS, shrinkage, pyknosis, karyolysis or karyorrhesis. The three-day duration of the acute study might have been too short for the drugs administered at the standard dosages to percolate the brain tissue and produce significant histopathologic reactions. This finding contradicted that of Ekong *et al*. (2009) who reported that amodiaquine combined with artesunate at graded doses administered between three and six days caused histological alterations in the cerebellum, such as shrinkage of the Purkinje cells and layer as well as loss of the Purkinje cells. The difference in the findings of the two studies may be due to the variation in the length of duration of administration of the drugs used.

Also, the analysis of the general cytoarchitecture of the hippocampus in the acute study following observation with the light microscope showed that the number of viable Cornu Ammonis 1 cells in each of the experimental groups (AS, AQ and AS+AQ) did not substantially vary from that of the control (Plate 3, Figure 4.4). This suggested that the hippocampal CA1 cells did not undergo neurodegeneration, shrinkage, pyknosis, karyolysis or karyorrhesis. Immunohistochemical results using GFAP, COX-2 and iNOS also revealed no sign of astrogliosis, neuroinflammation and OS in the hippocampus as the expression of GFAP (Plate 7, Figure 4.8), COX-II (Plate 11, Figure 4.12) and iNOS (Plate 15, Figure 4.16) in each of the experimental groups was not substantially different from the control group. Similarly, the three-day acute administration of the drug might be too short for the components of the drugs to percolate the brain tissue and cause pathological changes in the hippocampal neurons and surrounding neuroglia. This finding however contradicted that of Ubulom *et al*. (2015) and Ekanem *et al*. (2009), who reported that acute oral doses of Artesunate/Mefloquine combination (Artequin) and Mefloquine alone at graded doses of half-normal, normal and double-normal doses caused large and dense populations of astrocytes and astrocytes' processes as well as loss of population of pyramidal neurons and vacuolations in the hippocampus of rats. The variation in the current findings from the previous studies might be associated with the difference in the artemisinin combination therapies used in the two studies.

In the delayed post-acute phase, the substantial decrease in the population of viable Purkinje cells in the cerebellum of the AS and most especially AQ separate group compared with the control and combination (AS+AQ) groups (Plate 2, Figure 4.3), suggest that the two drugs caused significant loss of Purkinje cells probably through lipid peroxidation and OS as observed in the biochemical pathways. Further evidences through physical observation also revealed that AQ caused karyolysis of Purkinje cells (as shown by the red circle and arrow in plate 2). The denuded nature of the granular cell layer in the cerebellum of the AQ group also indicated loss of these cells probably through the same process of lipid peroxidation and oxidative damage. In karyolysis, the chromatin of the nucleus fades due to loss of the deoxyribonucleic acid by degradation (Kumar *et al*., 2010). The pathological changes observed with the artesunate and amodiaquine treated groups might have resulted from complication of the lipid peroxidation and OS recorded in the biochemical test of this study because lipid peroxidation had been reported to be responsible for damage to tissue *in vivo* shortly

after cell membrane disruption (Mayes, 2000). Karyolysis of the Purkinje cells may reduce the output signals emanating from the cerebellar cortex to other parts of the brain and alter body functions; this is because the Purkinje fibres are the major cortical output of the cerebellum. Consequently, cerebellar dysfunction may occur resulting in some motor problems like gait disturbances, convulsion and dizziness as opined by Ekong *et al*. (2009). Loss of Purkinje cells might also cause loss of motor coordination and may culminate into death (Stucki *et al*., 2016), hence the reduced activity and lesser grip strength recorded in the treated animals might also be consequence of cerebellar denudation. This finding compares favorably with that of Ekong *et al*. (2009) who reported that amodiaquine+artesunate (8.75+2.86mg/kg) caused histological alterations in the cerebellum such as shrinkage of the Purkinje cells and the Purkinje cell layer, as well as loss of the Purkinje cells in a dose and time dependent pattern.

The substantial reduction in the population of viable hippocampal pyramidal cells (CA1) in the AS and AQ separate groups coupled with physical evidence of pyknotic cells in the AS, AQ and AS+AQ groups (Plate 4, Figure 4.5) was a strong indication that AQ and AS caused death of CA1 cells. This might be hinged on the OS cum damage caused by the presence of significant higher population of reactive nitrogen specie such as NO as expressed by the inducible nitric oxide synthase (Plate 16, Figure 4.17). In the hippocampus, pyramidal cells process sensory and motor cues to form a cognitive map encoding spatial, contextual, and emotional information, which they transmit throughout the brain (Graves *et al*., 2013; Preston and Eichenbaum, 2014). Pyknosis of these cells might reduce the spatial memory of the animals as observed in the neurobehavioural test of this study.

Also, there was significantly higher expression of astrocytes in the AS and AQ groups of both cerebellum (Plate 6, Figure 4.7) and hippocampus (Plate 8, Figure 4.9), compared with CT and AS+AQ groups, suggesting astrogliosis, an indication of neuronal damage or injury and may alter the cerebellar and hippocampal function due to possible alteration in the uptake of neurotransmitter by neurons. Astrocytes had been earlier described as neuroprotective cells which usually proliferate and increase in number during injury to the central nervous system in order to fill the injury sites. This process helps to heal and recover neurons; thus, with this action, they are called reactive astrocytes (Ekanem *et al*. 2009). Abbas and Nelson (2004) also reported that the presence of reactive astrocytes is an indication of early signs of neuronal cell loss, an indication of pathologic process. Findings from this study was in consonance with that

of Udoh *et al*. (2014), who reported that Artesunate/Mefloquine combination (Artequin) and Mefloquine caused large and dense populations of astrocytes and astrocytes' processes in the HP of adult Wistar rats. This, according to the authors may alter neuronal environment and impair the uptake of neurotransmitter thus altering the hippocampal function. This again further buttress the loss of spatial memory recorded against the AQ group in the MWZ test of spatial memory (a function of the hippocampus) in this study.

Statistical analysis revealed no statistical substantial variation in the expression of COX- II by the cerebellum between the experimental (AS, AQ and AS+AQ) and the control groups of animals (Plate 10, Figure 4.11) in the delayed sub-acute study. This indicated that there was no inflammation in this part of the brain following consumption of the drugs. However, there was significantly higher expression of COX-II by the hippocampus of the AS and AQ separate groups when compared with the CT and the AS+AQ groups (Plate 12, Figure 4.13) of the delayed post-acute study. This strongly suggested that AS and AQ caused inflammation of the hippocampal pyramidal (CA1) cells. Inflammation had previously been described as a pathological process resulting from production of cytokines by cells exposed to chemical agents or toxins; this may lead to neuronal dysfunction and eventually death (Ellison *et al*., 2013). Inflammation of a cell or neuron is also considered as one of the causes of necrosis (Sembulingam and Sembulingam, 2012).

The cerebelli of the AS, AQ and AS+AQ groups had significantly higher expression of iNOS compared to the CT (Plate 14, Figure 4.15) in the delayed post-acute study. The same trend was observed for immunoexpression of iNOS by the hippocampi (Plate 16, Figure 4.17). This strongly suggested that the drugs generated free radicals including NO which has percolated into the brain tissues within the delayed post-acute period. This further explained the OS recorded in the biochemical analyses. Oxidative stress had earlier been implicated in tissue damage (Mayes, 2000) and defective function of the brain (Singh *et al*., 2013). It also lent an insight to the outcome of behavioural tests which recorded low spatial memory and reduced muscle strength in the drug-treated group of animals of the delayed post-acute phase. Hence the expression of iNOS as observed in the drug-treated groups of this study strongly indicates pathological reaction such as neurodegeneration which may have resulted from OS.

CHAPTER SIX

6.0 SUMMARY AND CONCLUSION

6.1 Summary

This study was designed to investigate the safety profile of a commonly used ACT antimalarial drug (artesunate and amodiaquine) on the brain. The study was conducted in two phases of acute and delayed post-acute. A total of eighty adult male Wistar rats were used and were divided into four (N=20). Each of the four groups were further sub-divided into two groups (n=10). The first sub-group represented the acute phase while the second group represented the delayed post-acute phase. The four groups consisted of one control (distilled water) and three experimental (treatment) groups (AS, AQ and AS+AQ). Freshly prepared artesunate (4mg/kg BW), amodiaquine (10mg/kg body weight) and artesunate+amodiaquine combination (4mg/kg + 10mg/kg body weight) were administered orally and once per day to the animals in the treatment groups while the animals in the control group were administered distilled water. Drug administration was done once daily at 09:00 hours for three days with the aid of oral gavage. Weights of the animals were monitored throughout the period of the experiment. The animals were subjected to neurobehavioural (Morris-water maze and Fore-limb grip) tests in the first three days (acquisition trials) and the last day (probe trial) of each experiment. Blood samples were collected by ocular puncture and taken to haematology laboratory for comprehensive blood analysis (PCV, RBC, WBC, Platelets and differential WBC counts). Rats were sacrificed by cervical dislocation and brain tissues were excised and immediately weighed. Thereafter, half (n = 5) of the brain tissues were fixed in 10% buffered formol-saline for histological processing while the remaining half (n = 5) were homogenized and processed for biochemical analyses (MDA, NO, SOD, CAT and GSH) in order to determine lipid peroxidation and OS. Routine H&E staining was conducted to study the morphology of the hippocampus and cerebellum. For immunohistochemistry, GFAP was employed to express astrocytic glial cells, COX- II for expression of neuronal inflammation and

iNOS for expression of OS.

Results showed that body weights of rats increased in a similar pattern in both the control and the experimental groups in the acute phase. However, in the delayed post-acute phase, the body weight of the combination group increased in a similar pattern with the CT, the weight of the AS group increased but in a significantly lesser rate than the CT while the weight of the AQ group decreased significantly compared to the CT. There were no substantial brain weights variations among all the groups in both the acute and the delayed post-acute phases. Spatial memory, cognition and muscle coordination were not significantly affected by the drugs in the acute phase but in the delayed post-acute phase, spatial memory was reduced significantly by AQ, cognition was not significantly affected by the two drugs while motor coordination was reduced significantly by the AS and AQ. Haematological analyses revealed that there was no substantial variations in the blood cell parameters between the CT and the experimental groups in the acute phase but in the delayed post-acute phase, there was a substantial increase in the RBC, WBC, Hb, PCV and neutrophil counts in only the AQ group compared to the CT group. Biochemical analyses showed that in the acute phase, only the AS group had substantial upsurge in the level of MDA compared to the CT while other markers revealed no significant difference compared to the CT group. In the delayed post-acute phase, there was significant higher levels of MDA and NO (except AS) in the treatment groups compared to CT group while the SOD, CAT and GSH decreased significantly in the experimental groups compared to the CT group.

Histological and immunohistochemical analyses with H&E, GFAP, COX-II and iNOS revealed no significant observable changes in the cerebelli and hippocampi of the treated animals when compared to the CT in the acute phase. In the delayed post-acute phase, however, results revealed that AS and AQ caused significant decrease in the Purkinje cell population. Amodiaquine caused pyknosis of Purkinje cells and denudation of granular cells. Hippocampal CA1 pyramidal cell population decreased significantly in both the AQ and AS groups. Immunohistochemical staining with GFAP showed significantly higher expression of astrocytes in the cerebelli of AQ and AS groups compared to the CT group and significantly higher expression of astrocytes in the hippocampi of AS and AQ treated groups compared to the CT. COX-II expression by the cerebellum was not substantially different between the experimental groups and the CT group but it was expressed significantly higher in the hippocampus of AS and AQ separate groups compared to the CT group. Expressions of iNOS were also higher in the

cerebelli and hippocampi of each of the experimental groups compared to the CT group.

6.2. Conclusion

This study showed that treatment with AS, AQ and AS+AQ within the acute phase had no significant effect on (i) the body and brain weights of the Wistar rats; (ii) the spatial memory, cognition and motor coordination of the rats (iii) the blood cell count and volume of the rats; (iv) the biochemical markers of OS in the brain tissues of the animals; (v) the cytoarchitecture of the hippocampus and the cerebellum of the animals. However, the delayed post-acute phase results showed that: (i) administration of AQ at its normal therapeutic dose caused significant decrease in the body weights of the animals; (ii) AQ reduced the spatial memory of the animals while AS and AQ significantly reduced the motor coordination of the animals; (iv) AQ significantly increased the blood cell parameters in the animals; (v) AS, AQ and AS+AQ caused OS in the brain tissue of the animals; (vi) AS and AQ caused reduced population of Purkinje cells while AQ alone caused denudation of granule cells in the cerebellum; (vii) AQ and AS+AQ caused decreased population of pyramidal CA1 cells in the hippocampus; (viii) AS and AQ caused astrogliosis in the cerebellum and hippocampus; (ix) Both AS and AQ also caused inflammation in the hippocampus (x) AS, AQ and their combination caused OS in both the cerebellum and the hippocampus of the animals.

Based on these findings, artesunate and amodiaquine and their combination may be reported to have some delayed post-acute deleterious effects in the HP and CB of adult Wistar rat.

This research has thrown light on the gray area of delayed post-acute effect of AS, AQ and AS+AQ on the hippocampus and cerebellum of adult Wistar rats. None of the previous researches had similar design with this work as most of the researchers either looked at the immediate acute or chronic effect of these drugs on the experimental animals. The current design is unique because, even the standard clinical recommendation for taking these antimalaria drugs is three days while the effect on the body may linger for days after use.

Based on the scope and limitations of this study, future researches should endeavour to:

i. explore the effects of these two drugs separately and combined on the hippocampus and cerebellum over a considerable long term, i.e sub-chronic and chronic.

ii. investigate the effects of these two drugs separately and combined on the hippocampus and cerebellum using higher/variable doses.

iii. conduct similar study using other forms of ACTs.

REFERENCES

Abbas, A. and Nelson, F. K. 2004. Robin and Cotran Pathologic Basis of Diseases. 7th ed. *New Delhi: Elsevier*.

Adebayo, J. O., Igunnu, A., Arise, R. O., Malomo, S. O. 2011. Effect of Co-Administration of Artesunate and Amodiaquine on Some Cardiovascular Disease Indices In Rats. *Elsevier Journal of Food and Chemical Toxicology* 49: 45-48.

Adjuik, M., Agnamey, P., Babiker, A., Borrmann, S., Brasseur, P., Cisse, M., Cobelens, F., Diallo, S., Faucher, J. F., Garner, P., Gikunda, S., Kremsner. P. G., Krishna, S., Lell, B., Loolpapit, M., Matsiegui, P. B., Missinou, M. A., Mwanza, J., Ntoumi, F., Olliaro, P., Osimbo, P., Rezbach, P., Some, E. and Taylor, W. R. 2002. Amodiaqune-Artesunate versus Amodiquine For Uncomplicated Plasmodium Falsiparum Malaria In African Children: A Randomized, Multi-Centre Trial. *Lancet* 359:1365-1372.

Afifi, A. K. and Bergman, R. A. 2005. Functional Neuroanatomy: Text and Atlas. 2nd ed. *New York: McGraw-Hill*.

Agoma, U. P., Merimikwu, M. M., Ismaila, M. W., Omalu, I. T., Oguche, V. I, and Odey, S. 2008. Efficacy, Safety and Tolerability of Plasmodium Falciparum Malaria in four geographical zones of Nigeria. *Malaria Journal*. 7: 172-180.

Akindele, M. O, Odejide, A. O. 1976. Amodiaquine-induced involuntary movements. *British Medical Journal.* 2.6029: 214–215.

Akpalu, A. K. N. P. and Dodoo, A. N. O. 2005. Amodiaquine-induced dystonic reactions: case reports and implications for policy change in Ghana. *International Journal of Risk and Safety in Medicine.* 17: 1–4.

Andersen, Per, et al. 2007. The Hippocampus Book. *Oxford University press*.

Aprioku, J. S and Obianime A. W. 2011. Structure Activity- Relationship (SAR) of Artemisinin on Some Biological Systems on Male Guinea Pigs. *Insight Pharmaceutical Science* 1.1:1-10.

Asimus, S., Elsherbiny, D., Hai, T. N. 2007. Artemisinin Antimalarials Moderately Affect Cytochrome P450 Enzyme Activity In Healthy Subjects. *Fundamental and Clinical Pharmacology.* 21:307-316.

Bancroft, J. D., and Gamble, H. 2008. Theory and practice of histological technique. 6th ed. *Philadelphia: Churchill Livingstone/Elsevier*. 725.

Bagchi, K. and Puri, S. 1998. Free radicals and antioxidants in health and disease. *East Mediterranean Health Journal.* 4: 350–60.

Baker, F. J, Silverton, R. E. and Pallistea, C. J. 1998. Introduction to Medical Laboratory Technology, Nigeria: Bouty press 354 – 370.

Berry, R. B., Matthews, D. B. 2004. Acute Ethanol Administration Selectively Impairs Spatial Memory In C57BL/6J Mice. *Alcohol* 32: 9-18.

Beuge, J. A, and Aust S. D. 1978. Microsomal Lipid peroxidation. *Method Enzymol*.30: 302-310.

Bliss, T. V. and Collingridge, G. L. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361: 31–39.

Blundell, J., Finlayson, G., Axelsen, M., Flint, A., Gibbons, C., Kvist, T. and Hjerpsted, J. B. 2017. Effects of once-weekly semaglutide on appetite, energy intake, control of eating, food preference and body weight in subjects with obesity. *Diabetes, Obesity and Metabolism* 19.9: 1242.

Broe, M., Kril, J. and Hallidal, G. M. 2004. Astrocytic degeneration relates to the severity of disease in frontotemporal dementia. *Brain.* 127: 2214–2220.

Centre for Disease Control (CDC). 2016. Recommendations for travel to areas with malaria. USA

Chelikani, P., Fita, I. and Loewen, P. 2004. Diversity of structures and properties among catalases. *Cell and Molecular Life Science.* 61.2: 192–208.

Clark, R. L., Arima, A. and Makori, N. 2008. Artesunate: Development Toxicity and Toxicokinetics in Monkeys. *Birth Defect Research.* 83: 418-434.

Clark, R. L. 2009. Embryotoxicity of the Artemisinin Antimalarials and Potential Consequences for Use In Woman In The First Trimester. *Reproductive Toxicology*.

Clarke, J. B, Maggs, J. L, Kitteringham, N. R, *et al*. 1990. Immunogenicity of

Amodiaquine in the Rat. *International Archives of Allergy and Applied Immunology.* 91.4: 335-42.

Danglot, L., Triller, A. and Marty, S. 2006. The Development of Hippocampal Interneurons in Rodents. *Hippocampus* 116: 1032-1060.

Dawson, T. M. and Dawson, V. L. 1995. Nitric oxide: Actions and pathological roles. *The Neuroscientist* 1: 7–18.

Delcambre, G. H., Lui, J., Herrington, J. M., Vallario, K. and Long, M. T. 2016. Immunohistochemistry for the detection of neural and inflammatory cells in equine brain tissue. *Peer Journal* 4: 1601.

Delion, M. Mercier, P. Brassier, G. 2016. Advances and Technical Standards in *Neurosurgery* 43: 185-216.

Durrani, N., Leslie, T., Rahim, S., Graham, K., Ahmad, F., Rowland, M. 2005. Efficacy of combination therapy with artesunate plus amodiaquine compared to monotherapy with chloroquine, amodiaquine or sulfadoxine-pyrimethamine for treatment of uncomplicated *Plasmodium falciparum* in Afghanistan. *Tropical Medicine and International Health* 10:521–529.

Duvernoy, H. M., Cattin, F. and Risold, P. 2005. *The Human Hippocampus: Functional Anatomy, Vascularization and Serial Sections with MRI*. 4th ed. New York: Springer Science & Business Media. 56-58.

Edgar, N. and Sibille, E. 2012. A putative functional role for oligodendrocytes in mood regulation. *Translational Psychiatry* 2:109.

Ekanem, T. B., Salami, E., Ekong, M. B., Eluwa, M. A. and Akpantah, A. O. 2009. Combination Therapy, Anti-Malaria Drugs, Mefloquine and Artequin induced Reactive Astrocyte Formation in the Hippocampus of Rats. *The Internet Journal of Health* 9: 2.

Ekong, M. B., Igiri, A. O. and Mesembe, O. E. 2008. The Effect of Administration of Amodiaquine on Some Parameters of Behavior of Wistar Rats. *Nigerian Journal of Physiological Sciences* 23.1-2: 51-54.

Ekong, M. B., Igiri, A. O. and Ekenam,T. B. 2009. Effect of artesunate treatment on

some brain biomolecular and its behavioural implication. *J. Bagla Soc Physiol* 4.2: 44 – 50.

Ekong, M. B., Igiri, A. O., Ekenam,T. B. and Ekeoma, A. O. 2010. Behavioural partterns of rats in an open field following treatment with artesunate and amodiaquine combination. *African scientist* 11.3: 195-202.

Eichenbaum, H. 2000. A cortical- hippocampal system for declarative memory. *Nature Reviews Neuroscience.* 1: 41–50.

El-Bermawy, Manal I., Salem, Maysa F. 2015. Histological changes of the albino rat cerebellar cortex under the effect of different doses of tramadol administration. *The Egyptian Journal of Histology* 38.1: 43-155.

Ellison, D., Love, S., Chilli, L., Harding, B. N., Lowe, J. S., Vinters, H. V., Brandner, S., and Yong., W. H. 2013. *Neuropathology: A reference text of CNS Pathology*. 3rd Ed. Edinburg: Elsevier. 130-135.

Eweka, A. E., and Adjene, J. O. 2008. Histological studies of the effect of artesunate on superior colliculus of adult Wistar rats. *Internet Journal of Tropical med*icine. 4.2: 22-35.

Faloju, I. E. and Lesi, F. E. A. 2010. Drug-Induced Acute Dystonia in a 7 Year Old Child Following the Use of Artesunate-Amodiaquine: A Case Report. *International Journal of Risk & Safety in Medicine* 22: 111-114.

Farombi, E. O. 2000. Influence of Amodiaquine treatment on microsomal lipid peroxidation and antioxidant defence systems of rats. *Pharmacology and. Toxicology* 87.6: 249-54.

Ferreira, J. F. S. and Janick, J. 2002. Production of artemisinin from *in vitro* cultures of *Artemisia annua* L. In: *Biotechnology in Agriculture and Forestry* Vol. 51, T. Nagata & Y. Ebizuka (Eds.). Springer Verlag. 1-12.

Gao, J. H., Parsons, L., Bower, J. M., Xiong, J., Li, J. and Fox, P. T. 1996. CB Implicated in Sensory Acquisition and Discrimination Rather Than Motor Control. *Science* 272: 545-547.

Gahr, M. 2002. What is the adaptive role of neurogenesis in adult birds? *Progress in Brain Research.* 138: 233-254.

Garavan, H., Kaufman, J. N. and Hester, R. 2008. Acute Effects of Cocaine on the Neurobiology of Cognitive Control. *Philosiphical Transaction of the Royal Society London B Biological Science.* 363: 3267-3276.

Genovese R. F., Newman D. B., Brewer T. G. 2000. Behavioral and Neurotoxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats. *Pharmacology, Biochemistry and Behaviour.* 67.1: 37-44.

Gibananada, R. and Hussain, S. A. 2002. Oxidants. *Indian Journal of Experimental Biology*. 40: 1213–1232.

Ginsburg, H., and Atamna, H. 1994. "The redox status of malaria affected erythrocyte: an overview with an emphasis on unresolved problems". *Parasite.* 1.1: 5 – 13.

Gonzalo A. 2012. Functional Anatomy and Physiology of the Hippocampus In: Introduction to epilepsy. 37-39.

Graves, A. R., Moore, S. J., Bloss, E. B., Mensh, B. D., Kath, W. L. and Spruston, N. 2013. Hippocampal pyramidal neurons comprise two distinct cell types that are countermodulated by metabotropic receptors. *Neuron* 76.4: 776-789.

Gupta, A. S., van der Meer, M. A., Touretzky, D. S. & Redish, A. D. 2012. *Segmentation of spatial experience by hippocampal theta sequences*. *Nat. Neurosci.* 15, 1032–1039.

Haynes, R. K., Cheu, K. W., Tang, M. M., and Wong, H. N. 2011. A partial convergence in action of methylene blue and artemisinin: Aptagonism with chloroqune, a reversal with verapamil, an insight into the antimalaria activity of chloroquine. *Chem.med,* 6.9: 1603 – 1615.

Herculano-Houzel, S. 2010. Coordinated Scaling of Cortical and Cerebellar Numbers of Neurons. *Front Neuroanat* 4:12.

Hill, M. A. 2016. Embryology: Neural-CB Development in the Rats by Use of Nonlinear Mixed Effects Modeling. *Biopharm Drug Dispos*. 24: 71-85.

Imosemi, I. O., Osinubi, A. A., Saalu, L. C. and Olagunju, J. A. 2010. Phenytoin-Induced Toxicity in the Postnatal Cerebellar Development in Rat: Effect of Calotropisprocera on Selective Biochemical and Haematological Variables. *International Journal of Biological and Chemical Sciences* 4.6: 2387-2396.

Izunya, A. M., Nwaopara, A. O. and Oaikhena, G. A. 2010. Effect of Chronic Oral Administration of Chloroquine on the Weight of the Heart in Wistar Rats.*Asian Journal of Medical Sciences* 2.3: 127-31.

Jimsheleishvili, S., Dididze, M. 2019. Neuroanatomy, Cerebellum. *https://www.ncbi.nlm.nih.gov/books/NBK538167* (*last accessed 19/1/2020).*

Kandel, E. and Schwartz, J. 2013. *Principles of Neural Science*. 5th ed. New York: McGraw-Hill.

Kappe, S. H., Vaughan, A. M., Boddey, J. A., and Cowman, A. F. 2010. "That was then but is now: malaria research in the time of an eradication agenda" *Science,* 328:5980: 75 – 94.

Kersaitis, C., Halliday, G. M. and Kril, J. J. 2004. Regional and cellular pathology in frontotemporal dementia: relationship to stage of disease in cases with and without Pick bodies. *Acta Neuropathology* 108: 515–523.

Kettenmann, H. and Ransom, B. R. 2004. *Neuroglia*. 2nd ed. Oxford, UK: Oxford University Press.

Kevin, L. 2007. A rat model for systemic chemotherapy in breast cancer. *JOUR:* 43.

Koshiba, T., Hosotani, R., Miyamoto, Y., Wada, M., Lee, J. U, Fujimoto, K., Tsuji, S., Nakajima, S., Doi, R. and Imamura, M. 1999. Immunohistochemical analysis of cyclooxygenase-2 expression in pancreatic tumors. *International Journal of Pancreatology.* 26: 69-76.

Kumar, V., Abbas, A. K., Aster, J. C. and Fausto, N. 2010. *Robbins and Cotran Pathologic basis of disease.* 8th ed. Philadelphia: Saunders/Elsevier.

Li, Y. and Wu, Y. L. 2002. An Over Four Millennium Story behind Qinghaosu (Artemisinin) - A Fantastic Antimalarial Drug from a Traditional Chinese Herb. *Current Medicinal Chemistry.* 10: 2197-2230.

Liwang, C. and Xin – Zhaun, S. U. 2009. Discovery mechanism of action and combination therapy of artemisinin. *Expert Review on Antimalaria therapy* 7.8: 99 – 1013.

Liwang, C, Chen, G., Zhao, J., Nie, X. and Wan, C. 2013. 2,3,7,8 - Tetrachlorodibenzo-P-Dioxin (TCDD) Induces Microglial Nitric Oxide Production and Subsequent Rat Primary Cortical Neuron Apoptosis Through P38 / JNK MAPK Pathway. *Toxicology* 312: 132 – 141.

Lyck, L., Dalmau, I., Chemnitz, J., Finsen, B., and Schroder, H. D. 2008. Immunohistochemical Markers for Quantitative Studies of Neurons and Glia in Human Neocortex. *Journal of Histochemistry and Cytochemistry* 56.3: 201-221.

Lyda, O., Iveth, G., Piero, O. and Walt, R. J. 2007. Artemisinin Based Combination Therapy for Uncomplicated Falciparum Malaria in Colombia. *Malaria Journal* 6: 25-33.

Maggs, J. L, Tingle, M. D, Kitteringham, N. R. 1988. Drug-Protien Conjugates—XIV. Mechanisms of Formation of Protein-Arylating Intermediates from Amodiquine, A Myelotoxin and Hepatotoxin in Man. *Biochemical Pharmacology.* 37.2: 303-11.

Malomo, A. O., Owoeye, O., Elumelu, T. N., Akang, E. E. U, Adenipekun, A., Campbell, O. B. and Shokunbi, M. T. 2005. The Effect of Dexamethasone, Metronidazole, and Ascorbic Acid on the Morphological Changes Induced By Gamma Rays on The Spinal Cord of Wistar Rats. *African Journal of Medicine and Medical Sciences.* 34.2: 161- 165.

Malomo, A. O., Imosemi, I. O., Osuagwu, F. C., Oladejo, O. W., Akang, E. E. U. and Shokunbi, M.T. 2004. Histomorphometric Studies on the Effect of Cyanide Consumption of the Developing Cerebellum of Wistar Rat (Rattus Novergicus). *West African Journal of Medicine* 23.4: 3323-328.

Martinez, S. and Armengol, J. A. 2015. The Cerebellum: From Development to Learning. Lausanne: *Frontiers Media.* Doi: 10.3389/978-2-88919-619-7.

Mayes, P. A. 2000. Lipid of physiologic significance In: Murray, R. K.; Granner, D. K., Mayes, P. A. and Rodwell, V. W. (eds) *Harper's Biochemistry*. 25th ed. Mecurant-Hall, New York. 160-171.

Meister, A., and Anderson, M. E. 1983. Glutathione. *Annual Review of Biochemistry* 52.7: 11–60.

Meister, A. 1994. Glutathione-ascorbic acid antioxidant system in animals. *The Journal of Biological Chemistry* 269.13: 397–400.

Meshnick, S. R. 2002. Artemisinin: mechanism of action, resistance and toxicity. *International Journal for Parasitology.* 32.13: 1655-60.

Miles, A. M., Wink, D. A., Cook, J. C and Grisham, M. B. 1996. Determination of nitric oxide using fluorescence spectroscopy. *Methods Enzymology* 268: 105-120

Miller, L. G. and Panosian, C. B. 1997. Ataxia and Slurred Speech after Artesunate Treatment for Falciparum Malaria. *New England Journal of Medicine.* 336: 1328.

Morris, R. 1984. Development of a Water-Maze Procedure for Studying Spatial Learning in the Rat. *Journal of Neuroscience Methods.* 11: 47-60.

Nair, V., O'Neil, C. L. and Wang, P. G. 2008. Malondialdehyde, Encyclopedia of Reagents for Organic Synthesis. *John Wiley & Sons, New York.*

National Library of Medicine, United State. 2015. Clinical Research Information on Drug-Induced Liver Damage.

National Research Council. 1996. International Guide for the Care and Use of Laboratory Animals.

Nontprasert, A., Pukrittayakamee, S., Dondorp, A. M, Clemens, R., Looareesuwan, S. and White, N. J. 2002. Neuropathologic Toxicity of Arteemisinin Derivatives in a Mouse Model. *American Journal of Tropical Medicine and Hygiene.* 67.4: 423-429.

Nontprasert, A., Nosten-Bertrand, M., Pukrittayakamee, S., Vanijanonta, S., Angus, B. J. and White, N. J. 1998. Assessment of the Neurotoxicity of Parental Artemisinin Derivatives in Mice. *American Journal of Tropical Medicine and Hygiene.* 59.4: 519-522.

Nontprasert, A., Pukrittayakamee, S., Nosten-Bertrand, M., Vanijanonta, S. and White, N. J. 2002. Studies of the Neurotoxicity of Oral Arteminsinin Derivatives in Mice. *American Journal of Tropical Medicine and Hygiene.* 62: 409 – 412.

Nosten, F. and White, N. J. 2007. Artemisinin-Based Combination Treatment of *Falciparum Malaria*. *American Journal of Tropical Medicine and Hygiene.*77.6: 181-192.

Nozik-Grayck, E., Suliman, H., Piantadosi, C. 2005. Extracellular superoxide dismutase. *International Journal of Biochemical and Cell Biology.* 37.12: 2466 –71.

OECD. 2000. Guidance Document on Acute Oral Toxicity. Environmental Health and Safety Monograph Series on Testing and Assessment. No.24.

Olliaro, P. and Mussano, P. 2006. Amodiaquine for Treating Malaria. *The Cochrane Database of System Reviews* 2: 16.

Olumide, S. A. and Raji, Y. 2011. Long-Term Administration of Artesunate Induces Reproductive Toxicity In Male Rats. *Journal of Reproductive and Infertility* 12.4: 49-65.

Omotosho, G. O. and Babalola, F. A. 2014. Histological Changes in the Cerebelli of

Adult Wistar Rats Exposed to Cigarette Smoke. *Nigerian Journal of Physiological Sciences.* 29: 043- 046.

Onaolapo, O. J, Onaolapo, A. Y, Awe, E. O, Jibunor, N. and Oyeleke, B. 2013. Oral Amodiaquine, Artesunate and Artesunate-Amodiaquine Combination Affects Open Field Behaviors and Spatial Memory in Healthy Swiss Mice. *Journal of Pharmacy and Biological Sciences* 3: 569-575.

Onaolapo, O. J, Onaolapo, A. Y, Awe, E. O. 2013. Oral Artesunate-Amodiaquine Combination Causes Anxiolysis and Impaired Cognitive In Healthy Swiss Mice. *Journal of Pharmacy and Biological Sciences* 7.2: 97-102.

O'Neill, P. M., Bray, P. G., Hawley, S. R., Ward, S. A. and Park, B. K. 1998. 4-aminoquinolines-past, present, and future; a chemical perspective. *Pharmacology and Therapeutics* 77:29-58.

Oreagba, I. A. 2010. Pharmacology of Artemisinin-Based Combination Therapies. *Pharmacology and Therapeutics* 363: 9-17.

Osuagwu, F. C., Owoeye, O., Avwioro, O. G., Oluwadara, O. O., Imosemi, I. O., Ajani, R. S., Ogunleye, A. A. and Oladejo, O. W. 2007. Reduction of Hippocampal CA1 Neurons in Wistar Rat Following the Administration of Phenytoin for Seven Days. *African Journal of Medicine and Medical Sciences* 36.2: 103-108.

Owoeye, O., Farombi, E. O. and Onwuka, S. K. 2011. Gross Morphometric with Vernonia Amygdalina Leaf Extract. *Romanian Journal of Morphology and Embryology* 52.1: 81-88.

Panossian, L. A., Garga, N. I. and Pelletier, D. 2006. "Toxic Brainstem Encephalopathy after Artemisinins Treatment for Breast Cancer," *Annals of Neurology* 58; 5: 812-813.

Papiya, B., Taranginees, and Vikalp, T. 2015. Hematology and Biochemistry Effects of Sub-Chronic Artesunate Exposure in Rats. *Elsevier Toxicology Reports* 280-288.

Pfizer, 2016. Larimal® Drug Leaflet.Ipca Laboratories Ltd. 48, Kandivi Ind. Estate, Mumba; 400067.

Pichon, X., Wattiez, A., Becamel, C., Ehrlich, I. and Bockaert, J. 2014. Disrupting 5-HT2A Receptor/PDZ Protein Interactions Reduces Hyperalgesia and Enhances SSRI Efficacy Neuropathic Pain. *Molecular Therapy journals.* 18.8: 1462-70.

Preston, A. R. and Eichenbaum, H. 2014. Interplay of Hippocampus and Prefrontal Cortex in Memory. *Current Biology.* 23.17: 9.

Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, W.C, Lamantia, A.S., Mcnamara, J.O., Williams, S.M. 2004. Neuroscience. 3rd edition. *Sinauer Associates, Inc. Publishers*, Sunderland, Massachusetts. U.S.A.

Purves, D. 2011. *Neuroscience.* 5th ed. *Sinauer Associates, Inc. Publishers*, Sunderland, Massachusetts. U.S.A.

Pussard, E. and Verdier, F. 2009. Antimalarial 4- aminoquinolines: mode of action and pharmacokinetics. *Fundamental & Clinical Pharmacology* 8.1: 1-17.

Roostaei, T., Nazeri, A., Sahraian, M. A. and Minagar, A. 2014. The Human CB: A Review of Physiologic Neuroanatomy. *Neural Clin* 32: 859-869.

Ross, M. H., and Pawlina, W. 2010. Histology: A text and atlas: With correlated cell and molecular biology. 6th edition. Philadelphia: Lip pincott Williams & Wilkins.

Schenk, F. and Morris R. G. (1985). Dissociation between components of spatial memory in rats after recovery from the effects of retrohippocampal lesions. *Experimental Brain Research.* 58: 11–28.

Schmitz, C., Born, M., Dolezel, P., Rutten, B. P, Saint-Georges, L., Hof, P. R, Korr, H. 2005. Prenatal protracted irradiation at very low dose rate induces severe neuronal loss in rat hippocampus and CB. *Neuroscience* 130.4: 935–948.

Schmuck, G., Klaus, A. M., Krötlinge, F. and Langewische, F. W. 2009. Current developments in reproductive toxicity testing of pesticides. *Development and Reproduction Toxicology* 86.2: 131-143.

Sembulingam, K. and Sembulingam, P. 2012. *Essentials of Medical Physiology*. 6th ed. New Delhi: Jaypee Brothers Medical Publisher Ltd.

Sinclair, D., Donegan, S., Isba, R., Lallo, D. G. 2012. Artesunate versus Quinine for Treating Severe Malaria. *The Cochrane Database of Systematic Reviews* 6: 6.

Singh, I. N., Gilmer, L. K, Miller, D. M, Cebak, J. E, Wang, J. A, Hall, E. D. 2013. Phenelzine mitochondrial functional preservation and neuroprotection after traumatic brain injury related to scavenging of the lipid peroxidation-derived

aldehyde 4-hydroxy-2-nonenal. *Journal of Cerebral Blood Flow and Metabolism.* 33: 593–9.

Smith, J. P., Hicks, P. S., Ortiz, L. R., Martinez, M. J. and Mandler, R. N. 1995. Quantitative measurement of muscle strength in the mouse. *Journal of Neuroscience Methods.* 62: 15–19.

Stucki, D. M., Ruegsegger, C., Steiner, S., Radecke, J., Murphy, M. P., Zuber, B. and Saxena, S. 2016. Mitochondrial Impairments Contribute to Spinocerebellar Ataxia Type 1 Progression can be ameliorated by the Mitochondria-targeted Antioxidant Mitoq. *Free Radical Biology and Medicine.* 97: 427-440.

Tester-Dalderup, D. B. M. 1984. Antiprotozoal drugs. In: Dukes MNG (ed) Meyler's Side Effects of Drugs. 10th ed. *Amsterdam: Elsevier Science Publishers* 966.

Tewari, A., Hasan, M., Sahai, A., Sharma, P. K., Rani, A., Agarwal, A. K. 2010. White core CB in nicotine treated rats: a histological study. *Journal of Anatomical Society of India.* 59.2: 150-153.

Tietze, F. 1969. Enzymatic method for the quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal. Biochem* 27: 502-522.

Tian, G. F, Azmi, H., Takano, T., Xu, Q., Peng, W., Lin, J., Oberheim, N., Lou, N., Wang, X., Zielke, H. R, Kang, J. and Nedergaard, M. 2005. An astrocytic basis of epilepsy. *Nature Medicine.* 11: 973–981.

Turner, D. A. et al. 1998. The Morphological functions of entorhinal - hippocampal connection. *Progress in Neurobiology*. 55.6: 537-562

Ubulom, P. M. E., Udobi, C. E., and Madu, M. I. 2015. Amodiaquine and Ciprofloxacin Combination in Plasmodiasis Therapy. *Journal of Tropical Medicine* 2015.

Udoh N. B, Ekanem T. B, Ekong M. B, Peter A. I, and Akpantah A. O. 2014. Hippocampal Glial Degenerative Potentials of Mefloquine and Artequin in Adult Wistar Rats. 2: 104785.

Utoh-Nedusa, P. A., Akah, P. A., Okoye, T. C. and Okoli, C. O. 2009. Evaluation of Toxic Effects of the Dihydroartemisinin on Vital Organs of Wister Albino Rats. *American Journal of Pharmacology and Toxicology*. 4.4: 169-179.

Van Agtmael, M.A., Cheng-Qing, R., Mill, C.J., and Van, B. 1999. Multiple dose

pharmacokinetics of artemether in Chinese patient with uncomplicated *falciparum* malaria. *International Journal of Antimicrobial Agents. 12:* 151 – 158.

Vane J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism ofaction for aspirin-like drugs. *Nature: New Biology.* 231: 232–235.

Verkhratsky, A., Parpura, V. 2015. Astrogliopathology in Neurological, Neurodevelopmental and Psychiatric Disorders. *Neurobiology of Disease* 3: 1-8.

Verkhratsky, A.,Rodriguez, J. J., Steardo, L. 2014b. Astrogliopathology: A Central Element of Neuropsychiatric Diseases. *Neuroscientist.* 20: 576-588.

Vorhees, C. V. and Williams, M. T. 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature Protocols.* 1.2: 848-858.

Wood, S. C, Fay, J., Sage, J. R. and Anagnostaras, S. G. 2007. Cocaine and Pavlovian Fear Conditioning: Dose-Effect Analysis. *Behavioural Brain Research.* 176: 244-250.

World Health Organization. 2015. WHO Guidelines for the Treatment of Malaria. 3rd ed. Geneva, Switzerland: WHO.

World Health Organization. 2001. Antimalarial Drug Combination Therapy; Report of a WHO Technical Consultation. *Geneva* 60-70.

World Health Organization. 2000. The Use of Antimalarial Drugs; Report of a World Health Organization Informal Consultation. *Geneva* 43-100.

Yassa, M. A. 2006. The Role of Hippocampus in Memory. *Trends in Neurosciences* 35.10: 515-525.

Zelko, I., Mariani, T. and Folz, R. 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med* 33.3: 337–349.

Zeng, Q., Qin, F. and Yuan, L. 2008. Production of Artemisinin by Genetically-modified Microbes. *Biotechnology Letters* 30: 581-592.

Zhang, S. J, Ye, J., Miao, C., Tsao, A., Cerniauskas, I., Ledergerber, D., Moser, M. B. and Moser, E. I. 2013. Optogenetic dissection of entorhinal-hippocampal functional connectivity. *Science* 340.6128:1232627.

Zhang, Y., Loh, H. H. and Law, P. 2016. Effect of Opioid on Adult Hippocampal Neurogenesis. *The scientific World Journal* 2016: 2601264.

Zhao, K. and Song, Z. 1989. Distribution and Excretion of Artesunate in Rats. *Chinese Academy of Medical Sciences and Peking Union Medical College* 4: 186-188.

APPENDICES

Appendix I

Table 1. Body and Brain weights of the animals in the acute phase

Group	Initial weight (g)	Final weight (g)	Brain weight (g)
CT1	187	192	1.85
CT2	204	188	1.74
CT3	180	210	1.67
CT4	226	216	1.73
CT5	210	254	1.60
CT6	220	235	
CT7	182	219	
CT8	176	190	
CT9	198	217	
CT10	208	225	
AS1	189	190	1.9
AS2	180	208	1.67
AS3	210	192	1.61
AS4	198	205	1.65
AS5	173	188	1.62
AS6	185	215	
AS7	239	251	
AS8	175	186	
AS9	209	220	

AS10	204	207	
AQ1	186	185	1.63
AQ2	188	190	1.53
AQ3	185	196	1.95
AQ4	190	185	1.63
AQ5	187	194	2.04
AQ6	205	192	
AQ7	189	215	
AQ8	185	203	
AQ9	195	199	
AQ10	200	215	
AS+AQ1	180	192	1.73
AS+AQ2	165	185	1.65
AS+AQ3	166	176	1.67
AS+AQ4	172	183	1.97
AS+AQ5	146	170	1.89
AS+AQ6	169	178	
AS+AQ7	186	177	
AS+AQ8	181	189	
AS+AQ9	165	202	
AS+AQ10	189	197	

Appendix II

Table 2. Body and Brain weights of the animals in the delayed post-acute phase

Group	Initial weight (g)	Final weight (g)	Brain weight (g)
CT1	115	175	1.66
CT2	110	168	1.74
CT3	107	140	1.58
CT4	106	133	1.52
CT5	112	136	1.64
CT6	111	150	1.71
CT7	109	145	1.68
CT8	110	155	
CT9	107	160	
CT10	113	170	
AS1	172	201	1.84
AS2	165	178	1.66
AS3	162	155	1.69
AS4	160	122	1.53
AS5	172	183	1.58
AS6	170	182	1.69
AS7	175	211	1.76
AS8	165	200	
AS9	170	190	
AS10	168	182	

AQ1	180	141	1.6
AQ2	177	121	1.58
AQ3	175	92	1.37
AQ4	185	105	1.4
AQ5	181	130	1.59
AQ6	182	128	1.57
AQ7	180	160	1.8
AQ8	188	125	
AQ9	170	130	
AQ10	175	140	
AS+AQ1	135	152	1.68
AS+AQ2	140	133	1.6
AS+AQ3	138	156	1.66
AS+AQ4	141	142	1.65
AS+AQ5	137	161	1.61
AS+AQ6	136	174	1.66
AS+AQ7	139	178	1.27
AS+AQ8	145	150	
AS+AQ9	135	155	
AS+AQ10	130	160	

Appendix III

Table 3. Results of Neurobehavioural Parameters of Study (Acute study)

Group	Swimming Time (sec)	Swimming Distance (m)	Quadrant Crossing	Fore-Arm Grip Duration (sec)
CT1	7	300	8	17
CT2	3	128	7	14
CT3	5	135	7	17
CT4	2	120	8	20
CT5	2	120	6	14
CT6	6	295	5	21
CT7	4	130	6	22
CT8	5	200	6	11
CT9	9	355	5	10
CT10	4	130	4	5
AS1	7	295	5	3
AS2	9	350	6	10
AS3	6	320	4	9
AS4	8	315	8	8
AS5	17	890	6	10
AS6	36	1200	8	8
AS7	20	967	5	9
AS8	15	800	5	6
AS9	27	998	6	4

AS10	20	970	4	5
AQ1	25	995	7	10
AQ2	7	305	4	18
AQ3	6	300	8	21
AQ4	8	350	4	12
AQ5	7	306	5	15
AQ6	6	302	6	15
AQ7	8	351	8	13
AQ8	5	298	7	14
AQ9	7	300	8	17
AQ10	3	128	7	14
AS+AQ1	5	135	7	17
AS+AQ2	2	120	8	20
AS+AQ3	2	120	6	14
AS+AQ4	6	295	5	21
AS+AQ5	4	130	6	22
AS+AQ6	5	200	6	11
AS+AQ7	9	355	5	10
AS+AQ8	4	130	4	5
AS+AQ9	7	295	5	3
AS+AQ10	9	350	6	10

Appendix IV

Table 4.Results of Neurobehavioural Parameters of Study (Delayed post-acute)

Group	Swimming Time (sec)	Swimming Distance (m)	Quadrant Crossing	Fore-Arm Grip Duration (sec)
CT1	5.00	369.00	6.00	7.00
CT2	6.00	247.00	8.00	8.00
CT3	8.00	318.00	5.00	6.00
CT4	7.00	285.00	5.00	10.00
CT5	6.00	247.00	6.00	6.00
CT6	9.00	315.00	4.00	8.00
CT7	5.00	325.00	9.00	5.00
CT8	8.00	332.00	7.00	7.00
CT9	4.00	289.00	8.00	6.00
CT10	5.00	300.00	6.00	9.00
AS1	7.00	247.00	8.00	7.00
AS2	6.00	369.00	7.00	6.00
AS3	5.00	247.00	7.00	5.00
AS4	2.00	225.00	8.00	7.00
AS5	2.00	318.00	6.00	10.00
AS6	3.00	250.00	9.00	8.00
AS7	4.00	275.00	5.00	6.00
AS8	5.00	310.00	4.00	7.00
AS9	7.00	320.00	5.00	5.00

AS10	6.00	290.00	7.00	5.00
AQ1	7.00	285.00	4.00	9.00
AQ2	6.00	363.00	8.00	7.00
AQ3	5.00	247.00	4.00	10.00
AQ4	5.00	247.00	5.00	6.00
AQ5	7.00	318.00	6.00	5.00
AQ6	6.00	340.00	8.00	8.00
AQ7	8.00	260.00	7.00	8.00
AQ8	8.00	355.00	6.00	5.00
AQ9	7.00	290.00	5.00	4.00
AQ10	6.00	300.00	4.00	7.00
AS+AQ1	7.00	247.00	6.00	9.00
AS+AQ2	6.00	369.00	5.00	7.00
AS+AQ3	8.00	247.00	4.00	6.00
AS+AQ4	7.00	225.00	5.00	5.00
AS+AQ5	6.00	318.00	6.00	5.00
AS+AQ6	8.00	260.00	7.00	4.00
AS+AQ7	5.00	315.00	4.00	10.00
AS+AQ8	7.00	275.00	8.00	8.00
AS+AQ9	8.00	324.00	5.00	7.00
AS+AQ10	6.00	295.00	6.00	5.00

Appendix V

Table 5. Haematological Result for Acute Study

Group	PCV [%]	Hb [g/dl]	RBC [10^6/µL]	MCV [fl]	MCH [pg]	MCHC [g/d]	PlAT [10^3/µ]	WBC [10^3/µL]	NEU [%]	LYM [%]	EOS [%]	MON [%]	BAS [%]
CT1	50	14.5	6.580	72	28	2.8	9.070	3.465	1.9628	6.8765	0.035077	0	0.4
CT2	49	14.6	7.700	75	26	2.6	9.064	3.475	3.3124	5.1696	0.035416	0	0.3
CT3	51	14.7	7.040	79	29	2.7	9.068	3.486	3.4088	6.4627	0.0357545	0	0.3
CT4	55	14.3	7.326	80	30	2.5	9.066	3.477	2.5412	7.7558	0.056093	0	0.4
CT5	49	14.7	7.590	77	27	2.4	9.067	3.476	2.6376	7.0489	0.0564315	0	0.3
AS1	46	14.8	7.184	75	28	2.9	9.370	3.455	2.252	6.9455	0.0316925	0.1	0.2
AS2	52	15.3	7.612	74	29	2.7	9.372	3.453	2.3484	7.2386	0.032031	0.3	0.1
AS3	54	14.9	7.128	76	25	2.8	9.362	3.457	2.4448	7.5317	0.0423695	0.2	0.3
AS4	54	14.6	6.260	77	26	2.5	9.369	3.451	3.5052	7.8248	0.032708	0.1	0.5
AS5	47	14.7	6.810	78	27	3.1	9.365	3.388	3.6016	8.1179	0.0430465	0.3	0.4
AQ1	51	14.9	7.612	69	33	2.8	9.129	3.390	2.734	5.411	0.033385	0.4	0.3
AQ2	53	14.7	7.128	67	31	3.0	9.132	3.386	2.8304	8.7041	0.0537235	0.5	0.2
AQ3	50	14.8	7.832	70	28	2.7	9.138	3.389	2.9268	8.9972	0.034062	0.2	0.2
AQ4	45	15.2	7.920	63	29	3.1	9.120	3.387	1.8664	7.2903	0.0444005	0.2	0.1
AQ5	46	14.9	6.776	66	29	2.9	9.126	3.406	3.216	6.5834	0.034739	0.2	0.2
AS+AQ1	55	14.7	6.776	62	29	2.9	9.293	3.403	1.77	7.48	0.03	0	0.2
AS+AQ2	47	14.5	7.128	64	28	2.8	9.285	3.395	3.0232	5.7731	0.0303385	0	0.4
AS+AQ3	48	14.6	7.216	65	29	2.5	9.286	3.396	3.1196	6.0662	0.040677	0	0.3
AS+AQ4	42	14.8	7.568	68	30	2.6	9.290	3.401	2.0592	6.3593	0.0310155	0	0.4
AS+AQ5	46	14.4	6.712	66	29	2.7	9.286	3.400	2.1556	7.6524	0.051354	0	0.2

Appendix VI

Table 6. Haematological Result for Delayed post-acute Study

Group	PCV [%]	Hb [g/dl]	RBC [10^6/µL]	MCV [fl]	MCH [pg]	MCHC [g/d]	PlAT [10^3/µL]	WBC [10^3/µL]	NEU [%]	LYM [%]	EOS [%]	MON [%]	BAS [%]
CT1	50	14.7	8.07	61	18	30	7.06	11.5	8	92	0	0	0
CT2	44	13.1	6.52	66	20	30	7.42	8.2	16	82	2	0	0
CT3	48	13.9	6.94	69	19	28	6.04	6.4	8	90	1	0	1
CT4	44	12.9	6.43	69	20	29	5.07	10.6	20	79	0	0	0.1
CT5	43	13.3	6.5	69	19	28	7.11	8.8	18	82	0	0	0
AS1	48	14.3	7.97	60	18	30	7.94	9.5	30	67	2	1	0
AS2	42	12.4	6.15	65	20	30	4.88	7.3	24	76	0	0	0
AS3	50	15	7.63	68	17	28	6.17	15	9	90	1	0	0
AS4	46	13.6	6.64	69	20	30	9.35	8	21	77	2	0	0
AS5	45	14	6.81	66	19	28	5.06	12.1	36	62	1	1	0
AQ1	60	16.9	8.63	69	20	28	5.52	24.1	78	17	5	0	0
AQ2	59	16.4	8.5	66	19	28	4.27	18.7	35	63	2	0	0
AQ3	53	14.7	7.91	68	18	27	8.70	11.8	30	70	0	0	0
AQ4	55	15.9	8.25	67	19	29	6.70	19.5	45	58	0	0	0
AQ5	58	16.2	8.45	68	18	27	7.20	20.2	58	67	0	0	0
AS+AQ1	55	16.9	8.24	64	19	29	8.21	8.6	4	96	0	0	0
AS+AQ2	52	14.4	7.41	71	19	28	7.16	9.3	7	93	0	0	0
AS+AQ3	51	14.4	7.22	69	19	30	4.61	5.9	14	85	1	0	0
AS+AQ4	49	14.2	7.1	68	20	29	8.33	8.6	36	64	0	0	0
AS+AQ5	47	13.8	7.01	67	19	29	7.38	7.7	22	73	3	2	0

Appendix VII

Table 7. Biochemical Result for Acute Study

GROUP	MDA (nmmol/g)	NO (µm/mg)	SOD (U/mg)	CAT (µm/min/mg)	GSH (U/L)
CT1	3.65E-06	58.94949495	5.462466937	2.32851E-05	6.24338624
CT2	5.85E-06	56.92929293	7.420169954	3.19507E-05	5.98941799
CT3	3.33441E-06	57.93939394	4.334729426	1.89736E-05	3.61375661
CT4	3.73117E-06	50.11111111	5.837801896	2.48011E-05	3.8042328
CT5	4.14255E-06	54.15151515	4.213003643	1.83174E-05	6.14814815
AS1	4.45503E-06	48.5959596	7.956383258	3.21349E-05	5.21693122
AS2	6.5014E-06	49.35353535	4.96729744	2.11433E-05	3.46031746
AS3	7.73638E-06	45.81818182	6.644998325	2.75674E-05	5.06349206
AS4	7.01111E-06	50.11111111	7.425279241	3.01078E-05	5.96825397
AS5	9.89991E-06	53.39393939	7.191993502	2.97525E-05	5.44973545
AQ1	5.45146E-06	58.19191919	7.73671326	3.18561E-05	4.77248677
AQ2	6.13593E-06	51.12121212	1.095701183	2.50794E-05	4.13756614
AQ3	5.94108E-06	46.82828283	4.733695845	2.01686E-05	6.24338624
AQ4	7.00966E-06	58.6969697	3.645021212	1.67928E-05	6.7989418
AQ5	7.45367E-06	40.26262626	4.204670235	1.81363E-05	5.85185185
AS+AQ1	5.75613E-06	56.67676768	3.003724662	2.47871E-05	5.98941799
AS+AQ2	4.90309E-06	46.07070707	2.878224423	2.39852E-05	6.91534392
AS+AQ3	6.62709E-06	45.81818182	3.084147829	2.46392E-05	4.5978836
AS+AQ4	2.56051E-06	59.70707071	6.286990248	1.87045E-05	4.38624339
AS+AQ5	4.48472E-06	48.09090909	4.730740931	1.9989E-05	5.89417989

Appendix VIII

Table 8. Biochemical Result for Delayed post-acute Study

GROUP	MDA (nmmol/g)	NO (µm/mg)	SOD (U/mg)	CAT (µm/min/mg)	GSH (U/L)
CT1	18.99	44.12	10.01	10.1	11.32
CT2	17.65	45.07	9.56	10.11	10.58
CT3	18.77	45.08	9.8	10.34	10.39
CT4	17.12	46.11	10.12	10.45	11.23
CT5	18.11	45.07	9.98	10.22	12.21
AS1	23.05	47.04	5.66	6.02	7.12
AS2	23.25	47.11	5.14	6.04	7.04
AS3	23.07	47.31	5.78	7.11	6.98
AS4	22.09	47.05	6.06	6.77	7.52
AS5	24.87	47.09	5.87	7.03	7.15
AQ1	25.76	52.44	7.12	7.44	5.35
AQ2	25.12	50.08	7.56	7.86	6.02
AQ3	25.65	56.11	7.02	7.24	5.56
AQ4	25.99	51.02	6.97	7.52	6.19
AQ5	26.18	53.07	7.35	8.94	5.86
AS+AQ1	22.24	47.8	7.46	6.12	7.89
AS+AQ2	22.06	47.01	7.56	6.24	7.34
AS+AQ3	23.01	48.06	8.01	6.02	6.44
AS+AQ4	21.45	47.87	7.22	6.43	6.7
AS+AQ5	22.85	48.99	7.44	6.23	7.01

Appendix IX

Table 9: Histology and Immunohistochemistry for Acute Study

GROUP	PURKINJE CELL COUNT	CA1 CELL COUNT	GFAP EXPRESSION	GFAP EXPRESSION	COX-2 EXPRESSION	COX-2 EXPRESSION	INOS EXPRESSION	INOS EXPRESSION
C1	4	12	56.41	65.56	65.96	92.55	60.14	97.22
C2	5	11	44.82	64.85	42.19	85.57	76.41	96.30
C3	4	12	46.07	45.67	50.24	81.46	68.43	94.41
C4	4	13	44.90	55.58	55.35	90.56	85.24	86.52
C5	5	13	48.25	60.62	47.29	88.43	72.35	91.46
AS1	5	15	47.91	50.29	80.12	94.10	72.24	85.02
AS2	5	10	31.30	37.92	61.25	86.89	79.45	91.15
AS3	4	11	34.66	51.13	59.14	80.55	86.85	93.24
AS4	5	13	33.87	55.38	45.26	84.46	80.34	88.35
AS5	4	13	40.55	49.45	67.18	87.94	62.46	90.05
AQ1	4	11	42.35	60.39	74.48	89.18	66.15	83.99
AQ2	4	13	38.72	54.19	79.42	89.23	62.43	90.08
AQ3	5	12	48.03	52.35	65.45	87.17	77.64	93.43
AQ4	4	13	43.19	58.49	56.42	85.53	85.46	95.56
AQ5	5	12	45.62	50.55	51.16	90.46	60.58	88.45
AS+AQ1	3	12	39.22	66.12	82.61	86.95	78.62	95.50
AS+AQ2	4	13	66.12	62.24	59.46	88.62	81.45	88.94

AS+AQ3	4	12	43.06	58.42	65.53	85.36	75.64	92.75
AS+AQ4	5	12	48.34	60.25	55.47	89.63	77.86	85.38
AS+AQ5	4	11	51.26	54.16	71.64	83.57	65.64	86.63

Appendix X

Table 10: Histology and Immunohistochemistry for Delayed post-acute Study

GROUP	PURKINJE CELL COUNT	CA1 CELL COUNT	GFAP EXPRESSION (%)	GFAP EXPRESSION (%)	COX-2 EXPRESSION (%)	COX-2 EXPRESSION (%)	INOS EXPRESSION (%)	INOS EXPRESSION (%)
C1	5	13	50.83	64.68	68.83	45.66	63.25	68.60
C2	6	15	58.79	50.18	79.87	64.34	69.04	70.83
C3	5	14	55.67	58.09	55.58	71.69	61.90	66.09
C4	7	14	58.50	47.79	63.79	55.75	56.90	77.65
C5	6	13	56.76	48.57	60.34	45.66	65.55	73.57
AS1	3	10	58.35	62.05	41.91	84.39	77.65	72.93
AS2	2	10	52.45	67.16	72.51	94.36	77.56	85.11
AS3	2	11	59.88	86.75	69.45	87.44	81.45	84.57
AS4	3	12	60.48	91.29	76.60	69.76	71.70	95.48
AS5	3	10	55.50	70.54	55.43	75.65	79.67	82.28
AQ1	1	8	31.28	73.71	75.55	87.61	84.70	85.40
AQ2	1	8	20.04	90.07	66.49	86.54	86.52	93.42
AQ3	1	7	21.34	89.04	57.99	90.70	87.62	83.92
AQ4	2	6	20.19	82.02	71.24	88.42	80.85	87.83
AQ5	2	7	22.56	77.43	68.42	89.95	85.56	82.37
AS+AQ1	5	14	15.19	67.18	68.83	45.66	81.00	79.70
AS+AQ2	5	13	26.48	56.90	79.88	64.34	84.97	77.41

AS+AQ3	6	13	23.61	61.59	55.58	71.69	89.79	89.53
AS+AQ4	5	13	39.11	55.77	63.79	55.75	90.86	94.03
AS+AQ5	6	14	25.24	55.64	59.45	60.04	88.53	79.76

Appendix XI

Some snap shots of the researcher during the laboratory experiments

The researcher administering drugs to the animals during the first experiment

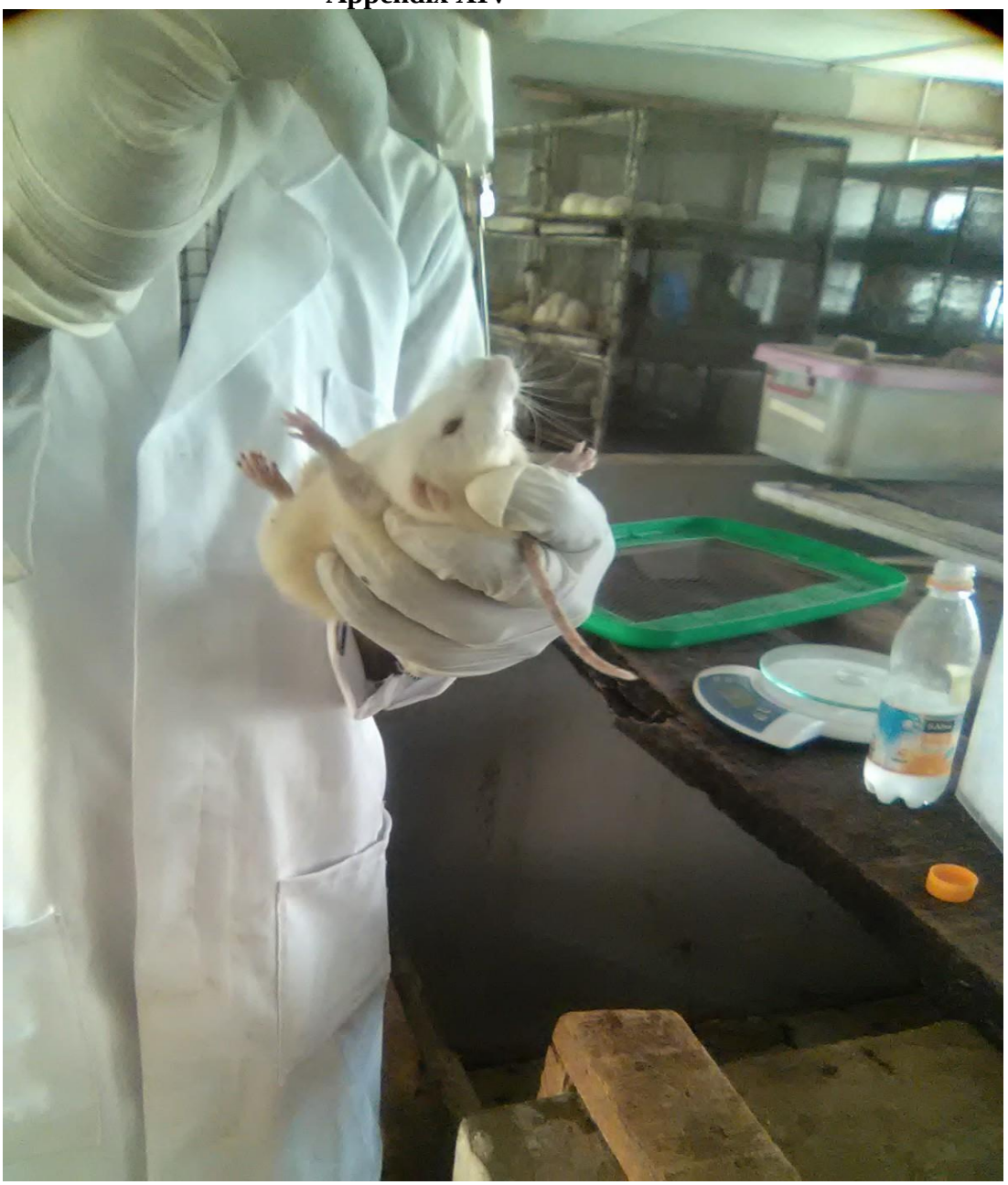
Appendix XII

The researcher administering drugs to the animals during the second experiment

Appendix XIII

The researcher while taking the weights of the animals during the experiment

Appendix XIV

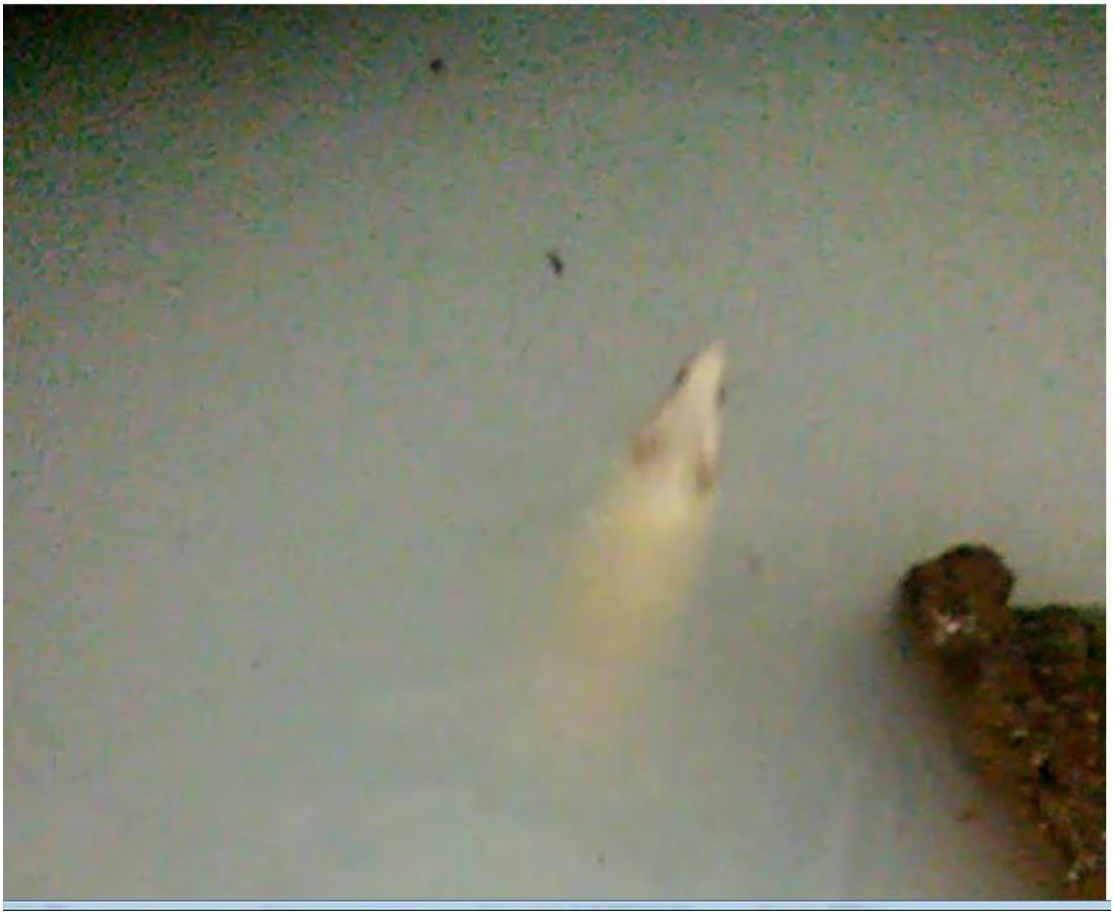


The researcher administering drugs to the animals during the second experiment

Appendix XV

Morriz-water maze test apparatus

Appendix XVI



A rat undergoing Morris-water maze test of spatial memory and cognition during the experiment

Appendix XVII

The researcher extracting blood sample from the animals during the experiment

Appendix XVIII

The researcher while sacrificing the animals for brain removal

Appendix XVIX

The researcher while extracting the brain during the experiment