ANTI-INFLAMMATORY POTENTIAL OF THE CORAL TREE (*ERYTHRINA ABYSSINICA)*: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVIDENCE IN CHRONIC TRYPANOSOMIASIS MOUSE MODEL

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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Master of Science in Veterinary Anatomy of the University of Nairobi.

Declaration

This thesis is my original work and has not been submitted for a degree in any University.

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Dedication

This work is dedicated to my wife Susan, and son Josiah. You are a part of me that I cherish. Grace Khwaka, my mother, and my brother, David Timbiti, who helped me and journeyed with me from my early life.

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List of Abbreviations

AA	Arachidonic acid
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of Variance
APCs	Antigen presenting cells
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CATT	Card agglutination test
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
DMSO	Dimethyl sulphoxide
DND*i*	Drugs for Neglected Diseases *initiative*
Dpi	Days post infection
ELISA	Enzyme linked immunosorbent assay
GFAP	Glial Fibrillary acidic protein
GSH	Glutathione
HAT	Human African trypanosomiasis
HRP	Horse radish peroxidase
IFN-γ	Interferon gamma

IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
ILRI	International Livestock Research Institute
iNOS	Inducible nitrous oxides
KD	Kilo daltons
LAMP	Loop mediated isothermal amplification
LD_{50}	Lethal dose 50
MCP-1	Monocyte chemoattractant protein
MHC	Major Histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MMP-9	Matrix metalloproteinase-9
MSF	Médecins sans Frontières
NGF	Nerve growth factor
Nrf2	Nuclear factor, erythroid-derived 2- related factor-2
NTD	Neglected tropical diseases
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PGE2	Prostaglandin E 2
PLA2	Phospholipase A2
PSG	Phosphate saline Glucose
PTRE	Post treatment reactive encephalopathy
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPSS	Statistical package for the social sciences
T.b. brucei	*Trypanosoma brucei brucei*
T.b. gambiense	*Trypanosoma brucei gambiense*
T.b. rhodesiense	*Trypanosoma brucei rhodesiense*
TGF-α,	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
Th1	T helper cell 1
Th2	T helper cell 2
TNF-α	Tumor necrosis factor alpha
Tris-HCl	Trisaminomethane hydrochloride
UNESCO	United Nations Educational, Scientific and Cultural Organization
VSG	Variant surface genes

Abstract

Human African trypanosomiasis is a protozoan disease prevalent in Sub-Sahara African countries that lie between 14° North and 29° south of the Equator. It is caused by a parasite of the genus Trypanosoma, which has several species and subspecies. *Trypanosoma brucei gambesience* occurs in West and Central Africa while *Trypanosoma brucei rhodesience* occurs in East and Southern Africa. In this region, close to 60 million people are at risk of infection. The neurological stage of the disease is characterized by neuroinflammation and 10% of patients treated with the recommended drug develop fatal post treatment reactive encephalopathy (PTRE). Several attempts have been made to scientifically evaluate plants for anti-trypanosomal effect including modulation of the adverse neuroinflammation associated with tissue trypanosomes and PTRE. This study aimed at establishing the potential activity of *Erythrina abbysinica* in reducing neuroinflammation following infection with *Trypanosoma brucei brucei*. Swiss white mice were divided into ten groups, two control groups (infected and non-infected) and eight infected groups treated with *Erythrina abyssinica* extracts. Infected groups were separately treated with methanol or water extract of *Erythrina abyssinica* at 12.5, 25, 50 or 100 mg/kg body weight. Parasite counts were monitored in peripheral circulation from the third day post infection up to the end of the study. Cerebrum samples were processed for histology, immunohistochemistry scanning and transmission electron microscopy. SDS-PAGE electrophoresis of the brain was also done to analyze brain proteins.

Following infection, trypanosomes were observed in circulation three days post-infection, with the parasitaemia occurring in waves. In the cerebrum, astrocytosis, perivascular cuffing, infiltration of inflammatory cells and protein degration were observed in infected mice. However, in animals treated with aqueous *Erythrina abyssinica* extracts, the neuro-inflammation was significantly reduced as noted by reduced astrocytosis, perivascular cuffing and infiltration by inflammatory cells, compared to non-treated mice. In addition, there was preservation of proteins by aqueous extract of *Erythrina abyssinica*. Trypanosomiasis degraded some brain proteins, which were conserved on treatment with *Erythrina abyssinica* extract. This study therefore documents anti-inflammatory and protein conserving properties of *Erythrina abyssinica* that may support its wide use as a medicinal plant by various communities in Kenya. Further studies are needed to ascertain the exact mode of action and whether the specific compounds act singly or in synergy.

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CHAPTER 1

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The functions of the brain include cognitive, learning, memory, sensory and motor processing (Strick *et al.*, 2009). It is has both physical and physiological protection; it is shielded by the bones of the skull, meninges, cerebrospinal fluid, and the blood brain barrier (BBB) (Mucke and Eddleston, 1993). The brain is immune privileged because of the tight control and restriction from peripheral immune reactions (Tansey *et al*., 2009), and it does not have a lymphatic system to capture potential antigens (Bentivoglio and Kristensson, 2007). Despite this protection, the brain can be invaded and damaged by a variety of micro-organisms (Mucke and Eddleston, 1993), and it is capable of dynamic immune and inflammatory responses to a variety of infectious agents (Rivest, 2003). Inflammation of the brain is caused by infections, stroke, toxins among other causes (Tansey *et al*., 2009). Microglia and astrocytes play a key role in brain inflammation (Suk, 2005). Interest in inflammation of central nervous system (CNS) has grown rapidly due to the role of neuro-inflammation in the development of several important neurodegenerative diseases such as Alzheimer's disease, stroke, traumatic brain injury, demyelinating disorders, Parkinson's disease and the pathology associated with CNS infections (Minghetti, 2004).

Medicinal plants are widely used for the maintenance of good health in developing countries (UNESCO, 1996). The demand for medicinal plants is increasing both in developing and developed countries because they are available at affordable prices, and

the belief that they are devoid of side effects (Okigbo and Mmeka, 2006) and sometime the only source of health care available to the poor. Plants used in traditional medicine therefore have an important role to play in the maintenance of health. Although plant extracts have been used in the treatment of diseases for a long time, their basis of use has largely not been scientifically validated. Understanding efficacy and safety of herbal preparations is a vital step in safeguarding the users and in developing them further for commercialization (Taylor *et al*., 2003). Considerable benefits are therefore possible for developing countries when the local medicinal plants are subjected to scientific methods of validation of traditional use (Houghton, 1995).

Human African Trypanosomiasis (sleeping sickness) is caused by *Trypanosoma brucei gambiense* in West and Central Africa, and by *T. b. rhodesiense* in East and Southern Africa (Boda *et al.*, 2009). The disease has two stages, the early stage (haemolymphatic) and the late stage (meningoencephalitic) stage (Amin *et al*., 2010). After inoculation by tsetse flies, the parasites multiply at the site before moving to lymph nodes through the lymphatics, from where they move into the blood stream. In the late stage, parasites enter into all organs including the brain (Enanga *et al*., 2002). The trypanosomes cross the blood-brain barrier near intercellular junctions and also through an active process (Masocha *et al*. 2007). This process is acute in *T.b. rhodesiense* infections, taking only a few weeks, but chronic in *T.b. gambiense* infection. Early stage sleeping sickness is treated with diminazine aceturate for *T.b. rhodesiense* and pentamidine isothionate for *T.b. gambiense* (Bouteille and Buguet 2012). Treatment of meningoencephalitic stage of the disease is very limited and

relatively few new formulations are currently available (Enanga *et al*., 2002). In addition, the organo-arsenical drug, melarsoprol, the drug of choice for use in the second stage of infection is toxic, causing a severe post treatment reactive encephalopathy (PTRE) in up to 10% of cases, with a fatality rate of about 50% (Pepin and Milord, 1994). Efforts have been put to develop new therapeutic agents for trypanosomiasis including those originating from medicinal plants (Adamu *et al.*, 2009). One of the approaches to treatment is to target immune reactions which are suggested as the trigger for the encephalopathy (Hunter *et al.*, 1992) associated with over expression of pro-inflammatory cytokines and their messenger RNAs (mRNA) (Quan *et al*., 1999; Amin *et al.*, 2009; Hunter *et al.*, 1991). There has also been much interest in the development of new drugs capable of preventing neuroinflammatory mediated brain injury (Vauzour and Minihane 2012) as increased production of proinflammatory cytokines in the central nervous system is a common occurrence in many neurodegenerative diseases (Griffin *et al.*, 1998; Mogi *et al.*, 1996; Maimone *et al.*, 1997). *Erythrina abyssinica*, a medicinal plant is used traditionally in for ear, nose and throat infections (Njoroge and Bussman, 2006), diarrhoea (Njoroge and Kibunga, 2007), pneumonia, sexually transmitted diseases and prostate cancer (Wagate *et al*., 2009). Several species of *Erythrina* have been shown to have anti-infammatory effects (Njamen *et al*., 2004). The species of erythrina produce many secondary metabolites, some of which function in defense systems against pathogenic fungi and bacteria (Kone *et al.*, 2011).

This study aimed at investigating the anti-inflammatory properties of *Erythrina abyssinica* in brain stage trypanosomiasis, using the mouse model.

1.2 LITERATURE REVIEW

1.2.1 General anatomy of the brain

The mammalian brain demonstrates approximate bilateral symmetry of anatomy, function, neurochemical activity and electrophysiology (Volkau *et al*., 2006). It is enclosed in its bony cranium (Guilherme, 2010) and is continuous with the spinal cord at the foramen magnum at the base of the skull. The brain is subdivided into five continuous regions, from rostral to caudal: the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. These regions are distinct during development, but as the brain grows, these regions fold and enlarge over each other making them less obvious, leaving only three regions visible. The regions are: cerebrum, cerebellum, and part of the brainstem. The regions are further divided into specific zones although there is increasing evidence that the borders between those zones are much blurrier than was previously thought (Guilherme, 2010).

1.2.2 Anatomy of the cerebrum

The cerebrum is large, oval and narrow on the anterior and posterior ends. It has a middle longitudinal cerebral fissure that separates the right and left hemispheres (Guilherme, 2010). The bottom of the fissure is formed by a large myelinated fiber tract that forms an anatomical and functional connection between the right and left

hemispheres, known as the corpus callosum (Patestas and Gartner, 2006). The surface is highly folded, with elevations (gyri) and depressions (sulci), which increase the surface area (Finger, 1994). The gyri and sulci are natural extensions of the subarachnoid space (Guilherme, 2010). Deep into the cortex is a central core of white matter that forms the bulk of the cerebrum and represents fiber tracts, supported by neuroglia, ferrying information destined for the cortex and cortical responses to other regions of the central nervous system. Within the white mater, there are aggregations of neuron cell bodies that form the basal ganglia. Other aggregations also occur in the diencephalon and include thalamus, epithalamus, hypothalamus and subthalamus (Patestas and Gartner, 2006). Each cerebrum has a cavity known as lateral ventricle, and the left and right ventricles are separated from each other by a membrane, septum pellucidum, and protruding into each ventricle is a mesh of special blood vessels known as choroid plexus that functions in the manufacture of cerebrospinal fluid. The cerebrum is arbitrarily divided into lobes named according to the overlaying skull bone (Türe et al., 2000). Each cerebral hemisphere is arbitrarily subdivided into five lobes: the frontal, parietal, temporal, and occipital lobes, and the insula (Patestas and Gartner, 2006). This sub-division is anatomic based (Guilherme, 2010). Although the geographic distributions of many of the sulci and gyri are relatively inconsistent, some regularly occupy specific locations. The sulci are generally smaller and shallower than the fissures, and one of these, the central sulcus (central sulcus of Rolando), separates the frontal lobe from the parietal lobe. The division between the parietal and occipital lobes is not readily evident when viewed from the lateral aspect because it is defined as the

imaginary line between the preoccipital notch and the parieto-occipital notch. However, it is clearly delimited on the medial aspect of the cerebral hemisphere, where the boundary between these two structures is the parieto-occipital sulcus and its continuation, the calcarine fissure.

1.2.2.1. Frontal lobe

On its lateral aspect, the frontal lobe extends from the frontal pole to the central sulcus, constituting the largest and most anterior part of the cerebral cortex (Guilherme, 2010). Its posterior most gyrus, the precentral gyrus, consists of the primary motor area and is bordered anteriorly by the precentral sulcus and posteriorly by the central sulcus. The region of the frontal lobe located anterior to the precentral sulcus is subdivided into the superior, middle, and inferior frontal gyri. This subdivision is due to the presence, though inconsistent, of two longitudinally disposed sulci, the superior and inferior frontal sulci (Patestas and Gartner, 2006). The inferior frontal gyrus is demarcated by extensions of the lateral fissure into three subregions: the pars triangularis, pars opercularis, and pars orbitalis. In the dominant hemisphere, a region of the inferior frontal gyrus is known as Broca's area, which functions in the production of speech. On its inferior aspect, the frontal lobe presents the longitudinally disposed olfactory sulcus. Medial to this sulcus is the gyrus rectus (also known as the straight gyrus), and lateral to it are the orbital gyri. The olfactory sulcus is partly occupied by the olfactory bulb and olfactory tract. At its posterior extent, the olfactory tract bifurcates to form the lateral and medial olfactory striae. The intervening area between the two striae is triangular in shape and is known as the olfactory trigone and it abuts the anterior perforated

substance. On its medial aspect, the frontal lobe is bordered by the arched cingulate sulcus, which forms the boundary of the superior aspect of the cingulate gyrus. The quadrangular-shaped cortical tissue anterior to the central sulcus is a continuation of the precentral gyrus and is known as the anterior paracentral lobule.

1.2.2.2 Parietal lobe

The parietal lobe extends from the central sulcus to the parieto-occipital Sulcus. The parietal lobe is interposed between the frontal and occipital lobes and is situated above the temporal lobe (Guilherme, 2010). On its lateral aspect, its anterior most gyrus, the post central gyrus, is the primary somesthetic area to which primary somatosensory information is channeled from the contra lateral half of the body. The post central gyrus by the post central sulcus is subdivided by the inconsistent intraparietal sulcus, into the superior and inferior parietal lobules (Patestas and Gartner, 2006). The former is an association area involved in somatosensory function, whereas the latter is separated into the supramarginal gyrus, which integrates auditory, visual, and somatosensory information, and the angular gyrus, which receives visual input. On its medial aspect, the parietal lobe is separated from the occipital lobe by the parieto-occipital sulcus and its inferior continuation, the calcarine fissure. This region of the parietal lobe is subdivided into two major structures, the anteriorly positioned posterior paracentral lobule (a continuation of the postcentral gyrus) and the posteriorly situated precuneus.

1.2.2.3 Temporal lobe

The temporal lobe is separated from the frontal and parietal lobes by the lateral fissure and from the occipital lobe by an imaginary plane that passes through the parieto-occipital sulcus (Patestas and Gartner, 2006), and therefore referred to as the anterior extension of the occipital region (Guilherme, 2010). The anterior most aspect of the temporal lobe is known as the temporal pole. On its lateral aspect, the temporal lobe exhibits three parallel gyri, the superior, middle, and inferior temporal gyri, separated from each other by the inconsistently present superior and middle temporal sulci. The superior temporal gyrus of the dominant hemisphere contains Bernice's area, which is responsible for the individual's ability to speak and understand the spoken and written word. Hidden within the lateral fissure is the superior aspect of the temporal lobe whose surface is marked by the obliquely running transverse temporal gyri (of Heschl), the primary auditory cortex (Patestas and Gartner, 2006). The inferior aspect of the temporal lobe is grooved by the inferior temporal sulcus that is interposed between the inferior temporal gyrus and the lateral occipitotemporal gyrus (fusiform gyrus). The collateral sulcus separates the fusiform gyrus from the parahippocampal gyrus of the limbic lobe (Duvernoy, 1999).

1.2.2.4 Occipital lobe

The occipital lobe extends from the occipital pole to the parieto-occipital sulcus. On its lateral aspect, the occipital lobe presents the superior and inferior occipital gyri, separated from each other by the horizontally running lateral occipital sulcus (Patestas and Gartner, 2006). On its medial aspect, the occipital lobe is subdivided into the

superiorly located cuneate gyrus (cuneus) and the inferiorly positioned lingual gyrus, separated from each other by the calcarine fissure. The cortical tissue on each bank of this fissure is known collectively as the striate cortex (calcarine cortex), and forms the primary visual cortex.

1.2.2.5 Limbic lobe

The limbic lobe includes the cingulate gyrus, parahippocampal gyrus, hippocampal formation, subcallosal gyrus, parolfactory gyrus, and the preterminal gyrus. On the medial aspect of the hemisected brain and the various regions of the corpus callosum are obvious landmarks (Patestas and Gartner, 2006). The anterior extent of the corpus callosum, known as the genu, bends inferiorly and turns posteriorly, where it forms a slender connection, the rostrum, with the anterior commissure. The posterior extent of the corpus callosum is bulbous in shape, and is known as the splenium. The cingulate gyrus is located above the corpus callosum and is separated from it by the callossal sulcus. As the cingulate gyrus continues posteriorly, it follows the curvature of the corpus callosum and dips beneath the splenium to continue anteriorly as the isthmus of the cingulate gyrus. The anterior continuation of the isthmus is the parahippocampal gyrus whose anterior most extent is known as the uncus. Above the parahippocampal gyrus is the hippocampal sulcus, which separates the parahippocampal gyrus from the hippocampal formation (composed of the hippocampus, subiculum, and dentate gyrus). Just beneath the rostrum of the corpus callosum is the subcallosal gyrus. The connection between the anterior commissure and the optic chiasma is the lamina terminalis and the

cortical tissue anterior to the lamina terminalis is the parolfactory gyrus and preterminal gyrus. The subcallosal, parolfactory, and preterminal gyri are referred to as the subcallosal area.

1.2.2.6 Histology of the cerebrum

The cerebral cortex is well endowed with neurons, neuroglia, nerve fibers, and a rich vascular supply. In most parts of the mammalian cortex, a six-layered pattern exists (Barr and Kiernan, 2009). The number of layers permits the classification of the cortex into three types: the archicortex, mesocortex and neocortex (Patestas and Gartner, 2006). The archicortex, phylogenetically the oldest region, is composed of only three layers and is located in the limbic system. The mesocortex, phylogenetically younger, is composed of three to six layers, and is located predominantly in the insula and cingulate gyrus. The neocortex is phylogenetically the youngest region of the cerebral cortex; it is composed of six layers and comprises the bulk of the cerebral cortex. Although the cerebral cortex is arranged in layers, superimposed upon this cytoarchitecture is a functional organization of cell columns (Patestas and Gartner, 2006). All neurons of a single column respond to like stimuli from the same region of the body. The organization of the six layers of the neocortex is known as its cytoarchitecture, where each layer has a name and an associated Roman numeral. Molecular layer I contain afferent fibers from the thalamus or from the cerebral cortex, external granular layer II contain stellate neurons, external pyramidal layer III has small pyramidal cells, internal

granular layer IV contains fusiform neurons, internal pyramidal layer V has larger pyramidal cells, while fusiform layer VI contains fusiform neurons.

The central core of white matter that forms the substance of the cerebrum is composed of myelinated nerve fibers of varied sizes and their supporting neuroglia. These fibers may be classified into the following three categories: commissural, projection, and association fibers (Patestas and Gartner, 2006).

Commissural fibers (transverse fibers) interconnect the right and left cerebral hemispheres. There are four bundles of commissural fibers, the corpus callosum, anterior commissure, posterior commissure, and hippocampal commissure. The largest group of the commissural fibers, the corpus callosum, is comprised of four regions: the anteriormost rostrum, the curved genu, the relatively flattened body, and its posterior most regions, the splenium. The corpus callosum connects the neocortex of the right hemisphere with that of the left. The anterior commissure connects the right and left amygdalas, the olfactory bulbs, and several cortical regions of the two temporal lobes. The posterior commissure connects the right and left pretectal region and related cell groups of the mesencephalon. The hippocampal commissure (commissure of the fornix) joins the right and left hippocampi to one another (Patestas and Gartner, 2006).

Projection fibers are restricted to a single hemisphere and connect the cerebral cortex with lower levels, namely the corpus striatum, diencephalon, brainstem, and spinal cord. The majority of these fibers are axons of pyramidal cells and fusiform neurons. These fibers are component parts of the internal capsule, which is subdivided into the anterior limb, genu, posterior limb, retrolentiform, and sublentiform regions. The projection

fibers may be subdivided into corticopetal and corticofugal fibers. Corticopetal fibers are afferent fibers that bring information from the thalamus to the cerebral cortex. They consist of thalamocortical fibers. Corticofugal fibers are efferent fibers that transmit information from the cerebral cortex to lower centers of the brain and spinal cord. They consist of the corticobulbar, corticopontine, corticospinal, and corticothalamic fibers.

Association fibers, also known as arcuate fibers, are restricted to a single hemisphere and are subdivided into two major categories, short arcuate fibers and long arcuate fibers. They are the axons of pyramidal cells and fusiform neurons. Short arcuate fibers, which connect adjacent gyri, do not usually reach the subcortical white matter of the cerebral cortex; most of them are confined to the cortical gray matter. The long arcuate fibers, which connect nonadjacent gyri, consist of the following fiber tracts: the uncinate fasciculus, cingulum, superior longitudinal fasciculus, inferior longitudinal fasciculus, and fronto-occipital fasciculus.

The basal ganglia, called ganglia even though they are nuclei, are a set of inter connected nuclei located in the cerebrum (Yin and Knowlton, 2006). The nuclei influence cortical control of voluntary movement (Pollack, 2001). The basal ganglia are composed of the caudate nucleus, lenticular nucleus (putamen and globus pallidus), subthalamic nucleus of the ventral thalamus, and the substantia nigra of the mesencephalon (the caudate nucleus and the putamen together are referred to as the striatum) (Patestas and Gartner, 2006). These nuclei have numerous connections with various regions of the CNS; some receive input and are categorized as input nuclei, some project to other regions and are referred to as output nuclei, whereas some receive

input, project to other regions of the CNS, and have local interconnections and these are known as intrinsic nuclei (Patestas and Gartner, 2006; Yin and Knowlton, 2006).

The diencephalon, interposed between the cerebrum and the midbrain, has four regions: the epithalamus, thalamus, hypothalamus, and subthalamus. The right and left halves of the diencephalon are separated from one another by a narrow slit-like space, the ependymal-lined third ventricle. Rostrally, the interventricular foramina lead from the lateral ventricles into the third ventricle, whereas caudally, the third ventricle is connected to the fourth ventricle by the cerebral aqueduct (Patestas and Gartner, 2006). The epithalamus composed of the pineal body, stria medullaris, and habenular trigone, constitutes the dorsal surface of the diencephalon. The right and left thalami compose the bulk of the diencephalon and form the superior aspect of the lateral walls of the third ventricle. The two thalami, structures composed of numerous nuclei, are connected to each other by a bridge of gray matter, the interthalamic adhesion. Some of the nuclei of the thalamus form distinctive bulges on its surface, namely the pulvinar (L., "cushion") and the medial and lateral geniculate bodies. The boundary between the thalamus and the hypothalamus is marked by a groove, the hypothalamic sulcus, located along the lateral walls of the third ventricle. Structures associated with the hypothalamus are the pituitary gland and its infundibulum, the tuber cinereum, and the two mammillary bodies. The subthalamic nuclei and fiber tract form the subthalamus.

1.2.2 Central nervous system (CNS) immunology

The CNS has evolved both anatomically and physiologically to protect its vital functions from damaging by immune-mediated inflammation (Aloisi, 2001). The brain has traditionally been regarded as immunologically privileged (Engelhardt and Coisne, 2011). The blood-brain barrier maintains physiological and immunological homeostasis from the varying milieu of the periphery (Engelhardt and Coisne, 2011) and restricts the entry of pathogens, plasma proteins and immune cells. The nature and number of cellular mediators of inflammation have been shown to be quite distinct in brain tissue compared to the periphery. During brain inflammation there is little neutrophil recruitment and the major resident inflammatory cells are microglia, which represent the brain's macrophages (Giselle *et al.*, 2009), and astrocytes. The CNS appears to show marked resistance to inflammation in response to a number of stimuli as compared with peripheral tissues. These differences reflect the tight regulatory environment of the brain and a balance between inflammation-induced tissue repair and tissue damage (Amor *et al.*, 2010). Other mechanisms that have been proposed to contribute to central nervous system immune privilege include local productions of anti-inflammatory mediators by activated glial cells (Stoll *et al.*, 1998; Oscar and Ignacio, 2012). These responses are crucial for the elimination of pathogens, removal of dead cells and tissue repair. However, prolonged inflammation can lead to both acute and chronic CNS diseases (Chavarria and Alcocer-Varela, 2004).

1.2.3 Immunocompetent cells in the central nervous system

Macrophages and dendritic cells have been identified in the meninges and choroid plexus, in strategic locations to guard the ventricular/subarachnoid compartment (McMenamin, 1999). Perivascular macrophages, which surround small and medium size cerebral vessels, ensure protection of the central nervous system at the level of the blood-brain barrier displaying phagocytic and immune regulatory functions (Thomas, 1999; Williams *et al*., 2001). However, if the inflammatory stimulus (e.g. pathogen or its components) crosses the BBB, the brain parenchyma itself contains glial cells that vigorously react to any immunological stimuli and to neuronal injury, and play an active role in the development of inflammation. Glial cells are generally classified into two groups: Macroglia, which include astrocytes and oligodendrocytes that are of ectodermal origin, and Microglia, of mesodermal origin. These invade the CNS during embryonic development at the time of vascularization (Cuadros and Navascues, 1998). Glial cells retain the ability to divide throughout life. Oligodendrocytes are restricted to the central nervous system; these cells produce a laminated, lipid-rich wrapping called myelin around some axons; which plays an important role in the conduction of action potential in neurons by increasing the speed of potential propagation (Zalc, 2006).

1.2.3.1 The role of microglia in inflammation

Microglia is the main resident inflammatory cell in the CNS. These are macrophage-like cells, derived from bone marrow stem cells that populate (Ling, 1979; Perry *et al.*, 1985 : Streit *et al.*, 1988) the central nervous system early during development (Chan *et al.*, 2007) and remain within the central nervous system as the resident macrophage population (Kettenmann *et al.*, 2011). Microglia comprises 5 to 20% of the total non-neuronal cell population (Davis *et al*., 1994; Nakajima and Kohsaka, 2001). These cells are especially important to guard the integrity and homeostasis of the brain. In normal conditions they are quiescent, but become activated by injury or infection and have been suggested to represent the first line of defense for the CNS (Nakajima and Kohsaka, 2001). The CNS normally lacks professional antigen presenting cells and intra parenchymal leukocytes. During inflammation, antigen presenting cells from the periphery are recruited to the brain by pro-inflammatory stimuli (Kreutzberg, 1996). Microglia has several morphological forms depending on their functional and developmental state (Miguel *et al.*, 1995). During embryonic development monocytes migrate to the CNS where they transform into intermediate cells (Nakajima and Kohsaka, 2001), or amoeboid cells that have a flat morphology and pseudopodia. These forms represent a transient population of microglia that is present during the late prenatal to early post natal period. Microglia in adult brain is reffered to as ramified microglia (Kettenmann *et al.*, 2011; Nakajima and Kohsaka, 2001). These cells have a small oval to oblong cell body with large nucleus and only a little amount of cytoplasm,

as well as numerous long, branched processes. Ramified microglia have been characterised as down regulated (or inactive) macrophages; as they lack most of the characteristic markers and activities of this group (lack of phagocytic and endocytic activity, low expression of leukocyte common antigen (CD45), low levels of membrane ligands and receptors that are essential for mediating or inducing typical macrophage functions (Davis *et al*., 1994).

Microglia is able to react to internal and external stimuli (in relation to the central nervous system) and to direct their reaction for the purpose of tissue repair and for further induction of protective immune responses (Gebicke-Haerter *et al.*, 1996). Following the stimulus, microglia migrates to the damaged sites of the central nervous system where they proliferate and become activated. They can assume two distinct forms – activated and reactive microglia (Davis *et al*., 1994). The activation of microglia is an important host defence mechanism. Activated microglia release various pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), chemokines (IL-8, MIP-1α, MIP-1β, MCP-1), proteases, as well as oxidative and nitrosative free radicals (Thomas, 1999; Kreutzberg, 1996; Peterson et al., 2004).

Microglia can also produce anti-inflammatory mediators, such as TGF-β, PGE2 and IL-1 receptor antagonist (Benveniste *et al*., 2001). Due to their ability of phagocytosis microglia play also the role of scavengers for macromolecules and apoptotic or

damaged brain cells as well as pathogens. Upon activation microglia can perform several innate immune functions, including the induction and regulation of T-cell responses. They are able to function as antigen presenting cells (APCs) and to influence the intra cerebral balance of T-helper Th1 and Th2 cell-mediated immune responses (Aloisi, 2001). In the normal brain parenchyma the expression of major histocompatibility complex (MHC) antigens is generally very low and resting microglia behave as poor APC, but in virtually all inflammatory and neurodegenerative conditions they become activated and express MHC class II (Kreutzberg, 1996). Although microglia represents the first line of defence in the brain, the activation of these cells can also have negative effects. The inflammatory mediators released from activated microglia can contribute to central nervous system damage as neurotoxins (Aloisi, 2001), and enhance the onset and progression of CNS diseases. Lipopolysaccharide (LPS), which is a strong activator of immune cells, has been shown to damage neurons *in vitro* (Bal-Price and Brown, 2001). Excessive or chronic microglial activation has been implicated in a number of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease (Blasko *et al*., 2004), as well as trauma, ischemia, brain tumours and infectious diseases (Neumann, 2003). Microglia are also involved in several other immune response processes including rejection of transplanted tissue, autoimmune central nervous system diseases (e.g. multiple sclerosis) and acquired immunodeficiency syndrome (AIDS) associated dementia complex (Garden, 2002).

1.2.3.2 The role of astrocytes in inflammation

Astrocytes make up a substantial proportion of the central nervous system and participate in a variety of physiological and pathological processes. In adults, astrocytes constitute about 70% of the total population of brain cells. Their primary function is to provide structural, metabolic and trophic support to other cells (Kimelberg and Nedergaard, 2010). Astrocytes act as a bridge to supply nutrients from blood capillaries to neurons and provide the major site of glycogen storage in the brain. Moreover, they are also able to synthesize and secrete a variety of neurotrophic growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and insulin-like growth factor-1 (IGF-1), which may be beneficial for neuronal survival (Rudge *et al*., 1994). Astrocytes are also responsible for maintaining a homeostatic environment in the brain and may also provide glutathione precursors to neurons as they protect neurons against the toxicity of reactive oxygen species (ROS) in a co-culture system (Dringen *et al*., 2000). These cells not only support neuronal survival, but they also modulate neuronal signaling (Hansson and Ronnback, 2003). Moreover, there is growing evidence that they play an active role in synaptic transmission, not directly forming synaptic contacts, but contributing to the physiological functioning of neurons by the integration of neuronal inputs, exhibition of calcium excitability and modulation of neighbouring neuronal responses (Araque and Navarrete, 2010). Apart from being involved in a variety of physiologic processes, astrocytes rapidly react to different neurological insults to become activated, causing

astrocytosis. The main feature of astrocytosis is the increase in the number and size of glial fibrillary acidic protein (GFAP) expressing astrocytes. GFAP is an intermediate filament cytoskeletal protein expressed primarily by astrocytes and it is considered as the marker of astrocytes (Sofroniew and Vinters, 2010). Reactive astrocytes form a glial scar in areas of tissue necrosis, excluding the non-neuronal cells from parenchyma and filling in the space which results from neuronal loss (McGraw *et al*., 2001). Astrocytes produce proteases and protease inhibitors which allow them to remodel the extracellular matrix at sites of neuronal damage and to clear the debris of degenerating cells (Gardner and Ghorpade, 2003).

One of the important functions of activated astrocytes is the involvement in the immune functions in the central nervous system. Several studies performed both *in vivo* as well as *in vitro* have demonstrated that activated astrocytes produce a large variety of molecules, which are involved in the initiation and regulation of the inflammatory response. These include several pro- and anti-inflammatory cytokines (IL-1, TNF-α-tumour necrosis factor, IL-6, TGF-α-transforming growth factor alpha, TGF-β, IL-8) and eicosanoids (Dong and Benveniste, 2001). Activation of astrocytes leads also to the expression of inducible nitric oxide synthase (iNOS), which is involved in tissue repair (Licinio *et al.*, 1999). Activated astrocytes also produce other molecules involved in immune responses (Brown, 2007; Höftberger *et al.*, 2004). The activated astrocytes present antigens to T lymphocytes (Cornet *et al.*, 2000). Moreover, they up-regulate the expression of several adhesion molecules (selectins, integrins, adherins), which play a

role in the migration of leukocytes through the blood-brain barrier into the CNS parenchyma (Dietrich, 2002).

Prominent reactive astrocytosis occurs in inflammation of the brain including AIDS dementia complex, a variety of CNS infections, prion-associated spongiform encephalopathies, inflammatory demyelinating diseases, acute traumatic brain injury, ischemia (Anderson and Swanson, 2000) and neurodegenerative diseases like Alzheimer's (Mrak and Griffin, 2005). Astrocytes are also essential for the morphological and physiological formation of a functional BBB in the CNS. *In vitro* studies have demonstrated that endothelial cells alone cannot provide a tight barrier without the presence of astrocytes (Shuler *et al*., 2005). Several studies suggest that astrocytes secrete soluble factors essential for the development of specific BBB properties (Ballabh *et al*., 2004).

1.2.4 *Erythrina abyssinica*

1.2.4.1 Distribution of *Erythrina abyssinica*

The genus *Erythrina* is one among several genera from the Fabaceae family. Also known as a sub-family of leguminosae, the fabaceae family is one of the largest botanical families and widely distributed around the world, spread out over temperate, tropical and cold regions. The family is composed of 32 tribes, whose genera are chemically represented by a variety of flavonoid skeletons, notably pterocarpans and isoflavones. There are about 650 genera comprising about 18,000 species (Polhill *et al*., 1981). The Fabaceae family produces valuable medicinal drugs, ornamental species,

fodders plants, oil producing plants, inseticides and species with various other functions (Wojciechowski, 2003). The origin of the name *Erythrina* comes from the Greek word "erythros" which means red, alluding to the bright red flowers of the trees of the genus (João *et al.*, 2009). Over 130 species of "coral tree" exist. The coral tree all belong to the genus *Erythrina*, which has been widely studied and are distributed in tropical and subtropical regions of the world. *E. abyssinica* is distributed in warm regions of southern Africa and the savannah of eastern Africa (Yenesew *et al*., 2009) and commonly referred to as red hot poker coral tree due to its red flowers (Figure 1). It is native to Botswana, Burundi, Central African Republic, Congo, Democratic Republic of Congo, Eritrea, Ethiopia, Gabon, Kenya, Lesotho, Mozambique, Namibia, Rwanda, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zambia and Zimbabwe. It has been exported to other regions also where it is exotic; this includes Afghanistan, Bangladesh, Bhutan, India, Nepal, Pakistan and Sri Lanka. It grows well in most climates but not in dry, high areas and forests.

Figure 1: Photograph of *Erythrina abyssinica* tree. Note the intense red flowers (Inset) that make it be commonly referred to as red hot poker tree (adapted from flora of Zimbabwe: cultivated plants)

1.2.4.2 Uses and Biological activity of *Erythrina abyssinica*

Erythrina abyssinica is a popular and commonly used medicinal plant in traditional medicine by several communities in the regions where it occurs. The plant extracts are used for treatment of meningitis (Aerts, 2008), malaria, allergy (Kareru *et al*., 2007) elephantiasis, trachoma, syphilis, burns and swellings (Hines and Eckman, 1993). In Uganda, the plant is also used to treat helminthoses in chicken (Lagu and Kayanja, 2010). Among the Meru community in Kenya, the plant is used in treatment of helminthosis and anaplasmosis (Gakuubi and Wanzala, 2012) Studies have demonstrated the presence of analgesic and anti-inflammatory effects in extracts obtained from *E. senegalensis*, *E. velutina* and *E. mulungu* (Vasconcelos *et al.*, 2003). In folk medicine, various species are utilized as tranquilizers, against insomnia and to treat inflammation (García -Mateos *et al.*, 2001). Biological activities of *E. abyssinica* have been broadly studied and reported. The various parts of the plant that have been studied range from leaves, roots, root bark and stem bark. Kloos and others reported the activity of root, bark and leaves' extracts against molluscs (Kloos *et al*., 1987). The plant also has mitogenic effects on cell cultures (Tachibana *et al*., 1993). Flavonoids from this plant have been reported to have antiplasmodial (Yenesew *et al*., 2004) and free radical scavenging activity (Yenesew *et al*., 2009), anti-bacterial effect against broad range of bacteria, muscle relaxing effects, antidiarrhoea and anti- fungal activities (João *et al*., 2009; Olila *et al*, 2007), it has also been shown to have activity against rifampicin resistant *Mycobacterium tuberculosis* (Bunalema *et al*., 2011). The plant has shown anti-glycaemic activity in swiss mice diabetes model (Piero *et al.*, 2011). Several

compounds have been isolated from *E. abyssinica* (Yenesew *et al*., 2009) and their structures elucidated. The compounds mostly belong to two main groups of alkaloids and flavonoids (João *et al*., 2009). These include: Abyssinone IV, Abyssinone V, Abyssinin III, Abyssinone IV, Abyssinone V, 4', 7-Dihydroxy-2', 5'-dimethoxyisoflav-3-ENE 62.0 and Quercetin 5.4. (Figure 2)

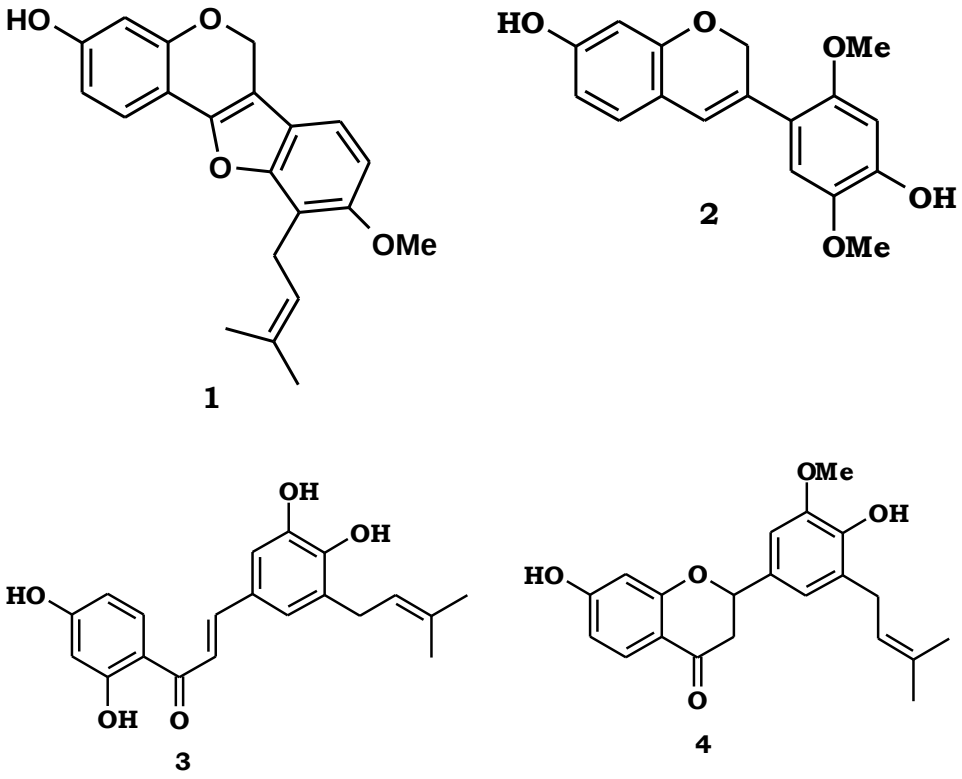


Figure 2: Chemical structure of active compounds isolated *from Erythrina abyssinica*. Erycrystagallin (1), Abyssinin III (2), abyssinone V (3) and abyssinone IV (4) (Yenesew *et al*., 2009: João *et al*., 2009)

1.2.5 Trypanosomiasis

Trypanosomiasis is an important zoonotic disease commonly referred to as Nagana in domestic animals and human African trypanosomiasis (HAT) also known as sleeping sickness in humans (Ngure *et al*., 2009). In animals, the disease is caused by *Trypanosoma brucei brucei, Trypanosoma vivax, Trypanosoma simiae* and *Trypanosoma congolense* while HAT is caused by two sub-species of *Trypanosoma brucei gambiese* and *Trypanosoma brucei rhodesiense*. The disease is lethal if left untreated (Stich *et al.*, 2002)

1.2.5.1 Distribution of trypanosomiasis

Human African trypanosomiasis is prevalent in Sub-Saharan African countries that lie between 14° North and 29° south of the equator (Rodgers, 2009). *T.b. gambiense* occurs in West and Central Africa while *T.b. rhodesiense* occurs in East and Southern Africa (Fig 3). In this region, close to 60 million people are at risk of infection, according to the world health organization (WHO, 2001). Cases of HAT are found only where tsetse flies are located. However some travelers have been reported as being HAT positive. In 2012, two HAT cases were reported amongst some tourists visiting Maasai Mara game reserve (Gobbi and Bisoffi, 2012). The effects of HAT are wide reaching, not only in Africa where it is an endemic disease, but also in non-endemic, developed areas.

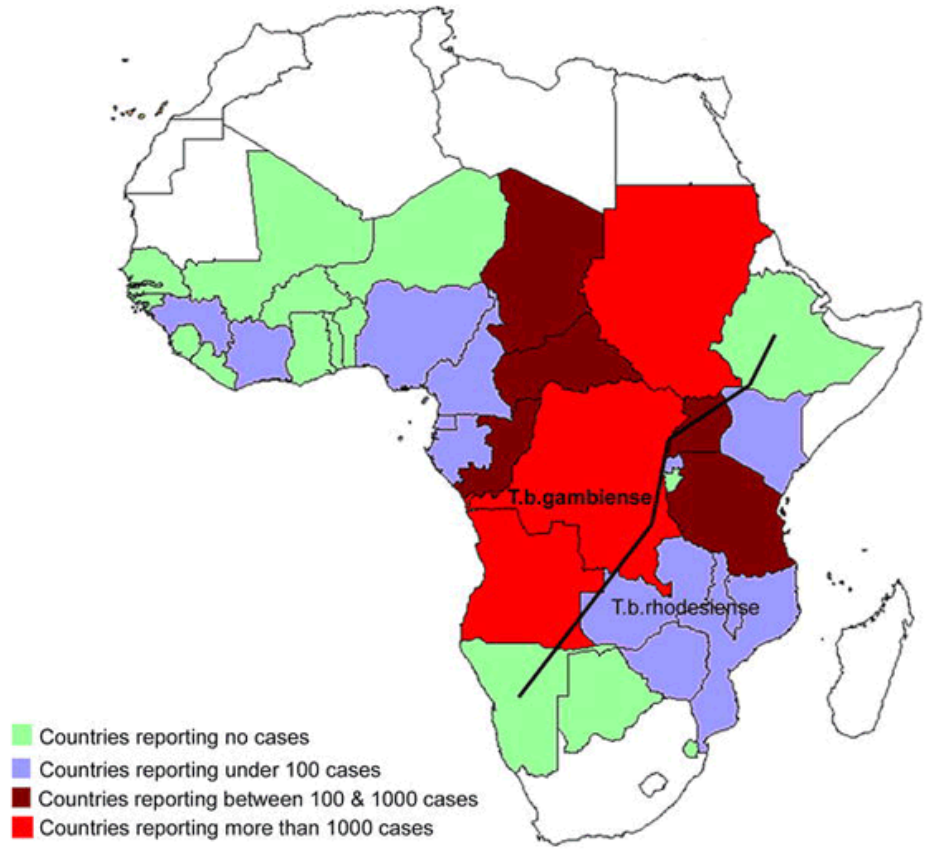


Figure 3: Map showing the distribution of Human African trypanosomiasis. The coloured part represents the prevalent region, confined between 14° North and 29° south of the equator. The black line demarcates regions with the East African and West African form of the disease (Adapted from Simarro *et al.*, 2008).

1.2.5.2 Pathogenesis of trypanosomiasis

The parasite is transmitted by Glossina species (tse tse flies), that has bitten an infected person or animal and ingesting infected blood meal, a phenomenon first described by David Bruce (Wolburg *et al.*, 2012). The parasites multiply in the fly's mid gut as metacyclics, transform into procyclic forms, and then travel to the salivary glands where they multiply again (Vickerman *et al*., 1988; Kennedy, 2005). When the fly takes its next blood meal, the parasites are transferred to the new host that becomes infected. In the animals, the parasites once again transform into trypomastigotes and multiply by binary fission in body fluids such as blood, lymph and spinal fluid. In humans the disease has two stages, the early haemolymphatic stage, and the late CNS stage. After the parasites are inoculated into man, they proliferate at the infection site, causing an inflammatory nodule known as a trypanosomal chancre (Zwart, 1989). Parasites then spread through lymphatic's to the lymph nodes and reach the blood stream, which marks the beginning of the hemolymphatic stage, as well as to the lymph nodes and other organs such as spleen, liver, heart, endocrine system and eye (Kennedy, 2005). Most patients will have swollen, palpable lymph nodes. The patient suffers from fever, headache, pruritus and generalized edema and malaise. Patients suffering from HAT will show generalized lymphadenopathy, usually on the back of the neck (Lejon *et al*., 2003), a condition known as winter bottom's sign. Parasites can, at this stage, be microscopically detected in blood, lymph and aspirates. Signs and symptoms may subside after the acute first stage.

In the meningoencephalitic (CNS) stage, parasites enter into the organs, including the central nervous system (Enanga *et al*., 2002). Disease stage can be determined by the presence of trypanosomes in the cerebrospinal fluid. As this requires a lumbar puncture, this is usually performed after an initial dose of suramin, to reduce the possibility of contaminating the CSF samples with trypomastigotes from the blood stream (Sinha *et al*., 1999). The trypanosomes cross the blood-brain barrier near intracellular junctions and also through an active process (Masocha *et al*., 2007). This process is acute in *T.b. rhodesiense* infections, taking only a few weeks, but chronic in *T.b. gambiense* infection. As the disease progresses, the classical signs of HAT arise: severe headaches, a disruption of the circadian rhythm, with night time insomnia and daytime somnolence, altered mental functions and personality changes (Buguet *et al.*, 2001). Generalized meningoencephalitis may lead to coma and death (Kennedy, 2006). Trypanosomes are entirely encapsulated in a variant surface glycoprotein (VSG) composed of a single protein molecule that protects the parasite from lytic factors in plasma (Taylor and Rudenko, 2006; Ferrante and Allison, 1983). After infection, the VSG is recognized by the host's immune system, which leads to non-specific B-cell activation and to the production of IgM and IgG antibodies to neutralize the parasites in an attempt to decrease parasitemia. A small percentage of trypanosomes, however, will have different surface coats and will escape detection, continuing to proliferate until new coat-specific antibodies are produced by the host's immune system. Over 200 VSG genes have been reported, including pseudogenes, but only one gene is expressed at a time (Marcello and Barry, 2007). This high degree of antigenic variation makes it extremely hard for a

vaccine to be produced (Stuart *et al*., 2008), and a high level of IgM can usually be detected in the patient. This increased measurement is considered a good indicator of trypanosomal infection (Louis *et al*., 2001).

1.2.5.3 Diagnosis of trypanosomiasis

Parasite numbers of below 100 trypanosomes / ml can be difficult to detect with microscopy alone (Chappuis *et al*., 2005a). Concentration methods such as microhematocrit centrifugation (Woo, 1970), quantitative buffy-coat analysis (Ancelle, 1997) or mini-anion exchange columns (Lumsden *et al.*, 1979) can be used to concentrate the parasites for easier microscopic detection. In West Africa, many endemic screening programmes rely on the card-agglutination test for trypanosomiasis (CATT), which tests the agglutination of trypanosomes antigens in the presence of specific antibodies, is a sensitive assay to detect *T.b. gambiense* antibodies in serum (Truc *et al*., 2002). *T.b. gambiense* antibodies can also be detected by ELISA or immunofluorescence although these are not the most practical methods to be used in the field due to their energy and reagent requirements. CATT is not effective for detecting *T.b. rhodesiense* infections (Matovu *et al*., 2010; Magnus *et al.*, 1978) and microscopic visualization remains the only test used to make a definitive diagnosis. Molecular techniques, such as PCR, have been developed and evaluated but have yet to be adopted in the field, due to the need for trained technicians, the proper equipment, a constant source of power, and the required storage methods for the supplies (Barrett *et al*., 2003). Furthermore, the tests must also be standardized and validated in a clinical setting

(Chappuis *et al*., 2005a). Some researchers report prolonged positivity after successful treatment, which could be misinterpreted as a continued infection (Solano *et al*., 2002). Recent innovations such as a novel loop-mediated amplification (LAMP) have led to the development of techniques for the detection of trypanosomes and may prove to be a useful alternative to PCR (Kuboki *et al*., 2003). LAMP has no need for a thermo cycler, with the whole reaction taking place between 60-65ºC, have a high specificity due to a complex set of primers and the products are easily detectable using fluorescent dyes and UV light (Kuboki *et al*., 2003).

1.2.5.4 Treatment of trypanosomiasis

There are only five licensed drugs for the treatment of HAT (Bacchi, 2009). Due to the fact that HAT is classified as a neglected tropical disease (NTD), there is little incentive for pharmaceuticals to invest in research, development or production of new anti-trypanosomal compounds. Those most in need of the drug are not able to pay for treatment and thus there is little financial enticement to produce these drugs. At the moment, all anti-trypanosomal drugs are currently donated to WHO by Sanofi-Aventis Paris, France (pentamidine, melarsoprol, eflornithine) and Bayer Leverkusen, Germany (suramin, nifurtimox) with storage and transport of the drugs overseen by Médecins Sans Frontières (MSF), however long-term availability of all trypanocides is still uncertain (Bouteille and Buguet, 2012). Pentamidine and suramin treat the haemolymphatic stage. Pentamidine is the recommended drug for treatment of first-stage of *T b gambiense*. It is administered intramuscularly for one week and is generally

well-tolerated (Bronner *et al*., 1991). Complications that arise from intramuscular injections include pain and swelling at the injection site, abdominal pain or gastrointestinal problems. Suramin is recommended for treatment against first-stage *T.b. rhodesiense* HAT. Nephrotoxicity, peripheral neuropathy and thrombocytopenia are frequent but mild side effects and can easily be treated. Because hypersensitivity is also quite common, leading to anaphylactic shock when there is concomitant filariasis, it is recommended that a low test dose be administered prior to beginning the long and complex drug course (Bouteille and Buguet, 2012). To fully treat trypanosomal infection in the CNS, the drug must be able to cross the blood-brain barrier (Kennedy, 2006). For treatment of second-stage HAT, melarsoprol remains the most widely-used drug; it is the only drug available to treat *T.b. rhodesiense* -caused HAT. This organ arsenic compound causes frequent adverse reactions that can be quite severe and even life-threatening. Post-treatment reactive encephalopathy (PTRE) occurs in 10% of all patients receiving melarsoprol and up to 50% of those receiving treatment may die as a result of complication with neuropathological analysis revealing acute hemorrhagic leukoencephalopathy (Adams *et al.*, 1986). The onset of headaches and fever can be indicative of possible complications (Blum *et al*., 2001). The cause of PTRE remains unknown, although many hypotheses exist. These include: sub-curative chemotherapy; immune complex deposition; aberrant immune response to glial cell-associated antigens; autoimmune mechanisms; and arsenical toxicity (Hunter *et al*., 1992).

Eflornithine is the most recent of the treatments against gambiense HAT. It has a much lower mortality than melarsoprol and therefore it is recommended as the drug of choice

to treat second-stage *gambiense*-disease (Chappuis *et al*., 2005b). *T.b. rhodesiense* shows an innate reduced susceptibility against eflornithine and thus treatment with melarsoprol is recommended (Iten *et al*., 1997). Treatment with eflornithine requires 2 weeks of injections repeated 4 times per day; bacterial infection at the site of the catheter can lead to sepsis but can easily be prevented with proper care (Chappuis *et al*., 2005b). Other potential side effects include anemia, gastrointestinal symptoms and convulsions (Burri and Brun, 2003). Recently the Drugs for Neglected Diseases *initiative* (DND*i*) recommended a combined nifurtimox-eflornithine treatment combining 7 days eflornithine (2 infusions per day) followed by 10 days of nifurtimox taken as an oral dose thereby reducing the number of injections (Priotto *et al*., 2009). Shorter drug regimens should translate to reduced admission times and greater patient adherence. For optimal treatment, skilled medical personnel are required, preferably ones familiar with the possible complications and treatment of HAT. While medical staff experience in dealing with HAT may be difficult to come by in non-endemic areas, patients are more likely to receive a more thorough treatment, including proper nutrition and complete monitoring (Barrett *et al*., 2003). An 18-month to two-year post-treatment follow up is necessary to declare a patient cured (Bouteille and Buguet, 2012).

Trypanosomes are developing resistance for the available drugs, these drugs were developed over fifty years ago, since then no new antitrypanosomal drug for treatment of animals have been introduced. Efforts have therefore been put to develop new therapeutic agents for trypanosomiasis including those originating from medicinal plants (Adamu *et al*., 2009). Medicinal herbs are moving from fringe to mainstream use

with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio friendly plant-based products for the prevention and cure of different human and animal diseases. The lack of product quality and consistency and the absence of compelling data on the safety and efficacy of most and traditional medicine approach present major challenges to any effort to optimize the distribution of precious health resources. Although the basis of the traditional use of some of the particular products has been validated or confirmed by scientific research and the compounds mainly responsible for the effects isolated, this is not the case for a large number of medicinal plants (Houghton, 1995). *E. abyssinica* is widely used as a medicinal plant; most of the conditions treated with the plant suggest an inflammatory involvement. This observation led to the question on whether the plant had anti-inflammatory properties. This study was therefore designed to test for this phenomenon.

1.4 OBJECTIVES OF THE STUDY

1.4.1 Broad objective of the study

The broad objective was to study the anti-inflammatory potential of *Erythrina abyssinica* by assessing the histological and immunohistochemical evidence in chronic trypanosomiasis mouse model.

1.4.2 Specific objectives of the study

1. Evaluate the parasitaemia and brain migration of trypanosomes in mice following experimental infection.
2. Determine the major active phytochemical groups in *Erythrina abyssinica*.
3. Evaluate perivascular cuffing and lymphocytic infiltration in cerebrum of trypanosome infected mice after treatment with *Erythrina abyssinica* extracts.
4. Evaluate astrocytosis in trypanosome infected mice after treatment with *Erythrina abyssinica* extracts.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Animals

Swiss white mice to be used for parasite passage were obtained from ILRI. The mice were irradiated using gamma radiation for 3 minutes, and passaged with *Trypanosoma brucei brucei* (GUTat 3.1). The irradiation was to immunosuppress the mice, inorder to have a fast parasite multiplication. Swiss white mice, males and females, 8-12 weeks of age, weighing 16-30 g acquired from the University of Nairobi, Department of Medical Physiology animal facility were used for the experiment, a total of 58 mice were used. The mice were acclimatized to experimental conditions for one week. The animals were housed in polycarbonate cages and fed on mice pellets (Mice pellets®, Unga feeds) and provided with water ad libitum through drinkers. Both food and water was provided ad libitum. Bedding (wood shavings) was changed daily.

2.2 Plant Material

2.2.1 Plant collection

Root bark of *E. abyssinica* was collected in Machakos County. Specimens of the plant were identified at the herbarium of the school of Biological Sciences, University of Nairobi. The root bark was washed to remove the soil and chopped into pieces of an approximate length of 1cm, air dried under a shade and ground into fine powder, which was stored in airtight container until it was processed.

2.2.2 Extraction and concentration of crude aqueous and methanol extracts

Methanol extraction was undertaken by soxhlet method as previously described (Ahmad *et al*., 2010). Briefly, One hundred (100) grams of the powdered Erythrina root bark sample was extracted with methanol in a Soxhlet extractor for 24 hours. The mixture was filtered in gauze and the filtrate centrifuged at 5000 revolutions per minute for 10 minutes. This was followed by further filtration with Whatman number 1 filter paper and the filtrate reduced *in vacuo*, in a rotary evaporator at reduced pressure at 40°C. Methanol was then removed from the extract by holding the extract in an oven at 40°C for 1 week. The sample was then weighed to determine the yield and stored in dark bottles at 4°C to avoid biological degradation before use.

Water extraction was undertaken by decoction method as previously described (Chebaibi and Filali, 2013). Briefly, 600 grams of the sample was soaked in 3 liters of water and boiled for two hours. After cooling, the extract was filtered in cotton gauze, and the filtrate centrifuged at 5000 revolutions per minute for 10 minutes. This was followed by further filtration with Whatman number 1 filter paper. The filtered extract was frozen followed by freeze drying. Finally, the sample was weighed to determine the yield then stored at 4^0C to avoid biological degradation.

2.2.3 Phytochemical analysis of *Erythrina abyssinica* extracts

Standard methods were used to determine presence of flavonoids and alkaloids as previously described (Prashant *et al*., 2011). The presence of alkaloids was tested using Hager's method, in which to one (1) ml of the extract was added to Hager's reagent (saturated aqueous solution of picric acid) and observed for formation of a prominent yellow precipitate. Presence of flavonoids was tested by two methods: sodium hydroxide method by adding sodium hydroxide to a small amount of the extract and observing for formation of yellow orange colour and Ammonium method where to one ml of the extract was added four ml of ammonium solution and observed for formation of greenish yellow colour.

2.2.4 Toxicity study.

The preliminary dose determining toxicity test of *E. abyssinica* extracts was performed in order to determine the lethal dose 50 (LD50). This would help in selecting the most appropriate dose range to be used during the experiment. *E. abyssinica* aqueous and methanol extracted were dissolved in normal saline and DMSO respectively. Toxicity test was performed as previously described (Randhawa, 2009). Briefly, female mice between 8-12 weeks of age were used to test toxicity of *E. abyssinica* extracts. Mice were divided into three groups of three mice each. The mice were administered with 100, 500 or 1000 mg/ kg body weight of the extracts through the intraperitoneal route, and thereafter closely observed for clinical signs including mortality and behavioral

change. Mortality data was used to calculate LD50. These observations were made for the first four (4) hours and thereafter daily for 14 days.

2.3 Trypanosome Infection and treatment with *Erythrina abyssinica* extracts

Cryopreserved *Trypanosoma brucei brucei* (GUTat 3.1) stabilate was obtained from International Livestock Research Institute trypanosome bank. The parasite was propagated and maintained in clean 4 weeks old Swiss albino mice. A single dose of 300 rads whole body X- irradiation was administered on these mice, using an X-ray machine operated at 250 kV and 15 mA, for 3 minutes. The irradiation was to immunosuppress the mice, so that the parasites could multiply faster, and also have a high parasite count, that was to be used to infect experimental mice. Frozen parasites were rapidly thawed at 37^0 C, diluted in 1 ml of phosphate buffered saline containing 10% glucose (PSG-mice), and then injected intraperitonealy. When parasitaemia in the tail blood reached a $6X10^7$ parasites/ml, the blood was collected by cardiac puncture. Parasite concentration was adjusted to $5x10^4$ parasites / ml in PSG-mice. Each mouse in experimental groups was infected with 0.2 ml of the diluted blood i.e. $1x10^4$ parasites, except the non-infected control. The mice were randomly divided into ten groups: non infected, non infected treated with saline, infected mice treated with *E. abyssinica* water extract (12.5, 25, 50 and 100 mg/kg dose groups) and infected mice treated with *Erythrina abyssinica* methanol extract (12.5, 25, 50 and 100 mg/kg dose groups). Treatment with *E. abyssinica* aqueous and methanol extracts was done every second day beginning on the 12^{th} day post infection.

2.4 Parasitaemia monitoring

2.4.1 Blood collection

Parasitaemia in *T.b. brucei* infected mice and controls were monitored daily for the duration of the study (28 days), beginning from day three post infection. The tail was snipped using small pair of scissors and a drop of blood placed on a glass slide and cover slipped.

2.4.2 Parasitaemia grading

The blood collected from the tail and coverslipped, was observed under a microscope at X 100 magnification. The field of view was matched to a chart (Fig 4) to give a corresponding score as described by Herbert and Lumsden (1976).

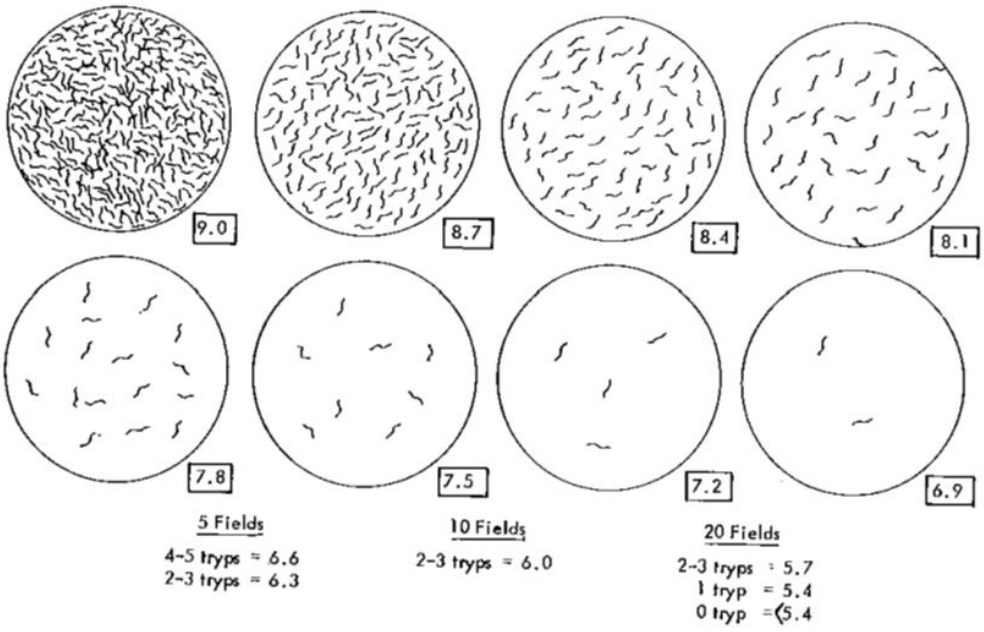


Figure 4: Chart for scoring parasite count in mice infected with *Trypanosoma brucei brucei*. The scores give an anti-log of parasites per/ml. X100 magnification. Adapted from Herbert and Lumsden (1976)

2.4.2 Blood smear staining.

During the course of *T.b. brucei* infection in mice, blood smears were prepared to observe the morphology of the parasites. For this purpose, two stains were used, Modified Hematoxylin eosin and giemsa. Blood was collected by snipping the tail with a small pair of scissors and a drop of blood placed on a clean glass slide, and a smear made. The smears were stained in either giemsa or modified hematoxylin eosin**.**

2.5 Monitoring brain migration of trypanosomes

At 21 days post infection, one mouse per group was sacrificed by cervical dislocation, and brain harvested. The brain was washed in PBS and homogenized in PSG-mice solution in a 5ml tube. Part of the filtrate was placed on several glass slides and either observed directly under a microscope or stained with giemsa.

2.6 Histology and electron microscopy

2.6.1 Tissue harvesting and fixing

At 29 days post infection, all mice were anaesthetized by placing them in a carbon dioxide chamber, and then pinned on a dissecting board before being exsanguinated. The skin covering the head was dissected and the underlying muscles scrapped out using a scalpel blade. With a mosquito scissors, two lateral incisions were made between the lateral margin of the eye orbit and occipital bone. An anterior incision was made to connect the two parallel incisions. The bone flap was then lifted to expose the underlying brain. Using a blunt small spatula, the brain was lifted from the cranium and

cut longitudinally with scalpel blade to divide into two. Cerebra were separated from cerebellum and placed either in 10% formalin or 2.5% glutaraldehyde for routine histology and electron microscopy respectively.

2.6.2 Tissue processing for histology

Cerebral tissues measuring $1cm^3$ were washed under running water for 24 hours. Tissues were then dehydrated in a series of increasing concentration of ethanol (70, 80, 95 and twice in 100%) and cleared in two changes of methyl benzoate for two hours each. This was followed by infiltration in two changes of molten paraffin wax two hours each at 58° C and thereafter embedded in paraffin wax. Tissues were sectioned at 5 µm in a Leitz rotary microtome, and mounted in albumin coated glass slides. Mounted tissue sections were deparaffinized in two changes of xylene, 10 minutes each, followed by rehydration with series of decreasing concentration of ethanol (100%- 50%), then washed in distilled water. The tissue slides were stained with hematoxylin and counterstained with eosin. They were then dehydrated in increasing concentration of alcohol, cleared in xylene and coverslipped. Tissue slides were examined under microscope Leica DM500, using various magnifications and photomicrographs taken. The tissues were analyzed semi quantitatively by modifying the technique used by Kennedy *et al*. (1997) for inflammation and graded as 0, 1, 2 or 3. The scoring was based on degree of perivascular cuffing, presence or absence of meningo-encephalitis and the extent of leucocytic infiltration in the brain parenchyma (Table 1).

Table 1: Criteria for grading brain pathology of chronic *Trypanosoma brucei brucei* mouse model. Adapted from Kennedy *et al.* (1997).

GRADE	CHARACTERISTICS
0	No pathological changes
1 (Mild)	Several mononuclear cells (2-3) in a few perivascular spaces.
2 (Moderate)	Up to 6 mononuclear cells in several perivascular spaces. Slight meningoenchephalitis
3 (Severe)	Heavy infiltration of mononuclear cells in perivascular spaces with complete occlusion. Mononuclear cell aggregates in parenchyma. Presence of meningoenchephalitis

2.6.3 Astrocyte immunohistochemistry

Formalin fixed paraffin wax embedded cerebral tissues were processed as described earlier (Yun *et al*., 1999). Tissue blocks were sectioned at 3 µ thickness and mounted onto gelatinized slides. Three pairs of serial sections per animal were processed. A random number between one and five was selected to determine the first pair of serial sections, thereafter; every third pair was picked to provide a total of three pairs. Tissue sections were dewaxed in xylene. Antigen retrieval was performed for 20 minutes in citrate buffer heated at 95˚ C. After washing with phosphate-buffered saline (PBS), each section was treated for 10 minutes with blocking buffer (Envision™ Flex, Dako) to inhibit endogenous peroxidase prior to incubation with a rabbit anti-GFAP polyclonal antibodv (1:300, Dako) for 30 minutes at room temperature. Following a brief rinse with PBS, a goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Envision™ Flex/HRP, Dako) was introduced for 20 minutes. Diaminobenzidine (Dako), a HRP substrate was introduced for 5 minutes followed by thoroughly washing in PBS. Finally, tissues were dehydrated in 100% ethanol, cleared in xylene and cover slipped.

2.6.4 Stereological counts of astrocytes

The numerical density of astrocytes was determined using the physical disector method (Sterio, 1984; Gundersen, 1986) in which the two consecutive serial sections, 3 µ apart were used, one being the look up section and the other the reference section. The slide was placed on the microscope and a randomly selected field of view was brought into

focus at X400 magnification, and a digital the image taken, the corresponding field in the reference section was selected and the image also taken, all images were stored in the computer memory.

The stored images were then processed by having a measuring frame (0.198 X 0.278 mm) drawn on them with the aid of Leica application suite program (LAS EZ version 1.8.0). The "forbidden line" rule was applied (Gundersen, 1977), where cells touching the forbidden lines were omitted from the count (Figure 5). Astrocytes appearing only on the look up section were counted and the numerical density obtained using the formula

$$Nv = \sum Q / h.\sum a$$

Nv- numerical density

Q- Number of cells counted

a-Area of counting frame = 0.055 mm^2 (0.198 X 0.278)

h- Distance between tissue section pairs = Section thickness (because they are consecutive sections) = 0.003 mm

NB. Therefore in a single frame, cells were counted within a volume of 0.000165 mm^3

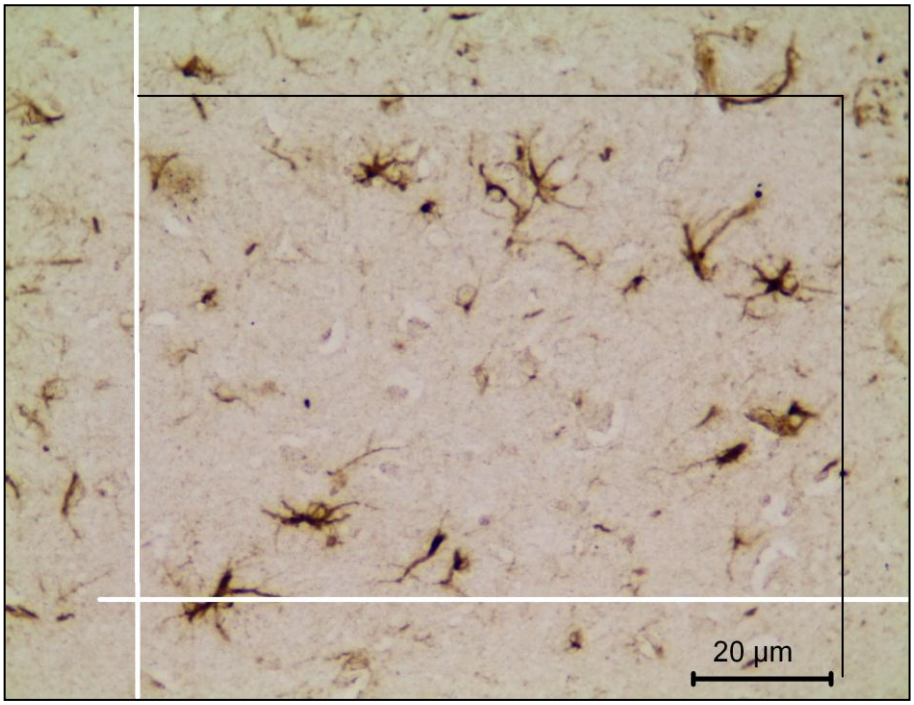


Figure 5: A frame used for counting GFAP stained astrocytes in the cerebrum of *Trypanosoma brucei brucei* infected mice. The frame measured 0.278 mm X 0.198 mm (Width X Height). The black and white margin lines are the forbidden and inclusion borders respectively. X400 magnification

2.6.4. Tissue processing for transmission electron microscope

Tissues were processed as described by Makanya et al. (2011); cerebral tissue blocks measuring 1x 1 mm were fixed in 2.5 % glutaraldehyde. the tissues were washed in three changes of 0.1M PBS and post fixed in 1% osmium tetroxide for one hour and rinsed in two changes of 0.1M PBS. Tissues were dehydration in increasing concentration of ethanol, followed by clearing and infiltration in propylene oxide. The tissues were embedded in araldite resin and polymerized at 60°C overnight. Semithin tissue sections were sectioned from the block with a microtome, stained with 1 % toluidine blue in borax solution and viewed under a LEICA DM 500 light microscope, to identify areas of interest for ultrathin sectioning. Ultrathin sections of the areas of interest were cut with a microtome, placed on copper grids, stained with uranyl acetate and lead citrate, before viewing in Phillips 301 transmission electron microscope.

2.6.5 Tissue processing for scanning electron microscope

This was processed as described by Kiama *et al*. (1994). Tissue blocks preserved in 2.5 % glutaraldehyde were washed in Millonig's buffer for 15 minutes while shaking. The tissues were stained with osmium tetroxide for 1 hour then washed in millonig's buffer for 20 minutes. This was followed by dehydration in a series of increasing concentration of alcohol followed by critical point drying. Tissues were mounted on aluminium stabs with silver conductive points and then sputter coated with gold palladium complex. Specimens were analyzed in JEOL-6010LA Scanning electron microscope. Digital images were obtained and stored in the computer memory.

2.7 Protein analysis.

2.7.1 Protein extraction.

Formalin fixed, paraffin wax embedded cerebral tissue were processed for protein extraction as described by Shan-Rong *et al*. (2006). This was to determine the difference in protein content of cerebrum, between different experimental groups. The tissues were dewaxed in xylene, rehydrated in distilled water, and. homogenized with use of a glass rod. Fifty ml of 20 mM Tris-HCl buffer (pH 7 or 9) containing 2% SDS was then added to the tissue homogenate followed by heating in a microwave for 5 minutes to free the proteins from formaldehyde cross linkage. Samples were stored at -20˚ C until use.

2.7.2 Protein quantification

Extracted proteins were quantified using Bradford method (1976). Bovine serum albumin (BSA) was used as standard and distilled water used as blank. Five concentrations of BSA were prepared; 5, 10, 15, 20 and 25 µg/µl. 30µl each of the sample, BSA standards, and blank were pipetted in duplicate into micro titer plates. Into each well, 200µl of Bradford reagent (coomassie blue 50mg, 100ml methanol, 100ml phosphoric acid, made to 1 liter with distilled water) was added and incubated for 30 minutes at room temperature. The results were obtained through a micro plate reader run using linear regression program at 595nm.

2.7.3 Electrophoresis

Brain proteins were separated using standard SDS PAGE method developed by Laemmli (1970). The samples of exracted proteins were heated for 5 minutes at 95°C. 10-30µg of sample was mixed with sample loading buffer containing 5% β-mercaptoethanol and loaded onto SDS-PAGE 10% SDS PAGE. The molecular weight standard (Sigma) was also loaded onto the gel. The running buffer was 25 mM Tris; pH 8.3, containing 0.1% SDS, and glycine. Electrophoresis was performed at 90 volts until adequate spread of the protein molecular marker was achieved. Thereafter, gels were removed and stained under slight agitation for 30 minutes with coomassie blue solution made up of 0.2% coomassie blue, 7.5% Acetic Acid and 50% Ethanol. The gels were then destained with a solution made up of 10% methanol and 7% glacial acetic acid. Gels were photographed using a digital camera and image processed with Adobe photoshop© Version 8 (Adobe Photoshop CS).

2.8 Data management and statistical analysis

Experimental data was recorded in excel spread sheet, archived in computer and also backup made in memory disks. Data for both parasitaemia and neuroinflammatory scores were recorded in excel data sheet (Ms Excel, USA), and line graphs plotted for parasitaemia. Data was then analyzed by comparing the means between the different groups using a one way ANOVA and a post hoc analysis using Tukey method for multiple comparisons. A 95% confidence interval was also calculated. This analysis was done with the aid of SPSS® (IBM) computer programme.

CHAPTER 3

3 RESULTS

3.1 Yields from *Erythrina abyssinica* extraction

Water extract had a yield of 7.66%, while methanol extraction had 6.13% yields. The methanol extract was a sticky, dark brown paste, while the aqueous extract was light brown powder.

3.2 Phytochemical screening

Qualitative phytochemical tests revealed presence of alkaloids and flavonoids in both water and methanol extracts of *E. abyssinica* (Figure 6 and Table 2). In Hager's method to test for alkaloids, addition of saturated aqueous solution of picric acid to both water and methanol extracts formed a strong yellow precipitate confirming presence of alkaloids. Sodium hydroxide or ammonia solution added to the extracts formed a yellow colour confirming the presence of flavonoids for both water and methanol extracts.

A B C

Figure 6: Photograph of phytochemical test results of alkaloids and flavonoids in *Erythrina abyssinica* root bark extracts.

(A) Positive Flavonoid test,

(B) Negative Control,

(C) Positive alkaloid test,

Table 2: Major phytochemical groups present in aqueous and methanol extracts of *Erythrina abyssinica*

	ALKALOIDS	FLAVONOIDS
Erythrina abyssinica aqueous extract	+	+
Erythrina abyssinica methanol extract	+	+

+ Denotes present

3.3 Toxicity study

Following intraperitoneal administration of *E. abyssinica* extracts, mice exhibited reduced motor activity, paw licking, sedation and tremors. One mouse dosed with 500 mg/kg died within 24 hours, two mice died within 24 hours in the 1000 mg/kg group (Table 3). The zero percent mortality data was corrected using the formula 100 (0.25/n) (Randhawa, 2009). A graph of probit against log dose was then plotted (Figure 7). The log dose corresponding to probit 5 (50% mortality) was 2.85 whose antilog is 708 which is the LD_{50} dose. Therefore, the intraperitoneal LD_{50} of *E. abyssinica* obtained was 708 mg /kg body weight. Therefore, 12.5, 25, 50 and 100 mg/kg doses were selected for the experiment, because they were way below the toxic doses.

Table 3: Survival data of mice dosed with crude extract of *Erythrina abyssinica* in preliminary dose determination toxicity test

Dose (mg/kg)	**log dose**	**No. of mice alive**	**No. of mice dead**	**% mortality**	**Probit**
100	2	3	0	8.33*	3.59
500	2.7	2	1	33	4.56
1000	3	1	2	66	5.41

*Corrected percentage mortality100 (0.25/n) where n is the number of animals per group = 3, (Randhawa, 2009)

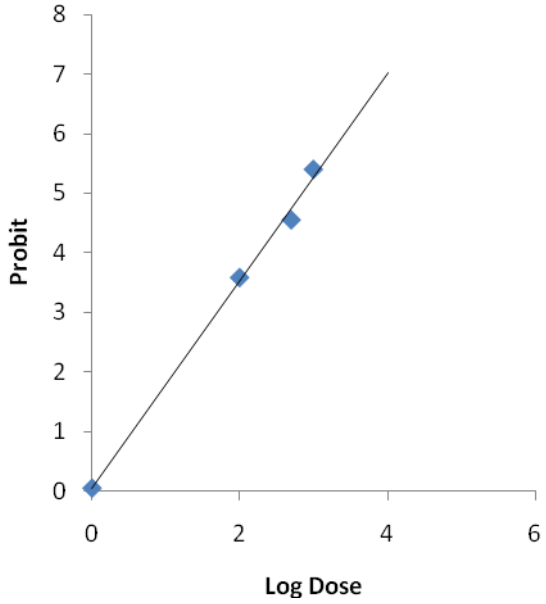


Figure 7: A plot of probit against Log dose of *Erythrina abyssinica* aqueous extract for determining LD50

3.4 Infection and parasitaemia

Parasites were visible in peripheral blood of all infected mice five days after intraperitoneal infection of mice with 1 x 10^4 trypanosomes (Figure 8). *E. abyssinica* did not have an effect on the parasites since all groups of animals had similar parasitaemia pattern (Figure 9). Some mice had moderate clinical signs of shivering and erect hair but none of the mice developed neurological signs.

Two distinct parasites morphology were observed in the course of the infection, there was a long slender form that had long flagella, a small undulating membrane and a centrally located nucleus (Figure 8). This form was prominent in the beginning of parasitaemic wave. The other type was broad blunt form that had a short or no flagella, broad undulating membrane coat and marginal nucleus. This form was prominent at the plateau phase of the wave. The brain homogenate of infected mice that were sacrificed on the twenty first day post infection was stained with giemsa and parasites observed. This homogenate was also injected intra peritoneally into clean mice that developed parasitaemia after three days.

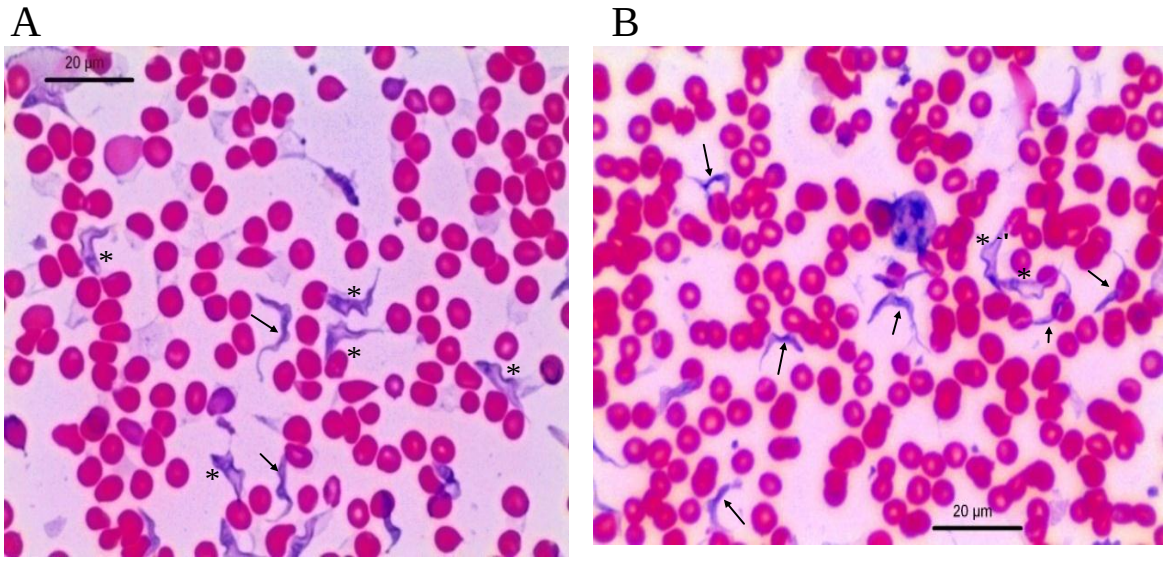


Figure 8: A) Late phase of infection B) Early phase of infection

Modified H and E stained micrograph of blood smears from mice infected with *Trypanosoma brucei brucei* (GUTat 3.1). Note the two forms of parasites, blunt (asterix) and sharp forms (arrow). The blunt forms were higher in number during the peak of parasitaemia (A), while the sharp form abundant in early phase (B).

In the positive control group (infected non treated), the parasites were first microscopically observed on the fourth day post infection, and by the fifth day post infection, all animals in the group had developed parasitaemia in peripheral blood. Parasitaemia score reached a peak on the seventh day post infection, anti-log 7.35 ± 0.315, this then subsided to a low score of anti-log 4.9 ± 2.493 on the nineth day post infection during the first wave. In the second wave, the peak occurred on day 16 post infection, with a score of anti-log 7.75 ± 0.122 and slightly decreased to anti-log 7.6 ± 0.155 on day 17 post infection. The parasitaemia then increased in the third wave to reach a peak of anti-log 7.8 ± 0.190.

In the mice that were injected with 12.5mg/kg dose of aqueous extract, the parasitaemia was observed first on the fourth day post infection and by the sixth day post infection; all the animals had developed microscopically visible parasitaemia. The first wave reached a peak of anti-log 6.04 ± 2.784 on the sixth day post infection and declined to a low of anti-log 5.66 ± 2.635 by the seventh day post infection. It then increased in the second wave to peak at anti-log 7.5 ± 0.245 by the sixteenth day post infection and subsided to a score of anti-log 7.46 ± 0.321.A third wave peaked at anti-log 7.76 ± 0.207 on the twentieth day post infection.

In the 25 mg/kg dose water group, parasites first appeared on the fourth day post infection and were present in all animals by fifth day post infection. In this group, the first wave peaked at anti-log 7.56 ± 0.251on day seven and decreased to anti-log 5.10

±2.909 by the ninenth day post infection. This was followed by a second wave that peaked at anti-log 7.74 ± 0.251by the fourteenth day post infection and decreased to a score of anti-log 7.62 ± 0.342. The third wave peak was observed on the twentieth day post infection with a mean score of anti-log 7.80 ± 0.212.

The middle 50 mg/kg group had the first observed parasitaemia on the fourth day post infection and all the animals in the group had developed microscopically visible parasitaemia by the fifth day post infection. The first parasitaemic wave attained a peak of anti-log 7.67± 0.160 by the seventh day post infection and declined to anti-log 6.04 ± 0.873 on the nineth day post infection. It then increased and to peak at anti-log 7.90 ± .245 by seventeenth day post infection and reduced to a score of anti-log 7.75 ± 0.226 in the second wave. A third and final wave peaked at anti-log 7.90 ± 0.310 on twentieth day post infection.

In the 100 mg/kg water group, the first animal developed parasitaemia on the fourth day post infection and by the fifth day post infection, all animals had developed parasitaemia. In this group, the first wave peaked at anti-log 6.90 ± 0.624 on the sixth day post infection followed by a decline to reach a low of anti-log 4.76 ±3.387by the eighth day post infection. The second wave then reached a peak of anti-log 7.56 ± 0.134on the fourteenth day post infection and reduced to a low of anti-log 7.26 ± 0.537. A third and final wave peaked at anti-log 7.68 ± 0.164on the twentieth day post infection.

In the 12.5mg/kg methanol dose group, parasites were first on the fourth day post infection and by the sixth day post infection, all the animals were positive for microscopically visible parasitaemia in peripheral blood. This group had a first wave peaking at anti-log 6.99± 0.641on the sixth day post infection followed by a decline to a low of anti-log 5.23 ± 2.387on the nineth day post infection. The second wave peak was at anti-log 8.01 ± 0.146occuring on the seventeenth day followed by a decline to anti-log 7.76 ± 0.270 by the eighteenth day post infection. A third and final wave peaked at anti-log 7.84 ± 0.207 on the twentieth day post infection.

The medium 25 mg/kg dose methanol group had the first observable parasitaemia on the fourth day post infection and all animals were positive by the sixth day post infection. In the group, the first wave peak was anti-log 6.86 ± 0.802 on the sixth day post infection and declined to anti-log 5.66 ± 2.595 by the seventh day post infection. A second wave peaked at anti-log 7.71 ± 0.285 on the sixteenth day post infection, and then declined to anti-log 7.54 ± 0.270 by the eighteenth day post infection. This low score of anti-log 7.54 ±0.270, 7.54 ± 0.472 persisted to the end of the study.

In the 50 mg/kg dose methanol group, the first microscopically visible parasitaemia was noted on the fourth day post infection, and all the animals were positive by the fifth day post infection. This groups' first wave peaked at anti-log 7.02 ± 0.268 on the sixth day post infection which then declined to anti-log 3.72 ±3.490 by the ninth day post infection. A second wave peaked at anti-log 7.80 ± 0.245 on the sixteenth day post

infection, followed by a decline to anti-log 7.73 ± 0.287 on the seventeenth day. On the twentieth day post infection, the third wave peaked at anti-log 8.03 ± 0.287.

Finally, in the 100 mg/kg dose methanol group, the first animal developed microscopically visible parasitaemia on the fourth day post infection, and all were positive by the fifth day post infection. The first wave had a peak of anti-log 7.11 ± 0.334 that occurred on the sixth day post infection and then declined to a low of anti-log 4.71 ± 3.344 by the eighth day post infection. A second wave peaked at anti-log 7.76 ± 0.113 on the sixteenth day post infection, which then declined to anti-log 7.75 ± 0.122 and anti-log 7.75 ± 0.226 on seventeenth and eighteenth days consecutively. A third and final wave peaked at anti-log 7.90 ± 0.155 on the nineteenth day post infection.

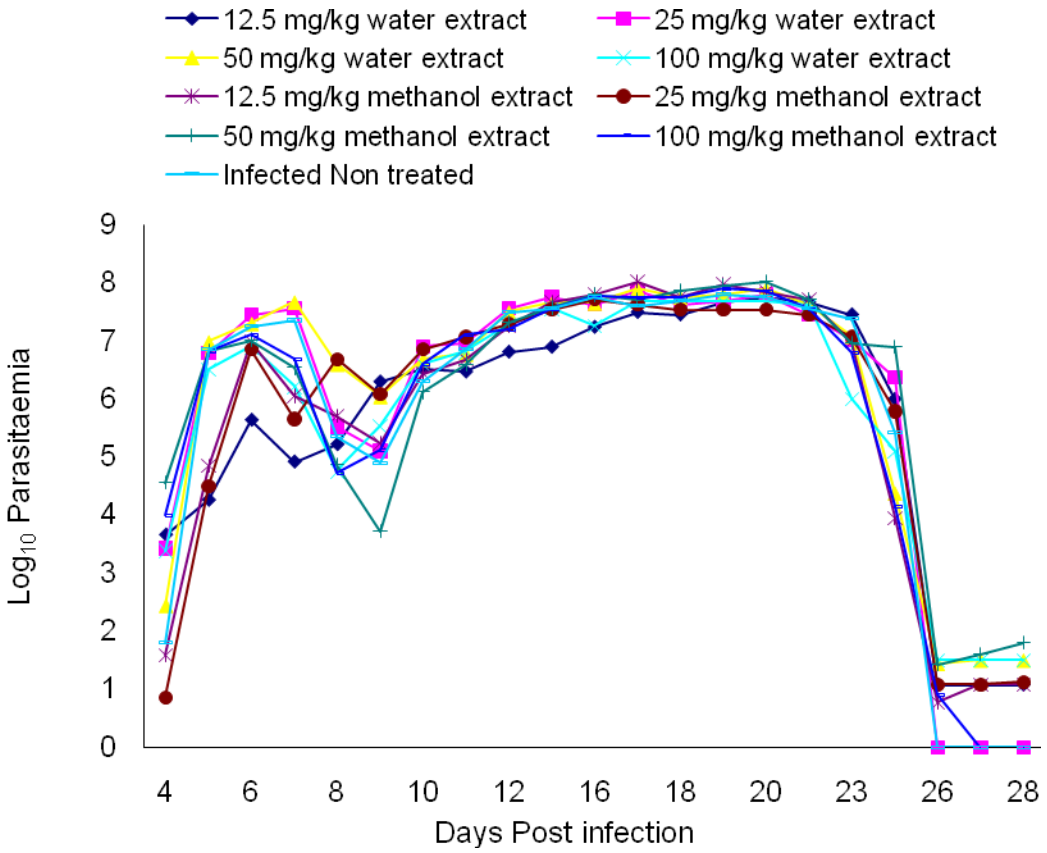


Figure 9: Growth pattern of *Trypanosoma brucei brucei* in mice treated with *Erythrina abyssinica* extracts. Mice were infected intraperitonealy with 1X10^4 blood stream forms of *Trypanosoma brucei brucei* (GUTat 3.1). A plot of parasitaemia against days postinfection shows fluctuation in parasite counts over the 28 days of the experiment in all experimental groups.

3.5 Derangement of cerebrum morphology

GFAP Immunohistochemistry and routine hematoxylin eosin staining showed astrocytosis and meningoencephalitis, which were greatly reduced in mice treated with aqueous *EA*. Infected non- treated mice had severe astrocytosis and meningoencephalitis, whereas infected - *E. abyssinica* extract treated mice had reduced astrocyte density and size, GFAP staining intensity and astrocyte processes (Figure 10 and Table 4). Astrocytosis was especially evident in paraventricular areas. There was also presence of parasites in cerebrum, and these were conspicuously noted to have a halo around them with glial cells forming a barrier around the halo (Figure 11). Parasites were also seen within the third ventricles, on choroid plexus and in cerebral parenchyma (Figure 11). There was severe perivascular cuffing in most blood vessels including those in hippocampus, with massive aggregation of mononuclear inflammatory cells in the brain parenchyma, these cell aggregates also varied in distribution, ranging from unifocal, multifocal, nodular or streak. The inflammatory cells comprised of lymphocytes, macrophages and plasma cells and formed perivascular cuffs in varying degree of severity. In infected-non treated mice, the cells completely obliterated perivascular spaces in the hippocampus, brain parenchyma and meninges, whereas in infected- *E. abyssinica* treated mice, there were only a few inflammatory cells in perivascular spaces in a few areas of cerebral parenchyma and the meninges (Figure 12).

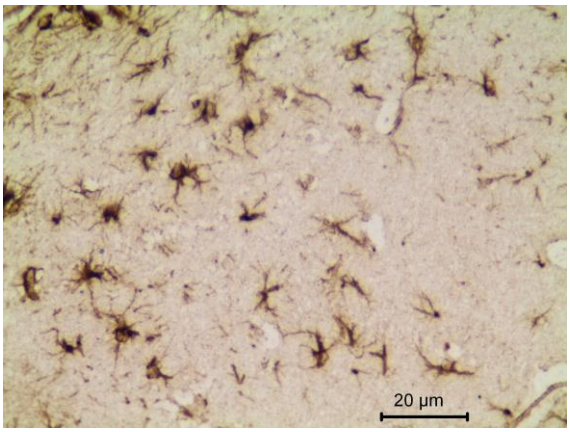
In infected non-treated mice, inflammatory cells were observed, dispersed all over the brain parenchyma especially in para-ventricular regions like the choroid plexus and

hippocampus. The severity of inflammation varied from single cells to multicellular aggregation (Figure 13). The cell aggregates varied inform, ranging from unifocal to multifocal. In infected-erythrina treated group, there was absence or minimal presence of inflammatory cell aggregates in the parenchyma.

Another notable pathological change observed was neuronal degeneration which occurred in all brain regions. This was characterized by shrinkage of the neurons, disintegration of cytoplasm that was acidophilic, condensation and strong basophilic chromatin material in the nucleus, some of which concentrated in the nuclear margin. The degeneration was more severe in infected – non treated mice compared to infected, *E. abyssinica* treated group. Degree of cerebral derangement was graded in all the animals (Table 5 and Figure 14).

Trypanosomes were observed on choroid plexus, in ventricles and within brain parenchyma especially white matter.

A



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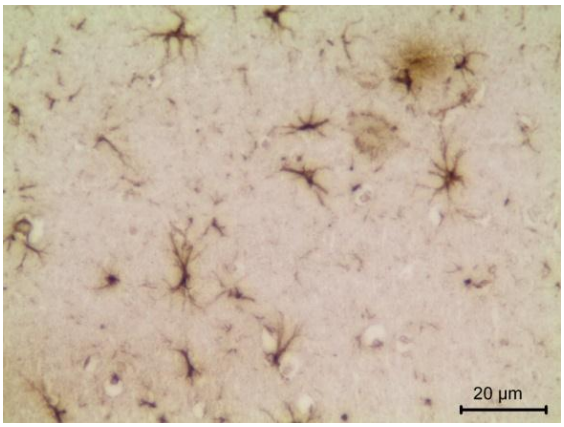

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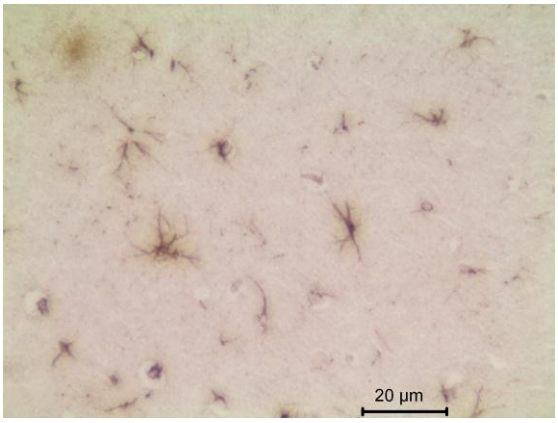

Figure 10: Micrograph of GFAP immunostained astrocytes in cerebrum of mice infected with *Trypanosoma brucei brucei (*GUTat 3.1*)* and treated with of *Erythrina abyssinica* extracts. (A) Infected mice without treatment showing intense staining of astrocytes that have enlarged with prominent cell bodies, increase in both number and staining intensity. (B) Infected mice treated with *Erythrina abyssinica* extract showing less intense staining and reduced cell count. (C) Non infected mice showing few astrocytes with less staining intesity.

Table 4: Mean numerical density of GFAP stained astrocytes per mm^3 volume of cerebrum, from mice infected with *Trypanosoma brucei brucei* (GUTat 3.1) and treated with different dosages of aqueous and methanol extracts of *Erythrina abyssinica*, compared to controls.

Dose Group (**mg/kg)**	**N= Animals in the group**	**Mean numerical density of** **Astrocytes/mm^3**
Non infected, saline treated	6	26,666 ± 1414
Water extract		
12.5	7	38,091 ± 1512
25	5	34,545 ± 1140
50	5	34,545±1140
100	5	36,363 ± 837
Methanol extract		
12.5	7	67,939 ± 1988
25	7	64,909 ± 1397
50	5	67,273 ± 3564
100	6	68,667± 1506
Control		
Infected, saline treated	5	69,886 ± 1967

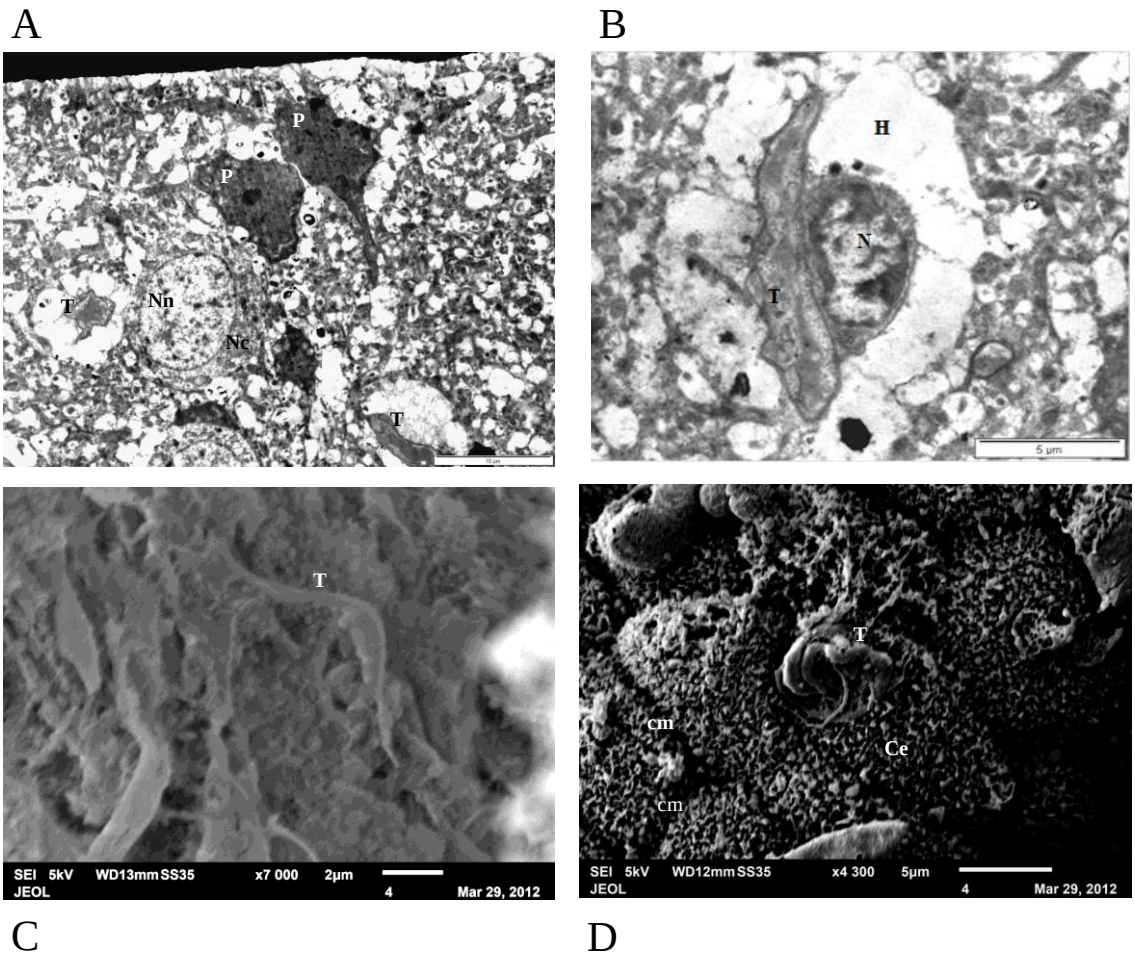

Figure 11: Electron micrograph of cerebrum from mice infected with *Trypanosoma brucei brucei* (GUTat 3.1). Trypanosomes (T) seen within cerebral parenchyma (A and B), in lateral ventricle (C) and on the surface of choroid plexus (D). Note the presence of halos (H) around parasites in cerebral parenchyma. N-neuron, Nc- Neuron cytoplasm, Nn-Neuron nucleus, Cc- choroid plexus cell, cm- choroid cell margins, P- degenerating neurons.

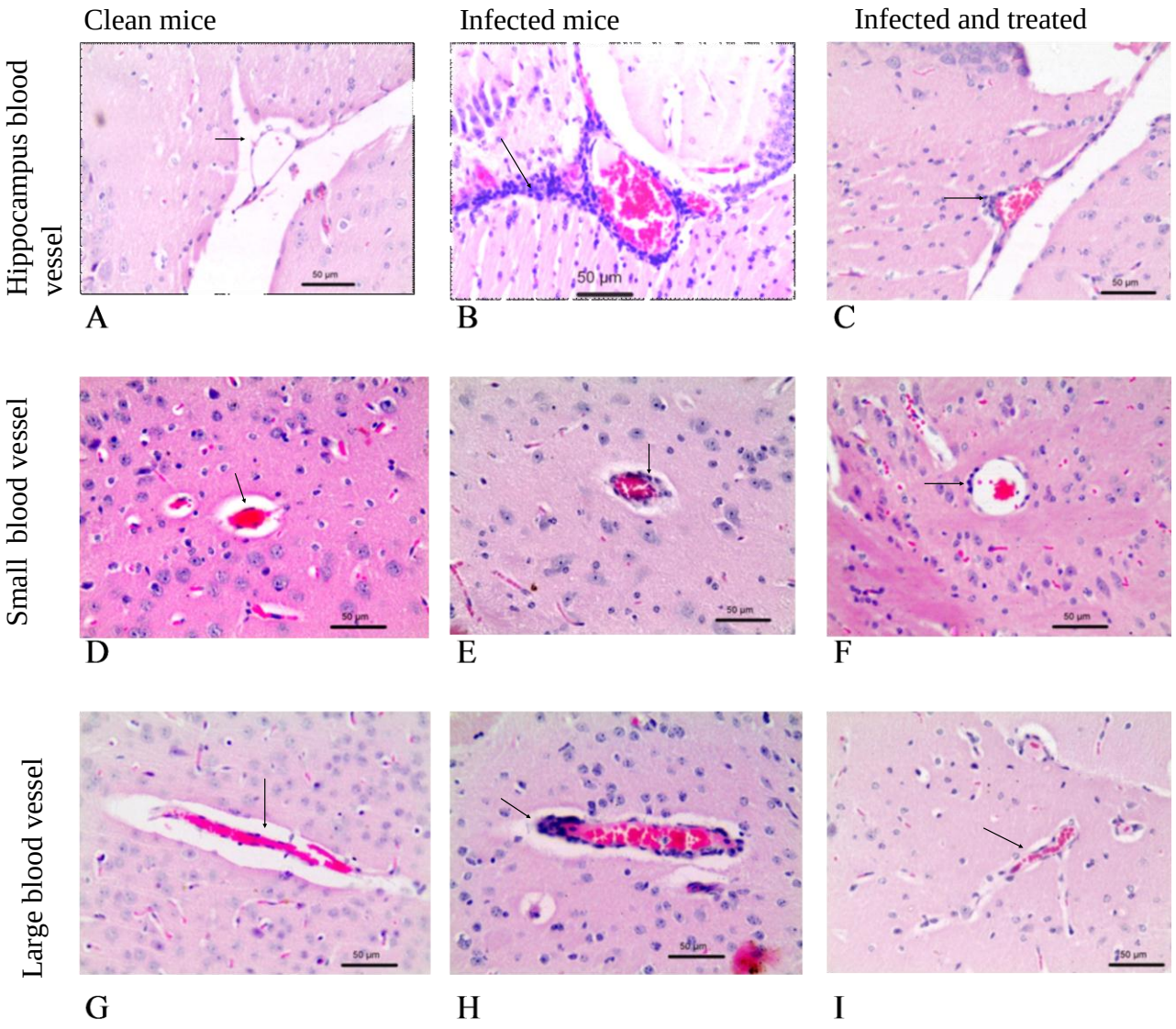

Figure 12: Micrograph of cerebrum from mice infected with *Trypanosoma brucei brucei* (GUTat 3.1). Perivascular cuffing in Hippocampal vessels (Row 1) and parenchymal blood vessels (Row 2 and 3) in, clean mice, infected mice, and those infected and treated. Not the reduced perivascular cuffing in treated mice (C, F and I) compared to those infected without treatment (B, E and H). A, D and G represent non-infected mice

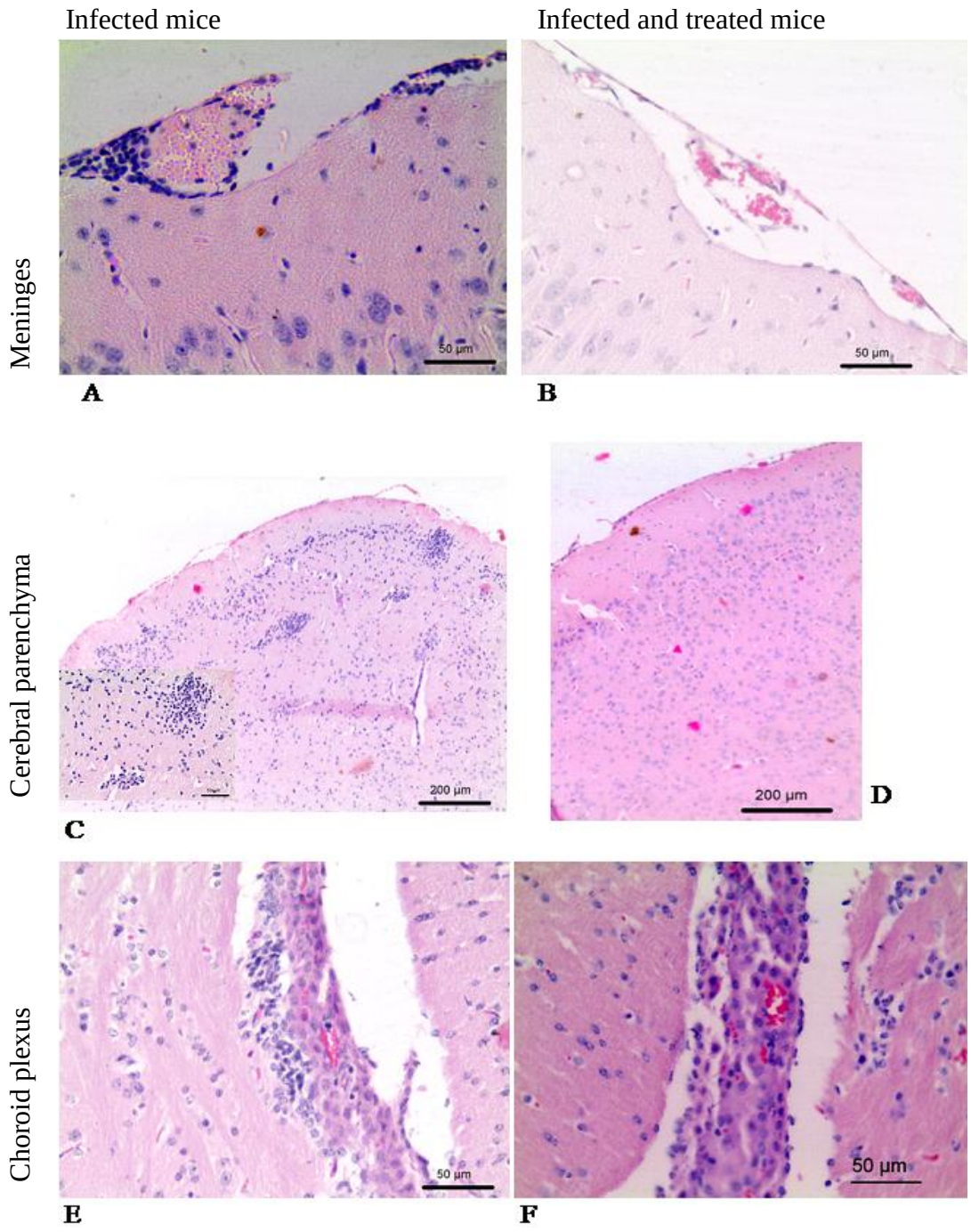

Figure 13: Micrograph of cerebrum from mice infected with *Trypanosoma brucei brucei* (GUTat 3.1). Lymphocytic infiltration in meninges (first row), parenchyma (second row) and choroid plexus (third row) between infected non-treated mice (A, C and E) and infected mice treated with *Erythrina abyssinica* extract (B, D and F). Note the severe infiltration in infected mice compared to the treated.

Table 5: Neuroinflammation scores in cerebrum of mice infected with *Trypanosoma brucei brucei* (GUTat 3.1) and treated with aqueous and methanol extracts of *Erythrina abyssinica*. Note that the water extract group had p- values less than 0.05 (at 95% confidence interval), therefore had a statistically significant improvement compared to infected, non treated mice

Dose Group (mg/kg)	**Mean Score**	**Difference with Control score**	**P-value 95% Confidence**
Infected, non-treated	2.8 ± 0.2		
Water extract			
12.5	1.14± 0.14	1.66	0.0*
25	1.40 ± 0.3	1.40	0.010*
50	1.40 ± 0.3	1.40	0.010*
100	1.20 ± 0.2	1.60	0.002 *
Methanol extract			
12.5	2.57 ± 0.28	0.23	0.999
25	1.86 ± 0.48	0.94	0.153
50	2.2 ± 0.5	0.80	0.450
100	2.33 ± 0.67	0.47	0.933

*Significant difference (P≤ 0.05)

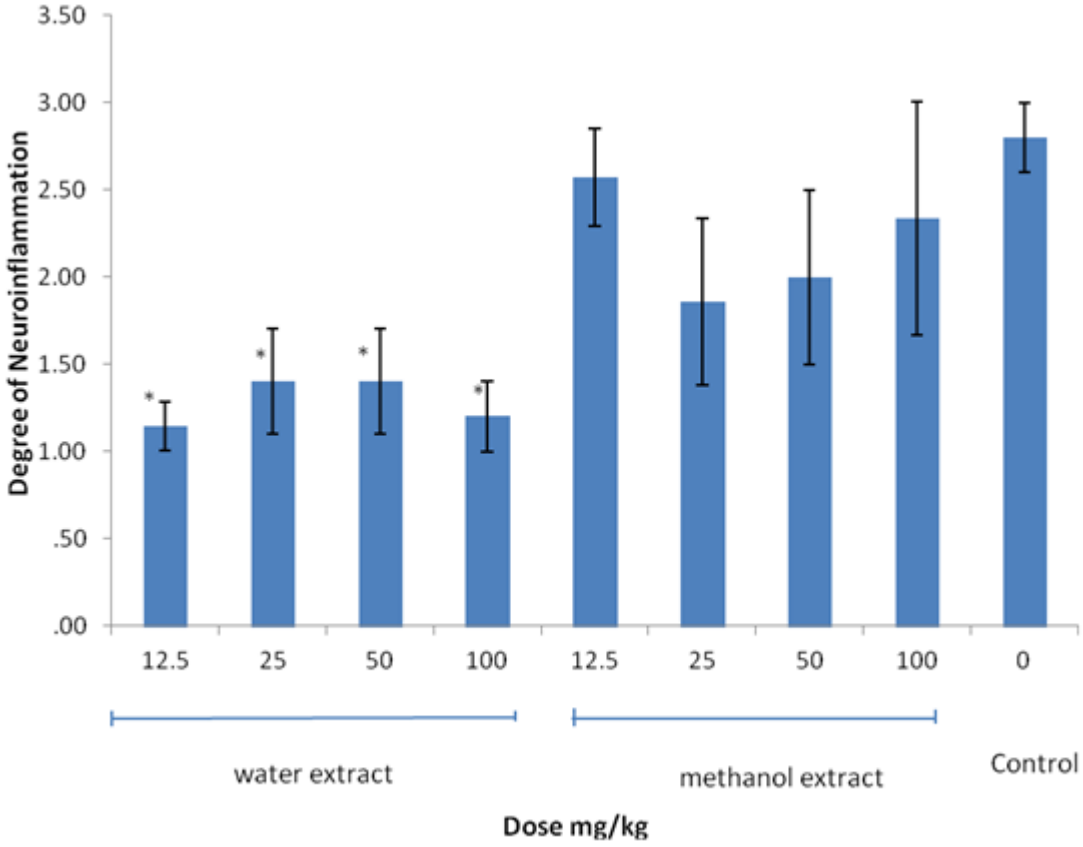

Figure 14: Graph showing degree of inflammation of various dosage groups and controls of mice infected with *Trypanosoma brucei brucei* (GUTat 3.1). Water extract groups had reduced neuroinflammation score that was statistically significant (*) compared to control group. Neuroinflammation was measured by degree of perivascular cuffing, lymphocytic infiltration into the cerebral parenchyma and meninges. * Significant difference (P≤ 0.05)

3.6 Protein analysis

Using the Bradford test, all extracts yielded proteins above 50 µg/ ml. The SDS-PAGE revealed a protein band coinciding with the 220 kilo daltons standard marker (Fig 15) in the high molecular weight range (30- 220 kDa).

This protein was present in the clean mice (Fig 15, lane 2), but missing in infected non-treated mice (Fig 15, lane 7). This indicates that the protein is degraded by trypanosomes. On treatment with aqueous extract of *Erythrina abyssinica,* the protein was protected from degradation with 12.5, 25, and 100 mg/kg dose groups (Fig 15, Lane, 3, 4 and 6). All the groups (Water extract and controls) had same amounts of the light mw proteins. For the heavy mw proteins, the positive control and the medium 1 water group had very small amounts based on the thickness of the bands, while low dose, medium dose 1 and high dose water groups had high amounts. The water treatment groups even had thicker bands compared to the non-infected group.

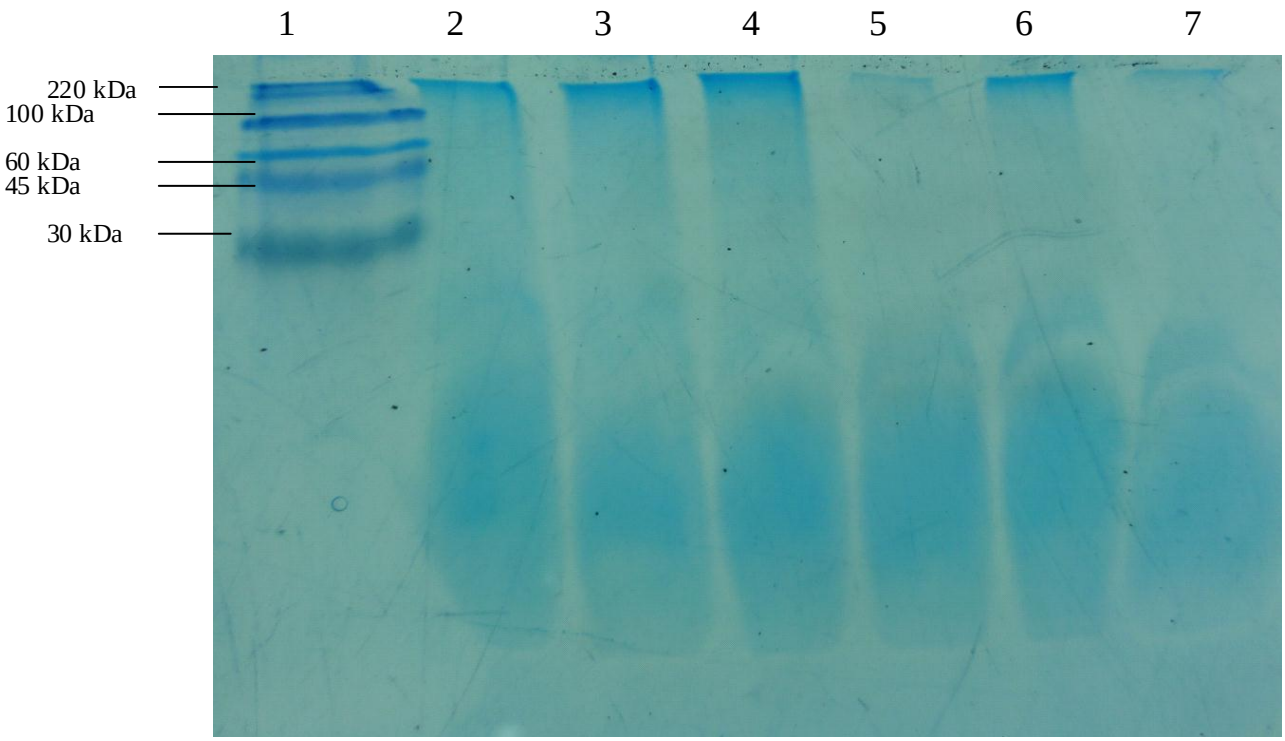

Figure 15: Protein profile of brain extracts from mice infected with *Trypanosoma brucei brucei* and treated with aqeous extract of *Erythrin abyssinica*. Standard protein molecular weight marker (1), Non infected mice (2), 12.5 mg/kg dose group (3), 25 mg/kg dose group (4), 50 mg/kg dose group (5) , 100 mg/kg dose group (6) and infected mice not treated (7). Note the thinness of the bands coinciding with the 230 kilo Daltons, standard on lane 5 and 7, note also the intensity and slight thickness of the same band in lanes 3 and 4 compared to that of lane 2.

CHAPTER 4

4 DISCUSSION

4.1 Phytochemical analysis and toxicity

The yields of 7.66% and 6.13% for aqueous and methanol extracts respectively, that was obtained in this study, and an LD50 of 708 mg/kg, compares fairly with other studies. Yields of 5- 12% were obtained by Lagu and Kayanja (2012), and an LD50 of 776.2 mg/kg obtained in another study done by Bunalema *et al.* (2011)

4.2 Parasitaemia and infection

The infection of mice by trypanosomes occurred in waves of increasing and decreasing parasite population, with two forms of parasites observed at different stages of the wave, a sharp and a short blunt form. This phenomenon is well known, where the blood forms are said to be heterogeneous/ pleomorphic, made up of the proliferative slender forms in ascending phase of parasitaemia and the non proliferative stumpy forms at the peak of parasitaemic wave (Baral, 2010). Nolan *et al* (2000) indicates that slender forms are characterized by the presence of a long free flagellum, an elongated centrally located nucleus, a sub terminal kinetoplast and a tightly attached undulating membrane. On the other hand, stumpy forms characterized by the virtual absence of a free flagellum, presence of a highly developed undulating membrane, a posterior rounded nucleus, a terminal kinetoplast and a large increase in the cellular volume (Nolan *et al*., 2000). Tyler observed the flagellar length may indicate slender to stumpy differentiation (Tyler *et al.*, 2001).The transformation of slender to stumpy form is controlled by

density sensing mechanism that leads to cell cycle arrest and it involves a series of biological and morphological transformation (Vassella *et al*., 1997). The stumpy forms are pre-adapted for transition to the procyclic forms that infect tsetse flies, with their key features being the arrest of their cell cycle, elaboration of some mitochondrial activities and resistance to lysis by host antibodies. Nolan *et al.* (2000), observed that the stumpy form of the parasite has the ability to regulate its internal pH, even in the face of a large, inwardly directed gradient of hydrogen ions, as well as a tolerance towards external proteolytic stress. These characteristics are thought to insure that the stumpy stages are available for transmission for a greater time period in the blood of their host (Seed and Wenck, 2003). The transition from slender to stumpy form has been hypothesized to be a mechanism of regulating self-growth in order to control their energy demand thus increasing host survival time (Seed and Wenck, 2003).

A total of three parasitaemic waves were observed with the interval between successive waves reducing as the disease progressed. The fluctuation of parasitaemia is due to a complex interaction between the host immune responses and parasite survival strategies (Namangala, 2011), the parasites reduce in number and a few that survive change their surface coat antigen and multiply to form a wave (Keith *et al*., 2004). Several other experimental studies have shown trypanosomiasis occurs in waves; this has been observed in trypanotolerant Ndama cattle and in mice infected with *T.b. gambiense, T.b. rhodesiense, T.b. brucei* and *T.vivax* (Agur and Mehr, 1997; Giroud *et al*., 2009; Ndung'u *et al*., 2008; Amole *et al*., 1982; MacGregor *et al*., 2011; Mahan *et al*., 1986).

4.3 Cerebral morphologic derangement

There was aggregation of activated astrocytes around trypanosomes in brain parenchyma. The current study is first to report a halo around trypanosomes and glial cells forming a barrier around the parasites.

At twenty one days post infection, the parasites were within the brain and were observed on choroid plexus, within ventricles and brain parenchyma 28 days post infection. This differs to what was observed by Giroud *et al*. (2009) where parasites reached the brain in four months. This difference may be attributed to the species of parasite and the breed of mice used, as *T.b. gambiense* was used to infect BALB/c J mice, while this study used the highly propagative *T.b. brucei* to infect Swiss white mice. These results are similar to what was observed in a study where *T.b. brucei* appeared in the brain after three to four weeks (Poltera *et al.,* 1981), Chamond *et al.* (2000), demonstrated presence of *T.vivax* in the brain on the twentieth day post infection in outbred and C57BL/6 mice. In *T.b. brucei* infected Sprague - dawley rats, Darsaud *et al*. (2003) observed presence of parasites and mononuclear inflammatory cells in the choroid plexus between day 13 and 15 post infection, they also observed neurological alterations represented by the altered parameters observed using open-field behavioral test at day 15 post infection. Schultzberg *et al*. (1988) observed nervous system pathology in the same animals and parasite strain at 21 days post infection, while Quan *et al*. (1999) reported nervous system pathology between day 10 and 21 post infection in Sprague- dawley rats. Several pathological changes were observed in the brain of infected mice including infiltration of mononuclear cells in perivascular

spaces within the white matter and meninges, neuronal degeneration and inflammatory cell aggregations within the brain parenchyma. These changes are the common pathological feature in late stage trypanosomiasis, first described in patients dying from the disease by Mott (1905). These pathological changes have been observed in natural infections in animals and used in developing experimental infection animal models for the late disease stage (Kagira *et al*., 2007, Ngotho *et al*., 2011, Thuita *et al*., 2008). Postmortem examination of trypanosome infected goats revealed the presence of mononuclear cell infiltration of the meninges (Losos and Ikede, 1972a). Neurologic lesions in goats infected with *T.b. gambiense* and brain pathology characterized by perivascular mononuclear-cell inflammatory reaction and associated degenerative changes have been reported (Losos and Ikede, 1972b).

Pathological changes in the brain of domestic and laboratory animals including sheep, goat, cattle, horses, dog cat, monkey and rodents has been reported (Losos and Ikede, 1972b). Poltera *et al.* (1981) first successfully developed the chronic trypanosomiasis mouse model and studied the neuropathology by histological and immunohistochemical techniques. In that study, they reported the presence of plasma cells and macrophages, some of which had foamy cytoplasms, trypanosomes and mononuclear cells in perivascular spaces both in the meninges and the white matter. They further observed that some trypanosomes were phagocytised by macrophages. In a rat model of *T.b. gambiense,* similar changes were observed that also included slight demyelination (Chirimwami *et al*., 1988). Areas around trypanosomes in the cerebrum were devoid of tissue reflected as a halo. The halo may be due to degradation of extracellular matrix by

trypanosomes, which have proteases linked onto their surface (Serrano-Luna *et al*., 2012). This is further supported by electrophoresis results that showed degradation of cerebral proteins by *T.b. brucei*.

The cause of these pathological changes is still not clear; it is suggested to be either due to a response to presence of the parasite in the brain or a response to circulating chemokines like TNF and IFN-γ invading the brain. The parasites have a variable surface antigen which activates the host immune system. TNF-α and IFN-γ produced peripherally, also appear to trigger the synthesis of cytokines inside the brain (Hansen *et al*., 1998; Quan *et al*., 1999; Rivest, 2009). Microglia and astrocytes are the resident immune cells in the brain (Oscar and Ignacio, 2012).

In the present study, large cells were observed in proximity to trypanosomes. These were considered activated microglia since microglia possess the potential to undergo dramatic morphological and functional changes from "resting" ramified forms to "activated" amoeboid cells. They constantly monitor their immediate environment by extending and retracting their projections over a minute-to-minute time scale, thus performing an immune surveillance role (Nimmerjahn *et al*., 2005). They have been implicated to play a very crucial part in most of the brain disorders (Streit *et al*., 2005) and neuroinflammation contributing to the severity of these diseases (Block and Hong, 2005). Activated microglia have been shown to secrete a host of pro-inflammatory soluble factors such as cytokines, free radicals and fatty acid metabolites (Smith *et al*., 1998), Rats infected with *T.b. brucei* were shown to have microglia with features of activation based on OX-42 and ED-1 immuno-reactiveness (Chianella *et al*., 1999),

with the degree of activation mirroring disease progression, these cytokines may then attract circulating lymphocytes that invade the brain. Microglia are capable of producing both pro and anti-inflammatory cytokines (Streit *et al*., 2005)), it is therefore possible that microglia are activated by circulating cytokines and in turn produce pro-inflammatory, leading to the observed pathology. Experiments done on rat brains infected with *T.b. brucei* demonstrated over expression of pro-inflammatory cytokines, interleukin1-β and tumor necrosis factor- α (Quan *et al*., 1999). Hunter found ribonucleic acid (RNA) transcripts of pro-inflammatory cytokines, Interleukin l-α, Interleukin 4, tumor necrosis factor -α, and macrophage inflammatory protein 1 in *T.b. brucei* infected mice (Hunter *et al*., 1991). The brain inflammation observed in the current study may be due to production of pro-inflammatory activity of microglia and astrocytes, as a result of either direct activation by the parasites surface antigens or indirectly by peripheral circulating cytokines.

4.4 Neuro-inflammation score

Aqueous *E. abyssinica* extract significantly reduced astrocytosis, which is a major key marker of neuro inflammation. This demonstrates its ameliorating effect on trypanosome induced neuroinflammation. All *E. abyssinica* treated groups were different from the control, with the aqeuos extract having statistically significant difference compared to control, with respect to astrocytosis, perivascular cuffing and infiltration of inflammatory cell.

This is the first study looking at potential anti-inflammatory effects of *E. abyssinica*, and especially neuro-inflammation.

Numerous studies have isolated different compounds including flavonoids from this plant and studied their various biological activities. Similarly, flavonoids isolated from other plants from the genus *Erythrina* have been studied for their biological activities including anti-inflammatory activities. *Erythrina addisoniae* bark extract showed marked anti inflammatory effects on phospholipase A(2)-induced paw edema and on the 12-O tetradecanoylphorbol 13-acetate-induced ear edema in mice, after systemic and local administration, respectively (Talla *et al*., 2003). Also, two flavonones isolated from *Erythrina sigmoidea* showed anti-inflammatory activity (Njamen *et al*., 2004). *Erythrina velutina* and *Erythrina crista-galli* extracts had activity against Carrageenan-induced pedal edema and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation respectively (Batista *et al*., 2011). Aqueous, ethanolic and ethyl acetate extracts of the bark and leaves of *Erythrina caffra, Erythrina humeana, Erythrina latissima, Erythrina lysistemon* and *Erythrina zeyheri* showed anti-inflammatory activity in work done by Pillay *et al*. (2001). Aqueous extract of *Erythrina senegalensis* stem bark also showed anti-inflammatory activity in egg albumin induced paw edema in a study done by Saidu *et al*. (2000). These observations therefore support the findings of the current study in which aqueous extract of *Erythrina abyssinica* root bark was observed to have anti-inflammatory effects in the cerebrum of trypanosome infected mice. It is not known if the anti-inflammatory activity may be associated to the groups

of compounds occurring in the genus *Erythrina*, the major groups of compounds isolated being flavonoids and alkaloids (João *et al*., 2009).

The selection of a particular food plant, plant tissue or herb for its potential health benefits appears to mirror its flavonoid composition (Hatti *et al*., 2009). Flavonoids from plants in the genus *Erythrina* have been reported to have anti- inflammatory effects by different mechanisms. Phospholipase A_2 (PLA2) is a lipolytic enzyme that specifically hydrolyzes the SN-2 ester bond of phospholipids (Zhou and Schulten, 1996) releasing polyunsaturated fatty acids like arachidonic acid (AA) for the subsequent biosynthesis of prostaglandins and eicosanoids, mediators of inflammation and pain (Meyer *et al.*, 2005). Due to the biological importance of PLA2 in inflammatory processes, the inhibition of its activity offers an attractive therapeutic target for the design of novel anti-inflammatory agents (Hegde *et al*., 1996). In his study, Hegde et al. (1996) isolated three flavonoids; abyssinone V, a new isoflavonone, and erycristagallin from *Erythrina variegate*, and Njamen *et al.* (2004) isolated Sigmoidin B from *Erythrina sigmoidea* (Njamen *et al*., 2004). In both studies all the isolated compounds inhibited Phospholipase A_2 activity, the same mechanism through which corticosteroids function as anti-inflammatories. Abyssinone V and erycristagallin have also been isolated from *Erythrina abyssinica* (Yenesew *et al*., 2009). It is therefore possible that *Erythrina abyssinica* may have ameliorated the trypanosome induced neuro-inflammation through inhibition of PLA2, but this was not tested in the study.

Sigmoidin A and Sigmoidin B, from *Erythrina sigmoidea* demonstrated inhibition of arachidonic acid metabolism by selectively inhibiting 5- lipoxygenase, an enzyme that breaks down arachidonic acid to form leucotrienes which are mediators of inflammation.

The mechanism through which *E. abyssinica* is able to ameliorate the trypanosome induced neuro-inflammation is not known, but future studies should explore the possibility of inhibition of 5-lipoxygenase. *Erythrina caffra*, *Erythrina humeana*, *Erythrina latissima*, *Erythrina lysistemon* and *Erythrina zeyheri* extracts showed cyclo-oxygenase inhibiting activity (Pillay *et al*., 2001), a pathway for synthesis of prostaglandins that mediate inflammation (Morteau, 2000). This is the same pathway that is inhibited by the non-steroidal anti-inflammatory drugs like paracetamol (Morteau, 2000). Considering that these plants are in the same genus as *E. abyssinica,* a genus that shares a group of isolated compounds, it is plausible to suggest that *E. abyssinica* inhibited the same pathway in the current experiment, leading to reduced inflammation in animals treated with the extract. (Figure 16)

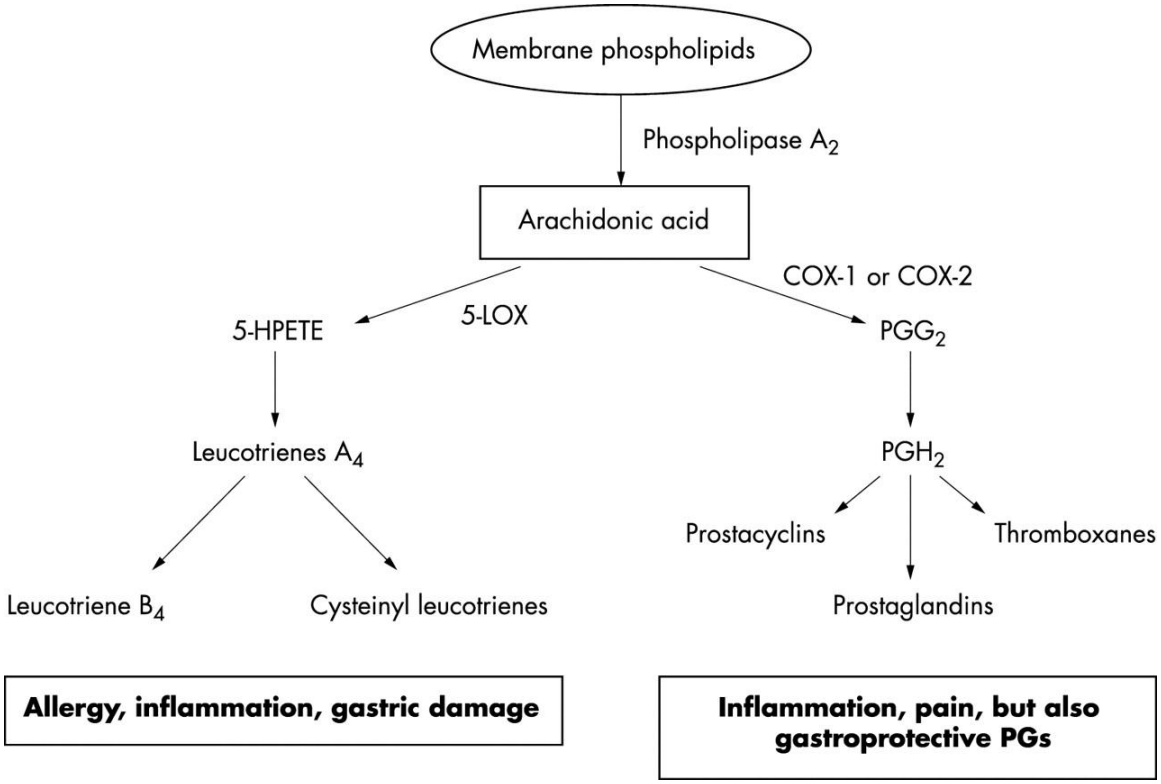

Figure 16: Inflammation pathway for production of prostaglandins (PG) and Leucotrienes, showing enzymes that are inhibited by flavonoids; which may be the way *Erythrina abyssinica* reduced neuroinflammation. Phospholipase A2, Cyclo-oxygenase-1 / 2 and 5-Lipo-oxygenase. Adapted from Martell-Pelletier (Martel-Pelletier, 2003)

4.5 Protein profile

In this study, it was observed that *E. abyssinica* conserved high molecular weight protein. Three protein bands were visible in this study. Considering the experiment was done using formalin fixed tissues, there might have been cross linking of several proteins which led to low protein extraction. Formalin forms cross linkages between proteins (Sameh and Tadashi, 2012) and separation in gels may yield less proteins profile as they are linked to each other compared to fresh tissues (Linda *et al.*, 2010).

It was noted in the study that *T.b. brucei* degrades a high molecular weight protein (250 KD), which was conserved and even slightly increased by water extracts of *E. abyssinica.* This effect may be due to parasite proteases since trypanosomes have proteases that are involved in host protein degradation; these proteases include cathepsin L- like cystein and cathepsin B- like cystein group of proteases (Zachary *et al.*, 2004). These enzymes are thought to function in disruption of blood-brain barrier or degradation of host immunoglobulins, extracellular matrix and mistargeted glycosyl-phosphatidyl-inisotol anchored proteins (Abdulla *et al*., 2008). This may also be supported by the observation made of halos surrounding trypanosomes in the brain; these may be areas in which parasite proteases attached on their surfaces break down extracellular matrix. Research done by Grab and others showed that brucipain causes activation of calcium signaling that leads to increase in permeability of blood brain barrier to *T.b. rhodesiense* transmigration leading to neuroinflammation (Grab *et al*., 2009). Results from the current study differ from what was reported by other workers, who observed that tight junction proteins were preserved in rat trypanosomiasis model

(Mulenga *et al*., 2001). However, the current study analyzed general proteins, and the degradation observed may be due to destruction of a wide range of proteins due to parasite proteases (Abdulla *et al*., 2008).

Another possible cause of protein degradation may be due to oxidative stress caused by reactive oxygen species produced either by the parasite or resident immune cells which led to lysosome damage, cytoplasmic protein degradation and neuron death. This idea is supported by evidence that oxidative stress causes lysosomal disruption and cytoplsmic degradation (Pivtoraiko *et al*., 2009). Microglias are also known to produce proteases (Von Bernhardi and Ramirez, 2001; Peterson *et al*., 2004) and a number of factors, including latent matrix metalloproteinase-9 (pro–MMP-9) on activation (Del Zopo *et al*., 2007). Cultured microglia produced proteases when treated with interleukin 1 (Colton *et al*., 1993), and a protease with chymotrypsin-like activity, thought to be localized in microglia was isolated in rat brain (Nelson and Siman, 1990).

Although trypanosome proteases are well known, their host brain protein degradation has not been well studied. So far, this is the first demonstration of brain protein degradation in chronic trypanosomiasis mice model.

E. abyssinica conserved the high molecular weight protein (250 KD). It also appeared to have slightly higher expression of the protein. From previous studies, flavonoids were shown to have the ability to interact with cell membranes and were protective against liposome destruction by Triton X-100 (Oteiza *et al*., 2005). Some flavonoids prevent leakage of n-acetyl glucosaminidase in lysosomes incubated in isosmotic glucose (Decharneux *et al*., 1992). Work by Coskun *et al*. (2005) showed quercentin, a

flavonoid, to prevent β-cell oxidative damage by streptozotocin, while quercentin and rutin had protective effects on rat peritoneal macrophages exposed to asbestos fibers (Kostyuk *et al*., 1996). In brain ischeamia model, quercetin significantly reduced ischaemic lesions (Dajas *et al*., 2003). Protease inhibition may also be another possible mechanism of action, as *Erythrina corallodendron* and *Erythrina cristagalli* seed extracts were shown to inhibit trypsin activity (Joubert and Sharon, 1985) indicating protease inbibiting effects of the genus *Erythrina*. The effect observed in this study may be due to the presence of flavonoids in *E. abyssinica*. Although not well understood, flavonoids are thought to act by linking with the hydrophilic head of the lamina bilayer of cell membranes, offering protection against disruption by both internal and external agents. (Oteiza *et al*., 2005)

Another probable mechanism of protein conservation by *E. abyssinica* is through expression of protective factors. The protein expression was slightly higher in the *Erythrina* extract treated groups compared to the negative controls. Johnson demonstrated activation of Nrf2 and increased expression of phase 2 genes in retinal cells by the flavonoid eriodictyol (Johnson *et al*., 2009). Nrf2 is a transcription factor that enhances phase 2 protein expression through up regulation of phase 2 protein genes. The slight protein conservation may be attributed to increase in phase 2 proteins which protect from oxidative stress. Fisetin is a flavonoid which has been shown to increase expression of brain derived neurotrophic factor (Maher *et al.*, 2006) increasing neuronal cell survival and differentiation. Flavonoids alter GSH metabolism, quench reactive oxygen species, and the inhibit calcium influx that signals the last step in the

cell death cascade induced by glutamate (Ishige *et al*., 2000). An increase in neurotrophic factors may be a probable reason for the slight increase in protein expression observed in *erythrina* treated animals. It is therefore possible that *E. abyssinica* preserved the structural proteins and at the same time caused an increase in phase 2 proteins, leading the observed results. However this needs to be studied further.

CHAPTER 5

5 CONCLUSION

Results of this study have provided evidence that extracts of *Erythrina Abyssinica:*

1. Contains alkaloids and flavonoids

2. Reduces perivascular cuffing

3. Reduces astrocytosis

4. Reduces infiltration of inflammatory cells in the cerebrum of chronic trypanosomiasis mouse model.

Erythrina abyssinica therefore has anti-inflammatory properties. It is also evident from this study that trypanosomes degrade proteins on invasion to the cerebrum of mice infected with *Trypanosoma brucei brucei* with an indication that *Erythrina abyssinica* extracts can prevent protein degradation in the cerebrum of *Trypanosma brucei brucei* infected mice.

RECOMMENDATION

Further studies are needed to ascertain the effects of *Erythrina abyssinica* extracts on other animal models of chronic trypanosomiasis. In addition, it should be ascertained whether these effects are as a result of a single molecule or a synergistic effect. The specific structural proteins degraded in the cerebrum by *Trypanosoma brucei brucei* need to be identified.

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