

**ANTITRYPANOSOMAL EFFECTS OF *BRASSICA OLERACEA* (CABBAGE)
METHANOLIC AND AQUEOUS EXTRACTS ON *RATTUS NORVEGICUS*
(ALBINO RATS) INFECTED WITH *TRYPANOSOMA EVANSI***

BY

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NOVEMBER, 2014

DECLARATION

I declare that the work in this thesis entitled ‘Antitrypanosomal effects of *Brassica oleracea* (Cabbage) methanolic and aqueous extracts on *Rattus norvergicus* (Albino rats) infected with *Trypanosoma evansi*’ has been carried out by me in the Department of Biological Sciences under the supervision of Prof. S. J. Oniye, Prof. P. I. Rekwot and Dr. O. O. Okubanjo. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for any degree or diploma at any university or higher institution.

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.....

.....

Signature

Date

CERTIFICATION

This thesis titled ‘ANTITRYPANOSOMAL EFFECTS OF *BRASSICA OLERACEA* (CABBAGE) METHANOLIC AND AQUEOUS EXTRACTS ON RATS INFECTED WITH *TRYPANOSOMA EVANSI*’ by Kingsley Onyekachi MOH meets the regulations governing the award of the degree of Master of Science in Biology of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to the loving memory of my mum, late Mrs Faith A. Moh.

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I return all glory to God Almighty for His endless love and mercy.

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ABSTRACT

The toxicity of synthetic drugs commonly used in the treatment of trypanosomiasis has become a problem in livestock management and there is an urgent need to provide a safer and a biological alternative in the treatment of the disease. Methanolic and aqueous extracts of *Brassica oleracea* were assessed for their antitrypanosomal effects on *Trypanosoma evansi* in laboratory rats. The phytochemical analysis of the plant extracts revealed the presence of carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, saponins, flavonoids alkaloids and anthracene. The Gas Chromatography Mass Spectrometry of chloroform fraction of the extract revealed the presence of the following bioactive compounds: 4H-Pyran- 4-one, Iberin nitrile, 9-Borabicyclo[4.2.1]nonane, dimer, 3-Formylhept-3-enedinitrile, Pyridine, Palmitic acid, Pentadecanoic acid, 13-Tetradec-11-yn-1-ol, Decane, 3-chloro-3-Chlorodecane, 5,8-Octadecadienoic acid, E-9-Tetradecenoic acid, n-Hexadecanoic acid, Heptadecane, 11-Tridecen-1-ol, 3,11-Tetradecadien-1-ol and n-Pentadecane. The acute toxicity test of the extracts showed no sign of toxicity in the rats. One- way analysis of Variance was used to determine the significant differences of the extracts using SPSS statistical program. There was an increase in the parasitaemia, body temperature, white blood cell count, plasma protein while there was a decrease in the packed cell volume and red blood cell count in the infected rats. There was an increase in the weights of the organs in the infected animals. Necrosis in the testis, lymphocyte hyperplasia in the liver, kidney, spleen and vacuolations in the brain were observed. Therefore, the methanolic extract of the plant was more effective than the aqueous extract both *in vivo* and *in vitro* which completely cleared the parasites at the dose of 800mg/kg body weight on day 10.

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LIST OF ABBREVIATION

Kcal= Kilocalorie

LMWC= Low- Molecular- Weight Carbohydrates

DNA= Deoxyrebonucleic acid

TLF= Trypanolytic Factors

CD4⁺= Cluster of Differentiation

VAT= Variable Antigen Type

VSG= Varriable Surface Glycoprotein

DEAE= Diethylamino ethylcellulose

PCR= Polymerase chain reaction

IFAT= Indirect Fluorescent Antibody Test

ELISA= Enzyme-Linked Immunosorbent Assays

WAD= West African Dwarf

GCMS= Gas Chromatography Mass Spectrometry

Mg= Miligram

Kg= Kilogram

PBS= Phosphate Buffer Saline

PCV= Packed Cell Volume

WBC= White Blood Cell

RBC= Red Blood Cell

RPM= Revolution Per Minute

ANOVA= Analysis of Varriance

SPSS= Statistical Package for Social Science

DMEM= Dulbecco's Modified Eagle Medium

CHAPTER ONE

1.0

INTRODUCTION

Trypanosomiasis (sleeping sickness in humans and nagana in domestic animals) is a parasitic disease caused by a protozoan blood parasite (Genus *Trypanosoma*) which affects both human and livestock (Omale and Joseph, 2011). About 60 million people and 48 million cattle are at risk of the disease (Samdi *et al.*, 2010). *Trypanosoma evansi* is a widely distributed flagellate protozoa that causes a disease called *surra* in domestic animals and is transmitted mechanically by biting flies such as *Tabanus* and *Stomoxys* spp. (Otte and Abuabara, 1991; Lun and Desser, 1995; Sumba *et al.*, 1998; Reid, 2002). *Trypanosoma evansi* is the most widespread pathogenic trypanosome globally (Luckins, 1988; Cadioli *et al.*, 2006). It has a wide range of hosts and is pathogenic to many species of domestic and wild animals (Franke *et al.*, 1994; Herrera *et al.*, 2002). In Africa, Camels are the most important host (Dia *et al.*, 1997), but cattle have also been reported as the next most highly susceptible animals (Mahmoud and Gray, 1980). In Nigeria, experimental studies have shown that donkeys, cattle, sheep, and goats are susceptible to *T. evansi* but undergo a protracted course of the disease (Ilemobade, 1971; Audu *et al.*, 1999; Shehu *et al.*, 2006).

There are considerable differences in the severity of syndromes caused by *T. evansi* infection in different geographical areas of its occurrence, depending on the virulence of the strain and the susceptibility of the host (Herrera *et al.*, 2004). More recently, the zoonotic potential of *T. evansi* has been stressed by several cases reported in humans in India (Powar *et al.*, 2006). A serological survey demonstrated a significant contact between humans and the parasite and contamination in humans could be due to insects, by either a

transcutaneous or peroral route (Sathaporn *et al.*, 2009). Trypanosomiasis due to *T. evansi* manifests itself both in acute and chronic forms; affected camels show fever, anorexia, marked generalized oedema and deteriorate rapidly and die; the chronic form is characterized by progressive loss of body weight, intermittent high fever, marked generalized muscular atrophy, pale mucous membranes and occasionally abdominal oedema. Affected camels also may exhibit a characteristic sweet odour due to an increase of urinary ketone bodies (Eyob and Matios, 2013). The chronic form is most common and is likely to present an association with secondary infection due to immune- suppression caused by *T. evansi* infection (Olaho- Mukani *et al.*, 1993; Ahmed, 2008). The main clinical signs of *T. evansi* infection include fever, anemia, weight loss, lethargy, swelling of the hind limbs, and hemostatic abnormalities (Hirohisa *et al.*, 2013). *Trypanosoma evansi* may exacerbate secondary infections and interfere with vaccinations because it suppresses the immunity of infected animals (Holland *et al.*, 2001; Holland *et al.*, 2003). In Nigeria, the most important trypanosome species are *Trypanosoma brucei*, *T. congolense*, *T. vivax*, and *T. evansi* in livestock, while *T. gambiense* infect human (Samdi *et al.*, 2010). Trypanosomiasis act as a hindrance to livestock production in endemic areas of Africa where the disease is common (Freiburghaus *et al.*, 1998; WHO, 2002; Kamuanga, 2003).

Several plants have been investigated for their efficacy against trypanosomiasis which include: *Moringa oleifera* (Atawodi and Shehu, 2010), *Cissus multistriata* (Omale and Joseph, 2011), and *Artemisia maritima* (Ene *et al.*, 2014). Natural products are valuable sources for new drug formulation (Shaba *et al.*, 2012) that are active against trypanosomes (Hoet *et al.*, 2004). Nigeria is naturally endowed with both savannah and tropical rainforest vegetations (Mann *et al.*, 2012) with diverse flora, offers a wide

spectrum of unique medicinal plants which are exploited for effective curing of various ailments (Burkill, 1985).

Cabbage (*Brassica oleracea* L. var. capitata) is one of the most important vegetables grown worldwide. It belongs to the family Cruciferae, which includes broccoli, cauliflower, and kale. The different cultivated types of cabbage show great variation in respect of size, shape and color of leaves as well as the texture of the head (Singh *et al.*, 2006). *Brassica oleracea* contains carbohydrates (5.8g), sugars (3.2g), energy (25kcal), dietary fiber (2.5g), protein (1.28g), vitamin C (44%), zinc (2%), potassium (4%), phosphorous (4%), magnesium 12mg (3%), calcium 40mg (4%), vitamin B6 0.124mg (10%), folate (Vitamin B5), 53µg (13%) (Oguwike *et al.*, 2014). Cabbage is consumed either raw or used in salads, etc. *Brassica oleracea* is still considered to be a primitive cultivar, being high yielding, less susceptible to pests and diseases and well adapted to a wide range of climates (Rosa, 1997). Due to its antioxidant, anti-inflammatory and antibacterial properties, cabbage has widespread use in traditional medicine especially in the healing and tumor protective effects (Yurtsever and Yardimic, 1999). Fresh cabbage juice, prepared either separately or mixed with other vegetables such as carrot and celery, is often included in many commercial weight-loss diets (Samec, 2011), diets that improve the bioavailable content of non- heme iron (Chiplonkar *et al.*, 1999), as well as alternative therapies for cancer patients (Maritess *et al.*, 2005). Clinical research has shown positive effects of cabbage consumption in healing peptic ulcers (Cheney, 1949), and facilitating the reduction of serum LDL levels (Suido *et al.*, 2002). Chemical components analysis has shown that the main constituents of cabbage are carbohydrates, comprising nearly 90% of the dry weight, where approximately one third is dietary fiber and two thirds are Low-

Molecular- Weight Carbohydrates (LMWC). Other characteristic components are glucosinolates (Wennberg *et al.*, 2006). *Brassica* species have been reported to possess cancer preventive properties (Beecher, 1994) that have been attributed to the glucosinolates and their derived products (Stoewsand, 1995).

1.1 Statement of Research Problem

Attempt to control trypanosomiasis in Africa is estimated to cost between \$600 million and \$1.2 billion yearly (Olukunle *et al.*, 2010). Diminazene aceturate (Berenil) and isomethamidium chloride are the most currently used trypanocides, used for prophylactics and curative purposes for the control of the disease in cattle (WHO, 1995). Unfortunately the parasites have developed resistance to these drugs (Schrevel *et al.*, 1996; Anene *et al.*, 2006; Geerts *et al.*, 2001) which makes the search for efficacious chemotherapeutic agents from locally available ethnomedicinal plants for use as trypanocidal agents necessary (Olukunle *et al.*, 2010). The existing treatments of trypanosomiasis is challenged with problems comprising drug resistance, toxicity and expensive/ limited drugs (Gutteridge, 1985; Atawodi *et al.*, 2002). *Brassica oleracea* is locally available in rural and urban communities in Nigeria, it is affordable and has been proven to have antitrypanosomal activity.

1.2 Justification

Resistance developed by trypanosomes to synthetic drugs has been reported (Schrevel *et al.*, 1996; Geerts *et al.*, 2001; Anene *et al.*, 2006) thereby compounding the issue of combating the scourge of trypanosomiasis. Several plants have been used to treat trypanosomiasis, some of these plants demonstrated potential trypanocidal activity

(Atawodi and Shehu, 2010; Omale and Joseph, 2011; Ene *et al.*, 2014). One of such plants is *Brassica oleracea* (cabbage) (Igwe *et al.*, 2002; Shaba *et al.*, 2012) which is basically available throughout the year in Nigeria. The active ingredients responsible for the trypanocidal activity of the plant is yet to be properly elucidated. Furthermore, the dosage of the extracts of cabbage that will suppress or eliminate trypanosomes in animals with limited or no damage to vital organs needs to be explicated. Prospects of introducing new trypanocidal drugs in the market are also not encouraging because of the antigenic variations of the parasite.

1.3 Aim

To assess the antitrypanosomal effects of methanolic and aqueous extracts of *Brassica oleracea* against *Trypanosoma evansi* in laboratory rats.

1.4 Objectives

- (i) To evaluate the *in vitro* antitrypanosomal effects of *B. oleracea* methanolic and aqueous extracts on *T. evansi*.
- (ii) To evaluate the *in vivo* antitrypanosomal effects of *B. oleracea* methanolic and aqueous extracts on laboratory rats infected with *T. evansi*.
- (iii) To determine the acute toxicity of *B. oleracea* methanolic and aqueous extracts in laboratory rats.
- (iv) To evaluate the histopathological effects of *B. oleracea* methanolic and aqueous extracts on some vital organs of laboratory rats infected with *T. evansi*.

1.5 Hypotheses

- (i) *Brassica oleracea* methanolic and aqueous extracts do not have any *in vitro* antitrypanosomal effect on *T. evansi*.
- (ii) *Brassica oleracea* methanolic and aqueous extracts do not have any *in vivo* antitrypanosomal effect on *T. evansi* infected laboratory rats.
- (iii) *Brassica oleracea* methanolic and aqueous extracts do not have any toxic effect in laboratory rats.
- (iv) *Brassica oleracea* methanolic and aqueous extract do not have any histological effect on some vital organs of *T. evansi* infected laboratory rats.

CHAPTER TWO

2.0

LITERATURE REVIEW

2.1 Morphology of *Brassica oleracea*

Brassica oleracea is a dicotyledonous crop that has fibrous and finely branched roots with an optimum temperature of 15-18°C (Ketseemang, 2006). It is a herbaceous, biennial dicotyledonous flowering plant distinguished by a short stem upon which is crowded a mass of leaves, usually green but in some varieties red or purplish, which while immature form a characteristic compact, globular cluster. The only part of the plant that is normally eaten is the leafy-head; more precisely, the spherical cluster of immature leaves, excluding the partially unfolded outer leaves (Oguwike *et al.*, 2014). Most cabbage measure about 12-36 inches in width and 12-24 inches in height (Emebu and Anyika, 2011).

2. 2 Distribution of *Brassica oleracea*

Brassica oleracea is distributed along the Atlantic coasts of northern Spain, France, Great Britain, and the Island of Helgoland (Gustafsson and Lanner- Herrera, 1997). Although essentially temperate, *B. oleracea* is now grown in other regions all over the world (Vaughan and Geissler, 1997).

2. 3 Uses of *Brassica oleracea*

Fresh market cabbage is used for cooking as main dish or mixed with other vegetables in stew and making of salads (Ketseemang, 2006).The vegetables are rich sources of many nutrients and antioxidant vitamins and can also be included in dieting programs, as it is a low calorie food (Mohammed *et al.*, 2012).

2. 4 Medicinal properties of *Brassica oleracea*

2. 4. 1 Anticancer effect

Several epidemiological studies have indicated an inverse association between consumption of vegetables from *B. oleracea* and a reduced risk of cancer (Peto *et al.*, 1981; Stoewsand *et al.*, 1988; Wattenberg *et al.*, 1989). This biological action is considered to be due in part to a group of phytochemicals termed glucosinolates. These secondary metabolites have been shown to break down into bioactive components that when added to experimental diets protect animals from cancer (Verhoeven *et al.*, 1997). Other researches have also shown the apparent anticancer activity of *B. oleracea* (Fowke *et al.*, 2000; Fowke *et al.*, 2003).

2. 4. 2 Antioxidant effect

Sulforaphane, indole- 3-carbinol, glucaric acid, and other isothiocyanates are antioxidants and potent stimulators of natural detoxifying enzymes in the body and these compounds are believed to be responsible for the lowered risk of atherosclerosis and cancer (Ames *et al.*, 1993; Hecht, 1999). Further researches have revealed that *B. oleracea* has also exhibited antioxidant and antimicrobial activities (Roy *et al.*, 2007; Ayaz *et al.*, 2008).

2. 4. 3 Anti- inflammatory effect

Varieties of cabbage heads could play an anti-inflammatory role in the prevention of chronic diseases associated to oxidative stress, such as in cancer and coronary artery disease (Sami *et al.*, 2013). *B. oleracea* is an excellent source of vitamin C and also contains significant amounts of glutamine, an amino acid that has anti- inflammatory properties (Mohammed *et al.*, 2012).

2. 4. 4 Anti tumor effects

Brassica oleracea had been demonstrated to possess a healing effect as well as a protective effect on Ehrlich ascites solid tumors of *Mus musculus* mice (Yurtsever and Yardimic, 1999).

2.5 *Trypanosoma evansi*

Trypanosoma evansi, the causative agent of the disease known as surra, was the first pathogenic trypanosome described (El Hassan, 2014). Infection with this parasite has an extremely wide geographical range in countries with hot warm- temperate climate (Hoare, 1972). Mostly, camels suffer from trypanosomosis caused by *T. evansi* that is transmitted mechanically, non-cyclically, by haematophagus flies such as horseflies (*Tabanus*) and stable flies (*Stomoxys*) (Eyob and Matios, 2013). Since the adaptation of the parasite to mechanical transmission by blood sucking insects (tabanids), the disease has spread beyond its original distribution in sub-Saharan Africa and is now also present in South America, North Africa and large parts of Asia (Vanhollebeke *et al.*, 2006).

2. 5. 1 Taxonomy and morphology of *Trypanosoma evansi*

Trypanosomes are unicellular flagellar protozoa belonging to phylum Sarcomastigophora, order of Kinetoplastidae, family Trypanosomatidae and the genus *Trypanosoma*, under the *Salivaria* group. The sub genus Trypanozoon includes the pathogenic species *T. evansi*, *T. brucei* and *T. equiperdum* (FAO, 2000). Because the trypanosome shows variation in its antigen coat, there are antigenic differences between isolates of *T. evansi*. There is limited, equivocal information concerning the existence of strains of *T. evansi* of different pathogenicity (Queiroz *et al.*, 2000). However, some strains are referred to colloquially as highly pathogenic‘ but this may be a result of host- vector factors, such as stock and insect

densities and the susceptibility of host species (Hoare, 1972). *Trypanosoma evansi* is morphologically identical with and indistinguishable from slender forms of other members of the subgenus *Trypanozoon* and is described as monomorphic but may be pleomorphic in some strains with length of 15 to 34 μm . Like *T. brucei*, *T. evansi* has a free flagellum and a small kinetoplast (about 0.6 μm) which is generally subterminal or marginal in the body. Leaf-like slender forms are characterized by a long free flagellum, which may be up to one half of the length of the organism with narrow and drawn out posterior end (Queiroz *et al.*, 2000).

2. 5. 2 Life cycle of *Trypanosoma evansi*

Replication of the trypanosome occurs by longitudinal binary fission both in the host and in the vector with the flagellum and kinetoplast dividing together (Liu and Liu, 2005), but in the noncyclically transmitted *T. evansi* developmental stages were not observed in any of the mechanical vectors. Consequently a procyclic or insect stage (epimastigotes) does not exist in *T. evansi* which is attributed to lack of maxi circles in the kinetoplast DNA (Ellie *et al.*, 1999). The non-cyclical transmission of trypanosomes is aided by biting flies and thus, in the absence of *Glossina*, the transmission is maintained in the ecosystem. Biting flies, such as *Tabanids* (horse flies), *Stomoxys* and *Hippoboscids* transmit *T. evansi* mechanically through their mouthparts when they feed on more than one host within a short interval because the trypanosomes remain infective for only a short period of time (Evans *et al.*, 1995).

2.6 Host range and geographic distribution of *Trypanosoma evansi*

Trypanosoma evansi occurs not only in Africa, but also in Central and South America, the Middle East, and Asia (Eyob and Matios, 2013). In Africa, beyond the northernmost limits

of the tsetse fly belt, and in parts of East Africa, camels are the most important host, while in Central and South America the horse is principally affected (Dia *et al.*, 1997). In Asia, a much wider range of hosts is involved, including the Bactrian camel and dromedaries, cattle, buffalo, horses and pigs (Pacholek *et al.*, 2001). This is contrary to observations in Africa and South America, where there is little evidence to suggest that domesticated livestock other than camels and horses, respectively, are clinically affected or infected with *T. evansi* (El-Sawalhy and Seed, 1999). The disease is most severe in horses, donkeys, mules, camels, dogs and cats. Camels, horses, dogs and Asian elephants are more susceptible than sheep and goats, which are more susceptible than bovines and pigs. Rats and mice are highly susceptible as experimental hosts for detecting subclinical (non patent) infection (Reid *et al.*, 2001). It has been suggested that, unlike in tsetse-transmitted trypanosomiasis, wildlife reservoirs of infection are unimportant with *T. evansi*, although it is possible that South American coatis and capybaras are an exception to this (Herrera and Davila , 2004).

2.7 Aetiology of surra

Surra is caused by *Trypanosoma evansi*, which is one of the salivarian group of trypanosomes. It multiplies by binary fission and it is found in the blood during the acute stages of the disease, but disappears rapidly after the death of the host (Hoare, 1972). There is limited, equivocal information concerning the existence of strains of *T. evansi* of different pathogenicity (Hoare, 1972; Queiroz *et al.*, 2000). Some strains are referred to colloquially as highly pathogenic but this may be a result of host vector factors, such as stock and insect densities and the susceptibility of host species (Hoare 1972).

2.8 Epidemiology of surra

Mechanical transmission by biting insects is the most important mode of transmission of *T. evansi* in camels, as well as in livestock and other large animals generally (Desquesnes *et al.*, 2013). Mechanical transmission is a nonspecific process, which can take place when a biting insect initiates a blood meal on an infected host, starts to feed on infected blood, is interrupted by the defensive movements of the host, flies off from the infected host, and lands on another animal to begin its blood meal again. When the insect first attempts to feed on blood, its mouthparts can contain a small amount of blood through capillary strength (Foil *et al.*, 1987). The residual blood may be partially inoculated into another animal during the early stage of the next attempt to bite, when the insect inoculates a small amount of saliva (necessary for its anticoagulant properties) prior to sucking the blood of the second host (Krinsky, 1976; Foil *et al.*, 1987; Desquesnes *et al.*, 2005; Desquesnes *et al.*, 2009). A mathematical model has recently been developed; for cattle carrying a mean burden of 20- 30 tabanids per head, the model indicated that the probability of transmission becomes significant when parasitaemia is above 10^6 trypanosomes/ ml (Desquesnes *et al.*, 2009). In biting insects, trypanosomes do not generally survive for long and their survival was estimated at 30 min with *T. vivax* in tabanids and even shorter in *Stomoxys* sp (Ferenc *et al.*, 1988). Experimental research shows that the transmission is efficient when there is a short time lapse between two interrupted blood meals, that is, less than 30 minutes (Mihok *et al.*, 1995; Sumba *et al.*, 1998).

2.9 History of trypanosomiasis caused by *Trypanosoma evansi* (surra)

Research has suggested that all Salivarian trypanosomes are separated from other trypanosomes approximately 300 million years ago (Haag *et al.*, 1998). After their emergence, Salivarian trypanosomes became gut parasites or commensals of early insects, which evolved around 380 million years ago (Dietmar, 2008). *Trypanosoma evansi*, the causative agent of surra, was discovered by Griffith Evans, in 1880 in infected camels and horses in India (Eyob and Matios, 2013). The local Indians had a local name for the disease- Surra, meaning emaciated (Al- Rawashdeh *et al.*, 2000). Since then, studies have shown that *T. evansi* is related to the African trypanosomes and is thought to have evolved from *T. brucei*, the cause of nagana in animals in Africa (Eyob and Matios, 2013). It is thought that *T. evansi* evolved from its ancestors along the edges of the tsetse fly belt in Africa and from there was spread through infected camels used for trade with India (Hoare, 1972). Domestic animals have yet been unable to develop tolerance or resistance to trypanosome infections within the 13000 years of their breeding (Dietmar, 2008).

2.10 Incubation period of *T. evansi*

The period between initial infection and the onset of clinical signs is extremely variable but generally ranges between 5 and 60 days, although longer periods have been recorded. The interval between infection and the demonstration of parasites in the blood is usually less than 14 days. Factors that affect the incubation period include the initial infective dose (equivalent to the number of infective insect bites), strain of parasite and stress. In non-clinical cases, the sensitivity of the method used to detect parasitaemia influences the time taken between infection and detection of the parasite.

2.11 Immune response to trypanosomiasis

2.11.1 Natural Immunity

Trypanolytic Factors (TLF) contained in normal human serum had been identified as high-density lipoproteins (Rifkin, 1978). Recently, two TLFs have been characterized in human serum. The first one (TLF1) belongs to a subclass of high-density lipoproteins and is inhibited by haptoglobin. In contrast, the second factor, TLF2, has a much higher molecular weight and does not appear to be a lipoprotein. Probably, the main trypanolytic effect is due to TLF2, which is not inhibited by haptoglobin (Raper *et al.*, 1996). The trypanocidal effect of cape buffalo serum has been attributed to xanthineoxidase (Muranjan *et al.*, 1997).

2.11.2 Chancre

Following inoculation of trypanosome into mammalian hosts, by the haematophagus fly, a local skin reaction is induced by trypanosome proliferation and appears a few days after inoculation (Luckins, 1992). In efferent lymphatic vessels, trypanosomes have been detected in lymph 1-2 days before the chancre and the number declined during development of the chancre (6 days) and later increased. They are detected in the blood 5days after inoculation. In *Trypanosoma congolense*- infected sheep, neutrophils predominate in the early days and then T and B lymphocytes infiltrate the chancre. Later, T lymphocytes predominate, especially CD8⁺ Tcells (Mwangi *et al.*, 1990). An early response due to an increase in CD4⁺ and CD8⁺ T cells was revealed by flow cytometry in the afferent lymph draining the chancre. As the chancres regressed there was an increase in lymphoblasts and surface immunoglobulin bearing cells (Mwangi *et al.* 1996). During this

first stage, trypanosomes expressed Variable Antigen Types (VATs) found characteristically in the tsetse fly, which changed after few days. An antibody response specific to these VATs appeared in the lymph and then in the plasma (Barry and Emery, 1984).

2.11.3 Complement

The appearance of Variable Surface Glycoprotein (VSG) on parasites prevents trypanosome lysis by this alternative pathway (Ferrante and Allison, 1983). For another strain of *T. b. gambiense*, it was demonstrated that the alternative pathway was incompletely activated without generation of the terminal complex (C5-C9) able to induce membrane lysis (Devine *et al.*, 1986). Immune complexes are constituted by antibodies specific to trypanosomes for example, antiVSG antibody leading to a rapid elimination of complement-fixing immune complexes (Russo *et al.* 1994). These immune complexes with complement activation are also involved in some adverse effects, especially in tissue damage mediated by immune complex deposits (Nielsen, 1985), such as thrombosis and glomerular involvement (Bruijn *et al.*, 1988, Van Velthuisen *et al.*, 1994).

2.11.4 Natural Killer Cells

Natural killer cells which lyse extracellular cells are considered to belong to the lymphocyte lineage and have functions in both innate and acquired immune responses. Research has shown that in *T. brucei* infected mice, natural killer cells activity was not modified in the early stages of infection, but was severely reduced from day 9 onwards (Askonas and Bancroft, 1984).

2. 11.5. T cells

Early researches have shown alterations in T cell functions in trypanosomiasis, both *in vivo* and *in vitro* (Mansfield and Wallace, 1974). In several studies, a transient proliferative T cell response to trypanosome antigens was noted in the first days of the infection followed by an absence of response (Gasbarre *et al.*, 1980). The kinetoplastid membrane protein 11 of African trypanosomes is a potent stimulator of T lymphocyte proliferation. Research has revealed that a strong trypanosome-specific T cell proliferation occurred in infected cattle following treatment (Lutje *et al.*, 1995). Human and mouse immune systems contain few $\gamma\delta$ T cells, in marked contrast to those of ruminants (Hein and Mac Kay, 1991). Research revealed that $\gamma\delta$ T cells, do not proliferate when stimulated with soluble VSG *in vitro* (Flynn and Sileghem, 1994).

2.11.6 B Cells

Research has shown that DNA from *T. brucei* stimulated B cell proliferation (Shoda *et al.*, 2001). Research has shown that antibodies directed against trypanosome VSG components appeared in sera and their binding to the surface coat of the trypanosomes was able to induce a decrease in parasitemia, both in the blood and extravascular spaces, specifically by immune lysis of parasites and their destruction by the Kupffer cells in the liver (Philippe and Bernard, 2006).

2. 11.7 Macrophages

The involvement of macrophages in experimental African trypanosomiasis has been comprehensively established in several experiments (Grosskinsky and Askonas, 1981; Fierer and Askonas, 1982; Namangala *et al.*, 2001) with different studies demonstrating

that macrophage activation can be detected within a few days of infection with African trypanosomes (Wellhausen and Mansfield, 1979; Scheleifer *et al.*, 1993). Experiments also stated that there is a severe increase in activated macrophages in the tissues of trypanosome infected animals (Grosskinsky *et al.*, 1983; Segal and Abo, 1993; Mabbott and Sternberg, 1995; Nishimura *et al.*, 2011) and lymphoid organs of infected mice become markedly enlarged during infection due to a disproportionate increase in macrophages within these tissues (Lopez *et al.*, 2008).

2.11.8 Antigenic variation and immune evasion

The blood stream form of African trypanosomes are entirely covered by 5×10^6 dimers of variable surface glycoproteins (VSG), which is the most abundant surface protein in the blood stream form of the trypanosomes (Eyob and Matios, 2013). It forms a dense surface coat of 12 to 15 nm over the entire surface of the trypanosome and accounts for about 15 to 20% of the total protein content of the bloodstream form of the parasite (Field and Carrington, 2009). This surface coat is attached to the outer membrane of the trypanosomes by glycosylphosphatid linositol (GPI) anchors, which make the variable surface antigen water insoluble and may contribute to the host's immune response to trypanosome infection (Pays and Nolan, 1998). It is likely that the VSG repertoire of *T. evansi* is smaller than that of trypanosomes with a tsetse fly intermediate host because exchange of genetic information and rearrangement of VSG repertoires occurs in this vector (Engstler *et al.*, 2007). During the ascending phase of the parasitemia, the majority of parasites are of the same antigenic type (called homotype). The host immune system recognizes this homotype and makes antibodies against it. As the parasites of the major variable antigenic type (VAT) are eliminated the parasitemia goes in descending phase but at the same time, the

parasites expressing the heterotype or the minor VATs are multiplying and one of them overgrows others. As a result, this one becomes the new homotype, leading to a new wave of parasitemia and resulting in a long-lasting chronic infection. So expression of the VSG is central in the antigenic variation process and eventually for exhausting the host immune system in the benefit of the parasite (Field *et al.*, 2009).

2.12 Pathology and clinical signs of surra

In any host, chancre is the earliest clinical sign of infection with *T. evansi* at the fly bite which is a cutaneous swelling in which the first trypanosomes multiply (Luckins, 1992). After formation of a chancre, the parasitaemia may remain high for 4 to 6 days after which it declines with remission of the temperature (Murray *et al.*, 1998). Anaemia is a major component of the pathology of surra and of African trypanosomosis, generally the degree of anaemia might be considered as an indicator of the disease severity. The infected animals become dull, anorexic with ocular discharges, and loss of body condition (Evans *et al.*, 1995). In the late stages, anaemia continues to be a major factor, with probably additional causes. However, irrespective of the cause of anaemia, the primary abnormality of function are the anoxic conditions created by the persistent anaemia tissue anoxia, which results in a fall in tissue pH and vascular damage (Connor, 1994). The central nervous system is reported to be most susceptible to anoxia with consequent development of cerebral anoxia (FAO, 2000). In acute cases, the spleen is enlarged but in chronic cases, it is atrophic, but these changes are not considered pathognomonic for disease. In addition, the oedema reported in the dependant parts of the body during the chronic stage could be due to a significant decrease in the albumin levels, resulting in alterations in osmotic pressure of the blood (Dargantes *et al.*, 2005). Some alterations in blood biochemistry,

including decrease in blood albumin and increase in globulin levels, hypoglycemia and increase in icterus index, have been reported in donkeys and horses (Soodan *et al.*, 1996; Marques *et al.*, 2000). Pathological changes detected in experimentally infected donkeys include enlarged spleen with hypertrophy of lymphoid follicles, congested lungs and neuronal degeneration and meningoencephalitis (Soodan *et al.*, 1996).

2.13 Diagnosis of trypanosomiasis

2.13.1 Wet blood films

This method makes use of a small drop of blood placed on to a clean glass slide and covered with a cover-slip to detect motile trypanosomes under a light microscope (200X). Improved visualisation can be obtained with dark-ground or phase-contrast microscopy (200- 400X). The sensitivity of this method is low, approximately 10 trypanosomes per μl , which is frequent in early or acute infections only. Examination of 20 μl of stained blood in a thick blood film slightly improves sensitivity, with a detection threshold of around 5,000 trypanosomes/ ml. It is the technique of choice for blood examination only when no centrifuge is available (Henry *et al.*, 1981), although it is quite time-consuming (10 to 20 min per slide) and requires expertise to recognize the parasite, which is frequently deformed in this preparation (Francois *et al.*, 2005).

2.13.2 Stained thick smears

This involves a large drop of blood (10 μl) placed on the centre of a microscope slide, spread with another slide, covered and stained with Giemsa stain to detect the presence of trypanosomes. The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites.

2.13.3 Stained thin smears

In this method, a small drop of blood (3- 5 μ l) is placed at one end of a clean microscope slide, another slide is used to spread the blood sample to form a thin film and stained with Giemsa's stain to detect the presence of trypanosomes. This technique permits detailed morphological studies and identification of the *Trypanosoma* species.

2.13.4 Haematocrit centrifugation technique

This involves the collection of blood sample with heparinised capillary tubes with one end sealed and centrifuged at 3000 g for 5 minutes at 12,000 rpm in a haematocrit centrifuge machine for trypanosome examination. Trypanosomes are large cells that concentrate at the junction between the buffy coat and the plasma, which is observed under the microscope (100- 200 $\times$). This technique can detect around 50- 200 trypanosomes/ml of blood (Desquesnes and Tresse, 1996).

2.13.5 Miniature-anion exchange centrifugation technique

This technique is mostly used for the purification of parasites from the blood but miniature systems have been developed, especially for diagnosis in humans (Lumdsen *et al.*, 1981). When a blood sample is passed through an appropriate DEAE-cellulose (diethylamino ethylcellulose) anion exchange column, the host blood cells, being more negatively charged than trypanosomes, can be adsorbed onto the anion-exchanger while the trypanosomes are eluted, retaining viability and infectivity (Lanham and Godfrey, 1970). The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood (Reid *et al.*, 2001).

2.13.6 Detection of trypanosomal DNA

Detection of minute amounts of trypanosomal DNA is a possible mean of identifying animals with active infections as the parasitic DNA does not remain for more than 24- 48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 1997).

2.13.7 Polymerase chain reaction (PCR)

PCR offers the sensitivity and specificity required for detection of trypanosome infection (Masiga *et al.*, 1992; Wuyts *et al.*, 1995), but it may give false-negative results. Experimental studies in sheep have shown that PCR can remain negative for long intervals during aparasitaemic periods (Bengaly *et al.*, 2001), while in buffalo the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation (Holland *et al.*, 2001).

2.13.8 Antigen detection

Circulating antigen detection in blood or serum is also a way to detect active infection. Research has revealed that several attempts to develop such tests have not yet reached a satisfactory level to be recommended for routine diagnosis (Morzaria *et al.*, 1996; Desquesnes, 1996; Monzon, 2006).

2.13.9 Serological tests

Two serological tests, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA), are routinely used to identify seropositive cattle (CFSPH, 2009). Because reactions to previous infections can be detected, serology is useful only for a presumptive diagnosis (CFSPH, 2009). The more easily standardised techniques of ELISA are used for serological tests (Rae *et al.*, 1989; Franke *et al.*, 1994; Tuntasuvan *et al.*, 1996; Davison *et al.*, 1999; Reid and Copeman, 2002; Reid and Copeman, 2003;) and

card agglutination test CATT (Bajyana Songa and Hamers, 1988; Njiru *et al.*, 2004). Attempts to develop new techniques such as latex agglutination tests have not been successful so far (Morzaria *et al.*, 1996; Gutierrez *et al.*, 2004; Holland *et al.*, 2005).

2.14 History of antitrypanosomal therapy

In the 20th century, Africa saw three severe sleeping sickness epidemics (Dietmar, 2008). The first one began in 1896 and lasted until 1906, and affected mainly Uganda and Congo (WHO, 2006). It was a devastating epidemic with 300,000 and 500,000 people estimated to have died in the Congo Basin and the Busoga focus in Uganda and Kenya, respectively (Hide 1999). The French physician Charles Louis Alphonse Laveran (1845- 1922) and the French biologist Felix Mesnil (1868- 1938) were the first to report in 1902 that sodium arsenite was effective in infected laboratory animals (Cox, 2004). In 1904, a paper was published by the Canadian doctor Harold Wolferstan Thomas (1875- 1931), and the Austrian doctor and zoologist Anton Breinl (1880- 1944), informing that the arsenical drug Atoxyl could cure experimentally infected animals. The German physician Robert Koch (1843- 1910), who investigated the trypanocidal activity of atoxyl on sleeping sickness patients on the Ssese Islands located in the northwest of Lake Victoria, found that the drug was by no means non- toxic; of 1622 atoxyl- treated patients Koch observed 22 cases of atrophy of the optic nerve with complete blindness. In 1907, Koch informed the German scientist Paul Ehrlich (1854- 1915) about these complications and advised him to improve the drug atoxyl. Already in 1904 Ehrlich had become interested in the chemotherapy of trypanosomiasis and had developed the dye trypan red, which proved to be both curative and prophylactic for *T. equinum* in mice but not for *T. brucei* (Vickerman, 1997). Eventually, it was Ehrlich's former assistant Wilhelm Roehl (1881- 1929) who in 1916,

with the help of a small team of chemists and the German chemical and pharmaceutical company Bayer, developed the first effective drug for treatment of sleeping sickness (Dietmar, 2008). The compound, Bayer 205, (later named suramin) is still in use in the therapy of early-stage *T. b. rhodesiense* infections. A year earlier, the American chemist Walter A. Jacobs (1883- 1967) and the American immunologist Michael Heidelberger (1888- 1991) discovered the organo- arsenical tryparsamide. This was the first drug to treat late-stage sleeping sickness alone, or in combination with suramin, and was also employed in the treatment of animal trypanosomiasis (Vickerman, 1997). In 1949, the arsenical melarsoprol, which was developed by the Swiss pathologist, microbiologist and chemist Ernst Friedheim (1899- 1989), was introduced for the treatment of late stage human African trypanosomiasis. It was the first and is still the only effective drug for late stage *T. b. rhodesiense* sleeping sickness (Dietmar, 2008). Since the 1950s, several drugs have become available for chemotherapy of animal trypanosomiasis. These include the phenanthridine derivatives homidium bromide (Ethidium, Novidium) and isometamidium chloride (Samorin, Trypamidium), the aminoquinaldine derivative quinapyramine (Anthrycid) and the aromatic diamidine diminazene aceturate (Berenil) (Kinabo, 1993). Melarsomine dihydrochloride (Cymelarsan) is the latest trypanocide to be developed and it was first available for commercial use in 1992 (Desquesnes *et al.*, 2013). It is used to control surra in camels through deep intramuscular injection at a dose rate of 0.25 mg/kg body weight (Desquesnes, 2004).

2.15 Treatment of surra

The most widely used curative trypanocide against surra is diminazene aceturate (Desquesnes, *et al.*, 2013). Other drugs in use include isometamidium chloride (both

curative and preventive), cymelarsan (so far, only recommended for curative treatment of camels), suramin, and quinapyramine (curative and/ or preventive) (Dia and Desquesnes, 2004). A curative dose of diaminazene aceturate is administered through intramuscular injection to obtain a high concentration of the chemical in the circulating blood. The withdrawal period for the consumption of produce from cattle injected with diaminazene aceturate is 21 days for meat and 3 days for milk (Peregrine and Mamman, 1993). Quinapyramine methyl- sulphate can be used to treat the infection by subcutaneous injection at a dose of 5 mg/kg body weight. A more effective combination of quinapyramine sulphate and quinapyramine chloride (Triquin) can be used as a curative/ preventive drug against *T. evansi* in horses and camels, administered by subcutaneous injection at a dose of 8 mg/kg body weight. However, the drug is quite efficient and the chemoprophylactic effect can last up to 4 months (Finelle, 1973).

2.16 Prevention and control of trypanosomiasis

2.16.1 Genetic control methods

Research has shown that a number of genes control infections of various pathogens by acting at the level of innate susceptibility, or at the level of acquired immunity (Philippe and Bernard, 2006). The existence of inbred susceptible and resistant strains of mice has made it possible to study the inheritance and mechanisms of host resistance. Resistance of mice to African trypanosomes is genetically determined. The control of resistance has been considered as dominant (Greenblatt *et al.* 1983) or recessive (De Gee *et al.*, 1988). In *T.b. rhodesiense*-infected mice, survival was not correlated with the height of the first peak of parasitemia, but a strong negative correlation between the second peak of parasitemia and

survival time was noted (Seed and Sechelski, 1995). However, in *T. congolense*-infected mice, the efficiency of clearance of the first peak of parasitemia was correlated with the survival period. Three loci influencing resistance of mice to *T. congolense* infection have been recently reported on chromosomes 5, 17 and 1 (Kemp *et al.*, 1997).

2.16.2 Trypanotolerance

Although evidences suggest that trypanotolerance is a relative rather than an absolute trait, and may be affected by nutritional status and stress of work as well as concurrent infections (Seck *et al.*, 2002), certain breeds of ruminants are found able to resist trypanosomosis more than others (Abenga and Vuza, 2005). Some African cattle breeds (N'Dama) are able to live and be productive in endemic areas and are considered to be trypanotolerant. In West Africa, shorter breeds of cattle, N'Dama and Muturu, and West African Dwarf (WAD) sheep and goats (Murray *et al.*, 1981) are known to exhibit different levels of trypanotolerance, while little is known about the tolerance status of many other animal species (Abenga and Vuza, 2005). Similarly, trypanotolerance has been described in Cape buffaloes (Black *et al.*, 2001). It has been documented that trypanotolerant cattle are found in 19 countries of West and Central Africa (FAO, 1987).

2.16.3 *Rattus norvegicus* (Albino rat)

A model organism is a non-human species studied to understand specific biologic phenomenon when experimentation on humans or other animals is not feasible or ethical (Fields and Johnston, 2005; Govind, 2011). *Rattus norvegicus* was the first mammalian species domesticated for biomedical research (Lindsey, 1979) and had remained a preferred model of human diseases because in many instances the physiology of the rat is a closer approximation to that of humans (Ihedioha and Daniel-igwe, 2014). The rat is also easier to

manipulate and the size makes it easier to handle and make serial blood draws (Gill *et al.*, 1989; Jacob, 1999; Ihedioha *et al.*, 2004; Iannaccone and Jacob, 2009; Gitig, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant material

Fresh leaves of *Brassica oleracea* were purchased from Samaru market, Zaria, Kaduna State, Nigeria, and authenticated in the Hebarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The leaves of the cabbage were gently removed from the stem, washed with tap water, air dried for three weeks at room temperature and pulverized using a mortar and a pestle.

3.2 Methanolic and Aqueous Extraction

The cold maceration method was employed in the preparation of the methanolic and aqueous leaf extracts of the plant in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. Twenty grams (20g) of the pulverised air- dried leaves of the plant were soaked in 200 ml of methanol and distilled water for methanolic and aqueous extractions respectively, in two clean extractor bottles and covered with cap. These were allowed to stand for 72 hours at room temperature in the case of the methanolic extraction and 24 hours in the case of aqueous extraction. Whatman's filter paper No. 1 was used to filter the resultant mixture of the extracts and the filtrates were concentrated using rotary evaporator under reduced pressure to separate the extracts from the methanol and the distilled water respectively.

3.3 Preparation of chloroform fraction of *B. oleracea* methanolic extract

Chloroform solvent was used for fractionation of the methanolic extract of *B. oleracea* (Githua *et al.*, 2010). Twenty grams (20g) of *B. oleracea* methanolic extract was dissolved

in 200ml of distilled water and was transferred into a separating funnel containing some cotton wools. 300ml of chloroform solvent was added to the mixture and shaken thoroughly then allowed to stand for 10 minutes. The filtrate was then separated from the residue of the extract. Another 300ml of chloroform solvent was added to the residue of the extract for a better fractionation, shaken thoroughly and allowed to stand for 10 minutes as described above. The above procedure was repeated the third time. The final residue obtained was concentrated using a rotary evaporator.

3.4 Phytochemical analysis

The phytochemical analysis of the plants was carried out in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria for the presence of bioactive constituents of the plant extracts. The following tests were carried out on each of the extracts using Trease and Evans (1996) method.

3.4.1 Test for carbohydrates (Molisch test)

To 500mg of each extract in a test tube, 3 drops of Molisch reagent was added and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer, reddish coloured ring at the at the interphase indicated the presence of carbohydrates.

3.4.2 Test for cardiac glycosides (Keller- kiliani test)

0.5g of each extract was dissolved separately in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. The interphase was carefully observed. A purple- brown- ring at the

interphase indicated the presence of deoxy sugars and a pale green colour in the upper acetic acid layer indicated the presence of cardiac glycosides.

3.4.3 Test for unsaturated steroids and Triterpenes (Liebermann- Bucchar test)

0.5g of the extract was dissolved in 2ml acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. Colour changes were observed immediately and over a period of one hour. Blue to green colour in the upper layer and a reddish, pink or purple colour in the lower layer indicated the presence of triterpene.

3.4.4 Test for Tannins (Ferric chloride test)

1ml of the extract was stirred with 1ml of ferric chloride. A greenish- black precipitate indicated the presence of condensed tannins while hydrolysable tannins gave a blue or brownish blue precipitate.

3.4.5 Test for saponins (Frothing test)

About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 minutes. A honey comb froth that persisted for 10- 15 minutes indicated the presence of saponins.

3.4.6 Test for flavonoids (Sodium hydroxide test)

5 drops of 10% sodium hydroxide were added to 200mg of the extract. A yellow colouration indicated the presence of flavonoids.

3.4.7 Test for alkaloids (Dragendoff's test)

To 200mg of the extract, 5 drops of Dragendoff's reagent were added. A reddish- brown precipitate indicated the presence of alkaloids.

3.4.8 Test for Anthracene (Bontrager's test)

To 100mg of the extract in a dry test tube, 5ml of chloroform was added and was shaken for 5 minutes. This was filtered and the filtrate shaken with equal volume of 10% ammonia solution. A bright pink colour in the aqueous (upper) layer indicated the presence of free anthraquinones.

3.5 Gas Chromatography Mass Spectrometric (GCMS) Analysis

The Gas Chromatography Mass Spectrometric (GCMS) analysis was carried out in National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria to determine the bioactive compounds responsible for any antitrypanosomal activity of the extract. The analysis was carried out on a GC-MS machine (Model: QP2010 Plus Shimadzu, Japan) under the following conditions: column oven temperature: 80.0°C; Injector temperature: 250.00°C; Injection mode: Split; Flow control mode: Linear velocity; Pressure: 108.0KPa; Total flow: 6.2mL/min; Column flow: 1.58 mL/min; Linear velocity: 46.3cm/sec; Purge flow: 3.0 mL/min; Split ratio: 1.0; High Pressure Injection: Off; Carrier Gas Saver: Off; Split Hold: Off; Ion Source Temperature: 230.00°C; Interface temperature: 250.00°C; Solvent cut time: 2.50 min; Detector Gain Mode: Relative; Start time: 3.00 minutes; End time: 28.00 minutes; Event time: 0.5 sec; Scan speed: 1250. The relative percentage of the extract was expressed as percentage with peak area normalization. The GCMS machine which was connected to a computer displayed the peaks, retention time, molecular weight, peak area and names of compounds present in the extract on the computer screen.

3.6 Experimental animals

The experiment was carried out with 45 adult male albino rats (*Rattus norvegicus*) weighing 190g. The animals were purchased from the Department of Veterinary and Public Health, Ahmadu Bello University, Zaria, Kaduna State. They were kept in a prefumigated pen in a well netted animal house that prevented flies and other haematophagous insects from feeding on them in the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The animals were acclimatized for two weeks and maintained with grower mash and clean water. Animals were handled in compliance with internationally accepted standards for the humane treatment of animals. Thirteen male and female albino rats weighing 190g were used for acute toxicity test of the extracts.

3.9 Trypanosomes

Trypanosoma evansi parasites were obtained from infected albino rats in the Protozoology Laboratory of Veterinary Parasitology and Entomology Department, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Blood samples from the infected donor rats were mixed together and the parasites counted using rapid matching” method of Herbert and Lumsden (1976).

3.8 Experimental Design

The animals were randomly separated into six different groups which were named as A, B, C, D, E and F. Groups A, B and C contained 10 animals each while groups D, E and F contained 5 animals each. Group A was further divided into two sub- groups, A1 and A2 which were infected and treated with the methanolic and aqueous extracts at 100mg/kg body weight respectively. Group B was further divided into two sub- groups: B1 and B2

which were infected and treated with methanolic and aqueous extracts at 400mg/kg body weight respectively. Group C was further divided into two sub- groups: C1 and C2 which were infected and treated with methanolic and aqueous extracts at 800mg/kg body weight respectively. Group D served as the positive control I, which were infected but untreated. Group E served as positive control II, which were infected and treated with standard drug (Diaminazene Aceturate) at a dose of 3.5mg/kg body weight. Group F served as negative control which were uninfected and untreated. Below is a flow chart showing the experimental design:

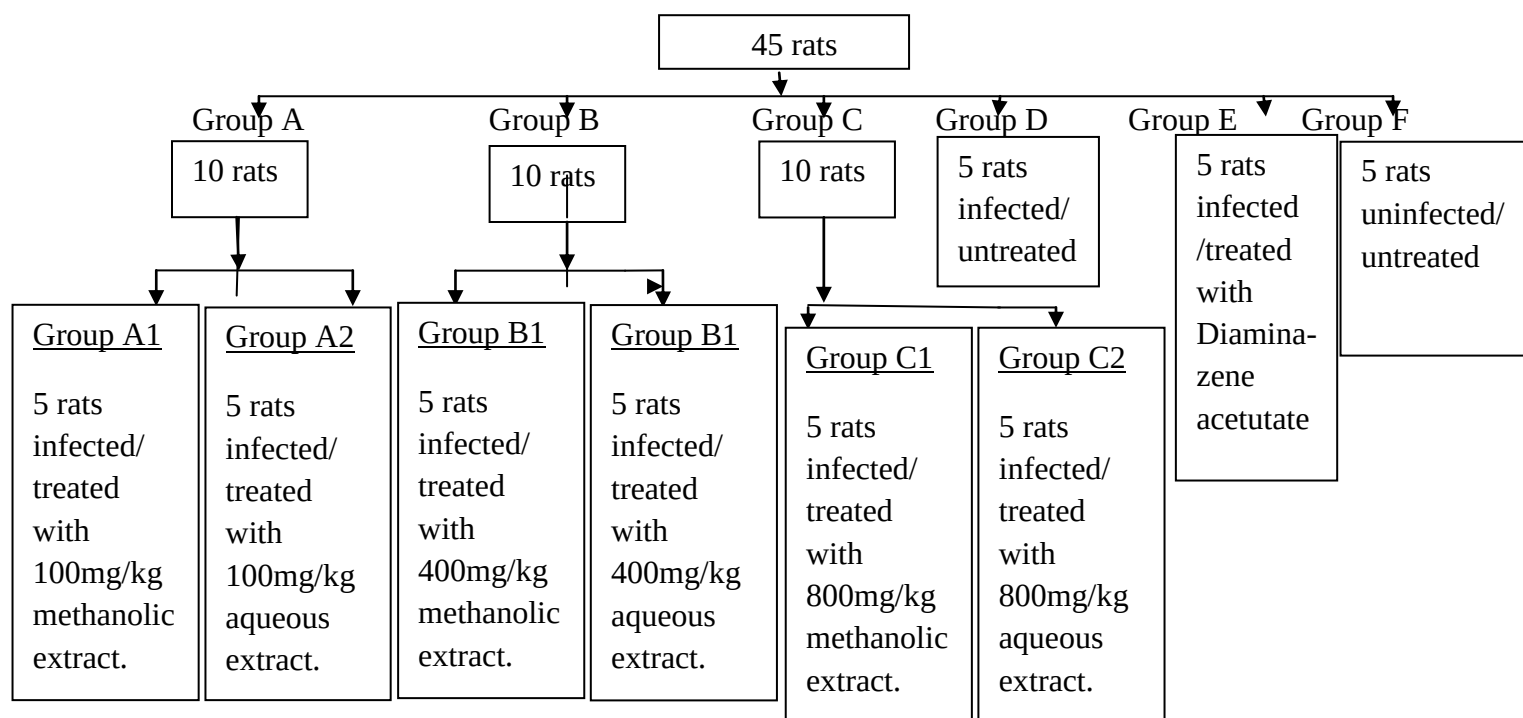


Figure 3.1: A flow chart showing the experimental design.

3.9 Acute toxicity test of the extracts on laboratory

Lorke's method was used to estimate the acute toxicity of the extracts. In the acute toxicity test, a total of 13 male and female laboratory rats (*Rattus norvegicus*) were used in two phases to determine acute toxicity of the extracts. In the first phase of the toxicity test, 9 albino rats were grouped into three consisting of 3 rats each. The plant extracts were administered to the rats in varying concentrations, 10mg/kg, 100mg/kg and 1000mg/kg body weight intraperitoneally. The animals in each group were observed for any immediate sign of toxicity and mortality within 24 hours. In the second phase of the toxicity test, different varying concentrations of the extract 1000mg/kg, 1600mg/kg, 2900mg/kg and 5000mg/kg body weights were administered intraperitoneally to four groups of rats consisting of one animal each for another 24 hours based on the results obtained from phase one (Lorke, 1983).

3.10 *In vitro* antitrypanosomal assessment of extracts

The assessment of *in vitro* antitrypanosomal activity was carried out in triplicates in 96 well micro titer plates. Methanolic and aqueous extracts initial doses of 40mg/ml, 20mg/ml and 10mg/ml were prepared by dissolving 40mg, 20mg and 10mg of extracts each in 1ml of Phosphate Buffer Saline (PBS) respectively to produce a final test mixtures of 20mg/ml, 10mg/ml and 5mg/ml. The test mixtures were incubated at 37°C for 20 minutes. In 96 wells of the microtitre plates, 10µl from each of the extract doses were mixed with 10µl of infected blood of rat containing about 32- 64 parasites per field (Herbert and Lumsden (1976). For negative and positive controls, the same concentrations of the extracts were replaced with normal saline and diaminazene aceturate (standard drug) respectively. 2µl each of the incubated test mixtures were placed on different glass slides covered with cover

slips and parasite motility was observed every 5 minutes for a total duration of 20 minutes under a microscope using x400 magnification. Cessation or drop in motility in percentage was used to check antitrypanosomal activity of the parasites when compared with the controls (Ene *et al.*, 2014).

3.11 *In vivo* antitrypanosomal assessment of extracts

Inoculation the animals was by intraperitoneal injection of 10,000 *T. evansi* in 0.5ml of blood using normal saline as diluents. Treatment with extracts and Diaminazene aceturate commenced on day 5 post- infection period when all the level of parasitemia reached up to 30 parasites per field for four to five different fields of view. Extracts and Diaminazene aceturate administration were done through intraperitoneal route (Ene *et al.*, 2014) for three days consecutively (Osho and Lajide, 2014). The body weight, temperature, packed cell volume, total blood protein, white blood cell count and red blood cell count of the animals were determined every 3 days while the level of parasitemia was checked daily by examination of the wet mount microscopically at x40 magnification using the “rapid matching” method of Herbert and Lumsden (1976).

3.12 Collection of blood samples

The site for the blood collection was prepared aseptically and thoroughly swabbed with 70% alcohol (Takeet and Fabemi, 2009). Blood samples were collected from the rats by making a snip on their tail tips using a pair of scissors.

3.13 Haematological examination

3.13.1 Plasma protein

Blood sample was collected using capillary tubes (length of 75 mm and diameter of 1 mm) by capillary action, leaving 15 mm unfilled. The tubes were sealed by flaming and spun in a microhaematocrit centrifuge at 3000 rpm for 5 minutes. The capillary tube was then gently broken at plasma part, then the plasma content poured on Golgberg hand held refractometer and viewed to determine the total blood protein (Bagger and Eriksen, 2003).

3.13.2 Packed Cell Volume

Blood was collected using microhaematocrit tubes (length of 75 mm and diameter of 1 mm) by capillary action, leaving 15 mm unfilled. The tubes were sealed by flaming one end and spun in a microhaematocrit centrifuge at 3000 rpm for 5 minutes. Packed Cell Volume was then measured using haematocrit reader.

3.13.3 White blood cell count (WBC)

A 1:20 dilution of blood was made by adding 0.02 ml of blood to 0.38 ml of Turks solution in a 75 mm x10 mm plastic tube. After tightly corking the tube the suspension was well mixed by rotation. The improved Neubauer counting chamber was loaded with the diluted blood by means of pasteur pipette. The loaded counting chamber was allowed for two minutes for cells to settle, after which the preparation was viewed under the microscope x10 mm objective. The cells were counted in the 4 large corner squares of the counting chamber as described by (Liang, 2010).

3.13.4 Red blood cell count (RBC)

A 1:200 dilution of blood was made in formol citrate solution by diluting 200 ml of blood into 4 ml of diluents in a plastic tube. A clean dry improved Neubauer counting chamber

with cover slip already in position was loaded with diluted blood using pasteur pipette. The chamber was left undisturbed for 2 minutes for the cells to settle. The cells were counted under the microscope using x10 mm objective. Cells were counted in 80 small squares in the central ruled area of the counting chamber as described by (Liang, 2010).

3.14 Histopathological examination

On day 14, the animals were sacrificed for histopathological analysis. The brain, kidney, liver, spleen, heart and testis of the experimental rats were excised and immediately transferred into 10% formalin for 28- 48 hours. The tissues were dehydrated in 70%, 80%, 95% and absolute alcohol (100%) for one hour in each of the solvents, then transferred into xylene to remove the alcohol for 3 hours. Thereafter, the tissues were transferred into paraffin wax and placed in an impregnating machine which was adjusted to 30-35⁰C for 4 hours for impregnation. A wax dispenser was used to run paraffin wax into an iron pan covered with an embedding ring and allowed to solidify. 1% acid alcohol was used for decolourizing, then transferred into a clean tap water and allowed to stay for 40- 45 minutes. After 40- 45 minutes in clean tap water, the tissues were transferred into eosin for 1-2 minutes and then rinsed with some clean tap water then transferred into 70%, 80%, 95%, absolute alcohol and xylene. Thin sections (5 μ) of the tissues stained with haematoxylin and eosin was made and mounted on clean glass slides before viewing using x400 magnifications for the histopathological examination (Takeet and Fagbemi, 2009).

3.15 Statistical analysis

One- way analysis of Variance (ANOVA) was used to determine the significant differences of the extracts using SPSS statistical program. Duncan multiple range test was used to separate their means at $P < 0.05$.

CHAPTER FOUR

4.0

RESULTS

4.1 Phytochemical analysis

The phytochemical analysis of the methanolic extract of *Brassica oleracea* showed the presence of the following secondary metabolites: carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, saponins, flavonoids and alkaloids. Tannis and anthracene were not detected (Table 4.1). The aqueous extracts of *B.oleracea* showed the presence of carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, flavonoids and alkaloids, but tannins and saponins were not detected (Table 4.1).

4.2 Gas Chromatography Mass Spectrometry (GCMS) analysis

The GCMS analysis of chloroform fraction of *B. oleracea* revealed sixteen different peaks (Fig. 4.1) representing sixteen different compounds detected by the GCMS machine as shown in figure 4.1. The following compounds were detected: 4H-Pyran- 4-one, Iberin nitrile, 9-Borabicyclo[4.2.1]nonane, dimer, 3-Formylhept-3-enedinitrile, Pyridine, Palmitic acid, Pentadecanoic acid, 13-Tetradecene-11-yn-1-ol, Decane, 3-chloro-3-Chlorodecane, 5,8-Octadecadienoic acid, E-9-Tetradecenoic acid, n-Hexadecanoic acid, Heptadecane, 11-Tridecen-1-ol, 3,11-Tetradecadien-1-ol and n-Pentadecane. Heptadecane had the highest molecular weight, E-9-Tetradecenoic acid had the highest peak area and n-Pentadecane had the highest retention time (Table 4.2).

4.3 Acute toxicity test of the methanolic and aqueous extracts of *B.oleracea*

The methanolic and aqueous extracts of *B.oleracea* did not show any sign of toxicity in the laboratory rats at the different dosages, in the first and the second phases of the acute toxicity test.

Table 4.1: The phytochemical constituents of methanolic and aqueous extracts of *Brassica oleracea*

S/N	Constituents	Methanolic	Aqueous
1	Carbohydrates	++	+
2	Cardiac glycosides	++	+
3	Unsaturated steroids	++	+
4	Triterpenes	++	+
5	Tannins	—	—
6	Saponins	+	—
7	Flavonoids	++	+
8	Alkaloids	++	+
9	Anthracene	—	+

Note: (-)= Absent; (+)= Present; S/N= Serial Number

GCMS ANALYSIS

KINGSLEY (SAMPLE -KS)

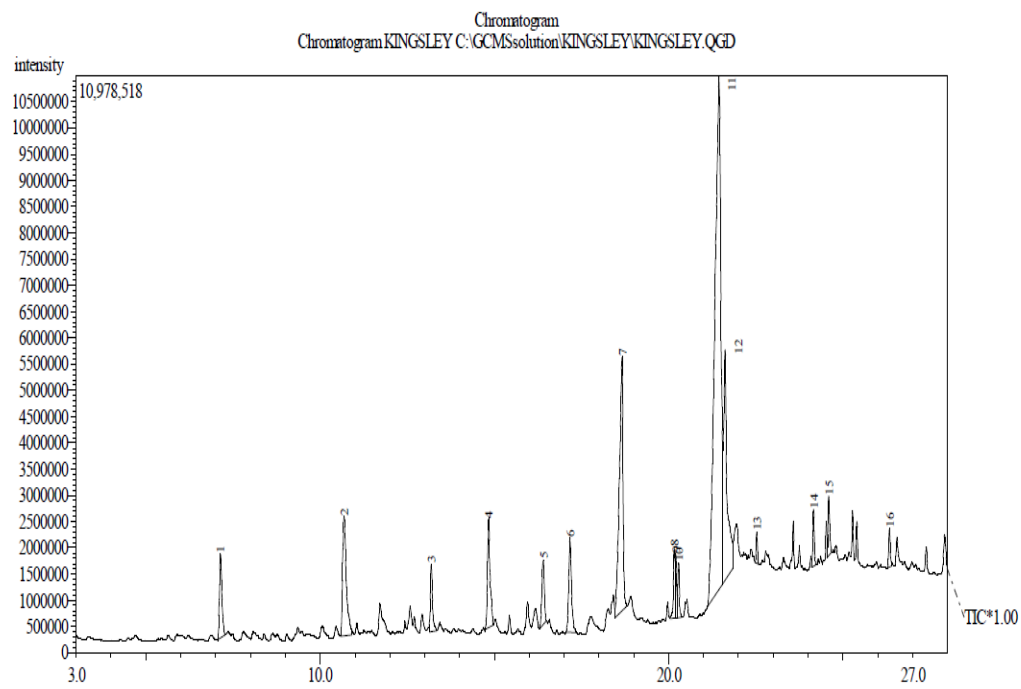


Figure 4.1: GCMS analysis of chloroform fraction of *B. oleracea* extract showing the different peaks of compounds present.

Table 4.2: The GCMS analysis of chloroform fraction of *Brassica oleracea* extract showing the different characteristics of the compounds

Peak No	Retention time (minutes)	Name of compound	Molecular weight(g/mol)	Peak Area (%)
1	7.144	4H-Pyran- 4-one	144	3.05
2	10.689	Iberin nitrile	131	6.24
3	13.189	9-Borabicyclo[4.2.1]nonane, dimer	244	2.11
4	14.839	3-Formylhept-3-enedinitrile	148	4.06
5	16.402	Pyridine	135	2.19
6	17.167	Palmitic acid	270	3.83
7	18.663	Pentadecanoic acid	242	13.62
8	20.160	13-Tetradecene-11-yn-1-ol	208	1.67
9	20.206	Decane, 3-chloro-3-Chlorodecane	176	1.28
10	20.288	5,8-Octadecadienoic acid	294	1.19
11	21.443	E-9-Tetradecenoic acid	226	45.09
12	21.618	n-Hexadecanoic acid	256	11.73
13	22.526	Heptadecane	296	0.55
14	24.156	11-Tridecen-1-ol	198	1.21
15	24.591	3,11-Tetradecadien-1-ol	210	1.36
16	26.332	n-Pentadecane	212	0.81

4.4 *In vitro* antitrypanosomal effect of *Brassica oleracea* aqueous extracts on *Trypanosoma evansi*

In the *in vitro* antitrypanosomal analysis of the *B. oleracea* aqueous extract, the following were recorded: 75% motility of parasites for 5mg/ml concentration at 20 minutes, 55% motility for 10mg/ml concentration at 20 minutes, 20% motility for 20mg/ml at 20 minutes. Diaminazene aceturate showed no motility of parasites at 15 and 20 minutes as presented in (Table 4.3).

4.5 *In vitro* antitrypanosomal effect of *Brassica oleracea* methanolic extracts on *Trypanosoma evansi*

In the *in vitro* antitrypanosomal analysis of the methanolic extract, 70% motility of parasites was recorded for 5mg/ml concentration at 20 minutes. 40% of parasite motility was recorded for 10mg/ml at 20 minutes. At 20 minutes, 0% motility was recorded at 20 mg/ml concentration. At 15 minutes, Diaminazene aceturate showed 0% motility of parasites (Table 4.4).

4.6 *In vivo* antitrypanosomal effects of *Brassica oleracea* methanolic extracts on *Trypanosoma evansi*

In the *in vivo* antitrypanosomal effects of *B. oleracea*, the methanolic extract showed more activity against the parasites while the aqueous extract showed little or no effect on the parasites (Figure 4.2). Gradual increase in the level of parasitaemia was recorded among all the infected groups until the treatment with extracts and standard drug commenced on day 5 post- infection. Three of the animals in the infected and untreated group died in the evening of day 7 while the remaining two animals died in the early hours of day 8 post-infection. The rest of the animals in other groups survived until day 14 of the experiment.

Table 4.3: The *in vitro* antitrypanosomal effect of *Brassica oleracea* aqueous extract

Time (minutes)	Motility (%) at 5mg/ml	Motility (%) at 10mg/ml	Motility (%) at 20mg/ml	Motility (%) for Diaminazene Aceturate	Motility (%) for Negative control
5	100	100	85	50	100
10	100	85	80	30	100
15	95	70	25	0	100
20	75	55	20	0	100

Note: The motility of the parasites were expressed in percentage(%)

Table 4.4: The *in vitro* antitrypanosomal effect of *Brassica oleracea* methanolic extract

Time (minutes)	Motility (%) at 5mg/ml	Motility (%) at 10mg/ml	Motility (%) at 20mg/ml	Motility (%) for Diaminazene Aceturate	Motility (%) for Negative control
5	100	80	65	50	100
10	90	80	60	30	100
15	90	65	10	0	100
20	70	40	0	0	100

Note: The motility of the parasites were expressed in percentage(%)

4.7 *In vivo* antitrypanosomal effects of *Brassica oleracea* aqueous extracts on *Trypanosoma evansi*

Brassica oleracea aqueous extract showed least antitrypanosomal activity *in vivo* as compared with the methanolic extract of the plant (Figure 4.2). This was demonstrated by the complete clearance of the parasites from the blood of the animals infected and treated with 800mg/kg body weight methanolic extract on day 10 when compared with the aqueous extract. As the experiment progressed, loss of body weight (Table 4.5), little or no effect on the body temperature (Table 4.6), PCV (Table 4.7), gradual increase in white blood cell count (Table 4.8), slight decrease in the red blood cell (Table 4.9), gradual increase in the total blood protein (Table 4.10) and increase in organ weight was recorded among the infected and untreated group of the animals (Table 4.11, Group D). Gradual increase in the level of parasitaemia was recorded among all the infected groups until the treatment with extracts and standard drug commenced on day 5 post- infection. All the animals in the infected and untreated group died on day 7 evening and day 8 during the experiment. Three of the animals in the infected and untreated group died in the evening of day 7 while the remaining two animals died in the early hours of day 8 post-infection. All the animals in other groups survived until day 14 of the experiment. Diaminazene aceturate dose of 3.5 mg/kg body weight was not able to clear the parasitemia in the *T. evansi* infected laboratory rats after ten days of post infection period as compared with those infected and treated with 800mg/kg body weight aqueous extract which reduced the parasitaemia considerably from day 9 post infection period. The animals which were infected and treated with 100mg/kg body weight aqueous extract showed least activity as compared with other infected and treated groups. The animals which were infected and treated with 400mg/kg body weight aqueous extract showed better antitrypanosomal activity next to those which were infected and treated with 100mg/kg body weight aqueous extract.

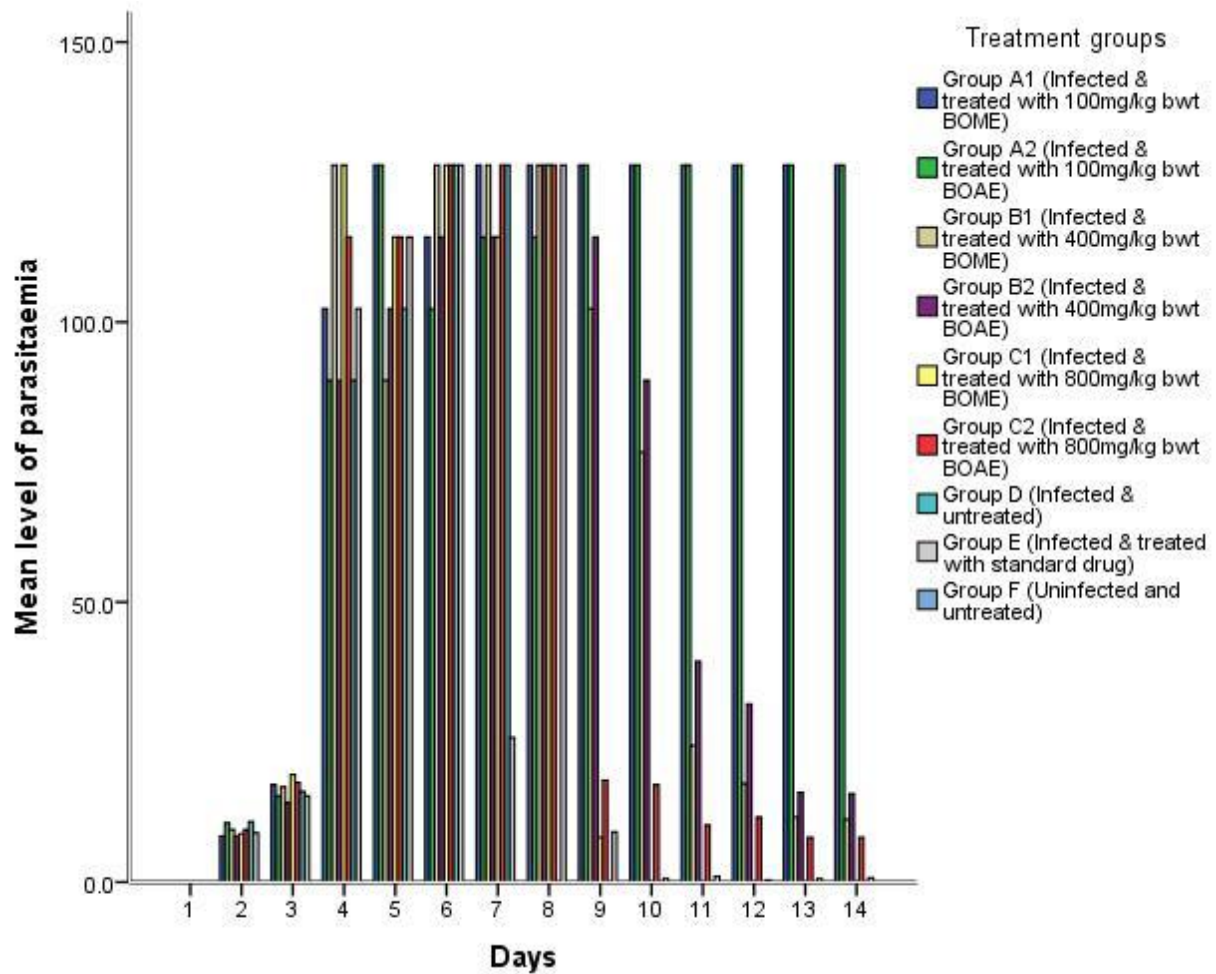


Figure 4.2: The effects of methanolic and aqueous extracts of *B. oleracea* on the parasitaemia in rats infected with *T.evansi*

Table 4.5: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the body weights of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	198.20±1.16 ^a	190.00±2.35 ^a	190.20±2.29 ^a	189.80±2.27 ^{ab}	190.40±2.91 ^{ab}
Group A2	195.60±0.93 ^{ab}	193.40±0.93 ^a	193.00±0.71 ^a	192.70±1.36 ^{ab}	193.60±1.21 ^{ab}
Group B1	197.20±0.86 ^a	195.80±0.80 ^a	195.60±0.87 ^a	198.60±1.33 ^a	196.00±0.84 ^a
Group B2	195.80±2.08 ^{ab}	193.20±2.42 ^a	192.60±2.38 ^a	193.40±2.16 ^{ab}	193.40±1.97 ^{ab}
Group C1	194.40±4.50 ^{ab}	193.40±3.97 ^a	193.40±4.07 ^a	195.80±3.64 ^{ab}	192.80±3.63 ^{ab}
Group C2	194.80±3.44 ^{ab}	191.20±3.73 ^a	192.20±3.46 ^a	191.40±3.52 ^{ab}	192.60±3.50 ^{ab}
Group D	192.40±3.42 ^{ab}	191.80±3.51 ^a	192.70±3.50 ^a	Died	Died
Group E	192.20±3.20 ^{ab}	191.60±2.64 ^a	197.40±2.40 ^a	191.60±2.14 ^{ab}	194.20±2.34 ^{ab}
Group F	188.00±2.21 ^b	187.80±1.50 ^a	187.20±1.46 ^a	183.60±2.16 ^b	187.00±1.52 ^b

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

Table 4.6: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the body temperatures of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	32.50±0.60 ^a	37.02±0.41 ^a	37.78±0.25 ^a	37.02±0.68 ^a	37.18±0.40 ^a
Group A2	33.72±0.70 ^a	37.28±0.25 ^a	36.58±0.58 ^a	37.20±0.36 ^a	37.40±0.29 ^a
Group B1	33.62±0.71 ^a	35.98±1.03 ^a	37.20±0.33 ^a	37.78±0.25 ^a	37.76±0.22 ^a
Group B2	35.70±0.81 ^a	36.90±0.42 ^a	37.10±0.45 ^a	37.76±0.27 ^a	39.04±0.62 ^a
Group C1	33.94±0.66 ^a	37.72±0.28 ^a	36.96±0.26 ^a	36.92±0.53 ^a	33.73±0.50 ^b
Group C2	32.28±0.75 ^a	37.44±0.43 ^a	37.26±0.40 ^a	35.54±0.45 ^b	34.44±0.73 ^b
Group D	33.54±0.75 ^a	36.86±0.55 ^a	37.12±0.48 ^a	Died	Died
Group E	33.48±1.03 ^a	37.01±0.54 ^a	36.90±0.29 ^a	34.98±0.54 ^{ab}	34.42±0.74 ^b
Group F	33.94±0.58 ^a	37.14±0.51 ^a	37.38±0.43 ^a	33.82±0.37 ^c	34.08±0.74 ^b

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

Table 4.7: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the packed cell volume (PCV) of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	40.80±0.73 ^a	32.20±0.86 ^b	32.80±1.40 ^a	34.60±0.75 ^b	32.80±1.36 ^b
Group A2	40.60±1.29 ^a	33.70±1.07 ^b	26.54±5.72 ^a	33.00±1.18 ^b	32.40±0.87 ^b
Group B1	38.90±0.66 ^a	33.86±1.46 ^b	33.40±1.21 ^a	33.60±1.21 ^b	34.00±0.71 ^b
Group B2	41.00±1.05 ^a	34.00±1.70 ^b	33.20±1.24 ^a	32.60±1.07 ^b	35.20±0.97 ^b
Group C1	35.65±0.75 ^a	33.40±1.20 ^b	32.80±1.59 ^a	38.20±1.07 ^a	39.20±0.86 ^a
Group C2	40.02±1.17 ^a	33.20±1.24 ^b	33.40±0.75 ^a	37.80±1.24 ^a	35.20±1.07 ^b
Group D	40.82±0.51 ^a	33.60±1.69 ^b	32.80±1.39 ^a	Died	Died
Group E	40.80±1.07 ^a	32.80±1.20 ^b	33.20±1.01 ^a	39.60±1.02 ^a	39.60±1.03 ^a
Group F	40.60±0.87 ^a	41.80±0.86 ^a	33.60±0.40 ^a	40.60±1.71 ^a	42.20±0.97 ^a

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene acetate. Group F=Uninfected & untreated.

Table 4.8: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the white blood cell counts(WBC) of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	10.98±1.12 ^a	8.18±0.46 ^{bc}	13.82±0.77 ^a	14.94±0.37 ^a	12.84±0.81 ^a
Group A2	7.70±0.55 ^b	9.94±0.67 ^{abc}	13.72±1.31 ^a	12.70±1.31 ^a	9.38±0.76 ^a
Group B1	11.02±0.55 ^a	10.99±1.12 ^a	11.92±0.76 ^{abc}	10.98±1.12 ^b	7.42±0.34 ^b
Group B2	9.36±1.14 ^{ab}	7.70±0.55 ^c	12.14±1.17 ^{ab}	8.70±0.55 ^c	7.60±0.40 ^c
Group C1	10.48±1.22 ^{ab}	11.02±0.55 ^a	11.10±1.21 ^a	8.62±0.47 ^{bc}	7.44±0.26 ^b
Group C2	7.70±0.55 ^b	9.36±1.14 ^{abc}	9.62±0.47 ^a	9.36±1.14 ^{bc}	7.40±0.22 ^b
Group D	11.70±0.55 ^b	10.48±1.22 ^{ab}	12.22±0.97 ^{ab}	Died	Died
Group E	9.36±1.14 ^{ab}	7.70±0.55 ^c	9.30±1.09 ^{bc}	8.24±0.58 ^c	7.22±0.26 ^b
Group F	9.20±0.70 ^{ab}	7.20±0.55 ^c	9.04±0.50 ^c	7.70±0.55 ^c	7.36±0.26 ^b

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

Table 4.9: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the red blood cell counts(RBC) of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	7.22±0.58 ^a	7.20±0.30 ^b	7.60±0.90 ^a	7.20±0.30 ^a	6.96±0.17 ^{ab}
Group A2	7.44±0.59 ^a	8.22±1.04 ^a	7.44±0.58 ^a	8.22±1.04 ^a	7.00±0.20 ^{ab}
Group B1	7.40±0.51 ^a	7.42±0.56 ^{ab}	7.42±0.76 ^a	7.22±0.60 ^a	7.20±0.20 ^a
Group B2	7.42±0.76 ^a	7.44±0.58 ^{ab}	7.80±0.90 ^a	7.44±0.59 ^a	7.16±0.26 ^a
Group C1	7.60±0.91 ^a	7.40±0.51 ^{ab}	7.62±0.59 ^a	7.40±0.51 ^a	7.12±0.23 ^{ab}
Group C2	7.24±0.59 ^a	7.42±0.76 ^{ab}	7.40±0.51 ^a	7.42±0.76 ^a	7.01±0.20 ^{ab}
Group D	7.26±0.59 ^a	6.26±0.91 ^{ab}	5.82±0.58 ^a	Died	Died
Group E	7.42±0.76 ^a	7.46±0.59 ^{ab}	7.36±0.57 ^a	7.60±0.90 ^a	6.16±0.61 ^c
Group F	7.42±0.61 ^a	7.82±0.48 ^{ab}	7.22±0.58 ^a	7.44±0.59 ^a	6.68±0.48 ^b

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

Table 4.10: The effects of methanolic and aqueous extracts of *Brassica oleracea* on the total blood proteins of rats infected with *Trypanosoma evansi*

Treatment groups	Day1	Day4	Day7	Day10	Day13
Group A1	6.08±0.23 ^a	7.28±0.30 ^a	6.96±0.17 ^{ab}	7.08±0.23 ^a	6.96±0.17 ^a
Group A2	6.28±0.18 ^a	7.32±0.11 ^a	7.00±0.20 ^{ab}	7.00±0.20 ^a	6.96±0.36 ^a
Group B1	6.16±0.38 ^a	7.00±0.28 ^a	7.20±0.20 ^a	6.68±0.48 ^{ab}	6.64±0.52 ^{abc}
Group B2	6.08±0.41 ^a	7.28±0.33 ^a	7.16±0.26 ^a	6.88±0.54 ^{ab}	6.76±0.36 ^{ab}
Group C1	6.16±0.17 ^a	7.08±0.23 ^a	7.12±0.23 ^a	6.40±0.58 ^{bc}	6.32±0.30 ^{bcd}
Group C2	6.28±0.33 ^a	7.06±0.23 ^a	7.00±0.20 ^{ab}	6.76±0.36 ^{ab}	6.88±0.30 ^a
Group D	6.20±0.53 ^a	8.00±0.40 ^a	7.82±0.33 ^c	Died	Died
Group E	6.16±0.17 ^a	7.24±0.43 ^a	6.68±0.48 ^b	6.16±0.22 ^c	6.20±0.35 ^{cd}
Group F	6.04±0.17 ^a	7.06±0.14 ^b	5.82±0.30 ^c	6.00±0.14 ^c	6.02±0.16 ^d

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

Table 4.11: The effects of methanolic and aqueous extracts of *B. Oleracea* on the weight of selected organs of rats infected with *T. evansi*

Treatment groups	Animal parts and organs weight (g)						
	Body	Brain	Liver	Testis	Kidney	Heart	Spleen
Group A1	193.00±2.08 ^a	0.76±0.03 ^a	5.62±0.26 ^a	1.99±0.09 ^a	0.82±0.08 ^{abc}	0.79±0.11 ^a	0.70±0.02 ^a
Group A2	193.00±0.58 ^a	0.67±0.03 ^b	5.48±0.15 ^a	1.97±0.07 ^a	1.01±0.07 ^a	0.86±0.05 ^a	0.78±0.06 ^a
Group B1	195.33±1.45 ^a	0.66±0.06 ^b	4.36±0.25 ^c	2.00±0.06 ^a	0.95±0.05 ^{ab}	0.82±0.07 ^a	0.55±0.02 ^b
Group B2	195.33±2.33 ^a	0.67±0.04 ^b	4.71±0.16 ^b	2.04±0.06 ^a	0.94±0.07 ^{ab}	0.75±0.07 ^{ab}	0.56±0.02 ^b
Group C1	187.67±3.84 ^a	0.65±0.03 ^b	3.88±0.06 ^{cd}	1.86±0.04 ^a	0.66±0.03 ^c	0.55±0.02 ^b	0.53±0.02 ^b
Group C2	187.67±3.28 ^a	0.70±0.02 ^b	4.63±0.21 ^b	2.10±0.02 ^a	0.83±0.07 ^{abc}	0.54±0.02 ^b	0.53±0.01 ^b
Group D	192.70±1.26 ^a	0.73±0.03 ^b	5.71±0.24 ^a	2.12±0.04 ^a	1.05±0.007 ^a	0.86±0.06 ^a	0.91±0.04 ^a
Group E	194.67±0.33 ^a	0.64±0.06 ^b	3.44±0.27 ^{de}	1.94±0.02 ^a	0.99±0.04 ^{ab}	0.72±0.07 ^{ab}	0.75±0.06 ^a
Group F	190.33±2.67 ^a	0.69±0.02 ^b	3.09±0.05 ^e	1.96±0.08 ^a	0.78±0.08 ^{bc}	0.75±0.07 ^{ab}	0.71±0.03 ^a

Notes: Values are expressed as mean±SD (Standard deviation). Means with different superscripted alphabets along columns are significantly different at P<0.05. Groups A1 & A2=Infected & treated with 100mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups B1 & B2=Infected & treated with 400mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Groups C1 & C2=Infected & treated with 800mg/kg body weight *B. oleracea* methanolic and aqueous extracts respectively. Group D=Infected & untreated. Group E=Infected & treated with Diaminazene aceturate. Group F=Uninfected & untreated.

4.8 Histopathological examination

Plates I-V show some histopathological changes which include vacuolations in the brains of the rats which were infected and treated with plant extracts as compared with the controls. There was no histological change in the hearts of the animals infected and treated with extracts and those infected and treated with Diaminazene aceturate (Plates VI and VII). Histopathological changes ranging from tubular necrosis, lymphocyte hyperplasia and tubular distortion were revealed in the kidneys of the animals infected and treated with plant extracts, compared with the controls (Plates VIII-XI). The spleens of the animals infected and treated with the extracts showed histopathological changes such as lymphocyte hyperplasia and sinusoidal congestions as compared with the controls (Plates XII-XIV). Plates XV-XVIII revealed histopathological changes in the liver of the infected and treated with extracts such as moderate lymphocyte hyperplasia, vascular congestion, slight perivascular necrosis, sinusoidal congestion and hepatocellular necrosis as compared with the controls. Plates XIX-XXII showed histopathological changes of the testes such as moderate and slight spermatogenic necrosis as compared with the controls.

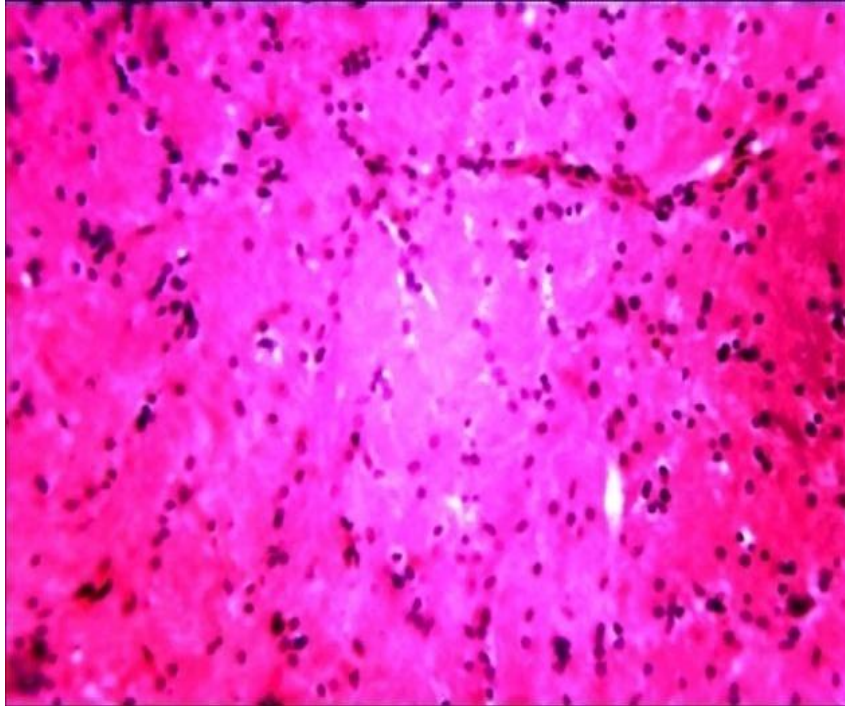


Plate I: Brain of rat uninfected/ untreated (H&E X400)

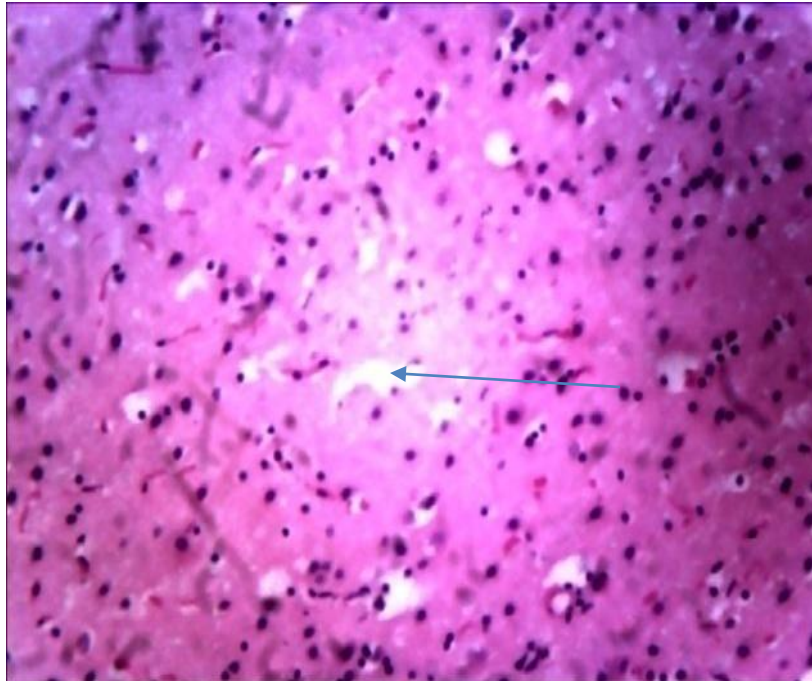


Plate II: Brain of rat infected/ untreated showing slight vacoulation (H&E X400)

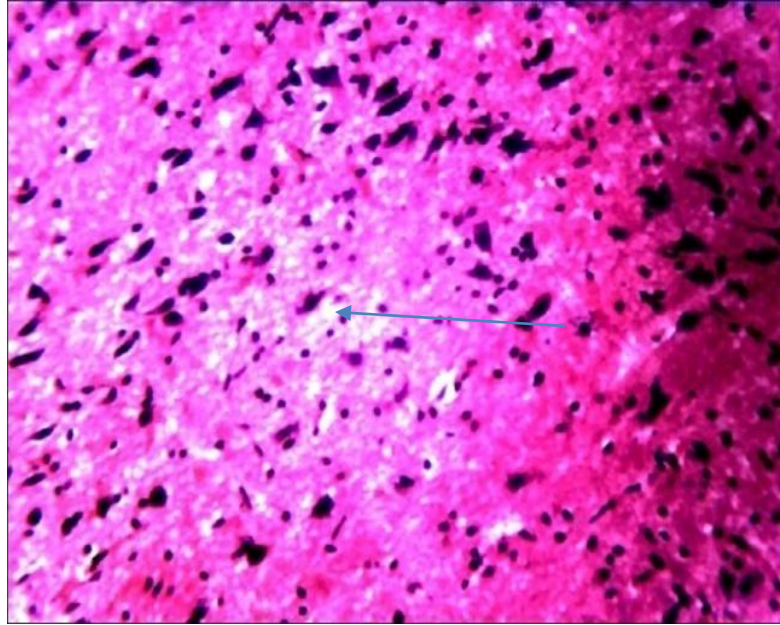


Plate III: Brain of rat infected/treated with 100mg/kg aqueous extract showing slight vacuolations (H&E X400)

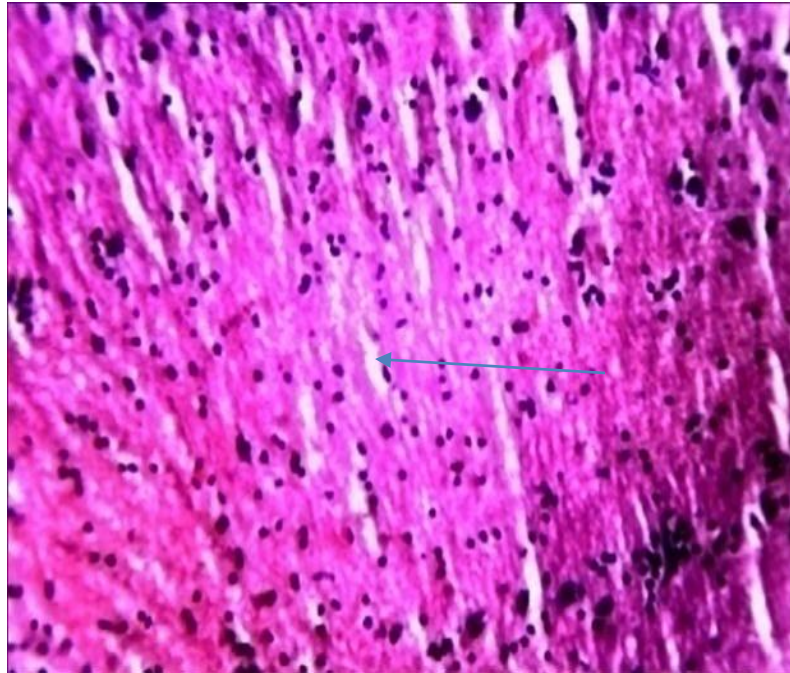


Plate IV: Brain of rat infected and treated with 100mg/kg methanolic extract showing slight vacuolation (H&E X400)

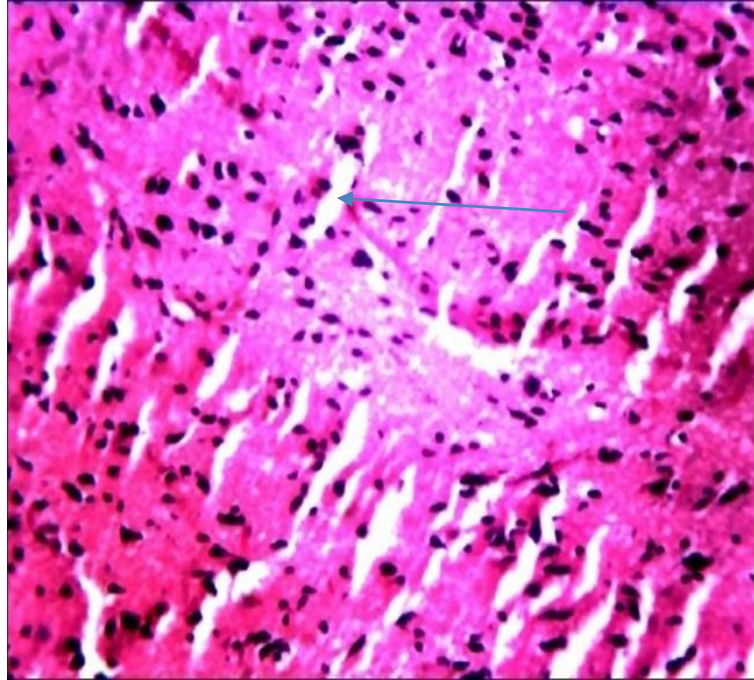


Plate V: Brain of rat infected and treated with 400mg/kg aqueous extract showing slight vacuolation (H&E X400)

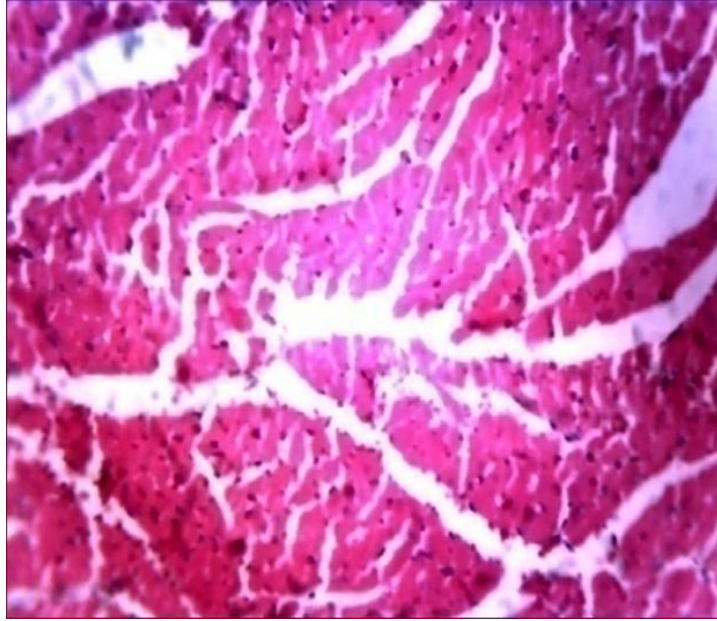


Plate VI: Heart of rat uninfected/untreated (H&E X400)

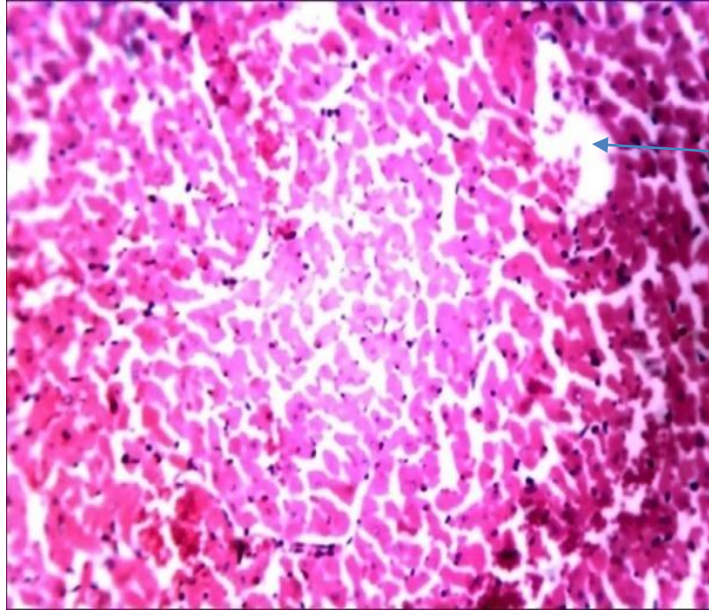


Plate VII: Heart of rat infected/untreated showing slight necrosis of muscular fibre (H&E X400)

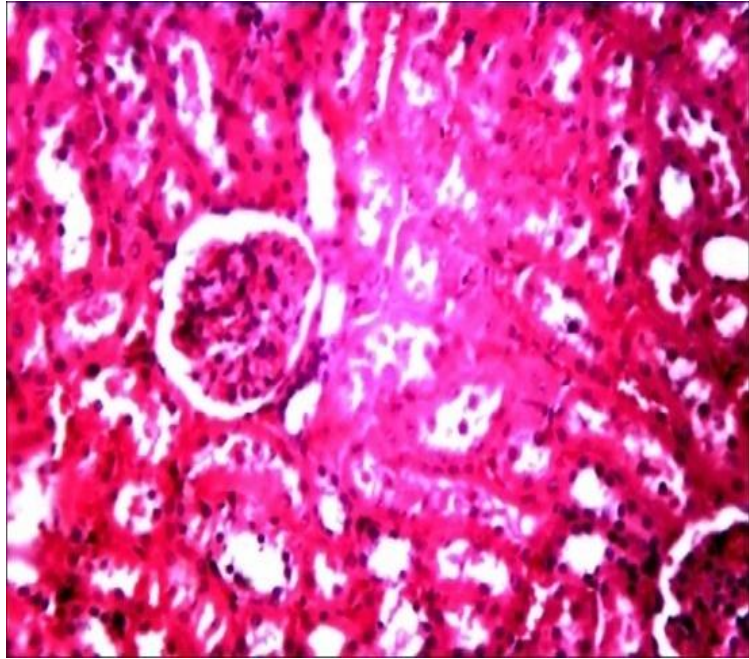


Plate VIII: Kidney of rat uninfected/untreated (H&E X400)

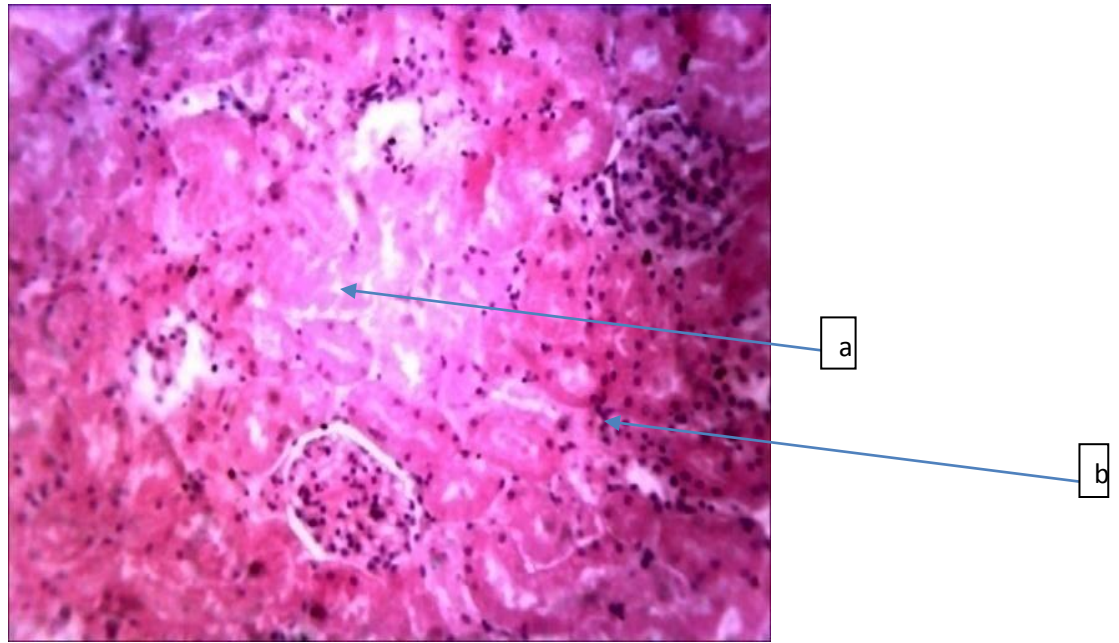


Plate IX: Kidney of rat infected/untreated showing intense necrosis of tubules (a) and lymphocyte hyperplasia (b) (H&E X400)

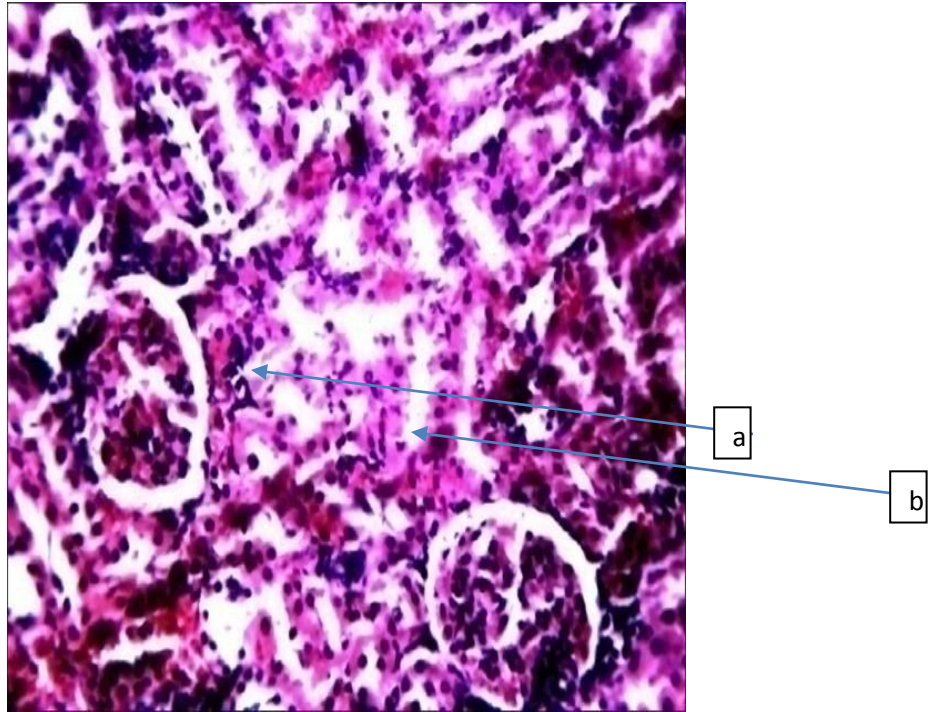


Plate X: Kidney of rat infected/treated with 100mg/kg aqueous extract showing intense nephritis (a) and slight tubular distortion (b) (H&E X400)

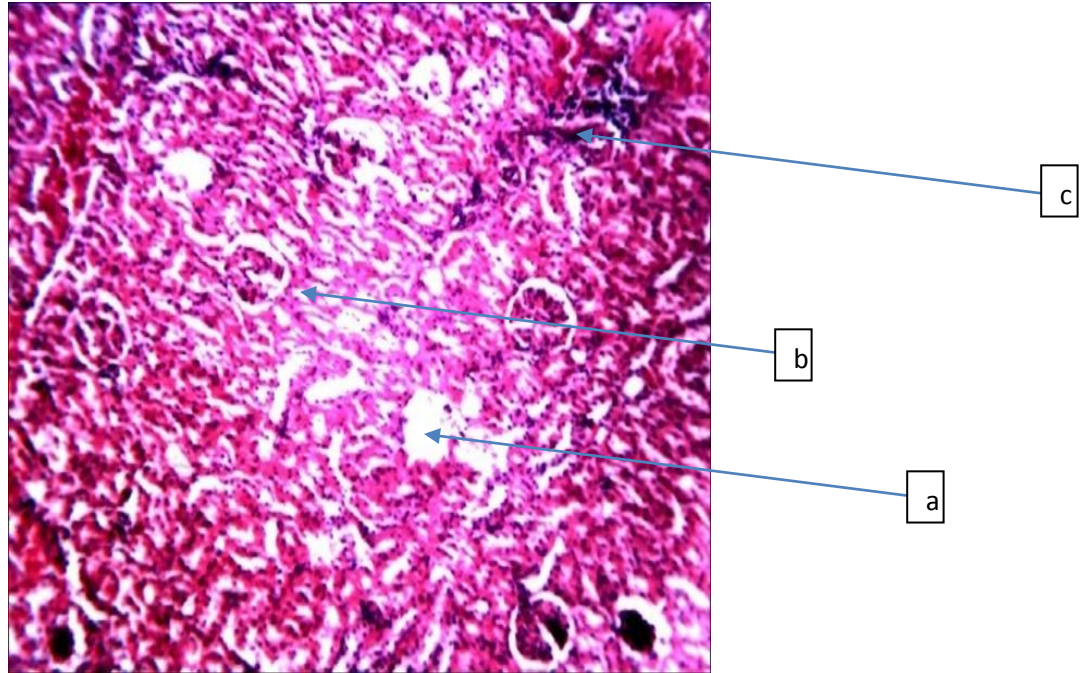


Plate XI: Kidney of rat infected/treated with 400mg/kg aqueous extract showing slight glomerular necrosis (a), tubular distortion (b) and lymphocyte hyperplasia (c) (H&E X400)

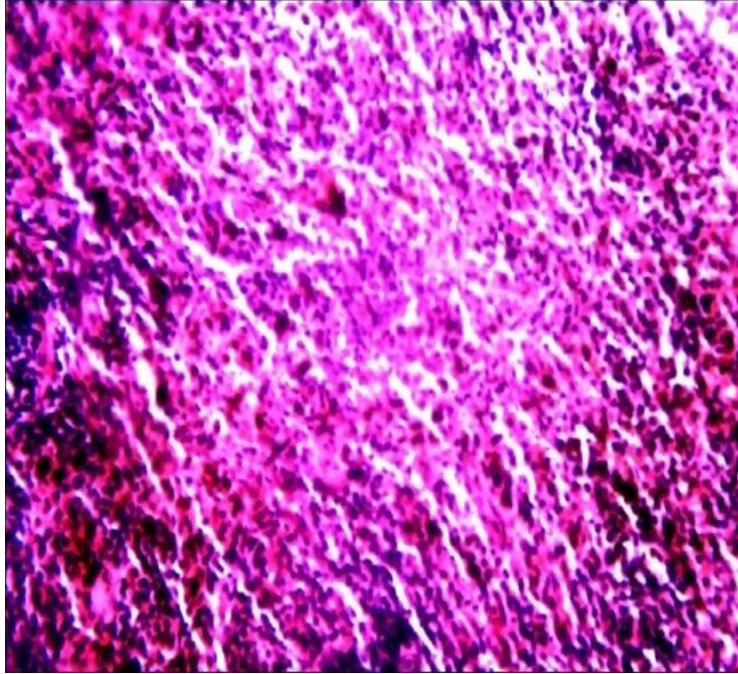


Plate XII: Spleen of rat uninfected/untreated (H&E X400)

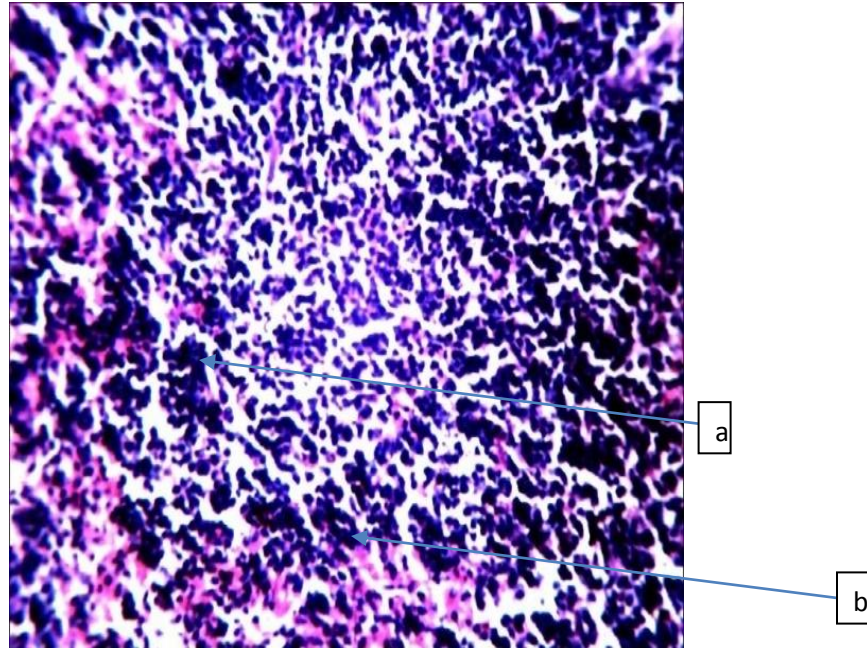


Plate XIII: Spleen of rat infected/untreated showing intense lymphocyte hyperplasia (a) with sinusoidal congestion(b) (H&E X400)

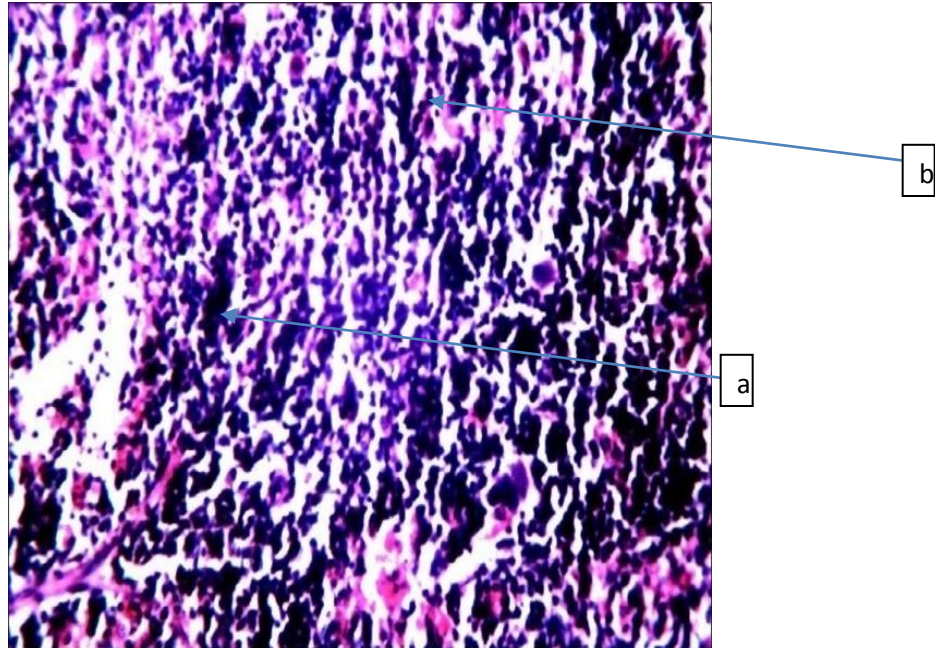


Plate XIV: Spleen of rat infected and treated with 100mg/kg aqueous extract showing lymphocyte hyperplasia (a) with sinusoidal congestion (b) (H&E X400)

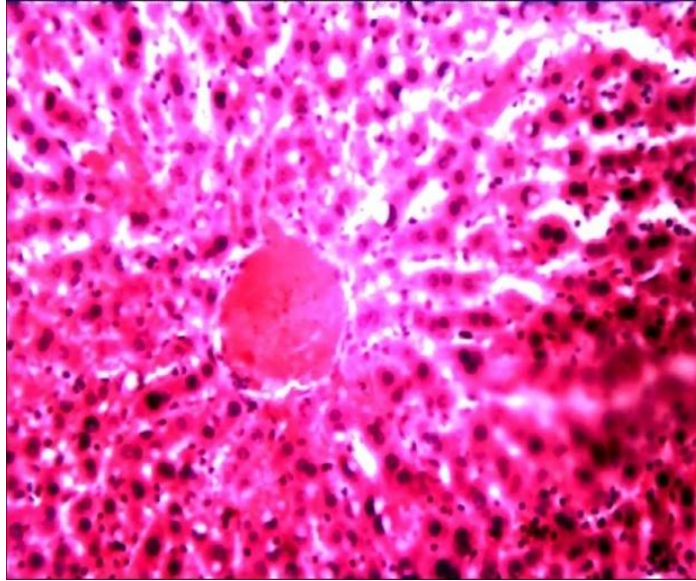


Plate XV: Liver of rat uninfected/untreated (H&E X400)

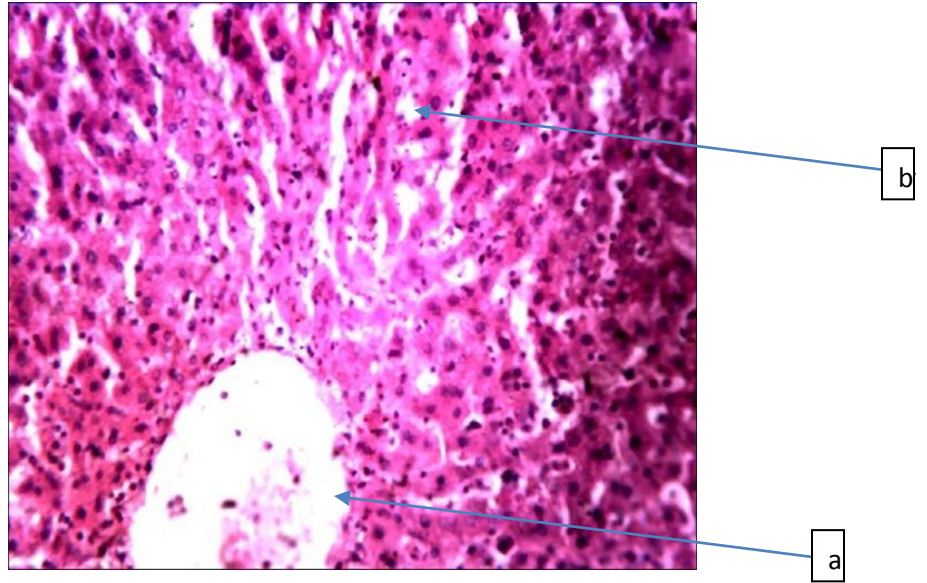


Plate XVI: Liver of rat infected/untreated showing slight perivascular necrosis (a) with vacoulation (b) (H&E X400)

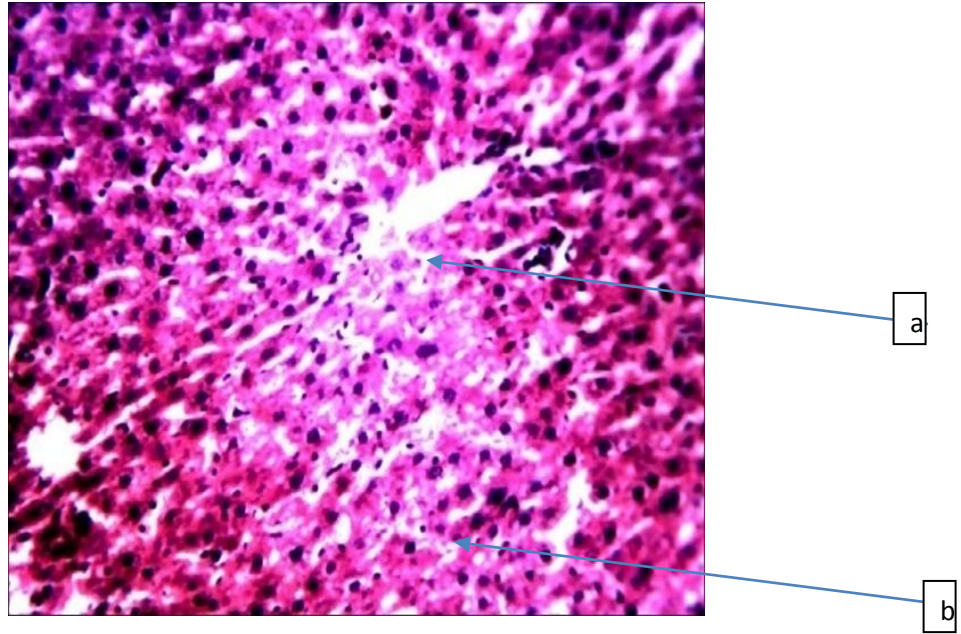


Plate XVII: Liver of rat infected/treated with Diaminazene aceturate showing slight vacoulation (a) with sinusoidal congestion (b) (H&E X400)

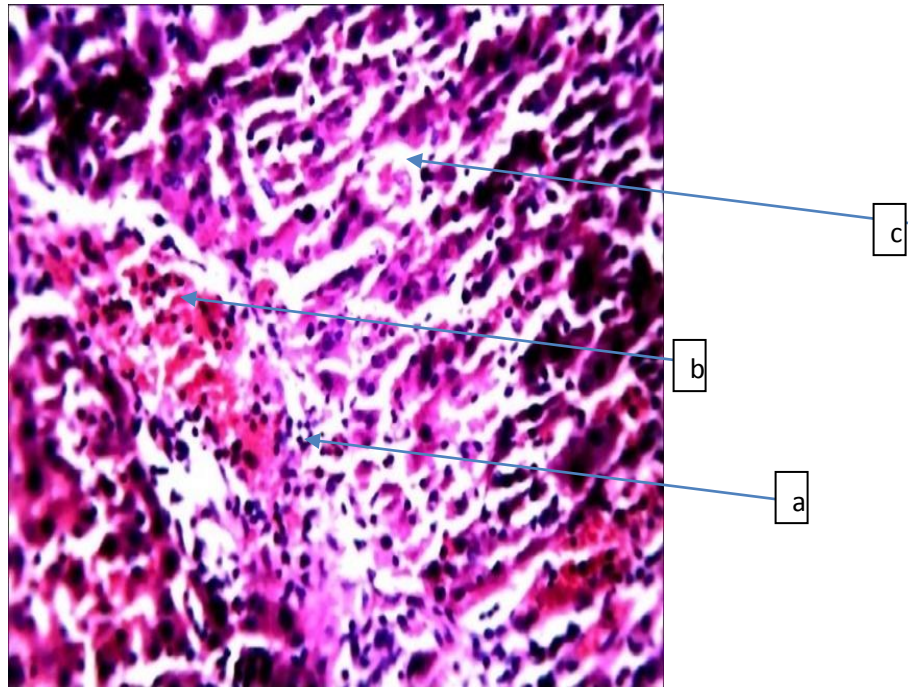


Plate XVIII: Liver of rat infected/treated with 100mg/kg aqueous extract showing slight lymphocyte hyperplasia (a) with vascular congestion (b) and slight hepatocellular necrosis (c) (H&E X400)

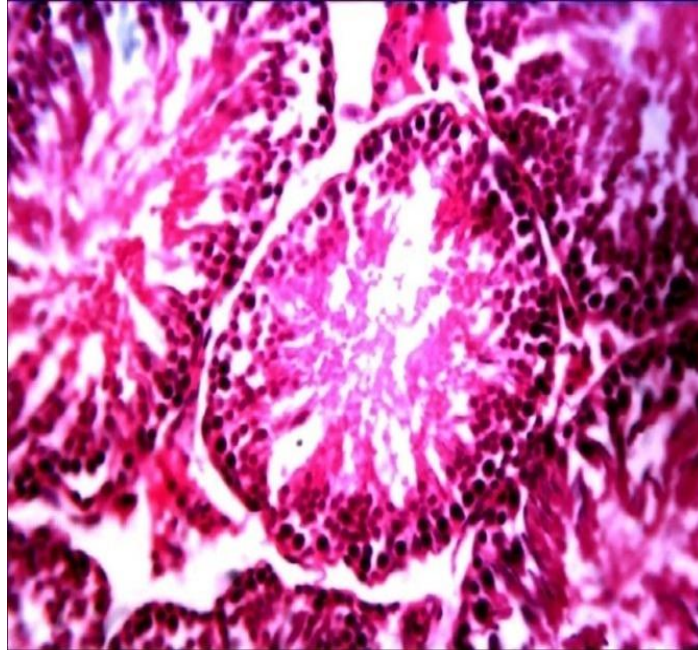


Plate XIX: Testis of rat uninfected/untreated (H&E X400)

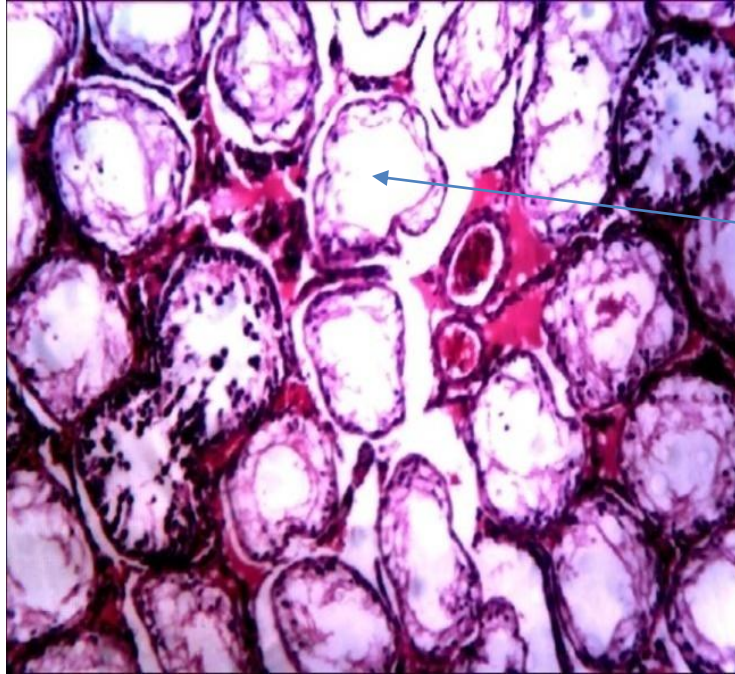


Plate XX: Testis of rat infected/untreated showing intense necrosis of spermatogenic cells (H&EX400)

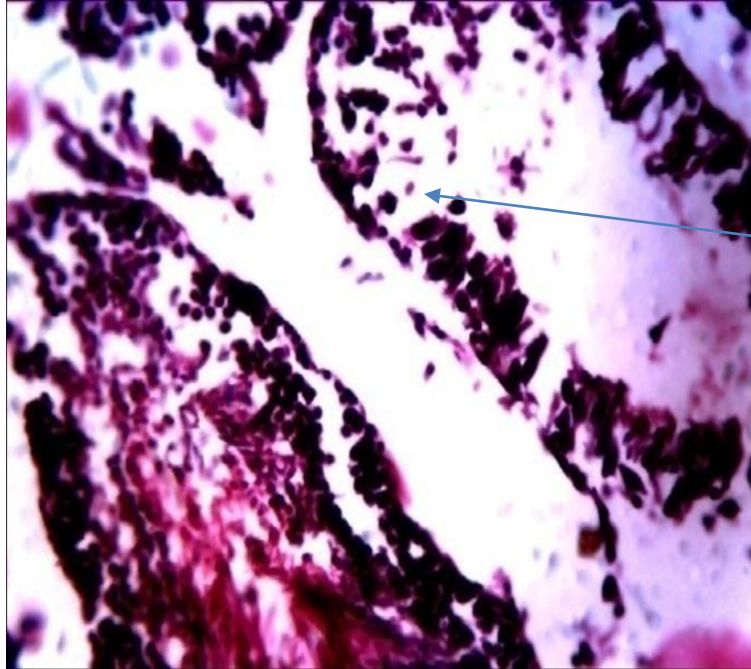


Plate XXI: Testis of rat infected/treated with 100mg/kg of aqueous extract showing slight necrosis of spermatogenic cells (H&EX400)

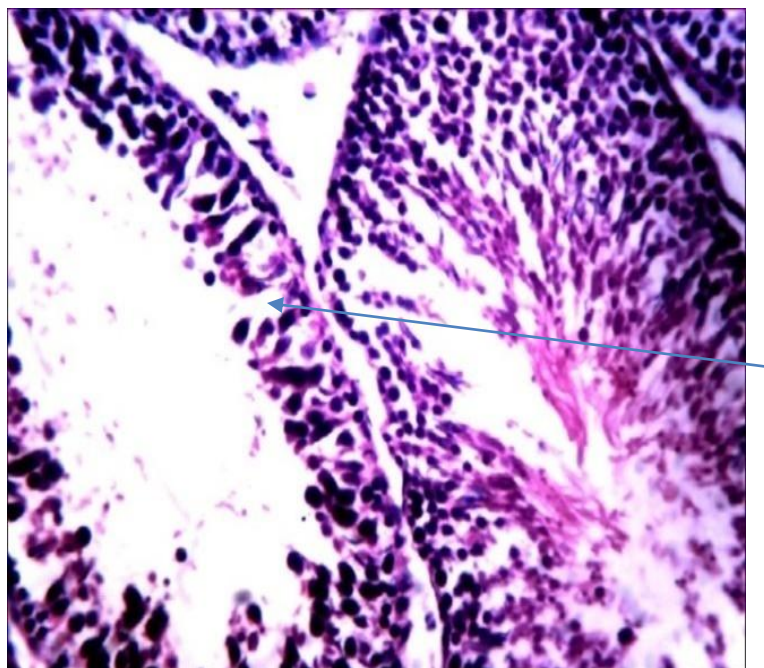


Plate XXII: Testis of rat infected/treated with 100mg/kg methanolic extract showing slight necrosis of spermatogenic cells (H&EX400)

CHAPTER FIVE

5.0

DISCUSSION

The phytochemical constituents of the methanolic extract of *B. oleracea* are higher in quality and quantity compared with the aqueous extract in this study. This demonstrates that methanol is a better extracting solvent compared with aqueous extract. For instance, carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, saponins, flavonoids and alkaloids were present in the methanolic extract while saponins was absent in the aqueous extract. In the *in vitro* analysis of the two extracts, 20mg/ml of the methanolic extract at 20 minutes produced a better antitrypanosomal result when compared with the aqueous extract by causing complete ceasation in the motility of the parasites. The antitrypanosomal effects of the methanolic and aqueous extracts of *B. oleracea* against *T. evansi* in this study suggests that the phytochemicals in the extracts may have played a synergistic role against the parasites. The presence of phytochemicals such as alkaloids, flavonoids, triterpenes and carbohydrates as revealed by the phytochemical screening of the aqueous extract is in agreement with the report by Atawodi and Shehu (2010) when they discovered the presence of alkaloids, flavonoids, triterpenes and carbohydrates from the methanolic leaf extract of *Moringa oleifera*.

Palmitic acid detected by the Gas Chromatography Mass Spectrometry(GCMS) may be among the specific compounds responsible for the *in vivo* and *in vitro* antitrypanosomal effects of the extracts against *T. evansi* as observed in this study. Palmitic acid detected by GCMS in the leaf of *Strychnos spinosa* played antitrypanosomal effects on *Trypanosoma brucei brucei* (Hoet *et al.*, 2006). Other compounds detected by the GCMS such as pentadecanoic acid, n-hexadecanoic acid, have also been reported by Bero *et al*,

(2013), among other compounds which were responsible for the antitrypanosomal effects of *Keetia leucantha* leaf extract against *Trypanosoma brucei brucei*. The compounds detected by the GCMS analysis in this present study may have played an inhibitory role on glycolysis in *T. evansi* (Chen *et al.*, 2007; Bero *et al.*, 2013).

There was no death or sign of toxicity among the laboratory rats by the extracts at the highest dose of 5000mg/kg body weight. The animals were feeding very well were active in movement which suggests that the plant extracts are safe to use. Thounaojam *et al* (1996) reported that there was no sign of toxicity of *B. oleracea* extract at 5000mg/kg body weight on mice.

In the present *in vitro* study of *B. oleracea* on *T. evansi*, the methanolic extract caused complete ceasation in the motility of the parasites at concentration of 20mg/ml at 20 minutes when compared with the aqueous extract in the parasites which were still motile at the same concentration and time (tables 4.3 and 4.4). This demonstrates that the methanolic extract of *B. oleracea* has higher antitrypanosomal activity on *T. evansi* than the aqueous extract. This observation is in agreement with that of Ene *et al.* (2014) when they reported complete ceasation in the motility of *Trypanosoma brucei* at 4mg/ml concentration at 25 minutes using *Picralima nitida* methanolic leaf extract. The ceasation in the motility of the parasites in this study suggests that the *B. oleracea* extracts might have affected certain enzymes involved in the metabolism of their cells. Igwe *et al.* (2002) repoted that aqueous extract of *B. oleracea* caused complete inhibition of motility on *Trypanosoma brucei brucei* at one hour using 0.0003mg/ml concentration.

The results of the *in vivo* antitrypanosomal activity of the extracts revealed that methanolic extract of *B. oleracea* had better antitrypanosomal effect than the aqueous extract. This was demonstrated by the complete clearance of the parasites from the blood of the animals infected and treated with 800mg/kg body weight methanolic extract on day 10 when compared with the aqueous extract. Two of the animals infected and untreated died on day 7 while the other three died on day 8. This may be attributed to excessive parasitaemia. All the animals in other groups survived till the end of the experiment. Shaba *et al.* (2012) reported the efficacy of methanolic extract of *B. Oleracea* leaves at different concentrations of 250-1000 µg/ml against *Trypanosoma evansi* on Vero cell line grown in Dulbecco's Modified Eagle Medium (DMEM) with foetal calf serum.

The loss of body weight amongst the animals in the infected and untreated group may be as a result of anorexia induced by the parasites which affected their feeding rate (Table 4.5). The fluctuating body temperature of the infected animals as observed in this study is a typical symptom of trypanosomiasis (Table 4.6) (Stephen, 1986). The PCV values among the animals infected and treated with 100mg/kg body weight aqueous and methanolic extracts decreased when compared with those infected and treated with 800mg/kg body weight methanolic extract which gradually increased at days 10 and 13 (Table 4.7). There was increased erythrophagocytosis of the parasites on the blood of the animals, hence the anaemia and low level of PCV. There was an increase in the white blood cell counts recorded among the animals infected and treated with 100mg/kg body weight aqueous extract when compared with the controls. This caused an increase in the production of the white blood cell to defend the body against the increased number of the parasites in the blood. There was a lower value of white blood cells among the animals

infected and treated with 800mg/kg body weight methanolic extract which resulted in less release of white blood cells, hence its best efficacy against the parasites. There was a slight decrease in the value of red blood cells among the animals infected and treated with 100mg/kg body weight methanolic extract when compared with the controls (Table 4.9). This is as a result of destruction of the cell membrane of the cells by the greater number of parasites in the blood thereby causing decrease in the red blood cell count. There was an increase in total blood protein level among the animals infected and treated with 100mg/kg body weight aqueous and methanolic extracts when compared with the controls. This may be attributed to the fact that proteins were released in the blood which suggests the presence of an infection. This disagrees with Sadique *et al.*, (2001) who reported decreased level of total protein in cattle infected with *T. congolense* which may be as a result of a different *Trypanosoma* species used. There was an increase in the weight of organs of the various infected groups of animals (Table 4.11). Splenomegaly observed among the infected animals in this study may be as a result of its hyperactivity in producing cells that play active roles in the body defense mechanism against the *T. evansi*, thereby causing its increased size. The brains of the animals which were infected and treated with 100mg/kg body weight of aqueous and methanolic extracts revealed slight and moderate vacuulations respectively while those infected and treated with 400mg/kg and 800mg/kg body weight of aqueous and methanolic extracts showed no histopathological changes compared with the controls (Plates I-V). The hearts of animals in all the treatment groups showed no histopathological changes except for those infected and untreated which showed slight necrosis of muscular fibre (VI-VII). This implies that the two extracts were able to provide protection against the parasites. The kidneys of the animals infected and treated with

400mg/kg body weight of the methanolic extract, 800mg/kg body weight of aqueous and methanolic extracts showed no histopathological change. There were histopathological changes in the kidneys of the animals infected and treated with 100mg/kg body weight of aqueous and methanolic extracts and 400mg/kg body weight aqueous extracts as compared with the controls (VIII-XI). The spleens of the animals infected and treated with 800mg/kg body weight of both the aqueous and methanolic extracts showed no histopathological changes compared with the control groups. The animals infected and treated with 400mg/kg body weight of both aqueous and methanolic extracts showed slight and moderate lymphocyte hyperplasia respectively (XII- XIV). However, the spleen of the animals infected and treated with 100mg/kg body weight methanolic extract revealed intense lymphocyte hyperplasia while 100mg/kg aqueous extract revealed both lymphocyte hyperplasia as well as sinusoidal congestion compared with those in the infected and untreated group. This explains its least *in vivo* antitrypanosomal efficacy in this study. There were marked histopathological changes observed in the liver of animals in all the treatment groups when compared with the control groups. This shows that both the aqueous and the methanolic extracts were not able to prevent any form of damage to the liver (XV- XVIII). Treatment dosages of 800mg/kg body weight of both extracts were able to prevent damage in the testis of the animals. Other treatment dosages such as 100mg/kg body weight aqueous and methanolic extracts and 400mg/kg body weight of both extracts revealed necrosis of the spermatogonic cells in the testis compared with the controls (XIX- XXII). The various forms of congestion and necrosis observed in the organs of the animals are in agreement with the reports of Taiwo *et al.*, (2003) and Takeet and Fagbemi (2009).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Trypanosomiasis is a parasitic disease of both human and livestock and it is one of the neglected diseases of Africa. In one of the efforts to fight this disease, *Brassica oleracea* methanolic and aqueous extracts were used to evaluate their antitrypanosomal effects on laboratory rats infected with *Trypanosoma evansi* both *in vitro* and *in vivo*. The plant extracts were found to be non-toxic to the laboratory rats thereby ascertaining its safety for consumption as herbal medicine. Presence of phytochemicals such as carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, saponins, flavonoids and alkaloids were found in the extracts. Bioactive compounds such as 4H-Pyran-4-one, Iberin nitrile, 9-Borabicyclo[4.2.1]nonane, dimer, 3-Formylhept-3-enedinitrile, Pyridine, Palmitic acid, Pentadecanoic acid, 13-Tetradecene-11-yn-1-ol, Decane, 3-chloro-3-Chlorodecane, 5,8-Octadecadienoic acid, E-9-Tetradecenoic acid, n-Hexadecanoic acid, Heptadecane, 11-Tridecen-1-ol, 3,11-Tetradecadien-1-ol and n-Pentadecane were revealed by the Gas Chromatography Mass Spectrometry (GCMS) of the extract. Final concentrations of 5mg/ml, 10mg/ml and 20mg/ml showed some effects on the parasites *in vitro*. The methanolic extract of plant was found to be more effective against the parasites than the aqueous extract both *in vitro* and *in vivo*. There was an increase in the parasitaemia, body temperature, white blood cell count, plasma protein while there was a decrease in the Packed Cell Volume and Red Blood Cell count in the infected rats. There was an increase in the weights of the brain, kidney, liver, heart, spleen and testis of the infected animals. No histopathological change was observed in the hearts of the infected rats treated with both extracts. Other organs in the infected animals revealed various degrees of necrosis in the testis, lymphocyte hyperplasia in the liver, kidney, spleen and vacuolations in the brain.

6.2 Conclusion

Carbohydrates, cardiac glycosides, unsaturated steroids, triterpenes, saponins, flavonoids and alkaloids are present in methanolic and aqueous extracts of *Brassica oleracea*. The

compounds detected by the Gas Chromatography Mass Spectrometry (GCMS) revealed the presence of 4H-Pyran-4-one, Iberin nitrile, 9-Borabicyclo[4.2.1]nonane, dimer, 3-Formylhept-3-enedinitrile, Pyridine, Palmitic acid, Pentadecanoic acid, 13-Tetradecene-11-yn-1-ol, Decane, 3-chloro-3-Chlorodecane, 5,8-Octadecadienoic acid, E-9-Tetradecenoic acid, n-Hexadecanoic acid, Heptadecane, 11-Tridecen-1-ol, 3,11-Tetradecadien-1-ol and n-Pentadecane in *B. oleracea*, and these compounds are the likely bioactive compounds responsible for the antitrypanosomal effects of the plant extracts against *Trypanosoma evansi*. This study showed that methanolic and aqueous extracts of *B. oleracea* are safe for use in herbal medicine. Methanolic and aqueous extracts of *B. oleracea* possess antitrypanosomal effect but the methanolic extract is more potent both *in vitro* and *in vivo* than aqueous extract of the plant.

6.3 Recommendations

- i. The use of *Brassica oleracea* leaves is recommended for livestock farmers as this provides a safer and a natural treatment alternative for trypanosomiasis than synthetic drugs which have been reported by researches to be toxic to animals.
- ii. It is suggested that the following compounds: 4H-Pyran-4-one, Iberin nitrile, 9-Borabicyclo[4.2.1]nonane, dimer, 3-Formylhept-3-enedinitrile, Pyridine, Palmitic acid, Pentadecanoic acid, 13-Tetradecene-11-yn-1-ol, Decane, 3-chloro-3-Chlorodecane, 5,8-Octadecadienoic acid, E-9-Tetradecenoic acid, n-Hexadecanoic acid, Heptadecane, 11-Tridecen-1-ol, 3,11-Tetradecadien-1-ol and n-Pentadecane played the antitrypanosomal role against *T. evansi*.
- iii. Further research is recommended to isolate the main active ingredient among other compounds detected by GCMS of *B. oleracea* extract.

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APPENDICES

1.ANOVA of body weights, temperature, PCV, WBC, RBC, total blood protein of rats

		Sum of squares	df	Mean square	F	Sig
Body weight	Between Groups	377.644	8	47.206	1.284	0.282
	Within Groups	1323.600	36	36.767		
	Total	1701.244	44			
Temperature	Between Groups	7.675	8	0.959	0.348	0.941
	Within Groups	99.368	36	2.760		
	Total	107.043	44			
PCV	Between Groups	4.400	8	0.550	0.127	0.998
	Within Groups	156.400	36	4.344		
	Total	160.800	44			
WBC	Between Groups	74.330	8	9.291	2.365	0.037
	Within Groups	141.408	36	3.928		
	Total	215.738	44			
RBC	Between Groups	0.372	8	0.047	0.105	0.999
	Within Groups	15.880	36	0.441		
	Total	6.252	44			
Total protein	Between Groups	0.288	8	0.036	0.368	0.931
	Within Groups	3.520	36	0.098		
	Total	3.808	44			

2.ANOVA of body weights, temperature, PCV, WBC, RBC, total blood protein of rats(cont'd)

		Sum of squares	df	Mean square	F	Sig
Body weight	Between Groups	211.378	8	26.422	0.742	0.654
	Within Groups	1281.600	36	35.600		
	Total	1492.978	44			
Temperature	Between Groups	9.658	8	1.207	0.838	0.576
	Within Groups	51.860	36	1.441		
	Total	61.518	44			
PCV	Between Groups	334.578	8	41.822	5.032	0.000
	Within Groups	299.200	36	8.311		
	Total	633.778	44			
WBC	Between Groups	80.716	8	10.090	3.059	0.010
	Within Groups	118.756	36	3.299		
	Total	199.472	44			
RBC	Between Groups	3.612	8	0.451	0.966	0.477
	Within Groups	16.820	36	0.467		
	Total	20.432	44			
Total protein	Between Groups	7.191	8	0.899	10.534	0.000
	Within Groups	3.072	36	0.085		
	Total	10.263	44			

3.ANOVA of body weights, temperature, PCV, WBC, RBC, total blood protein of rats(cont'd)

		Sum of squares	df	Mean square	F	Sig
Body weight	Between Groups	214.978	8	26.872	0.796	0.610
	Within Groups	1216.000	36	33.778		
	Total	1430.978	44			
Temperature	Between Groups	4.568	8	0.571	0.653	0.728
	Within Groups	31.480	36	0.874		
	Total	36.048	44			
PCV	Between Groups	197.687	8	24.711	1.013	0.444
	Within Groups	878.432	36	24.401		
	Total	1076.119	44			
WBC	Between Groups	119.948	8	14.993	3.248	0.007
	Within Groups	166.204	36	4.617		
	Total	286.152	44			
RBC	Between Groups	0.744	8	0.093	0.201	0.989
	Within Groups	16.640	36	0.462		
	Total	17.384	44			
Total protein	Between Groups	2992.160	8	374.020	327.068	0.000
	Within Groups	41.168	36	1.144		
	Total	3033.328	44			

4.ANOVA of body weights, temperature, PCV, WBC, RBC, total blood protein of rats(cont'd)

		Sum of squares	df	Mean square	F	Sig
Body weight	Between Groups	255.500	7	36.500	1.211	0.325
	Within Groups	964.400	32	30.138		
	Total	1219.900	39			
Temperature	Between Groups	72.567	7	10.367	10.093	0.000
	Within Groups	32.868	32	1.027		
	Total	105.435	39			
PCV	Between Groups	349.900	7	49.986	8.262	0.000
	Within Groups	193.600	32	6.050		
	Total	543.500	39			
WBC	Between Groups	274.978	7	39.283	11.224	0.000
	Within Groups	112.000	32	3.500		
	Total	386.97	39			
RBC	Between Groups	3.600	7	0.514	11.224	0.407
	Within Groups	15.428	32	0.482		
	Total	19.028	39			
Total protein	Between Groups	5.456	7	0.779	5.413	0.000
	Within Groups	4.608	32	0.144		
	Total	10.06	39			

5.ANOVA of body weights, temperature, PCV, WBC, RBC, total blood protein of rats(cont'd)

		Sum of squares	df	Mean square	F	Sig
Body weight	Between Groups	244.975	7	34.996		0.344
	Within Groups	952.800	32	29.775		
	Total	1197.775	39			
Temperature	Between Groups	103.926	7	14.847		0.000
	Within Groups	62.212	32	1.944		
	Total	166.138	39			
PCV	Between Groups	446.375	7	63.768		0.000
	Within Groups	158.400	32	4.950		
	Total	604.775	39			
WBC	Between Groups	245.068	7	35.010		0.000
	Within Groups	35.280	32	1.103		
	Total	280.348	39			
RBC	Between Groups	2909.216	7	415.602		0.000
	Within Groups	40.800	32	1.275		
	Total	2950.016	39			
Total protein	Between Groups	4.812	7	0.687		0.000
	Within Groups	3.504	32	0.110		
	Total	8.316	39			

6.ANOVA of Parasitaemia of rats

		Sum of squares	df	Mean square	F
Parasitaemia (Day 1)	Between Groups	0.000	8	0.000	
	Within Groups	0.000	36	0.000	
	Total	0.000	44		
Parasitaemia (Day 2)	Between Groups	415.778	8	51.972	8.662
	Within Groups	216.00	36	6.000	
	Total	631.778	44		
Parasitaemia (Day 3)	Between Groups	1310.000	8	163.750	6.374
	Within Groups	924.800	36	25.689	
	Total	2234.800	44		
Parasitaemia (Day 4)	Between Groups	8982.400	8	7372.800	9.529
	Within Groups	27852.800	36	773.689	
	Total	86835.200	44		
Parasitaemia (Day 5)	Between Groups	61895.111	8	7736.889	11.333
	Within Groups	24576.000	36	682.667	
	Total	86471.111	44		
Parasitaemia (Day 6)	Between Groups	68994.844	8	8624.356	27.071
	Within Groups	11468.800	36	318.578	
	Total	80463.644	44		
Parasitaemia (Day 7)	Between Groups	96520.711	8	12065.089	42.528
	Within Groups	10213.200	36	283.700	
	Total	106733.911	44		
Parasitaemia (Day 8)	Between Groups	70348.800	8	10049.829	98.143
	Within Groups	3276.800	36	102.400	
	Total	73625.600	44		

7.ANOVA of Parasitaemia of rats(cont'd)

Parasitaemia (Day 9)	Between Groups	123206.800	8	17600.971	67.285
	Within Groups	8370.800	36	261.588	
	Total	131577.600	44		
Parasitaemia (Day 10)	Between Groups	113767.500	8	16252.500	61.385
	Within Groups	8472.400	36	264.763	
	Total	122239.900	44		
Parasitaemia (Day 11)	Between Groups	106628.175	8	15232.596	513.530
	Within Groups	949.200	36	29.663	
	Total	107577.375	44		
Parasitaemia (Day 12)	Between Groups	108154.375	8	15450.625	2183.834
	Within Groups	226.400	36	7.075	
	Total	108380.775	44		
Parasitaemia (Day 13)	Between Groups	112851.975	8	16121.711	6614.035
	Within Groups	78.000	36	2.438	
	Total	112929.975	44		
Parasitaemia (Day 14)	Between Groups	112622.175	8	16088.882	6810.109
	Within Groups	75.600	36	2.363	
	Total	112697.775	44		

8.ANOVA of organ weights of rats

		Sum of squares	df	Mean square	F	Sig.
Brain	Between Groups	0.102	7	0.015	3.021	0.032
	Within Groups	0.077	16	0.005		
	Total	0.179	23			
Liver weight	Between Groups	17.131	7	2.447	21.675	0.000
	Within Groups	1.807	16	0.113		
	Total	18.938	23			
Testis weight	Between Groups	0.104	7	0.015	1.379	0.280
	Within Groups	0.172	16	0.011		
	Total	0.275	23			
Kidney weight	Between Groups	0.301	7	0.043	3.772	0.013
	Within Groups	0.182	16	0.011		
	Total	0.484	23			
Heart weight	Between Groups	0.300	7	0.043	3.306	0.023
	Within Groups	0.207	16	0.013		
	Total	0.507	23			
Spleen weight	Between Groups	0.245	7	0.035	9.880	0.000
	Within Groups	0.057	16	0.004		
	Total	0.301	23			