Evaluation of Anti-trypanosomal Activities of Fermented Wheat germ and Garlic bulb Extracts in *T. brucei*-infected rats

BY

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APPROVAL PAGE

The thesis has been read and approved as meeting the requirement of the Department of Biochemistry, University of Ilorin, Nigeria for the award of degree of Doctor of Philosophy (Ph.D.) in Biochemistry.

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DEDICATION

This work is dedicated to almighty Allah (S.W.T) who gives me life and strength to carry out this project and to my late mother, Mrs. Sherifat Omobolaji Yusuf, may the almighty Allah grant you al-janal.

ACKNOWLEDGEMENT

Praise be to ALLAH, the most gracious, the most merciful. He (alone) we worship and he alone we seek for help. The almighty that is never weary of protecting me from childhood to this present stage of life.

I wish to express my sincere and unreserved appreciation to my supervisor Prof. Clement O. Bewaji. Thank you for the painstaking and scholarly guidance.

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My profound gratitude goes to my parents, brothers and sisters for their love, care, support and encouragement not only in this course of this work. I love you all.

At this juncture, l acknowledge everyone that contributed immensely in one way or the other to the successfully completion of this work. Although, I cannot

mention everybody's name, Almighty God knows you and equally appreciates you all.

Finally to my beloved one Mr Olanrewaju Ibrahim Shittu, l pray Allah reward you accordingly.

YUSUF K.O

2010

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ABSTRACT

Ethyl acetate extract of wheat (*Triticum aestivum*) and methanolic extract of garlic (*Allium sativum*) were obtained by fermenting powdered wheat germ and garlic bulb. The powdered wheat germ and garlic bulb were extracted with ethyl acetate and methanol separately. The ethyl acetate extract of wheat and methanolic extract of garlic were partially purified by column chromatography to give fractions A-E and A-D respectively. Phytochemical screening of the extracts revealed that fermented wheat and garlic bulb contained high percentage of glycoside, saponins and alkaloids. Administration of the extracts shows reduced parasitaemia and extension of life span from 8 days in the infected untreated (control) group to 14 days of the infected wheat treated and 17days in infected garlic treated group.

The effects of ethyl acetate extract of wheat (*Triticum aestivum*) and methanolic extract of garlic (*Allium sativum*) on some serum and liver enzymes as well as some hematological and electrolyte profile in rats infected with *Trypanosoma brucei* were also studied. There were significant increases($p<0.05$) in the haemoglobin (Hb) concentration, packed cell volume (PCV) and red blood cell

(RBC) counts in infected treated groups when compared with infected untreated (control), while there was no significant difference in mean cell haemoglobin concentration(MCHC).. The results also showed significant increases ($p< 0.05$) in the activities of serum aspartate transaminase (AST) of the infected untreated group when compared with the infected treated groups as well as significant decrease in liver alanine transaminase (ALT) and serum alkaline phosphatase (ALP). There is also significant decrease in liver catalase activities of infected untreated compared with infected wheat treated groups while there were significant increase in serum and liver SOD of infected garlic treated when compared with infected untreated groups.

Crude extract of fermented wheat and garlic bulb were partially purified using column chromatography and characterized. N- Hydrocarbon and oxygenated hydrocarbon were conclusively identified in the extracts by gas chromatographic – mass spectral GC/MS analysis and ^{1}H- NMR experiments

Trypanosomal brucei ribonucleotide reductase was analyzed using bioinformatic program. R1 and R2 subunits of ribonucleotide reductase sequence were analyzed to contain transmembrane domain at five different positions for R1 and two

positions for R2. The N-terminal signal have been identified on length 11 for both subunits with possible cleavage site between 36 and 37 for R1 and 39and 40 for R2.

Therefore, the results suggest that the crude extracts (fermented wheat and garlic bulbs) could be potential agents in the management of African sleeping sickness.

CHAPTER ONE

INTRODUCTION/LITERATURE REVIEW

1.1 INTRODUCTION

Trypanosomiasis is a disease caused by the parasitic protozoa, trypanosomes of the genus *Trypanosoma*. They are microscopic elongated unicellular organisms that live and multiply in the blood and other body fluids of their host, causing sleeping sickness in humans and related diseases in domestic animals (ILRAD, 1991). They belong to five well differentiated subgenera which include all the ten less defined species of the genus *Trypanosoma* (Losos, 1986). Species of this parasite include*; T. brucei, T. congolense, T. vivax, T. equiperdum, T. envansi, T. enquinum* and *T. cruzi.*

Trypanosoma brucei, a distant relative of malaria parasite, is the principal scourge of Africa. It consists of three subspecies, two of which infect humans. *T. brucei rhodesiense* found in East Africa and southern Africa, *T. brucei gambiense* found in West Africa and *T. brucei brucei* which is not infectious to humans but in cattle causes a wasting disease called Nagana (Gull, 2005).This disease occurs in the northern part of Nigeria (Igwe and Onabanjo, 1989). The tsetse fly belts of sub-Sahara Africa are between 14^0N and 20^0S. Saliva of the blood sucking female tsetse flies found in Africa in a belt that stretches south of the Sahara and north of the kalahari (Gull, 2005).

Infection of the mammalian host starts with the bite by an infected tsetse fly (*Glossina spp*), which injects the metacyclic trypanomastigote form of the parasite in its saliva before taking its blood meal. The trypanosomes multiply locally at the site of the bite for a few days before entering the lymphatic system and the blood stream through which they reach other tissues and organs including the Central Nervous System (CNS) (WHO, 2001; Wellcome News,

2005). In the blood stream, the parasite can be attacked by the human immune system and get round this through a process of antigenic variation. In the mammalian host, the trypomastigote cell is completely covered by a dense monolayer of the identical glycoprotein that protect the parasite against direct lyses by complement (Borst and Fairlamb, 1998). Only when specific antibodies are present against the surface epitopes is the parasite destroyed. The antibodies kill most parasites, but small but sufficient fraction of the parasite population have switched coat and escaped the body's onslaught (Borst, 2002) and proliferate until the new generation of specific antibodies, mainly of the immunoglobulin M (IgM) type are developed by the host. Up to 1,000 different genes encoding the variant surface glycoprotein are present in the *T.brucei* genome (Vanhamme *et al.*, 2001). This phenomenon explains the fluctuating number of circulatory typanosomes in the patients blood (Ross and Thompson, 1910), which contributes to the limited sensitivity of parasite detection methods in clinical practice.

Chemotherapy available for control and eradication of trypanosomiasis is very limited at the moment. Effective trypanocidal drugs available are beset with problems of drug resistance and toxicity (Pepin and Milford, 1994). In addition to emerging cases of drug resistance, drugs require lengthy, parenteral administration and almost all trypanodical drugs have severe side effects, thus, underscoring the urgent need to develop more effective and safer trypanocidal drugs. Several reports on evaluation of different chemicals/drugs for trypanocidal activity have appeared just as interesting reports on the anti-trypanosomal effects of plant extracts (Asuzu and Chineme, 1990). Some of these reports have indeed shown that some plants possess trypanocidal activities that caused be used in the management of African Trypanosomiasis.

1.2 LITERATURE REVIEW

1.2.1TRYPANOSOMES DESCRIPTION AND CLASSIFICATION

Trypanosomes are unicellular protozoan (Fig 1) with a single flagellum that contains microtubules in the 9+2 arrangement typical of

other flagellates. It belongs to the class Zoomastigophora and order Kinetoplastida, so-called because of the large DNA containing structure, the kinetoplast, found at the base of the flagellum which aids them in movement. The kinetoplast encloses a large amount of mitochondrial DNA and for the parasite to survive, the kinetoplast and its DNA contents must be faithfully copied at each cell division and segregated into two daughter cells (Gull, 2005).

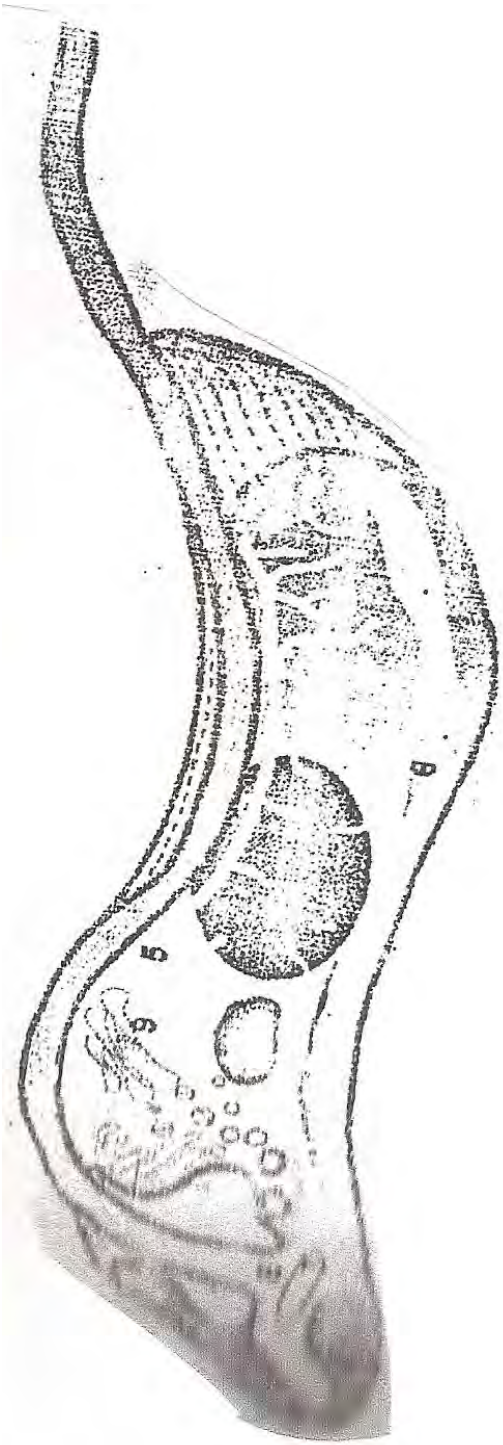

Fig 1: A simplified view of the trypanosome

Source: Wellcome News (2005)

The classification of trypanosomes is as shown below:

Phylum	Protozoan
Sub-phylum	Sarcomastigophora
Super class	Mastigophora
Class	Zomastigophora
Order	Kinetoplastida
Family	Trypanosometidae
Genus	*Trypanosoma*

1.2.2 TRYPANOSOMIASIS

Trypanosomiasis is a chronic disease caused by the protozoan blood parasite trypanosome of the genus *Trypanosoma*. In humans, it is referred to as sleeping sickness while in cattle and other animals which serve as a reservoir for the protozoa, the disease is called Nagana. The variation of the disease occurs in Central and Western Africa, both of them transmitted in the salivary glands of infected

tsetse flies. *T. brucei* consist of three sub-species, two of which infect humans, these include *T.brucei gambiense* being the most common and a more local version *T. brucei rhodesiense*. The third subspecie which is not infectious to humans but in cattle is the causative agent of bovine trypanosomiasis (Nagana) also known as *T. brucei brucei* (Treoberg *et al.,* 1999). In South America, another version of the disease known as chagas disease caused by T.cruzi which is transmitted by the triatomid bug of the family Rediviidae (Bailey *et al.,* 2005).

Generally, trypanosomiasis are present in three forms;

i. Human African trypanosomiasis (HAT) or African sleeping sickness
ii. Human American trypanosomiasis or chagas disease.
iii. African Animal Trypanosomiasis (AAT) or Nagana.

Human African trypanosomiasis (HAT) or sleeping sickness, is a disease caused by *T. brucei gambiense* or *T. brucei rhodesiense*. The two are transmitted by the genus *Glossina* (order Diptera) and are

restricted to Sub-Saharan Africa. Both are fatal if left untreated. HAT is the prototype of a neglected disease, affecting the poorest people of the poorest continent (Trouiller *et al.*, 2002).

1.2.3 EPIDEMIOLOGY OF THE DISEASE

It is estimated that 60 million people are exposed to HAT in nearly 200 separate active foci from 36 Sub-Saharan countries but only 4 million to 5 million are under surveillance (WHO, 1998). The location of endemic foci of HAT follows the patchy distribution of tsetse flies found in a belt that stretches south of the Sahara desert and north of the Kalahari Desert (Barrett *et al.*, 2003). Despite the absence of reliable epidemiological figures, the World Health Organization (WHO,1998; WHO, 2001) reports that 300,000 to 500,000 people might be infected by the *T.brucei gambiense* form of the disease in Western and central Africa. The most severely affected countries are the Democratic Republic of Congo, Angola, Central African Republic and Southern Sudan, where HAT has remerged during the last decades mainly due to long-standing geopolitical instability and subsequent erosion or collapse of control programmes (Moore and Richer, 2001). *T.b gambiense* is transmitted by the bite of tsetse flies of the *Glossina palpalis* or *G. fuscipes* groups. Human vector contact occurs mostly in

forested rivers and shores but is also peridomestic when huts are built in or near plantations (Burri and Brun, 2003).

T .b. rhodesiense HAT is a zoonosis present in Eastern and Southern Africa. Wild animals including game animals are usually affected, but epidemics occasionally occur in domestic animals and humans. The parasite is transmitted by the bite of tsetse flies of *Glossina morsitans* or *G. fuscipes* group. Human-vector contacts typically occur in savanna woodland but can be peridomestic during epidemics (Burri and Brun, 2003). The incidence of *T. b. rhodesiense* HAT is currently much lower than that of *T. b. gambiense* HAT, but large epidemics were observed in the past. Not more than 50 cases of HAT are diagnosed yearly outside Africa (Lejon *et al.,* 2003). Visitors to some game parks in Eastern Africa are at particular risk for *T. b. rhodesiense* HAT (Jelinek *et al.,* 2002).Migrants from countries where *T. b. gambiense* is highly endemic can have HAT that remain unrecognized for years (Sahlas *et al.,* 2002). Studies conducted in Western and Central Africa have failed to find an increased risk of

HAT among Human Immunodeficiency Virus (HIV) infected individuals, but no definite conclusion can be drawn from the available data (Louis *et al.*, 1991).

In Nigeria, trypanosomiasis has a severe impact on livestock and human. Economic losses due to tsetse flies and trypanosomiasis have never been fully quantified (PAAT, 2006).

1.2.4 TRANSMISSION AND LIFE CYCLE

There are two stages in the life cycle of trypanosome is the tsetse fly stage and the human stage, details of the two stages are shown in Figure 2 below. The trypomastigote is the only form to be observed in the mammalian host, whereas the epimastigote form occurs during the development phase in the tsetsefly. During the entire life cycle, *T. brucei* cells multiply by binary fission and are considered to be exclusively extracellular.

The cycle begins when the tsetse fly *(Glosinna spp*) feeds on blood from infected hosts (reservoir hosts). The trypomastigote

multiply in the mid-gut of the insect vectors for 10 – 15 days and then migrate toward the anterior portion of the gut. On reaching the salivary gland, few days later, the organism transforms into epimastigotes, which attached to tissue by their thin flagella and multplies by binary fission. After some days of infection, the epimastigote begins to transform back to metacyclic trypomastigotes and this form the infective parasite stage (WHO, 1998). Two different trypomastigote forms can be observed in the mammalian host: a long, slender proliferative form and a short, stumpy non-dividing form. Both forms are taken up by the tsetse fly, but only the later is able to complete the complex cycle (Fig 2).

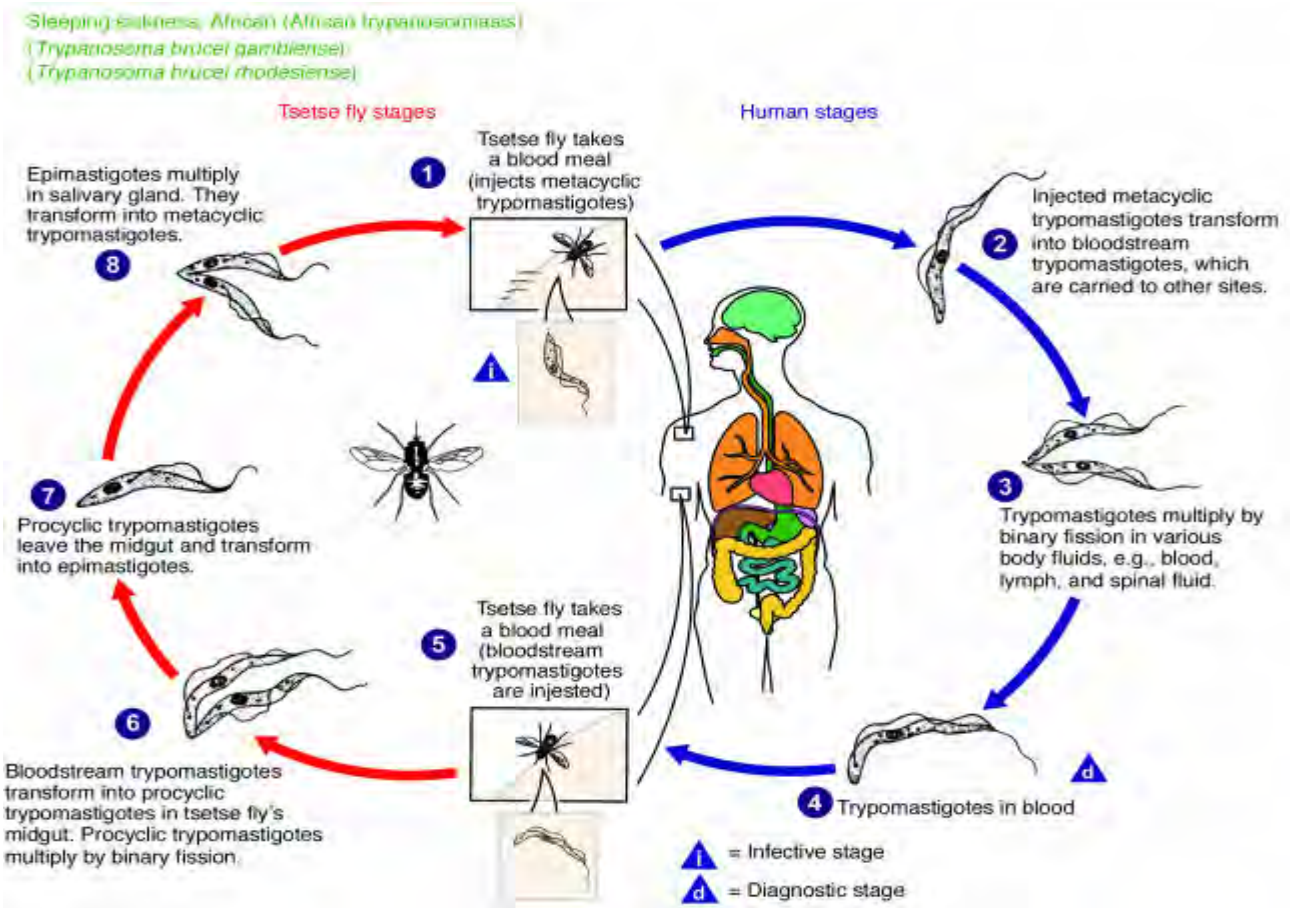

Fig 2: The life cycle of *Trypanosoma brucei* in the human and the tsetse fly.

Source: Alexander and Melanie (2008)

1.2.5 HOST-PARASITE RELATIONSHIP

Antigenic variation is a remarkable and an extraordinary phenomenon by which trypanosomes evade the host defence system. It is characterized by periodic fluctuation in parasitaemia during the course of the infection (WHO, 2001).

The blood stream trypanosomes are endowed with a 10-12mm thick surface coat (Vickerman, 1969), which consist of a single species of tightly packed glycoprotein, the variant surface gylycoprotein (VSG) (Cross, 1975) that is embedded in the outer leaflet of the lipid bilayer via a glycosyl-phosphatidayl-inositol (GPI) anchor. During the course of infection, the trypanosomes are able to express many kind of surface proteins known as variable surface glycoprotein's (VSG'S) (ILRAD, 1991). Thus, each trypanosome in the population expresses a particular surface variant antigen type (VAT) at any one point in time. Host antibodies produced in response to one VSG (=VAT) are ineffective against the antigenically different VSG'S, which appears in subsequent paratiaemic waves. Hence, each

successive and antigenically distinct trypanosome population which arises, can evade the immune responses (i.e VAT –specific antibodies) of the host to earlier VSG's. Antigenic variation is known to be antibody independent, non random and under genetic control; the number of possible VSG's, but not finite. The role of each successive VAT- specific and antibody produced by the host is selective rather than inductive, at any one point in time during infection of small number of new (heterotype) VATS are expressed by some few individual trypanosomes among the predominant homotype population.

The production of host antibodies in response to the numerous, successive homotypes results in their destruction, but allows the few new heterotypes to survive and proliferate in their place, thus producing a new parasitaemic wave. The greater the heterogeneity of VATS (i.e VSGS), sequentially expressed, the more likely is infection to persist, the response of an individual trypanosome to changing host conditions (antibodies) is far less important, if at all, than the

cumulative response of the heterotype/homotype population as a whole.

1.2.6 CLINICAL AND PATHOLOGICAL MANIFESTATION OF THE DISEASE

The clinical manifestations that characterize sleeping sickness are classified to the clinical progression of the disease that is the haematolymphatic or first stage and the meningoencephalitic or second stage (WHO, 1998). The clinical signs are generally unspecific and their frequency varies between individuals and disease foci.

(A) First stage signs and symptoms

Clinical manifestation of trypanosomiasis is hard and painful. At the site of inoculation, a painless skin lesion, the trypanosome chancre develops (Molyneux, 1984; WHO, 1998).

(ii) **Lymphadenopathy:** lymph glands swell as the parasites multiply within; in Gambian sleeping sickness swollen neck glands are useful sign. Lymph glads can be a useful source of parasites for diagnosis (WHO, 1998; Pentreath and Kennedy, 2004).

(iii) **Fever:** Waves of parasiteamia follow with fever; the pathological effect of these invasive waves on the composition of the blood (WHO, 1998; Pentreath and Kennedy, 2004).

(iv) **Headache:** Headache is the most frequent complaint and is typically severe and persistent (Dumas and Girard, 1978; WHO, 1998; Pentreath and Kennedy, 2004).

(v) **Hepatomegaly and splenomegaly**: Enlargement of the liver and spleen may be revealed by examination of abdomen (Dumas and Girard, 1978; WHO, 1998; Pentreath and Kennedy, 2004).

(vi**)** **Anaemia:** Anaemia is frequent and may be severe causing cardiac failure. It is often detected by examination of the conjunctive (Molyneux, 1984; WHO, 1998).

(vii) **Skin rash:** Skin rash known as trypanids occur as ring like patches with polycyclic contours of 1-10cm diameter. These patches are not easily recognizable on dark skin (WHO, 1998).

(viii) **Musculoskeletal pains:** Muscle aches and joints pains are also very frequent but are common in most febrile illness (WHO, 1998).

(B) Second stage signs and symptoms

(i) **Neurological signs**: Neurological signs and symptoms are specific to the second stage. These include cranial nerve dysfunction, abdomal reflexes, neuronegative disorders and deterioration of consciousness which may lead to coma (Legon *et al.*, 2003).

(ii) **Sleep disturbance:** The circadian rhythm of sleep and wakefulness disappears. Periods of sleep and wakefulness may occur at any time of the day and night; the periodicity varies according to the severity of the disease (WHO, 1998).

(iii) **Alteration of mental state:** Mental confusion and temporospatial disorientation may occur. Psychiatric disorders varied may include personality disorders, behavioural changes and alteration of mood such as euphoria or depression (Molyneux, 1984; WHO, 1998; Pentreath and Kennedy, 2004).

(iv) **Tone disorders**: Hypertonia (extrapyramidal origin) or hypotonia (cerebellar orgin due to sensory disorders) may occur (WHO, 1998).

Other neurological disorders include: Convulsion, cranial nerve dysfunction, deterioration of consciousness, which may lead to coma Molyneux, 1984; WHO, 1998; Pentreath and Kennedy, 2004).

1.2.7 **BIOCHEMISTRY OF DRUG TARGETS IN TRYPANOSOMES**

The biochemistry of trypanosome is centred on the survival in the host and selective elimination. The design of drugs based on different metabolic pathways and possible points for drugs target(Antitrypanosomal targets), which covers purine and pyrimidine metabolism; glycolysis; trypanothione and trypanothione reductase; tryparedoxins; polyamine and ornithine decarboxylase; mitochondrial DNA, antigenic variation in African trypanosome; protein farneylation, and cysteine proteases (Mansour,2002).

(1) Nucleic acid as a target for antitrypanosomal

Purine and pyrimidine metabolism are target for antitrypanosomal by the enzyme, ribonucleotide reductase.

Ribonucleotide reductase (RNR) is the enzyme which catalyses the rate limiting step in the *de novo* synthesis of DNA precursor molecules (Thelander and Graslund,1994). The enzyme catalyses the reduction of ribonucleotides to deoxyribonucleotides, the precursor for DNA synthesis.A reation assisted by a protein bound 5- deoxyadenosyl cobalamin- derived free radical. There are three different classes of RNRs, where most of the eukaryotic and some prokaryotic organisms belong to class I. Class I RNR is a heterodimeric enzyme of $\alpha_2\beta_2$ type. The large R1 subunit binds substrates and allosteric effectors, while the small R2 subunit contains a tyrosyl radical (Hofer *et al.*, 1997; Hofer *et al.*, 1998), generated by a binuclear ferric iron center. The tyrosyl radical in the R2 protein is linked to the active site in the R1 subunit through a hydrogen-bonded long-range electron transport chain. Class I and to some extent class III RNRs are inhibited by hydroxyurea, which acts as a specific radical scavenger. Since hydroxyurea inhibits the growth of *T. brucei* under aerobic conditions, the parasite presumably contains a class I enzyme.

(2) Glycolysis

Glycolysis is the major pathway for the utilization of glucose and is found in the cytosol of all cells. It is a unique pathway in that it can utilize oxygen if available (aerobic) or function in the total absence of oxygen (anaerobic). However, oxidized glucose beyond pyruvate, end stage of glycolysis requires not only molecular oxygen but also mitochondrial enzyme such as pyruvate dehydrogenase complex, the citric acid cycle and the respiratory chain (Mayes, 1996). The blood forms of African trypanosomes are entirely dependent on carbohydrate metabolism for their energy needs. This metabolism proceeds at an extremely high rate and differs in its organization from that found in other eukaryotes. The enzyme lactate dehydrogenase is absent (Dixon, 1966) and the NADH generated in glycolysis is reoxidized indirectly by molecular oxygen via dibydroxyacetone phosphate: glycerol-3- phosphate oxidase and NAD linked glycerol -3- phosphate are involved (Grant *et al.*,

1960). This pathway is target by suramin. The drug has their sensitivity because mammals do not use this metabolic pathway.

(3) Antigenic variation

Trypanosome lives extra-cellularly in evading immune response by a process known as antigenic variation. The variant antigen is the glycosyl phosphatidyl inositol (GPI), anchored variable surface glycoprotein (VSG), which coats the entire surface of trypanosomes (Milne, 1999) except for the flagella pocket, which is the only area that is involved in uptake of nutrients from the outside. Trypanosome has a unique pathway for fatty acid synthesis contrary to views that trypanosome could not synthesized fatty acids. The major product of this pathway is myristic acid which trypanosome require in massive amounts to produce their glycosyl phosphatidyl inositol (GPI) anchors on their surface proteins. Antigenic variation has a lot to do with the way the parasite grows in the host. The glycosyl phosphatidyl inositol (GPI) anchor fixes the coat to the cell surface is a potential drug target (Wellcome News, 2005).

1.2.8 TREATMENT FOR SLEEPING SICKNESS

The current chemotherapy of sleeping sickness depends on very old drugs (Atouguia and Costa, 1999; Keiser *et al.*, 2001). The present arrays of available drugs are limited based on the following problems including drug resistance and toxic side effects. Generic for variable efficacy and formulations below claimed level, lead to idea condition for resistance to develop. Poor infrastructure and political upheavals add to the difficulties of maintaining desired standards of use (Barrett *et al.*, 2004).

Drugs for the treatment of first stage of the infection

Pentamidine and suramin are registered for treatment of the first stage of the disease.

Pentamidine: Pentamidine (Fig 3)was introduced in 1939 for the treatment of first stage African trypanosomiasis caused by *T. b.* gambiense (Seebeck *et al.*, 1999). It is poorly absorbed from the

gastrointestinal tract and therefore has to be injected intramuscularly, which severely complicates its application under fluid conditions. Its side effects include abortion, nephrotoxicity, excessive insulin release followed by insufficiency confusion and hallucination.

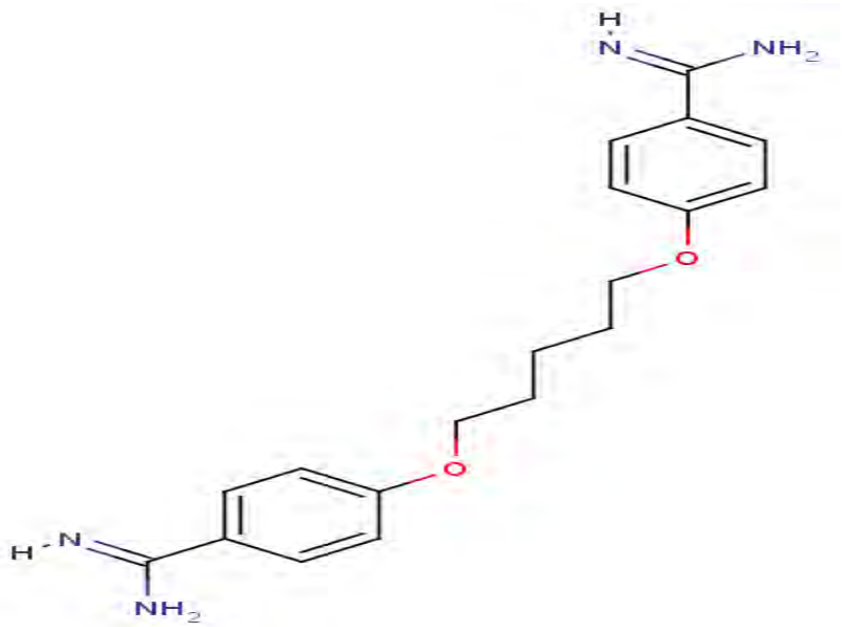

Fig. 3: Pentamidine

Source: Seebeck *et al.* (1999)

Suramin: Suramin sodium (Fig 4) was introduced in 1992 for the treatment of sleeping sickness (WHO, 1998). It is supplied as a white

powder for the injection that is readily soluble in water. It is also plagued by considerable side effects such as fatal collapse after the first injection, heavy proteinuria, stomal ulceration, exfoliative dermatitis, severe diarrhea etc (Seebeck *et al.*, 1999). Although, both drugs for first stage treatment are quite effective, they do not penetrate the blood-brain-barrier, and thus they are ineffective for the treatment of second or late stage trypanosomiasis (Seebeck *et al.*, 1999).

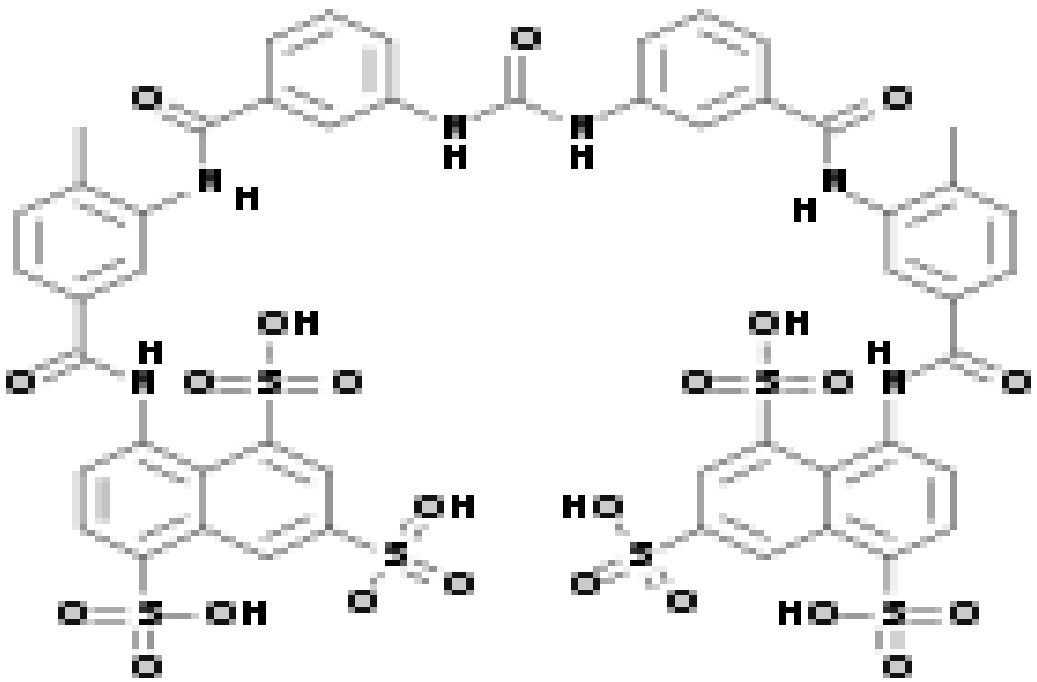

Fig. 4: Suramin

Source: Seebeck *et al* (1999)

Drugs for late stage infection

Melarsoprol: Melarsoprol is an arsenical compound that was introduced in 1949 (WHO, 1998). It is a mixture of melarsenoxide and dimercaprol. Melarsoprol is fairly effective but ifs use is prone to cause serious side effects, particularly a high risk of total

encephalopathy (Seebeck *et al.*, 1999). Another problem with melarsoprol is severe tissue damage caused by its solvent (3.6% propylene, glycol) upon injection (Ginoux *et al.*, 1984). The serious venous damaged provoked by melarsoprol treatment added to the problems of using intravenous injections in rural areas. Therefore, this was the reason for the development of tropical melarsoprol formulation (Atouguia and Costa, 1999).

Nifurtimox: Nifurtimox is a synthetic nitrofuran that was developed for the treatment of American trypanosomiasis (Chagas disease) caused by *T.* cruzi (WHO, 1998). The mechanism of action of nifurtimox is unknown. Toxic side effects involving the central and peripheral nervous system are common and patients may be tempted to discontinue treatment if not under supervision. Other side effects include: convulsion, psychiatric reactions, vertigo, disturb sense of balance, polyneuropathy, gastro-intestinal discomfort and skin rash (WHO, 1998).

Eflornithine (difluoromethyl-ornithine or DFMO) the most modern treatment was registered in USA, 1990 and Europe, 1991 and by the end of 1995 had been registered in seven African countries (WHO, 1998). DFMO is an irreversible inhibitor of the enzyme ornithine decarboxylase. The enzyme catalyzes the synthesis of putrescine from ornithine and it is a central player in the biosynthesis of polyamine and nucleotides in trypanosomes as well as animals. EEfornithine is the only new molecule registered for the treatment of human African trypanosomiasis over the last 50 years. It is the drug used mainly as a backup for melarsoprol refractory *T. b.* gambiense case. The most commonly used dosage regime for the treatment of *T. b.* gambiense sleeping sickness of 100mg/kg body weight at interval of 6h for 14 days (150 mg/kg body weight in children) of eflornithine given as short infusions. Its efficacy against *T. b.* rhodesience is limited due to the innate lack of susceptibility of this parasite based on a higher ornithine decarboxylase turnover. Adverse drug reactions during

efornithine therapy are frequent and the characteristics are similar to other cytotoxic drugs for the treatment of cancer. Their occurrence and intensity increases with the duration of treatment and the severity of the general condition of the patient. Generally, adverse reactions to efornithine are reversible after the end of treatment. They include convulsion, gastro- intestinal symptoms like nausea, vomiting and diarrhea, bone marrow toxicity leading to anaemia, leucopenia and thrombocytopenia, hearing impairment and alopecia. The drug arrests embryonic developments and rabbits but the extent of excretion into breast milk is unknown (Burri and Brun, 2003).

1.3 Medicinal plants

As source of remedies for the treatment of many diseases dated back to prehistory and people of all continents have this old tradition. Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from plants. In developing

countries where medicines are quite expensive, investigation on antimicrobial activities from ethanomedicinal plants may still be needed. It is obvious that these phytochemicals will find their way in the antimicrobial drugs prescribed by physicians (Cowan, 1999). Notably in West Africa, new drugs are not often affordable. Thus, up to 80% of the populations use medicinal plants as remedies (Kirby, 1996; Hostettman and Marston, 2002). Therefore, in this study, Wheat germ and garlic bulb which are natural food source and known to contain excellent nutrient for weak immune systems and antioxidant properties would be employed.

1.3.1 DESCRIPTION AND CONSTITUENT OF *Allium sativum .L.* (GARLIC)

Allium sativum L., commonly known as garlic, is a species in the onion family *Alliaceae*. Its close relatives include the onion, the shallot and the leek. Garlic has been used throughout recorded history for both culinary and medicinal purposes. It has a characteristics

pungent, spicy flavour that mellows and sweetens considerably with cooking (McGee, 2004).A garlic bulb, composed of 4-60 cloves, can be 1½-3 inches in diameter (4-7.5 cm.) and grow to a height of 10 inches to 5 feet (10 cm.-1½ m)(Fig 5). The flowers are white with a rose or green cast. The bulbs themselves are creamy white and may have a purplish hue, as may the paper-like covering that surrounds the bulb and encloses each clove.

Fig. 5: *Allium sativum*
Source: William (1793).

The cloves are used as seed for consumption (raw or cooked), and for medicinal purpose. A number of the Allium family, which also include onion, garlic is rich in a variety of powerful sulphur containing compounds including thiosulfinates (best known compound is ajoene). While these compounds are responsible for garlic characterically pungent odour, they are also the source of many of its health-promoting effects. In addition garlic is an excellent source of manganese, a very good source of vitamin B6 and Vitamin C and a good source of selenium (Balch and Phyllis, 2000).

One of the major health benefits of garlic is its cardiovascular benefits due to its effect on blood pressure, platelets aggregation, serum triacylglycerol level and cholesterol level (Tapsell *et al.*, 2006). Vitamin C is the body's primary antioxidant defender in all aqueous areas such as the blood stream where it protects low density lipoproteins (LDL) cholesterol from oxidation (Durak *et al.*, 2004). The selenium in garlic not only helps prevent heart disease, but also protect against cancer and heavy metal toxicity, a co-factor of

glutathione peroxidase (one of the most important internally produced antioxidants (Andorfer *et al.*, 2003). Selinium also works with Vitamin E in a number of vital antioxidant systems. Since vitamin E is one of the body defenders in all fat soluble areas, while Vitamin C protects water soluble areas. Manganese also functions as co-factor in a number of other important antioxidant defense enzymes, for example superoxide dismutase studies have found that in adults deficient in manganese, the level of High Density lipoprotein (HDL) (the "good form" of cholestrol) is decreased. Garlic also contains compounds that inhibit lipoxygenase and cyclooxygenase, which are the enzymes that generate inflammatory prostaglandins and thromboxins, thus reducing inflammation. One of the sulphur compounds *allicin* is a powerful antibacterial and antiviral agent that works in hand with vitamin C to help kill harmful microbes (Lee *et al*, 2003).

When crushed, *A. sativum* also yields an antifungal compound (phytonacide). However due to poor bioavailability it is of limited use

for oral consumption. It also contains alliin, ajoene, enzymes, vitamin B, minerals and flavonoids.

In modern naturopathy, garlic is used as a treatment for intestinal worms and other intestinal parasites, both orally and as anal suppository. Garlic cloves are used as a remedy for infection (especially chest problems), digestive disorders, and fungal infections such as thurch (Koch and Lawson, 1996).

1.3.2 DESCRIPTION AND CONSTITUENT OF WHEAT (*Triticum aestivum)*

Wheat is one of the most important domesticated crops grown around the world and plays major role among the few crop species extensively grown as food source (Harlan, 1981). Global wheat production is concentrated mainly in Australia, Canada, China, Pakistan, Russia, Turkey, Ukraine and the United States, accounting for over 80 percent production. Pakistan is the 8th largest wheat producer, contributing about 3.17% production from 3.72% of the wheat growing area. Wheat in Pakistan is a leading crop that occupies a central position in agriculture and its economy (Shuaib *et al.*, 2001). Wheat is considered a cereal largely due to the fact that it is grains contain protein with unique characteristic properties. Besides being a rich source of carbohydrates, wheat contains other valuable nutrients such as proteins, minerals (P, Mg, Fe, Cu and Zn) and vitamins like thiamine and vitamin E. However, wheat proteins are deficient in essential amino acids such as threonine (Adsule *et al.*, 1986). Globally

after maize, wheat grain is a staple food used to make flour for leavened, flat and steamed breads, cookies, cakes, pasta and for fermentation to make beer (Palmer and John, 2001). The phytochemical components of wheat include alkaloids, flavonoids, steroids and saponins. Wheat kernel contains 2-4% germ and most nutrients with the exception of starch are concentrated in the germ, the germ is the richest known natural source of tocopherol, abundant in B-group vitamins and protein of high biological value (Tsen, 1985) and its oil of favourable fatty acid pattern (Paul *et al.*, 1987). Because of their beneficial nutritional values, wheat germ and wheat bran are frequently used in human food supplements, breakfast, nutria-bars and various fibre drink mixture, therefore, they are part of the regular western diet. Avemar is the first fermented and concentrated wheat germ extract produced by an optimized process to yield 0.4 mg/g (on dry matter basis). 2, 6-dimethoxy-p-benzoquinone and given as a nutritional supplement for cancer patients. (Tian *et al.*, 1999 ; Suttle *et al.* 2000*).* Avemar is a natural fermented wheat germ extract with

no known toxicities, and it is a strong regulator of leukaemia tumor cell macromolecule synthesis, cell cycle progression, apoptosis and proliferation. Avemar regulates metabolic enzymes that are involved in glucose carbon redistribution between proliferation related structural and functional macromolecules (RNA, DNA) replication (Sukkar and Edoardo, 2004). Avemar treatment results in profound intracellular metabolic changes that bring devastating consequences for the proliferation of leukaemia cells of the lymphoid lineage. This fermented wheat germ extract has a clear and definite and anti-proliferative action that targets nucleic acid synthesis enzymes and induces cell cycle arrest and apoptosis through a cascade based mechanism as reported herein (Boros *et al.*, 2001; Nichelatti *et al.*,2002).

1.4 SERUM AND TISSUE STUDIED

1.4.1 Serum

Serum has been described as the plasma contents minus the clotting factor, the fibrin as well as fibrinogen (Prockop and Unlebruch, 1969). It contains enzymes, water, proteins, glucose, amino acids, lipids, salts, antigen, hormones, antibodies and urea. The serum proteins are of importance in the maintenance of osmotic relationship between the circulating blood and the tissue spaces and a reduction in the serum protein content could result to oedema. The proteins being amphoteric, combine with both acid and base and hence serve as buffers, maintain the level of acidity and alkalinity in the blood. The proteins are also important in the transportation of substances such as free fatty acids, bilirubin and drugs e. g barbiturates which are insoluble in plasma.

Wills (1985) reported that small alteration in the tissue composition could significantly affect the serum enzyme levels. They reported that many of the enzymes that play major roles in metabolism are only found in serum in significant quantities when the cell membranes becomes leaky and even completely haemolyzed. These

enzymes measurements are therefore valuable in clinical diagnosis, both in the initial stages of the disease and during the period of recovery and repair.

1.4.2 Tissue

The liver is the largest solid organ in the body weighing about 1200 and 1500 g (Fig 6). It lies in the right hypochondrium and is roughly wedged shaped with its apex to the left side and base to the right. It is glossy in appearance and dark red in colour due to the rich supply of blood flowing through it (Mayne, 1985, Robert, 1996; Macrae *et al.*, 1997). In the rat, liver consists of four lobes (Rowett, 1974) which are further organized into lobules. The lobules are the functional units of the liver. The liver cells are known as hepatic cells. The structural plan of the liver is adapted to the diversity of hepatocyte function. Every cell is oriented such that the plasma membrane is adjacent on one side to the sinusoids that receive blood from both portal and arterial circulation and on the other side to the

bile canaliculi that form a communicating and collecting system for the bile synthesized by the liver cells. The cells contain large numbers of lysosomes that phagacytose very actively and can therefore swell to block the passage of blood through the sinunoids, if ingested particles are not digested and dispersed.

Wright and Plummer (1974) reported that the functional mass of the liver contains four main components.

i. The hepatocyte make up about a 60% of the mass of the liver
ii. Recticulo-endothelial cell
iii. The biliary tracts
iv. The blood vessels

Fig.5 Superior (A) and Anterior (B) views of the liver structure

The anatomical site and cellular architecture of the liver enables it to perform many diverse metabolic functions that are essential to the body. These functions include; regulatory metabolism of nutrients and vitamins, formation and secretion of bile, bilirubin metabolism and excretion, inactivation/detoxification of various substances (steriods), hormones, toxins), synthesis of plasma protein and immunity (Ganon, 1997; Macrae 1997). This liver is the major site of plasma protein synthesis, including albumin, the α and β-globulins, clotting factors and transport proteins (Macrae *et al.*, 1997).

1.5 ENZYMES STUDIED

Enzymes are biological catalysts of protein nature without which no chemical process in the living organism can be effected (Tanner *et al.*, 2006). Enzymes are central to every biochemical process acting in organized sequences.

Many enzymes are present in plasma (or serum) and their activity can be easily assayed in serum with diagnostic reagents. Elevation or

reduction in the levels of activity of enzymes may indicate the presence of a disease or damage to a specific tissue (Nelson and Cox, 2005). Enzymes can be divided into serum specific and serum non-specific enzymes. Serum specific enzymes are those enzymes present in the highest concentrations in the plasma and have functional roles in plasma. They include enzymes concerned with blood coagulation, fibrin dissolution and processing of chylomicrons. Plasma non-specific enzymes are normally present in low levels and play no functional role in the plasma. In disease of tissue and organs, the non-plasma specific enzymes are most important. Normally the plasma levels of these enzymes are low, a disease may cause change in cell membrane permeability or increased cell death, resulting in release of intercellular enzymes into the plasma. Wilkinson (1962) reported that changes in enzymes levels are a good marker of soft tissue damage. They also noted that damage to body cells result in the alteration of membrane permeability and consequently release of enzyme into the

extracellular fluid (ECT). Elevated enzyme levels may result from host defense mechanism (Kennedy, 2004).

The enzymes employed in the present study are Aspartate transminase (AST), Alkaline phosphatase (ALP), Alanine transaminase (ALT), Catalse (CAT) and Superoxide dismutase. **1.5.1 Aspartate Transaminase**

Aspartate Transaminase (AST) is also known as Glutamate Oxaloacetate transaminase (GOT). It is represented as E.C. 2.6.1.1. AST is present in both cytoplasm and the mitochondria (Bell *et al.*, 1972). Fleisher and Schwartz (1971) succeeded in separating tissue and serum aspartate transaminase into a cationic and anionic fraction by paper electrophoresis using a phosphate buffer at pH 7.4, but could find no indication of heterogeneity in alanine transaminase.

Reaction Catalyzed

AST mediates reactive systems in which an amino group is transferred from an amino acid to an oxo- acid without the formation of ammonia

as an intermediate (Bell *et al.*, 1972). It catalyzes the formation of oxaloacetate and glutamate from aspartate and α – oxoglutarate as shown by the equation

α – oxoglutarate + L –aspartate $\xrightarrow{\text{GOT}}$ glutamate + oxaloacetate

Diagnosis Application

The measurement of activity of AST is very important in clinical diagnosis especially in the initial stage of the disease as well as during recovery period. The level of AST increases significantly over the normal in myocardial infection, hepatic necrosis and other disease such as active cirrhosis (Wroblewski and La Due, 1955a). In a sample, the ratio of AST to ALT is used in the differential diagnosis of jaundice (Latner, 1975).

1.5.2 AlanineTransaminase

Alanine Transaminase (ALT) is also known as Glutamate Pyruvat2 transaminase (GPT). It is represented as E.C. 2.6.1.1. ALT or GPT occurs in human blood plasma, bile, cerebrospinal fluid and saliva but it is never found in urine even with high activity of the blood plasma provided there is no kidney lesion.

Reaction Catalyzed

ALT catalyzes the formation of pyruvate and glutamate from alanine and α – oxoglutarate as shown by the equation

α – oxoglutarate + L –alanine $\xrightarrow{\text{GPT}}$ glutamate + pyruvate

Diagnosis Application

The increase, above normal in serum ALT is considered a more sensitive indicator of hepatitis and liver cell damage (Schmidt and Schmidt) than serum AST as the former is found in higher concentration in liver tissue than in heart muscles. Furthermore, unless AST is considered increased, ALT levels do not usually increased in myocardial infarction. Hence, AST and ALT activities can assist in the differential diagnosis of cardiac and liver disease (Schmidt and Schmidt).

1.5.3 Alkaline phosphatase (ALP)

Alkaline phosphatase (E.C 3.1.3.1) is known as orthophosphoric monoester phosphohydroxylase. It is a marker enzyme for endoplasmic reticulum and plasma membrane (Wright and Plummer, 1974). It is present in most tissues and organs (Summer and Somers,

1953). It is more widely distributed than the transaminases and present in liver, bones etc. It aids in the addition of phosphate groups. It has so May substrates because of its wide pH range.

Reaction Catalyzed

The optimum pH of alkaline phosphatase (ALP) varies from 8.5-10.0 and is influenced by the type of substrate employed, concentration of the type and concentration of activation and finally, the buffer employed (Ahmed and King, 1960).

ALP hydrolyses phosphate group in human liver and intestine (Helen-Easton and Moss, 1967). The reaction catalyzed is described by the following equation:

P-Nitrophenyl phosphate + H_20 $\xrightarrow{ALP}$ P- Nitrophenol + Inorganic phosphate

Diagnosis Application

Alkaline phosphatase is a marker enzyme for the biological membrane (Wright and Plummer, 1974). Therefore, the measurement of its activity in the tissues and body fluild provides a significant aid in diagnosis of several disease conditions. Two abnormal level of ALP in the serum has been proposed - increase or decrease in normal levels.

Damage to biological membrane may lead to any of the two abnormal levels of ALP in the serum or the tissue.

1.5.4 Catalase (CAT)

Catalases (EC 1.11.1.6) are haem-containing proteins that catalyse the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen, thereby protecting cells from the toxic effects of hydrogen peroxide. It is found in high concentrations in a compartment in cells called the peroxisome. It is a common enzyme present in most all living organisms and a very potent catalyst. It converts the harmful byproduct of metabolism, hydrogen Peroxide, into water and oxygen. It also oxidizes toxins in the body such as formaldehyde, alcohols, phenols and formic acid.

It is present in every known animal and is vital to life processes.

Reaction Catalyzed

It catalyzes the convention hydrogen peroxide to water and oxygen as shown by the equation:

$2H_2O_2 \rightarrow 2H_2O+O_2$

1.5.5 SuperOxide Dismutase (SOD)

Superoxide dismutases (**SOD**, EC 1.15.1.1) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. SOD enzymes were previously thought to be several metalloproteins with unknown function (McCord and Fridovich, 1988). Several common forms of SOD exist: they are proteins cofactored with copper and zinc, or manganese, iron, or nickel. Brewer (1967) identified a protein that became known as superoxide dismutase as an indophenol oxidase by protein analysis of starch gels using the phenazine-tetrazolium technique.

Reaction Catalyzed

It catalyzes the convention superoxide and protons as H+ to hydrogen peroxide and molecular oxygen as shown by the half- reaction equation:

$$M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$$

$$M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2.$$

Where M = Cu (n=1); Mn (n=2) ; Fe (n=2) ; Ni (n=2).

1.6 HAEMATOLOGICAL STUDIES

Blood is a highly specialized connective tissue made up of cells and extracellular liquid medium. African trypanosomiasis is characterized by haematological changes, which drastically influence the pathogenesis of the disease. Although many studies on this phenomenon have been conducted in the past, most of them have focused on livestock trypanosomiasis with only a few human infections (Stephen, 1986; Neiger *et al*, 2002). The disease is associated with decline in the red blood cell (RBC) counts, haemoglobin (Hb) concentration, and haematocrit or packed cell volume (PCV) and Pallo in mucour membrane in the infected hosts, confirming that anaemia is a critical feature in the pathogenesis of African trypanosomiasis. In livestock, the anaemia has been typed as either microcytic-microchronic or macrocytic - macrochromatic (Stephen, 1986). The studies on anaemia caused by the human infective *T.b. rhodesiense* and *T.b. gambiense* in rodents have shown that it ranges between macrocytic normachronic to microcytic

hypochromic anaemia (Stephen, 1986). The haemoglobin (Hb), Packed Cell Volume (PCV), Red blood cell (RBC) and White blood cell (WBC) counts are usually measured in a haematological study.

Haemoglobin, Packed Cell Volume and Red blood cells

The erythrocytes (Red blood cells), account for a large number of blood cells. The main function of the erythrocyte is to transport haemoglobin, which is essential for respiration. Haemoglobin is the major component of the red blood cells, with a single one having about one million molecules of haemoglobin (Stroev, 1989). A decrease in haemoglobin signifies anaemia.

The PCV is the number of packed erythrocyte per 100 volume of blood. This gives an indication of the oxygen carrying capacity of the blood. High PCV indicates good performance while low count of RBC could be as a result of factors such as disease (Thompson and Proctors, 1977).

White Blood Cell

White blood cells also referred to as leukocytes include lymphocytes, neutrophils, monocytes, eosinophils and basophils. The main function of white blood cells is to defend the body against infection and foreign bodies. An elevated while blood cell count is often associated with infection.

1.7 Electrolytes

Bectel (1970) defined electrolytes as the ions in the body fluid that serve function such as regulating water balance, osmotic pressure acid base balance and nerve and muscle irritability. The electrolyte includes: sodium, potassium, magnesium, chloride, calcium, bicarbonate, carbonic acid sulphate, organic and inorganic phosphate, organic acids and proteins. Alternatively electrolyte are purely chemical bases given as a substance which when either in molten state or in solution conduct electricity and it is decomposed in the process (Harold, 1980) electrolyte in the clinical laboratory is taken to refer to only the inorganic ions, but in practice, a request for electrolytes determination usually means the determination of sodium, calcium,

potassium, bicarbonate and chloride ions (Bectel, 1970). Two fundamental points about blood electrolytes must be clearly understood namely, the requirement of electroneutrality and independence of ions of strong electrolytes. The term requirement of electroneutrality implies that the total number of anionic charges must equal the total number of cationic to obtain an electrically neutral blood while independence of ions of strong electrolyte means that in solution, we have sodium ions (Na^+) chloride ions (Cl^-) and bicarbonate (HCO_3^-) ions not NaCl and N_aHCO_3.

Blood electrolyte level is dependent on many factors. The consumption of acidic or basic food elevates anions and cations in the blood. Starvation and diabetes mellitus that increase utilization of fat for energy with a concomitant increase in blood ketone bodies increase the levels of anions in the blood lungs. Failure of the respiratory control centre in brain and lungs results in CO_2 and HCO_3^- anions in the blood. Defect in kidney functioning also distorts the acid base balance of the blood (Holum, 1983).

1.7.1 Sodium

Sodium is the major cation of the extracellular fluid and it plays a central role in the maintenance of normal distribution of water and the osmotic pressure in the various fluid compartments (White, 1978). Normal American daily diet contains approximately 8-15 grams (130-250 mmol) of sodium chloride, which is nearly completely absorbed from the gastrointestinal (Norbert, 1976). The excess sodium is excreted by the kidneys which are the ultimate regulators of the sodium in the body. It has been shown that sodium is a threshold substance with a normal renal threshold of 110-130 mmoI/L (Baron, 1982). Sodium is initially filtered by the glomeruli, but 80-85% is reabsorbed in the proximal portion and an additional amount in the distal portion of the tubules. The total amount of sodium reabsorbed may reach as much as 99% the re-absorption is greatly controlled by some adrenal cortical hormones mainly aldosterone (which enhance the tubular re-absorption of sodium and indirectly decrease the tubular

re-absorption of potassium ion). Sodium threahold is the level in the serum below which any sodium present in the glomerular filtrate is completely absorbed by the tubules (White, 1978). Hypernatremia occurs as a result of various kinds of brain injury. Diabetic coma after therapy with insulin causes a retransfer of cellular sodium into the extracellular fluid in order to maintain equal osmotic pressure in both compartments.

The maximum possible concentration in urine even in healthy adult is about 350 mmol/L (Baron, 1982). Hence, when the sodium intake exceeds the possible rate of excretion, sodium intoxication develops. Where there is an increase in extracellular fluid volume, the rise in serum sodium and chloride concentration is small as a result of net acidosis. Water passes from the intracellular fluid to the extracellular fluid in an attempt to maintain the serum osmotic pressure.

Baron (1982) reported a normal range of 136-148 mmol/L. Sodium depletion or low serum sodium level is what is referred to as

hyponatremia. This syndrome develops when there is diminished intake of sodium with normal water intakes or when there is general loss of both water and sodium which is replaced by water. Renal tubular disease, in which there may be defect in either the re-absorption of sodium in the Na^+ - H^+ exchange that can lead to sodium loss from the serum. When sodium is lost from the body the extracellular fluid because hypotonic, water leaves the extracellular fluid in an attempt to restore the serum osmotic pressure (Kamath, 1972). The kidney can combat sodium deficiency by increasing tubular re-absorption, via aldosterone stimulation, more efficiently that the combat water deficiency (Claude, 1972).

1.7.2 POTASSIUM

The major intracellular cation is potassium having an average cellular concentration in tissue cells of 150 mmol/L and concentration in red cells of 105 mmol/L (Berry *et al.*, 1989). This is approximately 23 times higher than the concentration of potassium in extracellular

fluid. The maintenance of high intracellular concentration, and low extracellular concentration of potassium is believed to be maintained by active transport mechanism, that utilizes the oxidative mechanism of the cells though it has been observed that there are some movement of potassium, it is extremely low and rapid shifts of potassium in or out of the cells by diffusion have not been observed (Norbert, 1976).

Body requirements of potassium are satisfied by a normal dietary intake of 80-200 mmol/day. Potassium is partially removed from the serum by glomerular filtration and is reabsorbed in the proximal tubules but it is effectively re-excreted by distal tubule under the influence of mineral locoticoids. Hence, there is no threshold level for potassium only a slight and temporary increase in serum potassium levels is affected by the intake of any amount of potassium absorbed by the intestinal tract.

Hyperkalaemia or hyperpotassemia is generally observed in cases of oliguria, anuria or urinary obstruction. Renal failure due to shock results in decrease of renal potassium from serum. renal tubular

acidosis which interferes with Na^+ - H^+ exchange also result in retention of potassium in serum. Renal dialysis which is a treatment of renal failure has an important purpose in the removal of accumulated potassium from serum. The chief toxic effect of hyperkalaemia is on neuromuscular condition especially in the heart. Treatment of the cause of hyperkalaemia includes stopping the excessive intake, correcting the acidosis and any water and salt depletion. Low sodium levels cause excitatory changes in muscle irritability and myocardial function, which are also accompanied by characteristic cardiographic changes. For this reason, the serum potassium determination has become a most important diagnostic tool in situations in which extremely high or low serum potassium levels are suspected. The treatment of low potassium levels can be achieved by giving potassium orally as potassium chloride through intravenous route. The normal ranges of serum potassium are 3.5-5.3 mmol/L (Norbert, 1976) and 3.8 – 5.0 mmol/L (Baron, 1982).

1.7.3 CHLORIDE

Chloride is also major extracellular anion and it constitutes about 103 mmol/L of the total anion concentration of approximately 154 mmol/L (Wills, 1971). It is involved in the maintaining proper water distribution, osmotic pressure and normal anion – cation balance in the extracellular fluid compartment. Chloride ions ingested with food are almost completely absorbed by the intestinal tracts. They are removed from the blood by glomerular filtration and are passively reabsorbed by proximal tubule, (Norbert, 1976). Excessive sweating may also be a route through which chloride is lost during hot weather period. However, this stimulates aldosterone secretion and then causes the sweat gland to secrete sweat of lower sodium and chloride concentration than that excreted during normal temperatures (Olaniyi, 1992).

1.7.4 BICARBONATE

Bicarbonate is the second largest anion fraction in serum. It is important as a component of the bicarbonate buffer system and it also serve as a transport for carbon dioxide from the tissue to the lungs (Ganong, 2001).

This function is essential to life, as almost if not all biochemical processes, which occur in the body to maintain life requires enzyme, which are pH sensitive. It formed by the dissociation of carbonic acid, as shown in the equation.

$$H_2CO_3 \rightarrow H^+ + HCO_3^-$$

In order to keep the pH of the body fluids constant (a homeostatic function performed by the kidney), bicarbonate is absorbed along with sodium ion in the early portion of the proximal tubule (Ganong, 2001). The secretion of hydrogen ions into the tubular lumen is an important step in the removal of bicarbonate ions from the body (Guyton and Hall, 2000). Thus, the removal of bicarbonate in the blood could serve as an index in kidney function.

1.7.5 INORGANIC PHOSPHATE

Phosphorus is an abundant element that is widespread in its distribution. It is a major intracellular anion in mammals. Total body phosphorus in a (70-kg) man is about 700 to 800 mg, 85% of which is in skeleton in hydroxyapatite phase; the remaining 15% is in soft tissues. Almost all of the phosphorus found in the extracellular fluid space is in form of inorganic phosphate (Berkelhammer and Bear, 1984). Serum inorganic phosphate reflects only a very minor percentage of total body phosphorus; however it is easily measurable and gives a due to the status of body phosphorus stores. The majority of the phosphate in the body is in the organic form as a complex with carbohydrates, lipids and proteins. Phosphorus is an essential element in the cellular structure, cytoplasm, and mitochondrion. Pulmonary muscle weakness may result from hypophospatemia. Hypophosphatemia causes decreased absorption of calcium in the renal tubules, leading to hyper calciuria. Both hyper and hypophatemia can be caused by cellular shifts of phosphate. The three primary

conditions that lead to phosphate dysfunction are dietary intake, gastrointestinal and renal status. Hyper phosphatemia occurs in several endocrine conditions associated with increased tubular reabsorption of phosphate (Brautbar *et al.*, 1987). The most common risk is ectopic or metastatis calcification, which may occur if the product of the serum calcium and phosphorus exceeds 70. The normal serum phosphorus concentration is 3.4 to 4.5 mg/dl (1.12-1.45 mmol/L). This fluctuates with age (it is higher in children than adults), dietary intake, and acid base status.

1.8 AIMS AND OBJECTIVES

The aims and objectives of the experiments are to:

- Identify and quantify the phytochemicals present in fermented wheat germ and garlic bulb extracted using ethyl acetate and methanol respectively.
- Assess the bioactivity of the crude extracts for anti - trypanosomal activities.
- Assess the effect of the crude extracts on some enzymes, haematological and electrolyte parameters of the infected rats.
- Purify and characterized the active constituents of the crude extracts.
- Predict the specific features of ribonucleotide reductase (RNR) in the parasite.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLANT MATERIALS

Wheat germ (*Triticum aestivum*) and fresh bulbs of *Allium sativum L.*, commonly known as garlic were purchased from Minna Central Market, Niger State, Nigeria and authentication was carried out at Federal College of Forestry, Ibadan, Oyo state.

2.1.2 PARASITE STRAIN

Trypanosoma brucei was obtained from the Veterinary and Livestock Studies Department of the Nigerian Institute for Trypanosomiasis Research, Vom, Plataeu State of Nigeria. The parasite was maintained through passaging to other rats. 0.5ml of parasite suspension in 0.9% saline solution was inoculated

intraperitoneally into uninfected rats. The suspension contained 3 or 4 trypanosomes per view at X40.

2.1.3 EXPRERIMENTAL ANIMALS

Albino rats weighing between 150 – 200 g were obtained from the Animal Breeding Unit of the Department of Biochemistry, University of Ilorin, Nigeria and fed with animal feed obtained from Bendel Feeds and Flour Mill Ltd, Ewo, Edo state.

2.1.4 ENZYME AND PROTEIN DETERMINATION

Assay kits for Transaminases (AST and ALT) were obtained from Randox laboratories Ltd., United Kingdom and alkaline phosphatase was obtained from Teco Ltd, United Kingdom.

2.1.5 CHEMICALS

Suramin and berenil were obtained from Sigma Aldrich, United State.

2.2 METHODS

2.2.1 PREPARATION OF WHEAT EXTRACT

Wheat germ powder (70g) was fermented using 30g of Baker's yeast (*Saccharomyces cerevisiae*) for 24 hours and the paste was extracted with 250ml ethyl acetate. The filtrate was then concentrated using rotary evaporator and stored at room temperature.

2.2.2 PREPARATION OF GARLIC EXTRACT

Garlic bulbs (*A. sativum*) were peeled to reveal its fleshy sections called cloves. The cloves were peeled and blended. Garlic bulbs (100g) was soaked in 250ml of methanol for 48 hours and filtered. The solvent was removed using rotary evaporator and stored at room temperature.

2.2.3 PHYTOCHEMICAL ANALYSIS

2.2.3.1QUALITATIVE ANALYSIS ON PHYTOCHEMICAL CONSTITUENTS

Qualitative analysis were carried out on the crude extracts (Wheat and garlic) using the method of Sofowora, 1993.

Test for Alkaloids

Confirmatory test for alkaloid was done by adding few drops of Drange droffs reagent to the extract. The development of deep brown precipitate confirmed the presence of alkaloid (Sofowora, 1993)

Test for Anthraquinone

The plant extract was shaken with equal volume of chloroform, then 10% NH_3 solution was added to the chloroform layer. Formation of brick red precipitate indicated the presence of anthraquinone derivatives

Test for Tannins (Ferric Chloride test)

Portion of the extract was diluted with distilled water and few drop of 10% ferric chloride solution was added. A blue or green colour indicates the presence of tannins

Test for flavonoids

The extract (0.2g) was diluted with sodium hydroxide solution. The appearance of a yellow solution which disappeared on addition of hydrochloric acid indicates the presence of flavonoids.

Test for Saponins

Distilled water (10ml) was added to 0.2g of the extract. The content was shaking vigorously in the test tube for 2 minutes. The presence of frothing or bubbling indicates the presence of saponin.

Test for Steroids

Five drops of concentrated H_2SO_4 was added to 0.2g of the extract. A reddish brown colour indicates the presence of steroids.

Test for Glycosides (keller-killian test)

The extract (0.2g) was dissolved in 3ml of $FeCl_3$ in glacial acetic acid and leave for a minute. 1.5ml of H_2SO_4 was added with a pipette so that it runs down the side of the test tube. A positive test is a clear inter-phase with a blue layer.

Test for Phlobatannins

The extract (0.2g) was boiled with 5ml of 1% HCl in a test tube or conical flask. The deposition of a red precipitate indicates the presence of phlobatannins.

Test for terpenoids

The extract (5ml) was mixed with 2 ml of $CHCl_3$ in a test tube. Concentrated H_2SO_4 (5ml) was carefully added to the mixture to form a layer. An interface with a reddish brown coloration is formed if terpenoids constituent is present.

2.2.3.2 QUANTITATIVE ANALYSIS ON PHYTO- CHEMICAL CONSTITUENTS

TANNIN

Sample (0.2g) was measured into a 50ml beaker. 20ml of 50% methanol was added, covered with Para film and placed in a water bath at 77-80^{o}C for 1 hour. It was shaked thoroughly to ensure a uniform mixing. The extract was quantitatively filtered using a double layered Whatman filter paper into a 100ml volumetric flask, 20ml of water added, 2.5ml Folin-Denis reagent and 10ml of 17% Na_2CO_3 was added and mixed properly. The mixture was made up to mark with water mixed well and allowed to stand for 20minutes.A bluish –green colour developed at the end of range 0-10 ppm. The absorbances of the Tannic acid standard solutions(Appendix 3a) as well as samples were read after colour development on a spectronic 21D spectrophotometer at a wavelength of 760 nm. (A.O.A.C, 1984)

Calculation:

$$\% \text{ Tannin} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

ALKALOIDS

This was done by distillation and titrimetric procedure. Sample (2g) was weighed into a 100ml beaker and 20mls of 80% absolute alcohol added to give a smooth paste. The mixture was transferred to a 250ml flask and more alcohol added to make up to 100ml, 1g magnesium oxide was added. The mixture was digested in a boiling water bath for 1.5hrs under a reflux air condenser with occasional shaking. The mixture was filtered while hot through a small bucher funnel. The residue was returned to the flask and re-digested for 30min with 50ml alcohol after which the alcohol was evaporated. Hot water was added to replace the alcohol lost. When all the alcohol has been removed, 3 drops of 10% HCl was added. The whole solution was later transferred into a 250ml volumetric flask, 5ml of zinc acetate and 5ml of potassium ferrocyanide solution was added, thoroughly mixed to give a homogenous solution.

The flask was allowed to stand for a few minutes, filtered through a dry filter paper and 10ml of the filterate was transferred into a separating funnel and the alkaloids present were extracted vigorously by shaking with five successive portions of chloroform. The residue obtained was dissolved in 10ml hot distilled water and transferred into a kjeldahl tube with the addition of 0.2g of sucrose, 10ml Conc.H_2SO_4 and 0.02g selenium for digestion to a colorless solution to determine %Nitrogen by Kjeldahl distillation method (Henry 1993).

Calculation:

%Nitrogen =Titre value x 0.56

% Total alkaloid = %Nitrogen x 3.26

FLAVONOIDS

Sample (0.5g) was weighed into a 100ml beaker and 80ml of 95% Ethanol was added and stirred with a glass rod to prevent lumping. The mixture was filtered through a Whatman No.1. filter paper into a 100ml volumetric flask and made up to mark with Ethanol. 1ml of the extract was pipette into 50ml volumetric flask, four drops of

Concentrated HCl added via a dropping pipette after which 0.5g of magnesium turnings added to develop a magenta red coloration. Standard flavonoid solution of range 0-5 ppm were prepared from 100 ppm stock solution and treated in a similar way with HCl and magnesium turnings like sample. The absorbances of magenta red coloration of sample and standard solutions were read on a Spectrophotometer at a wavelength of 520 nm (Appendix 3b) (Allen's commercial Organic Analysis, 1979)

Calculation:

$$\% \text{ Flavonoids} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

SAPONIN

The Spectrophotometric method of Brunner (1984) was used for Saponin analysis.1g of sample was weighed into a 250ml beaker and 100ml of isobutyl alcohol was added. The mixture was shaken on a shaker for 5 hours to ensure uniform mixing. Thereafter the mixture was filtered through a Whatman No1 filter paper into a 100ml beaker and 20ml of 40% saturated solution of magnesium carbonate was added. The mixture obtained with saturated magnesium carbonate was again filtered through a filter paper to obtain a clear colorless solution. 1ml of the colorless solution was pipette into 50ml volumetric flask and 2ml of 5% ferric chloride solution was added and made up to mark with distilled water. It was allowed to stand for 30min for blood red color to develop. 0-10 ppm standard Saponin solutions were prepared from saponin stock solution. The standard solutions were treated similarly with 2ml of 5% $FeCl_3$ solution as done for the sample above. The absorbance of the sample as well as

standard saponin solutions (Appendix 3c) were read after color development with a Spectrophotometer at a wavelength of 380 nm.

Calculation:

$$\% \text{ Saponins} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

GLYCOSIDE

Ten milliliters (10ml) of extract was pipetted into a 250ml Conical Flask.50ml Chloroform was added and shaken on a Vortex Mixer for 1hr. The mixture was filtered into 100ml conical flask and 10ml pyridine; 2ml of 2% sodium nitroprusside were added and shaken thoroughly for 10 minutes. 3ml of 20% NaOH was later added to develop a brownish yellow colour.

Glycoside standard of concentrations ranging from 0-5 mg/ml were prepared from 100 mg/ml stock Glycoside standard. The series of standards 0-5 mg/ml were treated similarly like sample above. The absorbance of sample (Appendix 3d) as well as standards was read on a Spectrophotometer at a wavelength of 510 nm (Method of Analytical Committee of Royal Society of Chemistry).

Calculation:

$$\% \text{ Glycoside} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

STEROIDS

Sample (0.5g) extract was weighed into a 100ml beaker. 20ml of Chloroform-Methanol (2:1) mixture was added to dissolve the extract upon shaking for 30minutes on a shaker. The whole mixture was later filtered through a Whatman filter paper into another dry clean 100ml beaker. The resultant residue was repeatedly treated with Chloroform-Methanol mixture until its free of Steroids.1ml of the filtrate was pipette into a 30ml test tube and 5ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath set at 37^0C-40^0C for 90minutes.It was cooled to room temperature and 10 ml of petroleum ether added followed by the addition of 5ml distilled water. This was evaporated to dryness on the water bath. 6ml of Liebermann Burchard reagent was

added to the residue in dry bottle and absorbance taken at a wavelength of 620 nm on a Spectrophotometer. Standard Steroids concentration of 0-4 mg/ml were prepared from 100 mg/ml stock steroid solution and treated similarly like sample (Appendix 3e). (Methods Analytical Committee of Royal Society of Chemistry).

Calculation:

$$\% \text{ Steroids} = \frac{\text{Absorbance} \times \text{Gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 10{,}000}$$

PHLOBATANNIN

Sample (0.5g) extract was weighed into 50ml beaker. 20ml of 50% Methanol was added, covered with parafilm and placed in a water bath set at 77-80^{0}C for 1 hour. The mixture was properly shaken to ensure uniform mixing and later filtered through a filter paper into a 50ml volumetric flask using aqueous methanol to rinse, and make up to mark with distilled water.1ml of the sample extract was pipetted into a 50ml volumetric flask, 20ml water 2.5ml Folin-Dennis reagent

and 10ml of 17% Sodium carbonate were added to the solution in the 50ml Flask. This mixture was homogenized thoroughly for 20mins. 0-5 mg/ml of Phlobatannin standard concentration were prepared from 100 mg/ml phlobatannin stock solution and treated like sample. The absorbances of standard solutions (Appendix 3f) as well as sample were read on a spectrophotometer at a wavelength of 550 nm (Methods of Analytical Committee of Royal Society of Chemistry).

Calculation:

$$\% \text{ Phlobatannin} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

ANTHRAQUINONES

Sample (0.5g) was weighed into 250ml beaker and 60ml of benzene added, stirred with a glass rod to prevent lumping. This was filtered into 100ml volumetric flask using filter paper.10ml of the filtrate was pipetted into another 100ml volumetric flask and 0.2% of Zinc dust was added followed by the addition of 50ml hot 5% NaOH solution. The mixture was heated below boiling point for five minutes

and then rapidly filtered and washed once in water. The filtrate was again heated with another 50ml of 5% NaOH to develop a red colour. Standard Anthraquinone solution of range 0-5 mg/l were prepared from 100 mg/l stock Anthraquinone and treated in a similar way with 0.2% Zinc dust and NaOH. The absorbances of sample as well as that of standard concentrations (Appendix 3g) were read on a Digital Spectrophotometer at a wavelength of 640 nm and the percentage anthraquinone is calculated (Lewis and Elvin - Lewis, 1977).

Calculation:

$$\% \text{ Anthraquinone} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

TERPENE

Sample (0.5g) was weighed into a 50ml Conical Flask,20ml of 2:1 Chloroform-Methanol mixture was added, shaken thoroughly and allowed to stand for 15minutes.The mixture was later centrifuged for another 15minutes.Supernatant obtained was discarded, and the

precipitate was re-washed with another 20ml chloroform-methanol mixture for re-centrifugation.The resultant precipitate was dissolved in 40ml of 10% sodium dodecyl sulphate solution. 1ml of 0.01M Ferric Chloride solution was added to the above at 30s interval ,and allowed to stand 30minutes.Standard terpenes of concentration range 0-5 mg/ml were prepared from 100 mg/l stock Terpenes solution from Sigma-Aldrich chemicals, U.S.A. The absorbances of sample as well as that of standard concentrations of Terpenes (Appendix 3h) were read on a Digital Spectrophotometer at a wavelength of 510 nm (Methods of Analytical Committee of Royal Society of Chemistry).

Calculation:

$$\% \text{ Terpenes} = \frac{\text{Absorbance x Gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

PHENOL

Sample (0.2g) was weighed into a 50ml beaker, 20ml of acetone was added and homogenize properly for 1hr to prevent lumping. The mixture was filtered through a filter paper into a 100ml Volumetric

Flask using acetone to rinse and made up to mark with distilled water with thorough mixing.1ml of sample extract was pipette into 50ml Volumetric flask, 20ml water and 3ml of phosphomolybdic acid added followed by the addition of 5ml of 23% sodium carbonate and mixed thoroughly, made up to mark with distilled water and allowed to stand for 10min to develop bluish-green colour. Standard Phenol of concentration range 0-10 mg/ml was prepared from 100 mg/l stock Phenol solution from Sigma-Aldrich chemicals, U.S.A. The absorbances of sample as well as that of standard concentrations (Appendix 3i) of Phenol were read on a Digital Spectrophotometer at a wavelength of 510 nm (Harborne, 1978).

Calculation:

$$\% \text{ Phenol} = \frac{\text{Absorbance} \times \text{Gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 10{,}000}$$

2.2.4 FRACTIONATION OF ACTIVE CONSTITUENTS

The crude extracts i.e wheat (5.84g) and garlic (8.66g) were subjected to column chromatography using silical gel (60 – 120 mesh), eluting with a step gradient of n-hexane, n-hexane – ethylacetate, ethyl acetate, ethyl acetate – methanol and methanol. Thin layer chromatography was performed with precoated silical gel GF- 25- UV 254 plates and detection was done by spraying with sulphuric acid for garlic and iodine for wheat.

2.2.5 EXPERIMENTAL DESIGN

The experiment was carried out in 2 stages. Each grouping of the experiment in the 2 stages contains five rats. In the first stage, infected rats were administered with 300mg/kg body weight of each extract on daily basis from the day parasite was first sighted in the blood. The control rats were infected untreated.

In the second stage, whole blood, serum and liver was obtained at the late stage (Day 10 post- infection) of infection from all the groups and control for this stage was uninfected not treated (normal) and infected untreated group under the same experimental condition.

2.2.6 PARASITE COUNT

Evaluation of parasitaemia was carried out at 24 hours interval to monitor infection progress until the animals died. This was done by counting the number of parasites under the light microscope at x40 magnification from thin blood smear freshly obtained from the tip of the tail of infected rats.

2.2.7 COLLECTION OF BLOOD AND ISOLATION OF LIVER

Blood was collected from chloroform anaesthesized rats through cardiac puncture. About 0.5ml of the blood was first collected into sample bottles containing an anticoagulant (EDTA), while about 4.5mls of the blood was collected into a centrifuge tube, allowed to coagulate and centrifuged at 1000 rpm for 20 minutes to get serum by decanting. The rat was then dissected and liver was encapsulated into sample bottle containing ice-cold sucrose solution (0.25M) to maintain a normal physiological environment. Blood collected inside anticoagulant was used for determination of the haematological parameters.

2.2.8 PREPARATION OF LIVER HOMOGENATE

Know weight of the liver were sliced into small pieces and then homogenized using pre-cooled pestle and mortar placed in a bowl of ice chips. The homogenized tissue were diluted with 0.25M sucrose solution to give a final volume (1:5 w/v) and centrifuged at 1000 rpm for 20minutes. The supernatant were stored for analysis.

2.2.9 ESTIMATION OF PACKED CELL VOLUME (PCV) HAEMATOCRIT

When anticoagulated whole blood is centrifuged, the space occupied by the packed red blood cells is termed PCV or haematocrit. This was estimated using anticoagulated whole blood. The haematocrit capillary tubes were 2/3to ¾ filled with blood. One end of the tube was sealed and then placed in the radial grooves of the centrifuge with the sealed end away from the centre of the centrifuge. The centrifugation was at a speed of 11000rpm for 30min after which the haematocrit tubes were removed and the PCV read from a micro haemoatocrit reader (Catwright, 1968).

2.2.10 DETERMINATION OF TOTAL WHITE BLOOD CELL COUNT (WBC)

This is the total white blood cell in a stated volume of blood which is expressed as number of cells per litre. Heparinized blood was used; the whole blood was mixed with a weak acid solution to dilute the blood and haemolyse the red cells. The white blood cell was then counted using improved Neubauer counting chamber as described by Dacie and Lewis (1991).

Principle: Whole blood is mixed with a weak acid to dilute the blood and haemolyze the red cells. Gentian violet is added to stain the nuclei of white cells. A dilution of 1 in 20 is used.

Procedure:

0.4ml of diluents (2% acetic acid and few drop of gentian) was dispense into small tube and mixed. 20µL pipette was used to blow the 20µL of blood into diluents and rinse by sucking up and down through the rubber tubing of the mouth piece. The mixture was mixed and allows to stand for 2-3minutes for complete lyses of the red cells. The chamber was filled by drawing some diluted blood from the tube. The red cells was allowed to settle in the counting chamber for 1-2 minutes and all the white cells were counted in the four corner of 1mm x 1mm squares of which each contains 16 smaller squares. The counts were added together and divide by 4 to get the average count per 1mm x 1mm square.

Calculation:

Since the depth of the counting chamber is 0.1mm, then the volume of blood in a 1mm x 1mm squares is 1 x1 x 0.01mm^3. If N

cells is the average count per 1mm x 1mm square, then 0.1 mm^3contains N cells and 1µl contains N x 10 cells.

$1mm^3$ (or 1µl) of whole blood contains N x 10 x 20 cells and 1 litre of whole blood contains N x 10 x20 x 10^6 cells.

2.2.11 DETERMINATION OF RED BLOOD CELL COUNT (RBC)

The red cell count is the number of red blood cells in a stated volume of whole blood and expressed as number of cells per litre of blood.

Blood was suitably diluted with an isotonic diluting fluid (normal saline) to prevent lyses of red cells and it was counted in a Neubauer counting chamber as described by Dacie and Lewis (1991). The convenient dilution facto used was 1: 200

Procedure:

0.4ml of diluting fluid (3% formol – citrate) was dispensed into a tube. The blood sample was mixed using a 20µL pipette by drawing up blood slightly above the mark, the outside of the pipette was wiped and bring the blood to the 20µL level by applying the pipette tip to a

filter paper or other similar surface. 20 µL of blood was blown into the diluting fluid and rinse by sucking up and down. The chamber was filled by drawing some diluted blood from the tube and allowing red cells to settle in the counting chamber for 3-5minutes. The red cells were counted in five groups of 16 small squares in the central ruled area of the chamber.

Calculation:

There are 25 groups of 16 small squares making up the 1mm x 1mm central ruled areas.

400 small squares occupy an area $1mm^3$. Each small squares has a volume of $1/400\ mm^3$

Since the depth of the counting chamber is 0.1mm, each small squares has a volume of $1/400 \times 1/10\ mm^3$

If N cells are counted in 80 such squares then $1/400 \times 1/10 \times 80\ mm^3$ of diluted blood has N cells.

$1mm^3$ of diluted blood has 400 x 10 x N/80 (cells)

Since the blood was diluted 1 in 200

$1mm^3$ of whole blood has 400 x 10 x N x 200/80 cells

1L of whole blood has N x 10,000 x 10^6 cells.

2.2.12 DETERMINATION OF HAEMOGLOBIN (Hb)

Haemoglobin is a coloured substance which can be converted into other molecules. This is calculated as:

Hb concentration = Packed cell volume/3

2.2.13MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

This was calculated using concentration of haemoglobin in the average erythrocyte dividing by the value of packed cell volume.

MCHC = Hb (g/100ml)/ haematocrit (PCV)

It is expressed in percentage (%).

2.2.14 TOTAL PROTEIN DETERMINATION

The protein um and liver were determined using the Biuret method as reported by Gornall *et al.*, 1949 and as modified by Henry *et al.*, (1974).

Principle

Cupric ions in alkaline medium interact with protein peptide bonds resulting in the formation of purple complex. The colour intensity is a measure of the protein content in the sample.

Procedure

A protein standard curve was first prepared in series. Cleaned test tubes were measured 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 of standard protein, Bovine Serum Albumin (BSA). The volumes were then made up to 1ml with distilled water and 4mls of biuret reagent were added. The optical density was taken at 540nm after 30 minutes using spectrophotometer. A standard calibration curve of optical density versus protein concentration was then made (Appendix 3J). Protein contents of test homogenates were determined after1 in 30 dilutions with 0.25M sucrose. 0.1ml of each homogenate was taken into separate test tubes and 0.9ml of distilled was added to each of the test tube to make up to volume of 1ml. 4ml of biuret reagent was also

added and the optical density was taken at 540nm using spectrophotometer.

Calculation

The protein content in each homogenates was obtained from the protein standard calibration curve (Appendix 3) taking into consideration, the appropriate dilution where necessary.

2.2.15 SPECIFIC ENZYMES ACTIVITIES

The enzymes assayed include; Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Catalase (CAT) and Superoxide Dismutase (SOD).

2.2.15.1 ASPARTATE TRANSAMINASE

Aspartate transaminase (AST) is also known as Glutamate Oxaloacetate transminase (GOT). The enzymes mediates reactive systems in which amino group is transferred from an amino acid to an oxo-acid without formation of ammonia as an intermediate. This is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4 – dinitrophenylhydrazine based on the procedure of

Reitman and Frankel (1957) that was modified by Schmidt and Schmidt (1963).

PRINCIPLE:

α – oxoglutarate + L –aspartate $\xrightarrow{\text{GOT}}$ glutamiate + oxaloacetate

Procedure

Wavelength: 546 nm

Curvette: 1cm light path

Incubation Temperature: 37^{o}C

Solution 1: Buffer (Phosphate buffer)

L – aspartate

α – Oxoglutarate

Solution 2: 2, 4 – dinitrophenylhydrazine

Pipette into test tubes:

	Sample Blank (ml)	**Sample (ml)**
Sample	-	0.05
Solution 1	0.25	0.25
Mix, incubate for exactly 30min at 37^{o}C Solution 1 Distilled water	 0.25 0.05	 0.25 -
Mix allow to stand for exactly 20 min at 20 to 25^{o}C NaOH (0.1)	 2.50	 2.50

Mix, read the absorbance at 546 nm (530 – 550 nm) after 5minutes

Calculation

The activity of AST in the sample was read off the standard curve (Appendix 3) and was used to calculate the specific activity using the equation:

$$\text{Specific activity (U/l)} = \frac{\text{Activity}}{\text{Protein concentration (mg/l)}}$$

2.2.15.2 ALANINE TRANSAMINASE (ALT)

Alanine transminase (ALT) or Glutamate pyruvate transaminse (GPT). It Catalyze the formation of pyruvate and glutamate from alanine and α – oxoglutarate. This was assay using the procedure of Reitman and Frankel (1957) that was modified by Schmidt and Schmidt (1963).

PRINCIPLE:

A – Oxoglutarate + alanine $\xrightarrow{\text{GPT}}$ L-glutamate + pyruvate

Procedure

Wavelength: 546nm

Curvette: 1cm light path

Incubation Temperature: 37^{o}C

Solution 1: Buffer (Phosphate buffer)

L – alanine

α – Oxoglutarate

Solution 2: 2, 4 – dinitrophenylhydrazine

Pipette into test tubes:

	Sample Blank (ml)	**Sample (ml)**
Sample	-	0.05
Solution 1	0.25	0.25
Mix, incubate for exactly 30min at 37^{o}C Solution 1 Distilled water	 0.25 0.05	 0.25 -
Mix allow to stand for exactly 20 min at 20 to 25^{o}C NaOH (0.1)	 2.50	 2.50

Mix, read the absorbance at 546nm (530 – 550nm) after 5minutes

Calculation

The activity of ALT in the sample was read off the standard curve (Appendix 3) and was used to calculate the specific activity using the equation:

Specific activity (U/l) = $\frac{\text{Activity}}{\text{Protein concentration (mg/l)}}$

2.2.15.3 Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is known as orthophosphoric monoester phosphohydroxylase. The optimum pH of alkaline phosphatase (ALP) varies from 8.5-10.0 and is influenced by the type of substrate employed, concentration of the type and concentration of activation and finally, the buffer employed. It was assayed as described by Wright *et al.*, 1972.

PRINCIPLE:

P-Nitrophenyl phosphate + H_20 $\xrightarrow{ALP}$ P- Nitrophenol + Inorganic phosphate

Reagents

Dieethanol amine Buffer, (pH 10.2)

Magnessium choride

P-Nitrophenyl phosphate

Procedure:

Working Reagent	1000ml
Samples	20ml

Mix and incubate for 1 minute at 37^0C. Measure the change in absorbance per minute (ΔOD/min) during 3 minutes.

Calculation:

ALP activity (U/L) = (ΔOD/min) x 2750.

Specific activity (U/l) = $\frac{\text{Activity}}{\text{Protein concentration (mg/l)}}$

2.2.15.4 QUANTITATIVE DETERMINATION OF CATALASE ACTIVITY

This was carried out by the method described by Bock *et al.*, 1980.

Principle:

Dichromateacetate $\xrightarrow{}$ H_2O_2 $\xrightarrow{}$ perchloric acid chromic acetate

Procedure:

A standard curve of hydrogen peroxide was first prepared by dispensing various volumes (0.00, 0.05, 0.10, 0.15, 0.30 e.t.c) of H_20_2 into test tubes and 2ml of dichromate/acetic acid was added to each. It was heated for ten minutes in a boiling water bath after cooling the volume of the reaction mixture was made up to 3ml with distilled water and absorbance was read at 570 nm on a spectrophometer.

A 50 dilution of the liver homogenate was prepared. 1ml of the diluted homogenate was dispensed into a test tube, 4ml of H_20 solution and 5ml of phosphate buffer was added. The reaction mixture was thoroughly mixed again by a gently swirling motion. 1ml portion

of the reaction mixture was withdrawn and blown into 2ml of dichromate acetic acid reagent at 60 seconds intervals. The absorbance of the reaction mixture was determined at 570 nm and the H_20_2 content was extrapolated from the standard curve.

2.2.15.5 DETERMINATION OF SUPEROXIDE DIMUTASE (SOD) ACTIVITY

Superoxide dimutase (SOD) is an enzyme responsible for the removal of suproxide formed from oxygen in tissues. This was done by the method of Winterbourn *et al.*, 1975.

Principle:

The ability of superoxide dimutase (SOD) to inhibit the antioxidation of epinephrine at pH 10.2 forms the basis for a simple assay of dimutase. Oxygen generated from xanthine oxidase reaction causes the oxidation of epinephrine to adrenochrome produced per O^{2-} introduced increase with increasing pH, and with increasing concentration of epinephrine.

PROCEDURES:

A 10 dilution of the supernatant was prepared. An aliquot of 0.1ml of the diluted tissue supernatant was added to 1.25ml of 0.05M phosphate buffer (pH 7.8) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.15ml of freshly prepared 0.3M adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 1.25ml of the phosphate buffer, 0.15ml of substrate (adrenaline) and 0.1ml of distilled water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation:

Increase in absorbance per minute = $\frac{A_3 - A_0}{2.5}$

Where A_o = absorbance after 30 seconds

A_3 = absorbance after 150 seconds

% inhibition = $\frac{\text{Increase in absorbance of substrate x 100}}{\text{Increase in absorbance of blank}}$

2.2.16 DETERMINATION OF SERUM SODIUM AND POTASSIUM

Sodium was determination using sodium light filter was inserted and the galvanometer of the instrument, switched on with the gas supply fully on, the flame was ignited and the air supply was turned on. The air pressure was regulated to 101b/sq inch. The gas was then adjusted to obtain discrete concentration of the flame. The galvanometer was set to absorbance of zero with distilled water and it was then set to an absorbance of 70 percent using the working Na+ and K+ standard (STD) then the prepared serum solution were then read in turn on the galvanometer of the flame photometer (Kaplan and Pesce, 1996).

Calculations

$$\text{Sodium } Na^{+}(\text{mmol/L}) = \frac{\text{Reading of unknown x concentration of STD x 500}}{\text{Reading of STD}}$$

For potassium determination, the potassium filter was inserted and the galvanometer was then set at an absorbance of 50 percent, using the working potassium standard (STD). Then, the prepared serum

solutions were read in turn on the galvanometer of the flame photometer (Kaplan and Pesce, 1996).

Calculation

$$\text{Potassium } K^+ \text{ (mmol/L)} = \frac{\text{Reading of unknown} \times \text{Concentration of Std} \times 50}{\text{Reading of Standard}}$$

2.2.17 Determination of Serum Inorganic Phosphate

1ml of serum was pipette into test containing 9ml of 5% trichloroacetic acid & shake for five minutes. Then filter into a clean dry tube. 5ml of the clear filtrate was pipette another tube marked “T” and 0.4ml perchloric acid ($HClO_3$) followed by 0.4ml of 5% ammonium molybdate solution and 0.2ml of 0.2% ascorbic acid solution was added similarly a standard (STD) potassium dihydrogen phosphate solution (5ml = 0.2g) was titrated. Contents of tubes were mixed well and allowed to stand for ten minutes. Then read the colour in a colorimeter using a red filter and setting water blank at zero (Kaplan and Pesce, 1996).

Calculation

$$\text{Phosphate P (mmol/L)} = \frac{\text{Reading of test} \times 0.02 \times 100}{\text{Reading of STD } 0.5}$$

2.2.18 Determination of Chloride Concentration

The chloride was precipitated by the addition of an excess of volhard’s silver nitrate solution and he excess nitrated with ammonium

thiocyanate, using iron alum as indicator. Thiocyanate standard was prepared by dissolving 1.3g ammonium thiocyanate (NH_4CNS) in 100ml of distilled water and tritrated a mixture of 100ml Volhards $AgN0_3$ 5ml. conc. $HN0_3$ and 5ml. 4% ferric ammonium sulphate with the thiocyanate until the first permanent pink colour is obtained. Note the volume of NH_4CNS used was noted and 2ml of serum was put in a 10ml volumetric flask and 10ml concentrated nitrate and volhard's $AgN0_3$ solutions were added. The mixture was allowed to stand for a few minutes and filter. After make up to the mark with distilled water. Then, 100ml of the filtrate was measured into a flask and 5ml ferric alum and nitrate with the standard ammonium thiocyanate were added (Varly, 1962).

Calculation

1ml of standard $AgN0_3$ + 0.01g NaCl if n is the number of ml. of thiocynate used in the titration, other (25-n) x 0.01g

NaCl are present in 10ml serum i.e (25-n) 0.1g NaCl in 100ml serum.

2.2.19 Determination of Serum Biocarbonate

Acid was added in excess to serum in distilled water. Carbondioxide was liberated from $HC0_3^-$ and equivalent of H^+ being removed by the formation of water. The excess acid was then titrated with standard alkali using an indicator sensitive to it. Indictors that could be used include neutral red and phenol sulphonephthalein.

1ml of 0.05M Hcl was transferred into a conical flask by means of pipette and 0.2ml of serum sample was added. The content of the flask was then stirred for one minute and 5ml of $C0_2$ free distilled water was added. Two drops of phenol red indicator was then added. Back titration was carried out with 0.010M sodium hydroxide until the color of the solution changed to reddish purple. The same type of titration as explained above was carried out on a blank (Varly, 1962).

Calculation

Biocarbonate = 1000(M ml acid x M of acid)

(Yml of NaOH x Y NaOH]

= 1000[0.05 – 0.01g]

Where x = volume of acid used; Y = volume of base used for titration.

2.2.20 CHARACTERIZATION OF ACTIVE CONSTITUENTS

2.2.20.1 Nuclear Nagnetic Resonance (NMR) spectrometry

Fractions were subjected to 1H- spectra measured on a Varian (Palo Alto, CA, USA) Inova 500 Spectrometer at 499.87 MHz with TMS (tetramethylsilane) as internal standard.

2.2.20.2 Gas Chromatography/ Mass Spectrometry (GC/MS)

The Gas Chromatography/ Mass Spectrometry analysis was performed on an Agilent model 6890 gas chromatography with split/ splitless injector interfaced to a 5973 mass selective detector separated at 70eV with a mass range of m/2 50 -500. The same temperature programme as for the gas chromatography was used.

2.2.21 Database search and clustering of ribonucleotide reductase

A key word search was performed using the phase “Trypanosome brucei ribonucleotide reductase” against Pubmed database. Protein sequences with R1 (ACCESSION U80910) and R2 (ACCESSION U80911) were retrieved. These were used for BLASTP search using the default setting. The retrieved R1 and R2 sequences were aligned with BLOSUM 62. For the clustering, the alignment outcomes were submitted for phylogenetic tree and analyzed with the use of neighbor – joining algorithm. Specific features of the sequenced R1and R2 were predicted by the use (bioinformatics analysis) of online databases and relevant software including Protein families databases (Pfam), PROSITE (Bateman *et al.*, 2000), InterPro (http://www.ebi.ac.uk/InterProScan/), PSORT II and TMpred (Nakai and Horton, 1999).

2.2.22 Statistical analysis

The groups mean ± S.E.M was calculated for each analysis and significant difference between means was evaluated by analysis of variance (ANOVA). Post test analysis was done using the Duncan multiple comparism tests. Values of $p < 0.05$ were considered as statistically significant (Adamu and Johnson, 1997).

CHAPTER THREE

3.0 RESULTS

3.1 Phytochemical constituents of on fermented wheat and garlic bulb extracts.

The result of phytochemical analyses of 24hr fermented ethylacetate wheat extract showed appeciable amount of glycoside, alkaloids and saponins; moderate amount of phenol, tannins, flavonoid, steroids, terpenes and anthraquinone and trace amount of phlobatannins (Table 1).

Also, phytochemical analyses of 48hr fermented methanolic garlic extract show appreciable amount of glycoside, alkaloids and saponins; moderate amount of phenol, tannins, flavonoid, steroids, terpenes and anthraquinone and trace amount of phlobatannins (Table 1).

Table 1: Phytochemical constituents of fermented wheat and garlic bulbs extracts.

PHYTOCHEMICAL	**WHEAT (%)± S.D**	**GARLIC (%)± S.D**
TANNINS	0.071 ± 0.001	0.058 ± 0.000
PHENOL	0.074 ± 0.005	0.075 ± 0.001
FLAVONOIDS	0.0790± 0.000	0.052 ± 0.001
STEROID	0.073± 0.001	0.086± 0.004
SAPONINS	**7.992± 0.031**	**0.696± 0.184**
PHLOBATANNINS	0.020 ± 0.002	0.025 ± 0.001
TERPENES	0.0844 ± 0.002	0.063 ± 0.001
ALKALOIDS	**4.017± 0.259**	**3.570± 0.014**
GLYCOSIDES	**19.513± 0.111**	**21.088± 0.877**

ANTHRAQUINONE	0.150 ± 0.001	0.092 ± 0.001

Each value is a mean of four determinations.

3.2 Fraction of fermented wheat and garlic bulbs extracts.

Plate 1 showed the thin - layer chromatogram of fermented wheat extract. The result of fermented ethylacetate wheat extract (5.84g) submitted to silical gel column chromatography, eluted with step gradient of hexane: ethylacetate: methanol (A with 60:40:0, B with 40:60:0, C with 20:80:0, D with 0:100:0 and E with 0:60:40) give different spot, which implies five (5) fractions (A---E) are presents. The result show that fraction C and E have the same distance moved with the solvent of dissolution, which implies that fractions C and E are for solvent of dissolution.

Plate 2 showed the thin - layer chromatogram of garlic extract.

The result of methanolic garlic extract (8.66g) submitted to silical gel column chromatography, eluted with step gradient of hexane: ethylacetate: methanol (A with 90:10:0, B with 80:20:0, C with 0:80:20, and D with 0:70:30) to give four (4) fractions (A---D). Fraction B has the same distance moved with the solvent of

dissolution, which implies that fractions B are the fraction for solvent of dissolution.

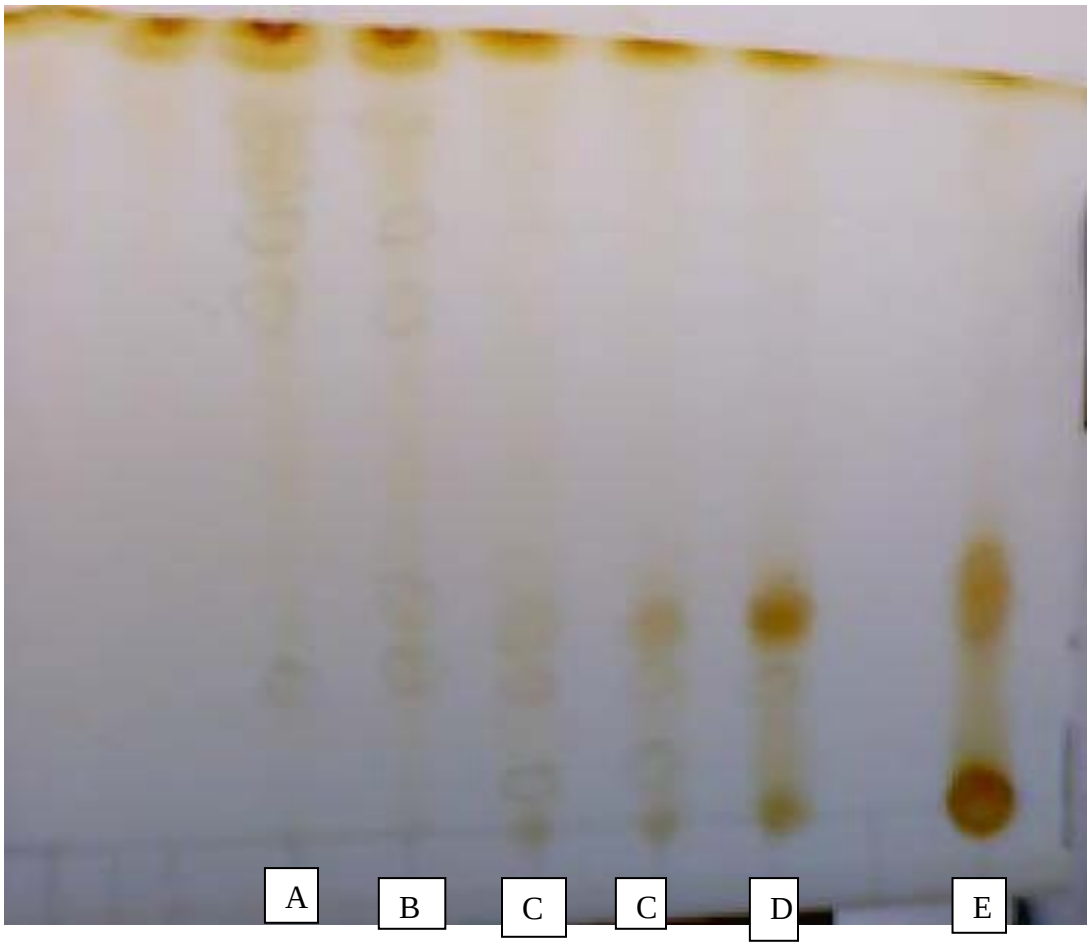

Plate 1: Thin layer chromatogram for fermented wheat extract fractions.

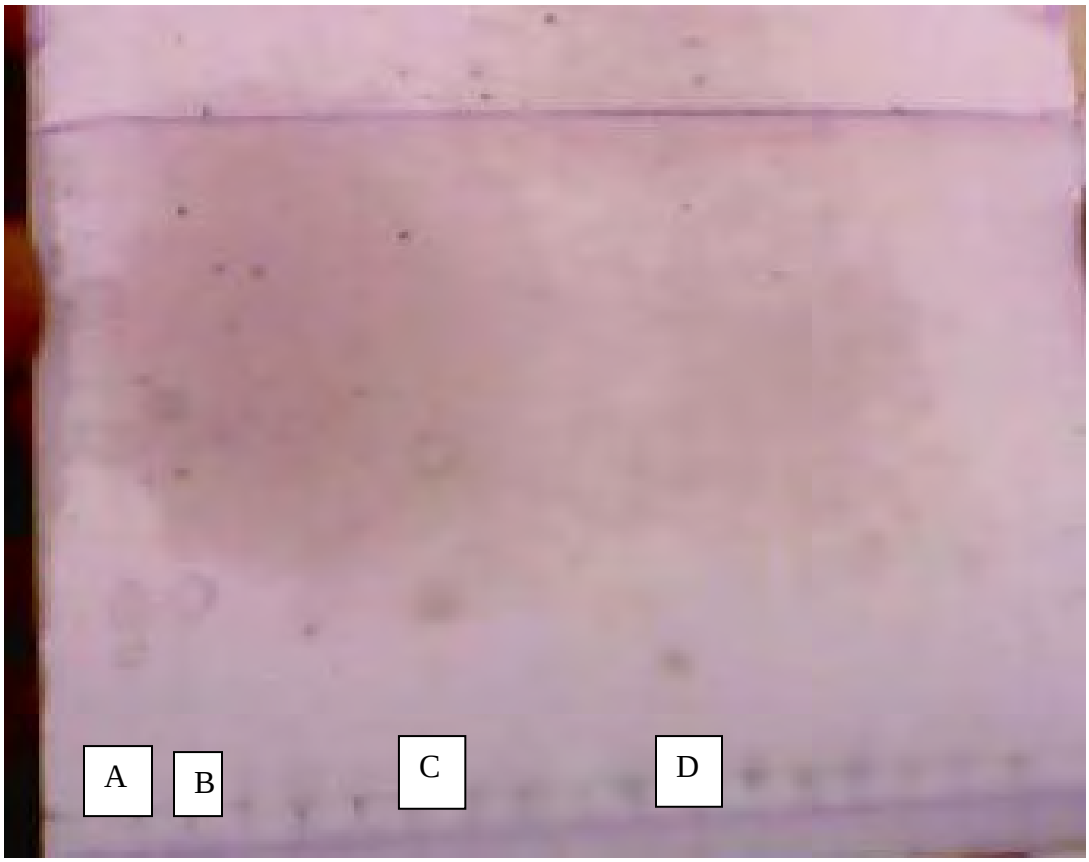

Plate 2: Thin layer chromatogram for garlic bulbs extract fractions.

3.3 Anti-trypanosomal properties of Fermented wheat germ and garlic bulbs extract in *T. brucei* – infected rats

Figure 1 showed the result of parasite count in infected rats treated with suramin, berenil (registered standard drugs), wheat and garlic bulbs extract at 300mg/kg body weight compared with the control (infected untreated) rats.

Suramin, berenil, wheat and garlic bulbs extracts were administered separately to infected rats to assess its activities against *T. brucei* infection. Suramin and berenil shows total clearance of parasite from the bloodstream after some days of treatment (24hrs with suramin and 48hrs with berenil) (Fig 1).The parasitaemia count of infected untreated group increased infinitely while infected treated with wheat extract shows a decrease in the proliferation (Fig 1). The graph shows low replication of parasite and extension of surviving days of rats treated with wheat extract from 8 days of the control (infected untreated) to 14 days for the infected treated group.

Also, the parasitaemia of infected treated with garlic bulbs extract of 300mglkg showed a decrease in the proliferation of parasite and extension of surviving days

of rats from 8 days of the control (infected untreated) to 17 days for infected garlic treated rats (Fig 1).

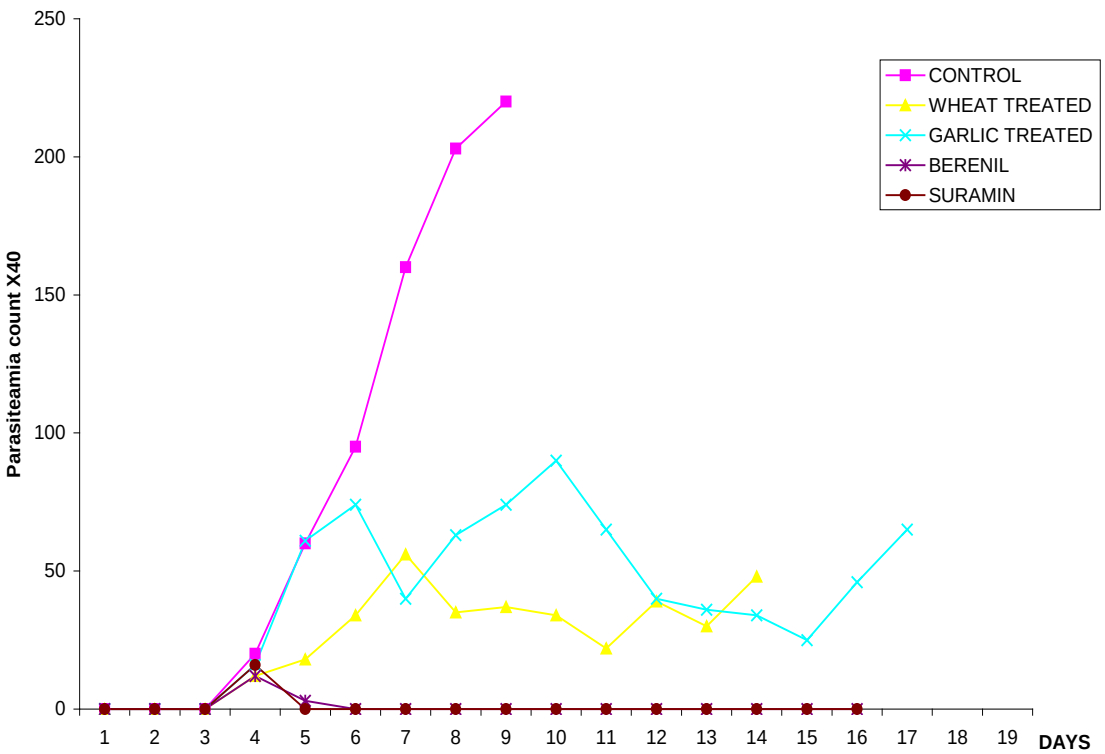

Fig 1: Parasiteamia count of rats infected with T.brucei and treated with standard drugs, fermented wheat and garlic extract.

Each point is an average count from five infected rats.

3.4 ENZYME STUDIES

The results of various enzymes studied on wheat extracts are presented in Figures 2 - 6 representing the specific activities of aspartate transaminase (AST), alanine transaninase (ALT), catalase (CAT), alkaline phosphatase (ALP) and superoxide dismutase (SOD) respectively.

Aspartate transaminase (AST)

The liver AST activities in the infected untreated, uninfected treated and infected treated groups were significantly increase ($p < 0.05$) than the uninfected not treated (control) group (Fig 2). The serum AST activities show significant increases ($p < 0.05$) in the uninfected treated and infected untreated when compared with the infected treated groups (Fig 2).

Alanine transaminase (ALT)

The results of the ALT activities in the serum and liver are presented in Fig 3. There was no significant difference ($p<0.05$) in liver enzyme activities of uninfected not treated (control) and other experimental groups. The serum ALT activities in the infected untreated and infected treated groups are not significantly different ($p<0.05$) when compared with the uninfected not treated

group, while the uninfected treated group are low when compare with other experimental groups.

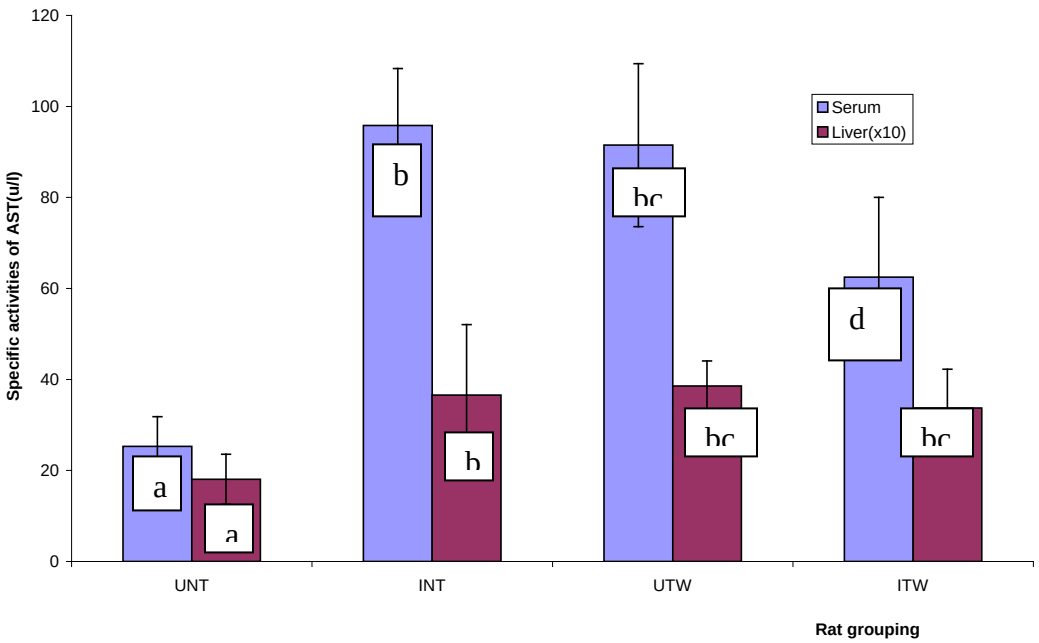

Fig 2: Specific activities of Aspartate Transaminase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated (control)
INT: Infected untreated
UTW: Uninfected treated with wheat
ITW: Infected treated with wheat

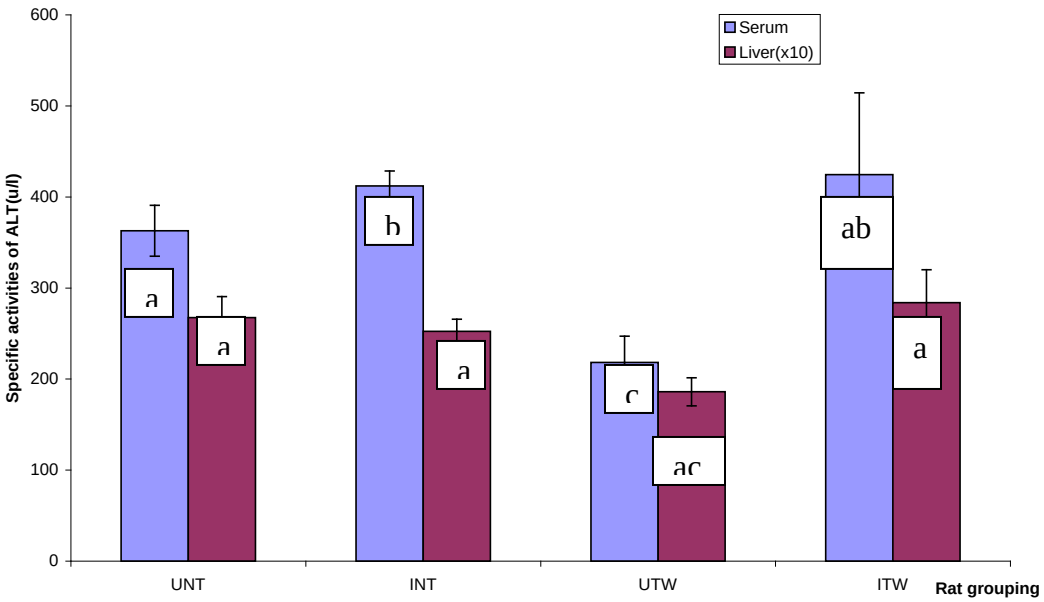

Fig 3: Specific activities of Alanine Transaminase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated (control)

INT: Infected untreated

UTW: Uninfected treated with wheat

ITW: Infected treated with wheat

Alkaline phosphatase (ALP)

Results of serum and liver ALP assays are shown in Fig 4. There was significant decrease ($p<0.05$) in the liver enzyme activities infected untreated, uninfected treated group and infected treated group when compare with uninfected not treated (control) group. While at $p<0.05$, serum ALP activities were significantly lower in infected untreated, uninfected treated and infected treated groups when compared with uninfected not treated (control) group.

Catalase (CAT)

The specific activities of catalase in serum and liver are shown in Figure 5. Serum and liver catalase activities of uninfected not treated (control) were significantly higher ($p<0.05$) than the other experimental groups.

Superoxide dismutase (SOD)

The results of serum and liver SOD activities are presented in Figure 6. The liver SOD activities of infected untreated and infected treated groups were significantly lower ($p<0.05$) when compared with uninfected treated group which was significantly lower than the uninfected not treated (control) group. The serum SOD activities of infected untreated were significantly lower ($p<0.05$) when compared with uninfected treated, infected treated and uninfected not treated (control) groups.

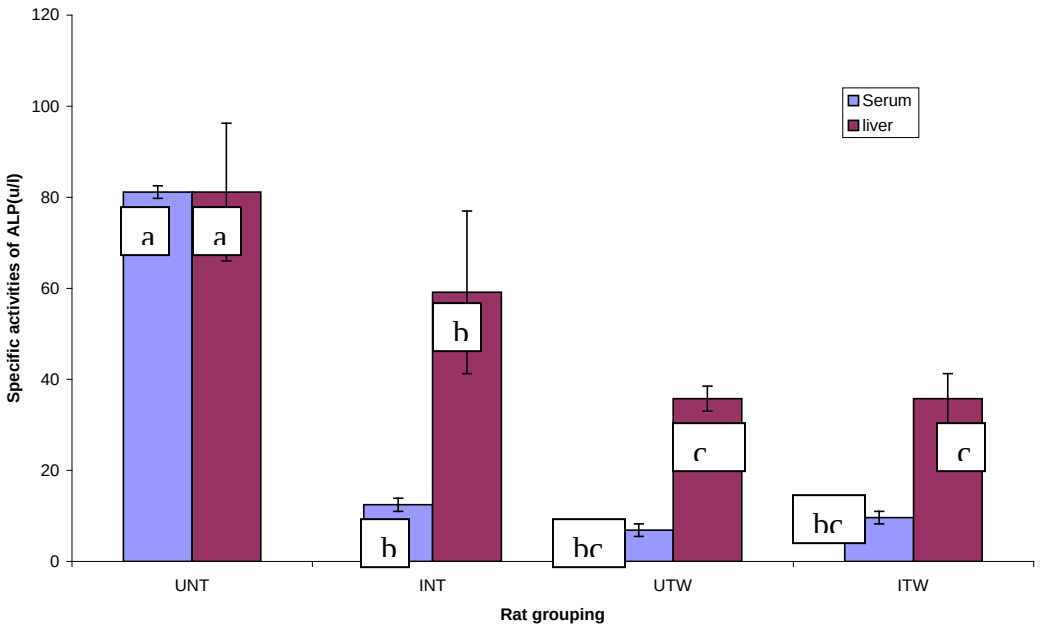

Fig 4: Specific activities of Alkaline Phosphatase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated (control)

INT: Infected untreated

UTW: Uninfected treated with wheat

ITW: Infected treated with wheat

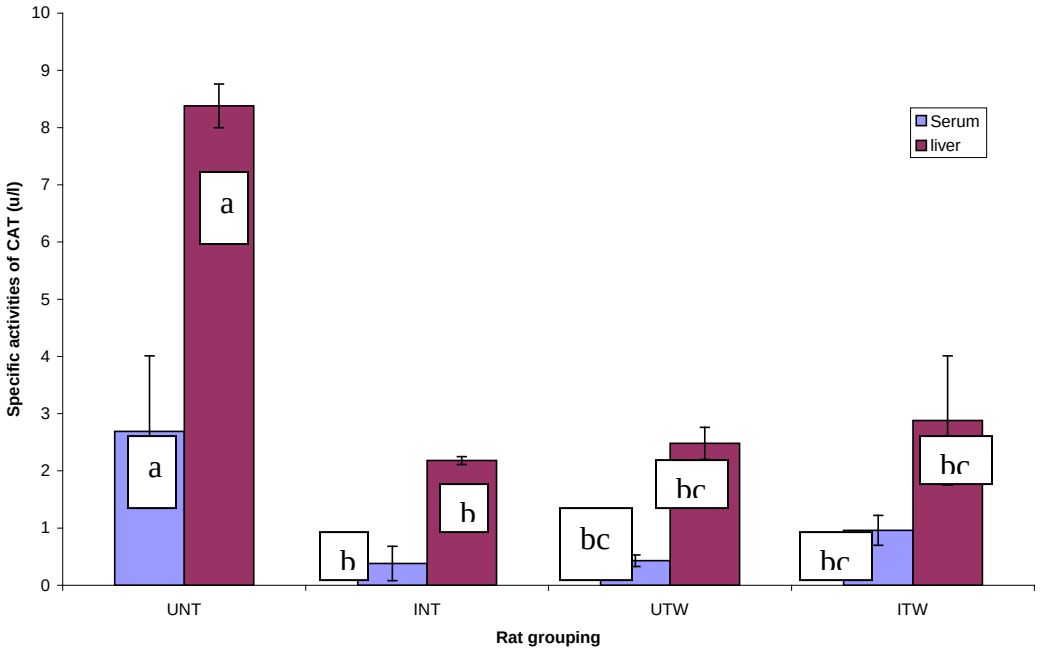

Fig 5: Specific activities of Catalase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated (control)

INT: Infected untreated

UTW: Uninfected treated with wheat

ITW: Infected treated with wheat

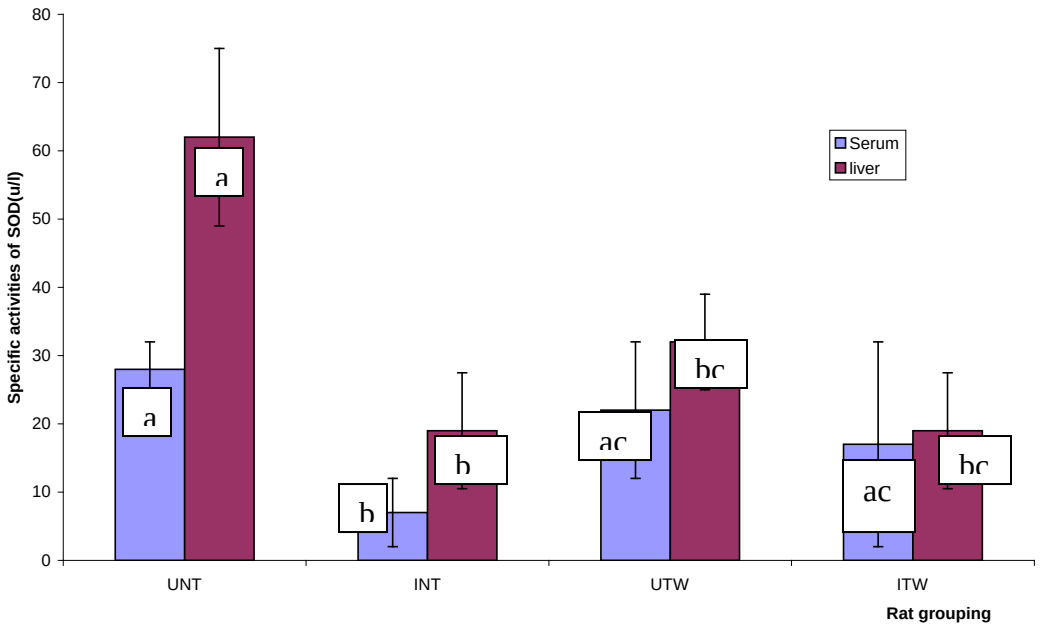

Fig 6: Specific activities of Superoxide Dismutase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated (control)

INT: Infected untreated

UTW: Uninfected treated with wheat

INT: Infected treated with wheat

The results of various enzymes studied of garlic extract are presented in Figures 7 - 11 representing the specific activities of aspartate transaminase (AST), alanine transaninase (ALT), catalase (CAT), alkaline phosphatase (ALP) and superoxide dismutase (SOD) respectively.

Aspartate transaminase (AST)

There was significant increase ($p<0.05$) in liver AST of infected untreated group when compared with uninfected not treated (control), uninfected treated and infected treated with garlic (Fig 7).Also, serum aspartate transamimase shows significant increases ($p<0.05$) in infected untreated and uninfected treated with garlic when compared with uninfected not treated (control) and infected treated with garlic groups.

Alanine transaminase (ALT)

Liver alanine transaminase activities of infected untreated shows significant decrease ($p<0.05$) when compared with uninfected not treated (normal), uninfected treated and infected treated groups (Fig 8).While serum specific ALT activities (Fig 8) of infected untreated rats was significantly higher

($p<0.05$) when compared with infected treated, uninfected treated and control groups.

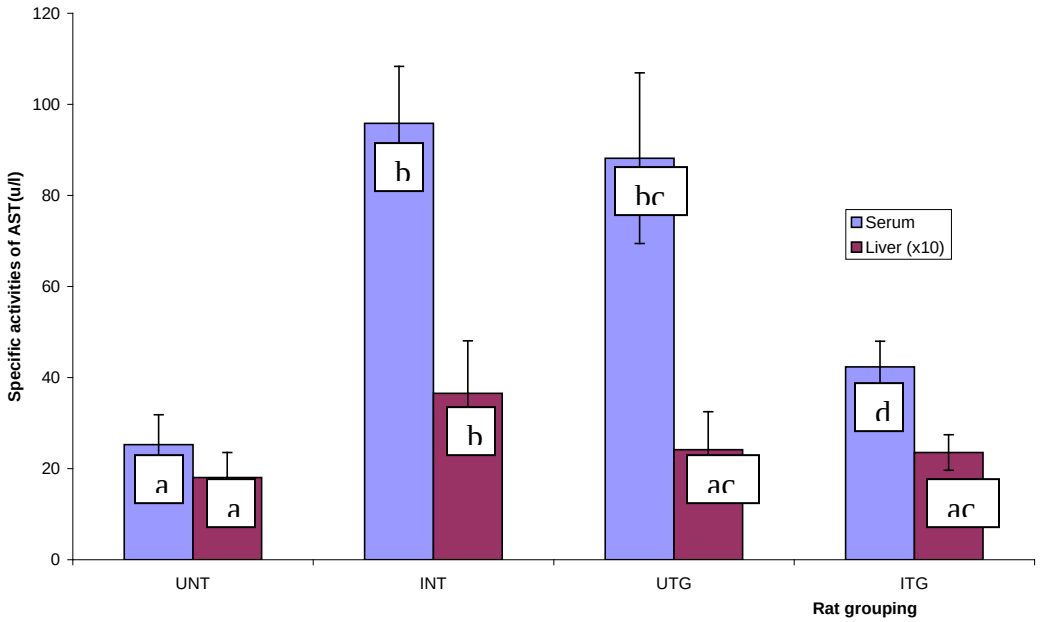

Fig 7: Specific activities of Aspartate Transaminase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT:

Uninfected not treated

INT: Infected untreated

UTG: Uninfected treated with garlic

ITG: Infected treated with garlic

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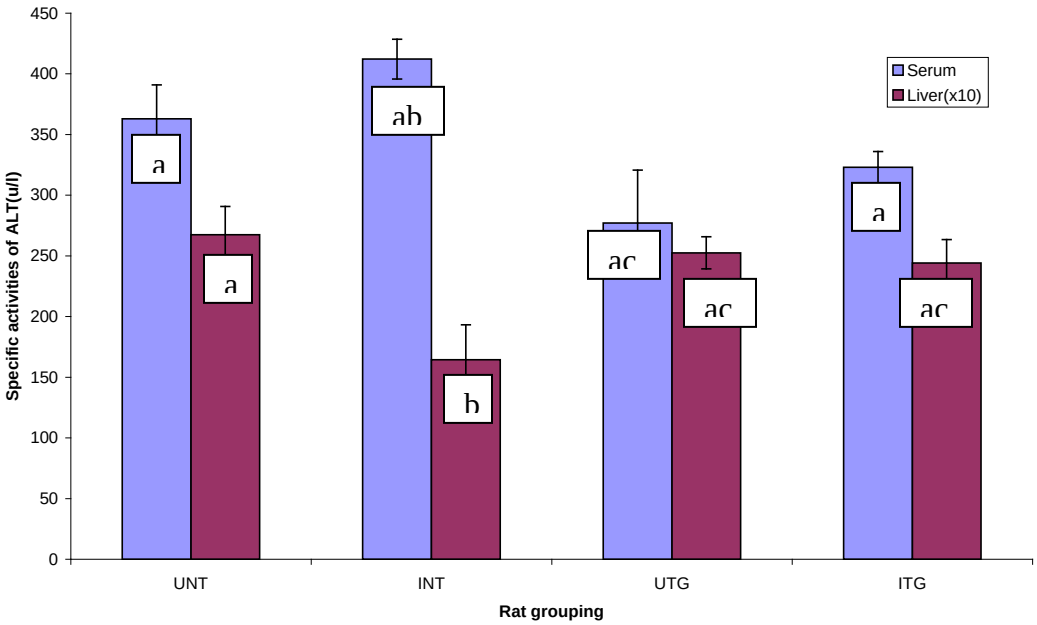

Fig 8: Specific activities of Alanine Transaminase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated

INT: Infected untreated

UTG: Uninfected treated with garlic

ITG: Infected treated with garlic

Alkaline phosphatase (ALP)

Results of serum and liver alkaline phosphatase assay are shown in Fig 9. The liver alkaline phosphatase activities in infected untreated and infected treated as well as uninfected treated group were significantly lower ($p<0.05$) when compared with uninfected not treated (control) group. At $P < 0.05$, serum alkaline phosphatase activities were significantly lower in infected untreated and uninfected treated groups when compared with infected treated and uninfected not treated (control) groups.

Catalase (CAT)

The specific activities of catalase in serum and liver assays are shown in Fig 10. Liver and serum catalase activities of uninfected not treated (control) are significantly higher ($p<0.05$) than the other experimental groups.

Superoxide dismutase (SOD)

The liver specific activities of superoxide dismutase (SOD) of infected untreated (control) group was significantly lower ($p<0.05$) than the other group while uninfected treated and infected treated groups show no significant difference (Fig 11). The serum SOD activities of infected untreated were significant lower

(p<0.05) than uninfected treated, infected treated and uninfected not treated (control) groups.

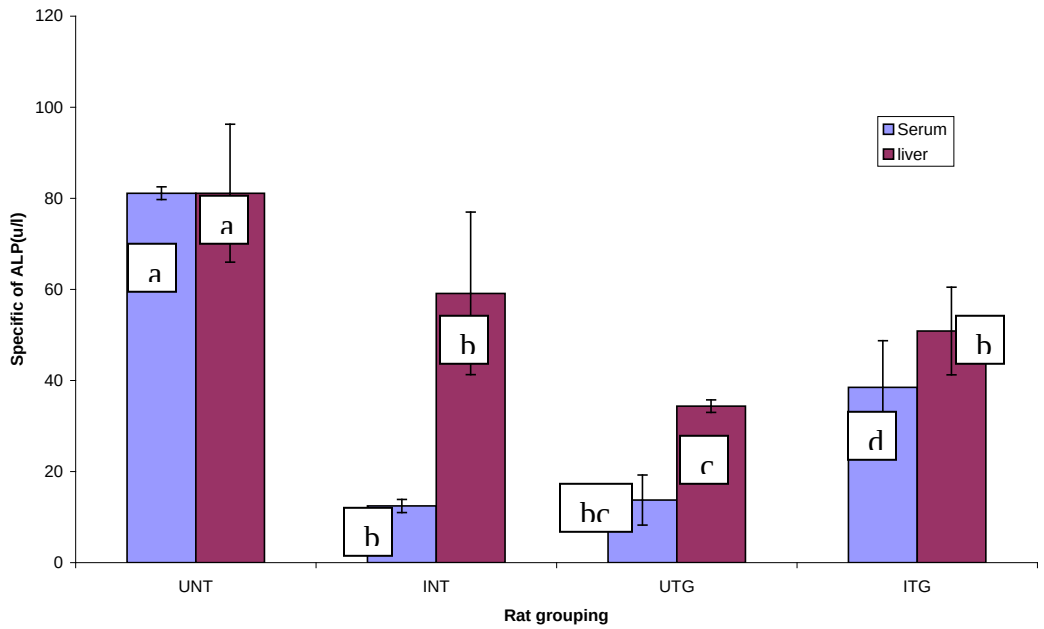

Fig 9: Specific activities of Alkaline Phosphatase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated

INT: Infected untreated

UTG: Uninfected treated with garlic

ITG: Infected treated with garlic

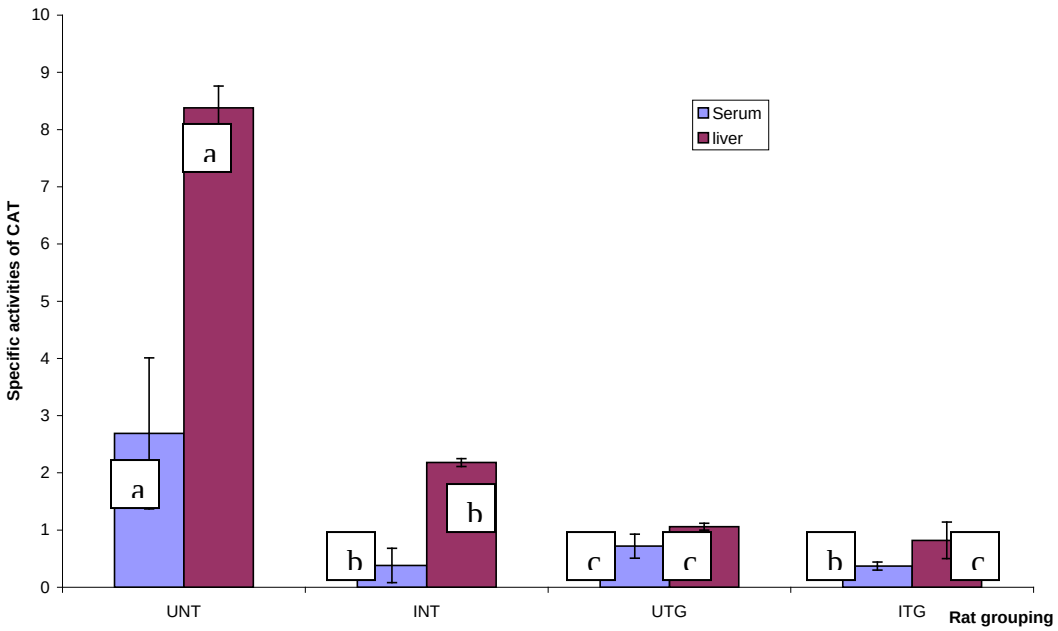

Fig 10: Specific activities of Catalase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT: Uninfected not treated

INT: Infected untreated

UTG: Uninfected treated with garlic

ITG: Infected treated with garlic

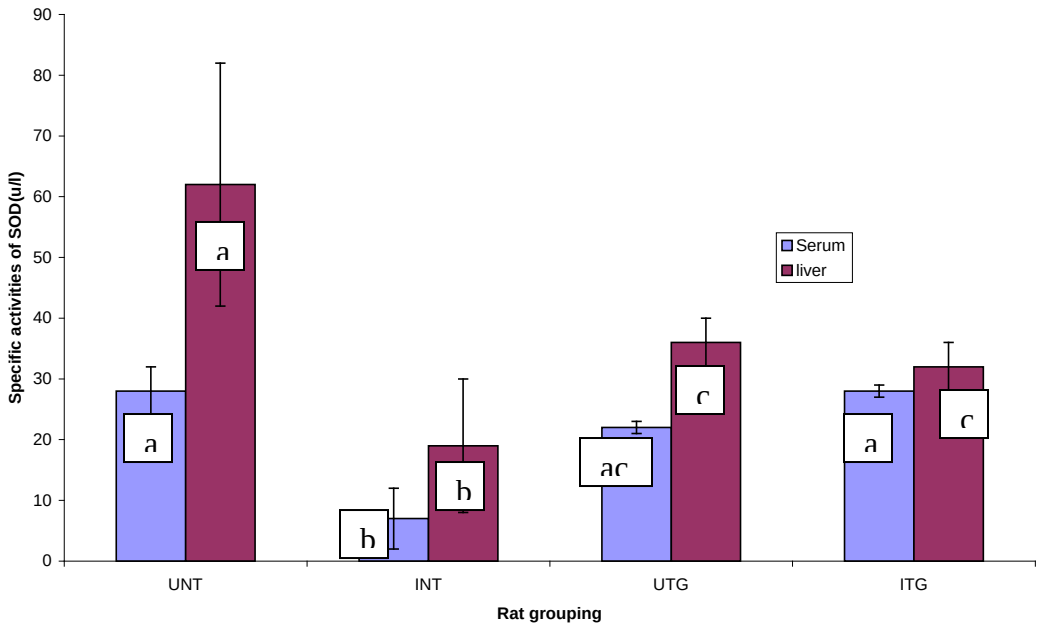

Fig 11: Specific activities of Superoxide Dismutase in the serum and liver of rats. Result are mean of four determinations + S.E.M. Bars carrying different letters are significantly different at p<0.05

UNT:

Uninfected not treated

INT: Infected untreated

UTG: Uninfected treated with garlic

ITG: Infected treated with garlic

3.5 Haematological studies

The results of haematological studies for the period of 10days post infection are presented in Table 2. There were significant decrease ($P<0.05$) in the values of packed cell volume (PCV), haemoglobin concentration (Hb) and red blood cell (RBC) count of the infected untreated groups in comparison with the uninfected not treated, uninfected treated and infected treated groups. Also, significant increase ($P<0.05$) was observed in white blood cell (WBC) count of the infected untreated groups when compared with the uninfected not treated, uninfected treated and infected treated groups. There was no significant difference ($P<0.05$) in the mean cell haemoglobin concentration (MCHC) of all the experimental groups.

Table 2: Haematological studies of *T. brucei* infected rats at 10 days post infection.

Rat Grouping	PCV (%)	Hb (g/dl)	RBC ($x10^{12}$/l)	WBC ($x10^{12}$/l)	MCHC (%)
Grp1	53.33±0.00[a]	17.76±0.00 [a]	4.95±0.38 [a]	2.44±0.06 [a]	33.31±0.00 [a]
Grp2	34.50±4.68[b]	11.50±1.57 [b]	2.99±0.45 [b]	8.19±0.38 [b]	33.39±0.07 [a]
Grp3	57.50±5.83[ac]	18.83±1.95 [ac]	6.75±0.63 [c]	2.57±0.22 [ac]	33.39±0.43 [a]
Grp4	56.50±6.83 [ac]	18.85±1.51 [ac]	6.15±0.44 [c]	2.72±0.40 [ac]	33.36±0.05 [a]
Grp5	45.00±8.30 [a]	15.00±3.25 [ac]	5.08±0.44 [ac]	4.12±0.16 [acd]	33.33±0.04 [a]
Grp6	47.50±10.20 [a]	15.85±3.39 [ac]	5.17±0.48 [ac]	4.59±1.81 [acd]	33.38±0. 61 [a]

Means along the same column with different superscript are significantly different in comparison with a control, b infected-untreated and c uninfected treated ($P<0.05$), values are means of four determinations ± S.E.M

Group1: Uninfected not treated (control)

Group2: Infected untreated

Group3: Uninfected treated with wheat

Group4: Uninfected treated with garlic

Group5: Infected treated with garlic

Group 6: Infected treated with wheat

3.6 Electrolyte profile

The effects of the extract on some serum electrolyte are presented in Table 3. There was significant increase ($p<0.05$) in Na^+ concentration of infected untreated group when compared with the uninfected not treated (control), uninfected treated and infected treated groups. Also, Cl^- ($p<0.05$) concentration was significantly elevated in all other experimental groups when compared with uninfected not treated (control) group. Whereas there was no significant difference ($p<0.05$) in all other electrolyte (P, HCO_3^-, K^+) compared to the uninfected not treated (control) group.

Table 3: Serum electrolyte profile of *T. brucei* infected rats at 10 days post infection.

Rat Grouping	Na^+ (mmol/L)	K^+ (mmol/L)	P (mmol/L)	HCO_3- (mmol/L)	Cl^- (mmol/L)
Grp1	50.00±0.00 [a]	0.55±0.00 [a]	48.67±0.38 [a]	22.62±0.06 [a]	18.00±0.00 [a]
Grp2	91.67±16.67 [b]	0.83±0.28 [a]	46.67±10.69 [a]	25.00±0.33 [a]	24.33±1.76 [b]
Grp3	62.5±19.09 [ac]	0.83±0.14 [a]	59.00±13.47 [a]	24.50±1.04 [a]	25.00±1.73 [bc]
Grp4	75.00±14.43 [ac]	0.83±0.14 [a]	65.00±13.47 [a]	24.50±0.60 [a]	26.00±1.73 [bc]
Grp5	75.00±14.43 [ac]	0.62±0.25 [a]	47.00±27.23 [a]	24.00±2.03 [a]	28.00±1.73 [bc]
Grp6	50.00±28.87 [a]	0.41±0.14 [a]	64.00±8.74 [a]	22.00±1.05 [a]	28.50±1.80 [bc]

Means along the same column with different superscript are significantly different in comparison with a control, b infected-untreated and c uninfected treated ($P<0.05$), values are means of four determinations ± S.E.M

Group 1: Uninfected not treated (control)

Group 2: Infected untreated

Group 3: Uninfected treated with wheat

Group 4: Uninfected treated with garlic

Group 5: Infected treated with garlic

Group 6: Infected treated with wheat

3.7 Gas chromatography/ Mass spectroscopy analysis of fermented wheat and garlic bulbs extracts.

The results of Gas chromatography/ Mass spectroscopy (GC/MS) analysis of the various fractions of wheat extract are presented in Tables 4 - 6 representing fraction A, B and D respectively.

Fraction A

The result of GC/MS analysis of the fractions A of wheat extract showed the presence of twelve (12) compounds corresponding to 64.90% of the total fraction (Table 4). The compound comprises of n- hydrocarbon (36.90%), oxygenated hydrocarbon (19.50%), aromatic hydrocarbon (8.50%). The major compound: Novacosane (9.10%), 1-Eicosene (13.77%) and Benzene (5.64%). The prominent among the oxygenated hydrocarbon are: Hexadecyl acetate (12.19%), Ethyl undecanoate (3.64%) and Octadecan -1-ol (3.64%). While that of aromatic hydrocarbon is. Methyl-z-(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate (8.50%)(Table 4).

Table 4: COMPOUNDS PRESENT IN FRACTION A OF FERMENTED WHEAT EXTRACT

KI	COMPOUND	%
642	Benzene	5.64
1329	1,3- Benzodioxole-5- Carbaldehyde	1.27
1224	5- hydroxymethyl-2-furancarboxaldehyde	1.09
1494	Ethyl undecanoate	3.64
1676	Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate	8.55
1786	Butyl dodecanoate	1.27
1994	1-Eicosene	13.77
2009	Hexadecyl acetate	12.19
2500	Pentacosane	4.91
2900	Nonacosane	9.10
2370	9-Tetracosene	3.46
2082	1-Octadecan-1-ol	3.64
	n-hydrocarbon	36.90
	Oxygenated hydrocarbon	19.50
	Aromatic hydrocarbon	8.50
		64.90

Each value is a mean of four determinations.

Fraction B

The GC/MS analysis of the fractions B of wheat extract revealed the presence of fourteen (14) compounds corresponding to 82.60% of the total fraction (Table 5). The compound comprises of n- hydrocarbon (28.10%), oxygenated hydrocarbon (49.50%), aromatic hydrocarbon (5.00%).The major compound 9-Novacosane (7.19%), 1-Eicosene (5.62%) and 5- Octadene (5.75%). The prominent among the oxygenated hydrocarbon are: Manool (15.05%), Butyl dodecanoate (10.15%) and Octadec -9-enoic acid (7.98%). While that of aromatic hydrocarbon is. Methyl-z-(1R, 2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate (5.03%)(Table 5).

Table 5: COMPOUNDS PRESENT IN FRACTION B OF FERMENTED WHEAT EXTRACT

KI	COMPOUND	%
1494	Ethyl undecanoate	4.24
1676	Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate	5.03
1922	Dibutyl phthalate	5.32
1786	Butyl dodecanoate	10.15
1994	1-Eicosene	5.62
2056	Manool	15.05
2200	Octadec-9-enoic acid	7.98
2456	Tetracosan-1-ol	5.41
2370	9-Tetracosene	5.03
2195	1-Docosene	3.15
2900	Nonacosane	7.19
2830	10- Demethyl squalene	1.28
2852	Hexacosan-1-ol	1.28
	5- Octadene	5.75
	n-hydrocarbon	28.10
	Oxygenated hydrocarbon	49.50
	Aromatic hydrocarbon	5.00
		82.60

Each value is a mean of four determinations.

Fraction D

The GC/MS analysis of the fractions C of wheat extract showed the presence of twenty three (23) compounds corresponding to 72.10% of the total fraction (Table 6).

The compound comprises of n- hydrocarbon (35.70%), oxygenated hydrocarbon (29.60%), aromatic hydrocarbon (5.20%) and nitrogen containing hydrocarbon (1.60%).The major compound 1-Eicosene (6.03%), Pentacosane(5.535) and Tetracosene (4.79%). The prominent among the oxygenated hydrocarbon are: Butyl dodecanoate (5.78%) Octadecanoic acid (4.98%) and 5-Tetradecencyl acetate (3.49%). While that of aromatic hydrocarbon is. Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate (5.19%) and nitrogen containing hydrocarbon is 2-phenyl acetonitrile(1.60%)(Table 6).

Table 6: COMPOUNDS PRESENT IN FRACTION D OF FERMENTED WHEAT EXTRACT

KI	COMPOUND	%
642	Benzene	3.29
1065	Phenyl ethanone	5.00
1197	1,2-Benzenediol	1.20
1140	2- Phenyl acetonitrile	1.60
1224	5- hydroxymethyl-2-furancarboxaldehyde	0.80
1274	1-(4-ethylphenyl)-ethanone	1.40
1392	1-Tetradecene	2.80
1494	Ethyl undecanoate	3.29
1676	Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate -	5.19
1790	(z)- 5- Tetradecenyl acetate	3.49
1786	Butyl dodecanoate	5.78
1793	1- Octadene	4.59
1994	1- Eicosese	6.03
2200	Octadecanoic acid	4.89
2370	9-Tetracosene	4.09
2400	Tetracosene	4.77
2900	Nonacosane	3.59
2862	Hexacosan-1-ol	0.47
2456	Tetracosan-1-ol	1.70
2600	Hexacosane	1.00
2500	Pentacosane	5.53
	4-hdroxy-2,5-dimethylfuran-3(H) one	0.47
	3,4,5-Trimethoxybenzaldehyde	0.98
	n-hydrocarbon	35.70
	Oxygenated hydrocarbon	29.60
	Aromatic hydrocarbon	5.20
	Nitrogen containing hydrocarbon	1.60

		72.10

Each value is a mean of four determinations.

The results of Gas chromatography/ Mass spectroscopy (GC/MS) analysis of the various fractions of garlic extract are presented in Tables 7 - 9 representing fraction A, C and D respectively.

Fraction A

The GC/MS analysis of the fractions A of garlic extract showed the presence of sixteen(16) compounds corresponding to 71.80% of the total fraction(Table 7).The compound comprises of n- hydrocarbon(21.70%), oxygenated hydrocarbon(45.50%) and unknown compound(4.60%).The major compounds in n- hydrocarbon are: Octacosane (7.10%),Hexacosane (4.8%) and 5-Octadecene(3.26%). The prominent among the oxygenated hydrocarbon are: Octadecanoic acid (15.92%),Hexadecanoic acid (13.74%) and 9-Octadecen -8-olide(12.47%)(Table 7).

Table 7: COMPOUNDS PRESENT IN FRACTION A OF FERMENTED GARLIC EXTRACT

KI	COMPOUND	%
701	2,3 –Pentanedione	0.77
792	1- octane	1.15
1593	1- hexadecane	2.50
1900	Nonadecane	2.88
1984	Hexadecanoic acid	13.74
2009	Octadecanoic acid	15.92
2800	Octacosane	7.10
2600	Hexacosane	4.80
2456	Tetracosan-1-ol	5.57
	9-Octadecen-18-olide	12.47
	5-Octadecene	3.26
	Unknown compound	4.61
	n-hydrocarbon	21.70
	Oxygenated hydrocarbon	45.50
	Unknown compound	4.61
		71.81

Each value is a mean of four determinations.

Fraction C

Determination of compounds in fraction C of garlic extract by GC/MS identified sixteen (16) compounds corresponding to 72.70% of the total fraction (Table 8).The compound comprises of n- hydrocarbon (41.70%), oxygenated hydrocarbon (24.10%) and aromatic hydrocarbon 6.90%).The major compounds in n- hydrocarbon are: 9- Tetracosene (11.05%), Tetracosane (4.75%) and 5-Octadecene(9.06%). The prominent among the oxygenated hydrocarbon are: Hexacosan-1-ol (8.63%), Octadecyl acetate (4.64%) and 1-Phenyl ethanone (3.44%) while prominent among aromatic hydrocarbon includes 2-methoxy-4-(2-propenyl) – phenol acetate (2.71%) andMethyl-z-(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate (4.17%)(Table 8).

Table 8: COMPOUNDS PRESENT IN FRACTION C OF FERMENTED GARLIC EXTRACT

KI	COMPOUND	%
1065	1- Phenyl ethanone	3.44
1392	1-Tetradecene	1.84
1481	Tridecan-2-one	1.12
1524	2-methoxy-4-(2-propenyl) – phenol acetate	2.71
1676	Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate	4.17
1841	2-Phemethyl benzoate	2.33
1994	1-Eicosene	5.91
2128	Octadecyl acetate	4.65
2195	1-Docosene	5.43
2370	9-Tetracosene	11.05
2400	Tetracosane	4.75
2800	Octacosane	3.68
2848	Hexacosanol	2.91
2852	Hexacosan-1-ol	8.63
	2S,3S-Methyi-2-amino-3-methyl pentanoate	1.07
	5-Octadecene	9.06
	n-hydrocarbon	41.70
	Oxygenated hydrocarbon	24.10

	Aromatic hydrocarbon	6.90
		72.70

Each value is a mean of four determinations.

Fraction D

The GC/MS analysis of the fractions D garlic extract showed the presence of seventeen (17) compounds corresponding to 87.04% of the total fraction(Table 9).The compound comprises of n- hydrocarbon(42.640%), oxygenated hydrocarbon(27.50%), aromatic hydrocarbon(10.30%) and nitrogen containing hydrocarbon(6.60%).The major compounds in n- hydrocarbon are: 1-Docosene (14.310%), 1-Octadene (11.90%) and Hexacosane (6.02%). The prominent among the oxygenated hydrocarbon are: 9-Octadecen -8-olide (7.60%), Hexadecyl acetate (6.55%) and Serkirkine acetate (4.81%). While that of aromatic hydrocarbon are. 2-methoxy-4-(2-propenyl) – phenol acetate (3.74%) and Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate (6.55%) and the nitrogen containing hydrocarbon are Integerrimine (2.64%) and Senkirkine (3.88%)(Table 9).

Table 9: COMPOUNDS PRESENT IN FRACTION D OF FERMENTED GARLIC EXTRACT

KI	COMPOUND	%
2600	Hexacosane	6.02
2402	Integerrimine	2.67
2470	Senkirkine	3.88
2800	Octacosane	4.55
2500	Pentacosane	3.08
2456	Tetracosan-1-ol	3.61
2195	1-Docosene	14.31
2009	Hexadecyl acetate	6.55
1793	1-Octadene	11.90
1676	Methyl-z(1R,2S)-3-oxo-2-(z)-pent-2-cyclopentyl-acetate	6.55
1524	2-methoxy-4-(2-propenyl) – phenol acetate	3.74
1383	E-(3,7-Dimethyl-2,6-Octadienyl acetate	2.94
1116	Tetradecane	1.60
1094	Methyl-2-butenoate	1.74
642	Benzene	0.94
	Serkirkine acetate	4.81
	9-Octadecen-18-olide	7.62
	n-hydrocarbon	42.64
	Oxygenated hydrocarbon	27.50
	Aromatic hydrocarbon	10.30
	Nitrogen containing hydrocarbon	6.60
		87.04

Each value is a mean of four determinations.

3.8 ^{1}H - Nuclear Magnetic Resonance analysis of fermented wheat and garlic bulbs extracts.

The results of ^{1}H - Nuclear Magnetic Resonance (NMR) analysis of the various fractions of wheat extract are presented in Figures 12 - 14 representing fraction A, B and D respectively.

Fraction A

^{1}H - NMR experiment of the fractions A wheat extract indicated the presence of methyl protons at δ0.9 and cluster of methylene protons adjacent to double bond at δ1.8, 2.0 and 2.3. There is also characteristic signal of double bond methines at δ5.3 and acidic signal at δ8.1 (Fig 12).

Fraction B

^{1}H- NMR experiment of the fractions B wheat extract indicated the presence of methyl protons at δ0.9 and cluster of methylene protons adjacent to double bond at δ1.6, 2.1 and 2.6. There is also characteristic signal of double bond methines at δ5.4. ^{1}H NMR also indicated the presence of hetero- atom between δ 3.0 (Fig 13).

Fraction D

^{1}H NMR experiment of the fractions D wheat extract indicated the presence of methyl protons at δ0.9 and cluster of methylene protons adjacent to double bond at

δ1.3. There is also characteristic signal of double bond methines at δ5.3. ^{1}H NMR also indicated the presence of hetero- atom between δ 3.5 and 3.7(Fig 14).

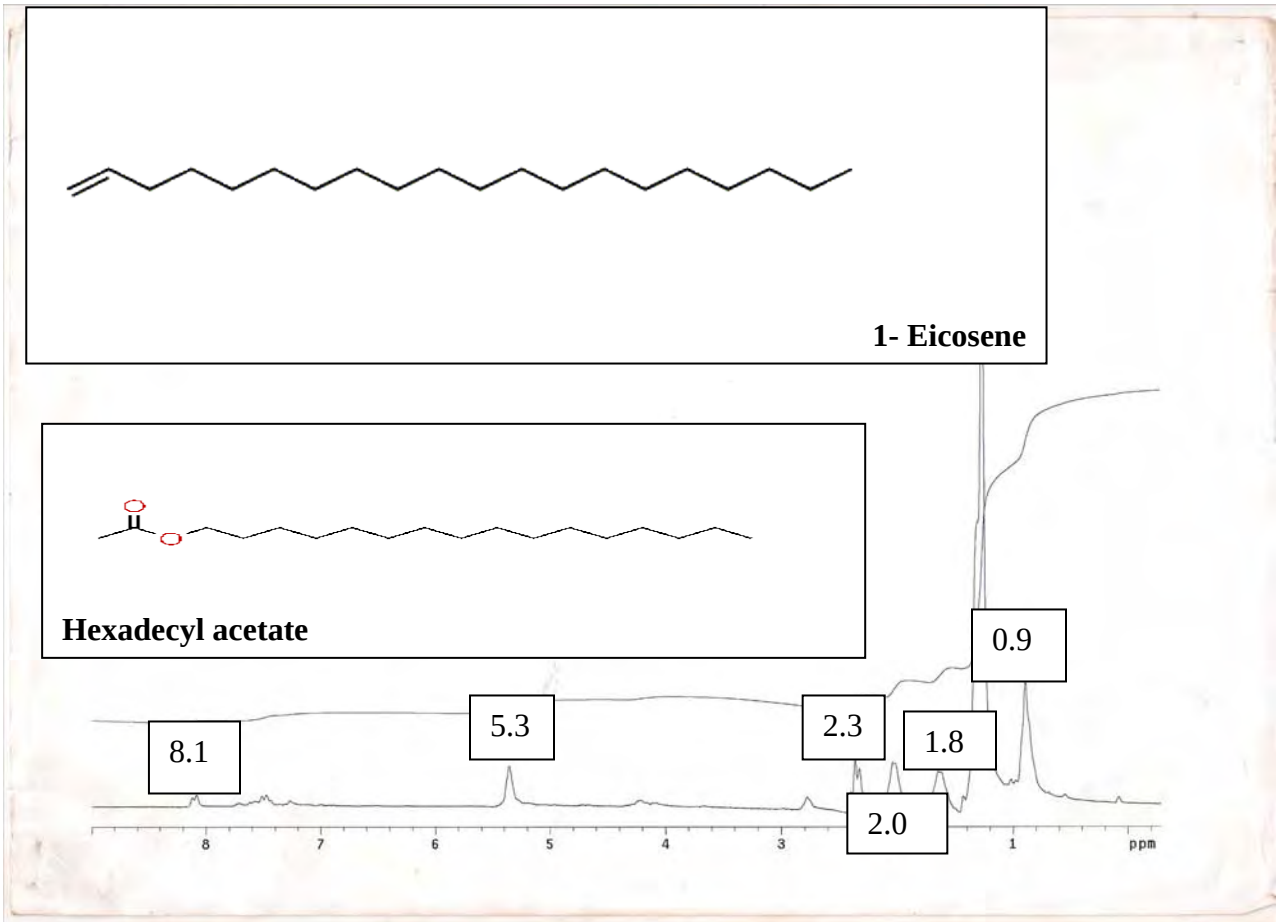

Fig 12: COMPOUNDS PRESENT IN FRACTION A OF FERMENTED WHEAT EXTRACT

Manool

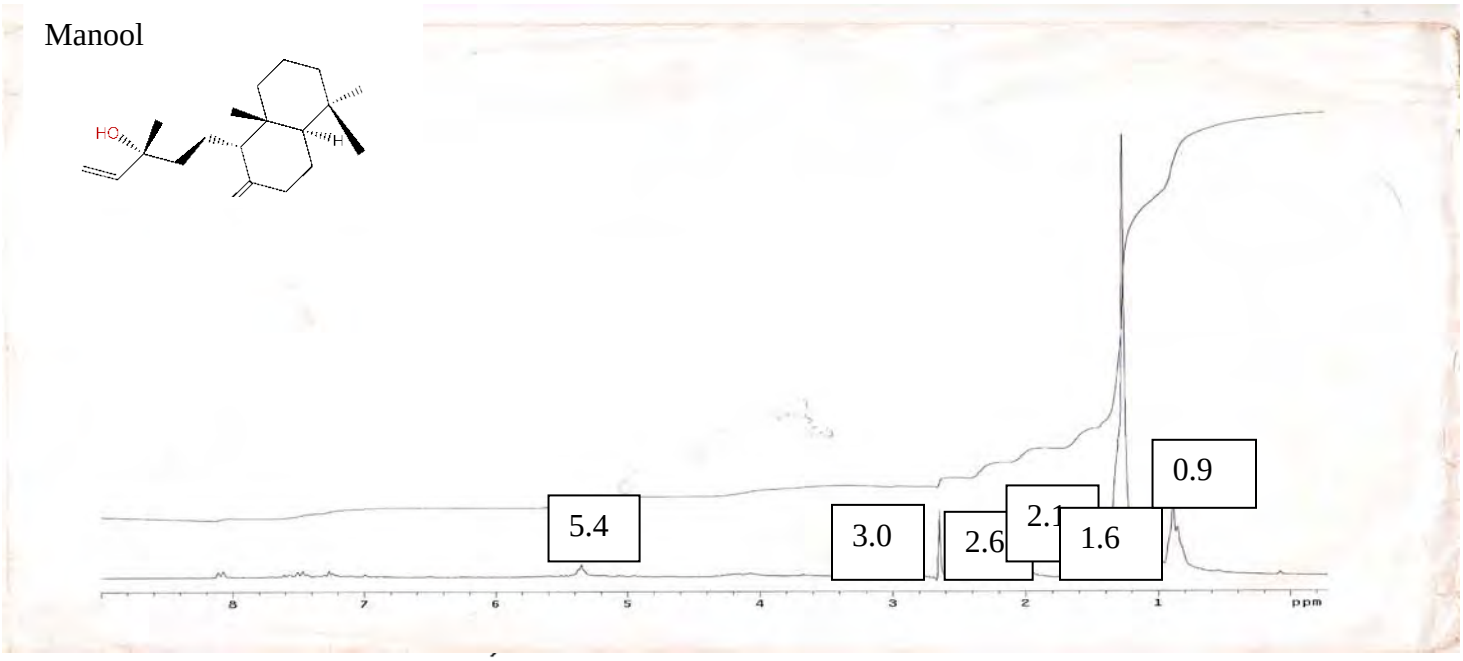

Fig 13: COMPOUND PRESENT IN FRACTION B OF FERMENTED WHEAT EXTRACT

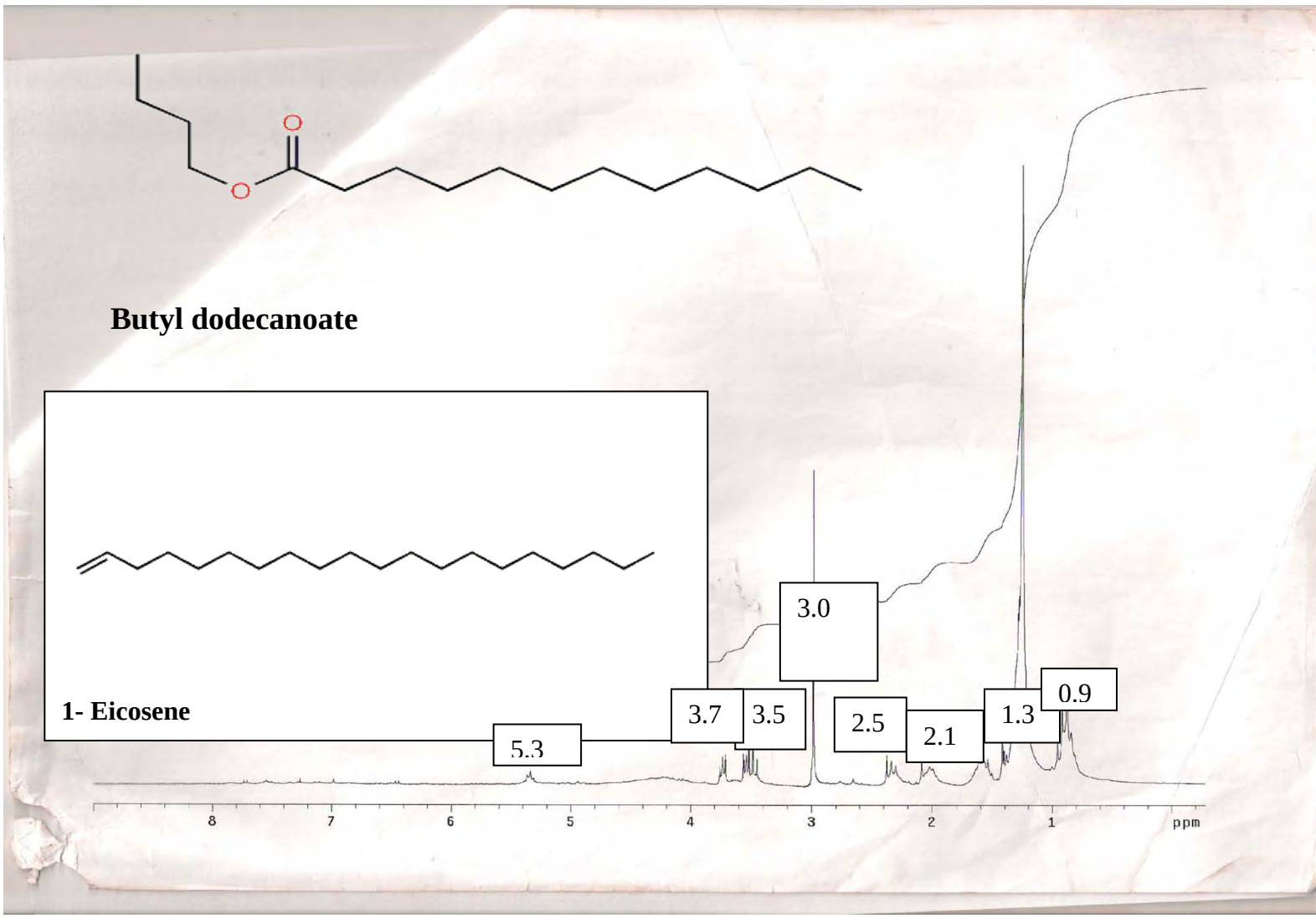

Fig 14: COMPOUNDS PRESENT IN FRACTION D OF FERMENTED WHEAT EXTRACT

The results of ^{1}H - Nuclear Magnetic Resonance (NMR) analysis of the various fractions of garlic extract are presented in Figures 15 - 17 representing fraction A, C and D respectively.

Fraction A

^{1}H NMR experiment of the fractions A garlic extract indicated the presence of methyl protons at δ0.9 and cluster of methylene protons at δ1.3, 2.0 and 2.3. The experiment also indicated the presence of hetero – atom at δ3.7. There is also characteristic signal of double bond methines at δ5.3. The acidic signal was noted at δ8.1 (Fig 15).

Fraction C

^{1}H NMR analysis of the fractions C garlic extract observed the presence of signal of methyl protons at δ0.9 and cluster of methylene protons at δ1.3. The experiment also indicated the presence of hetero – atom at δ3.5 and 3.7. There is also characteristic signal of double bond methines at δ5.3 (Fig 16).

Fraction D

^{1}H NMR experiment of the fractions D garlic extract indicated the presence of methyl protons at δ0.9 and cluster of methylene protons at δ1.3, 1.7 and

2.0. The experiment also indicated the presence of double bond methines at δ5.3 (Fig 17).

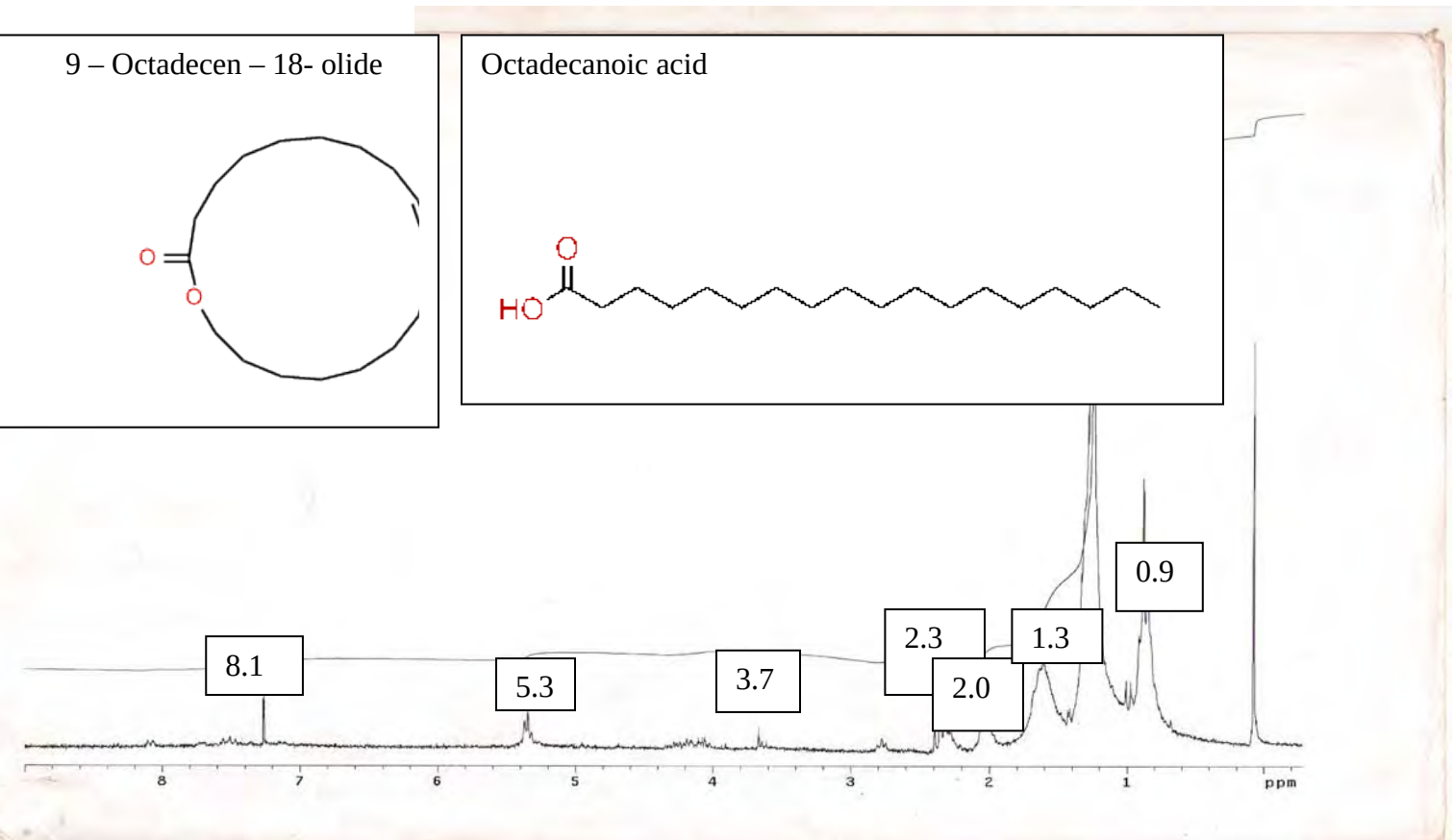

Fig 15: COMPOUNDS PRESENT IN FRACTION A OF FERMENTED GARLIC EXTRACT

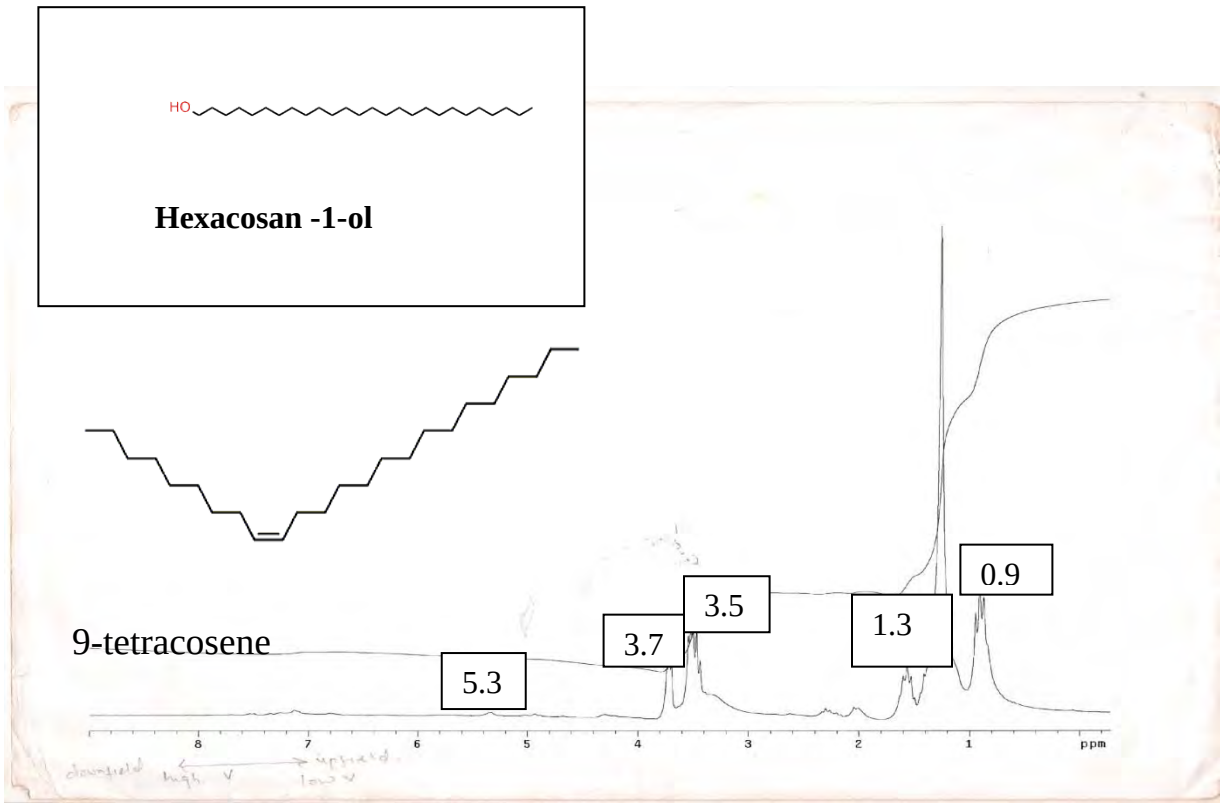

Fig 16: COMPOUNDS PRESENT IN FRACTION C OF FERMENTED GARLIC EXTRACT

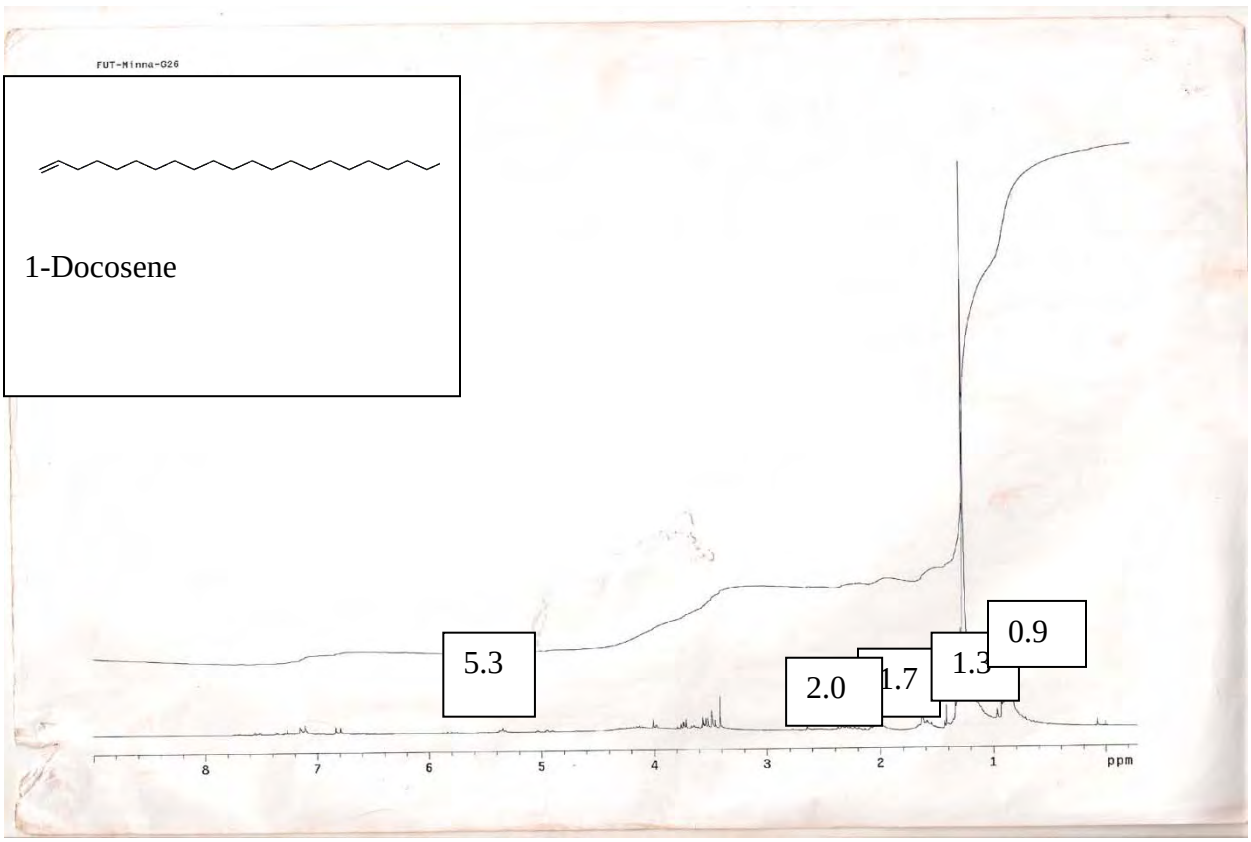

Fig 17: COMPOUND PRESENT IN FRACTION D OF FERMENTED GARLIC EXTRACT

3.9 BIOINFORMATIC ANALYSIS

The result of transmembrane domains for R1 and R2 subunit of ribonucleotide reductase are presented figures 18- 19.TMpred program makes prediction of membrane – spanning regions and orientation based on
the statistical analysis of TMbase. TMpred identified internal transmembrane domains, predicting possible transmembrane helices location at five (5) different amino acids position for R1 (Fig 18)

(1) 215- 231 (3) 511 -527 (5) 732 – 748

(2) 267 -286 (4) 611 -633

TMpred indicate membrane – spanning regions and orientation for R2 subunit to have two (2) transmembrane spanning at the following amino acids positions (Fig 19);

(1) 73 -91 (2) 173 -190

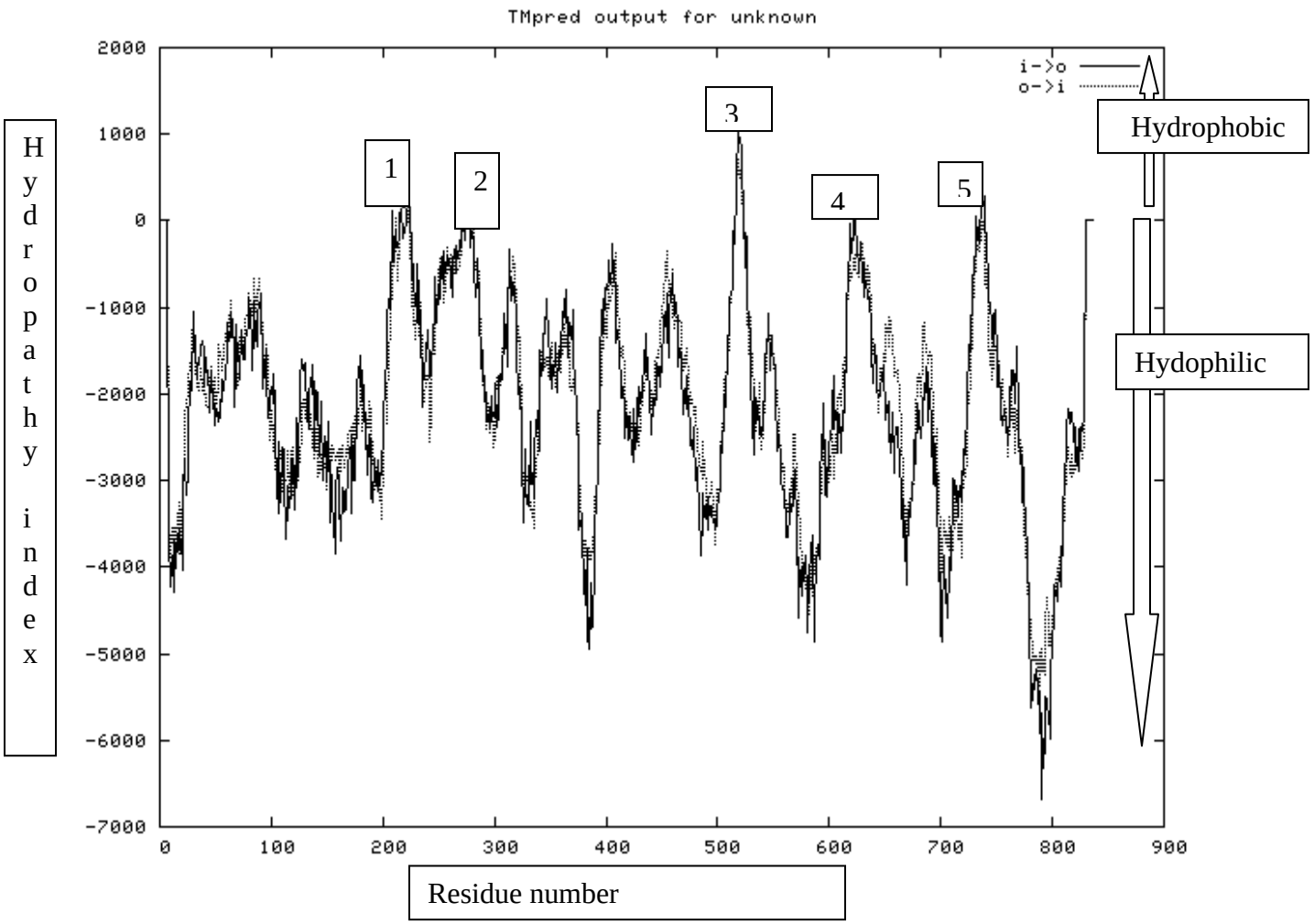

Fig 18: TMpred program analysis of R1 subunit

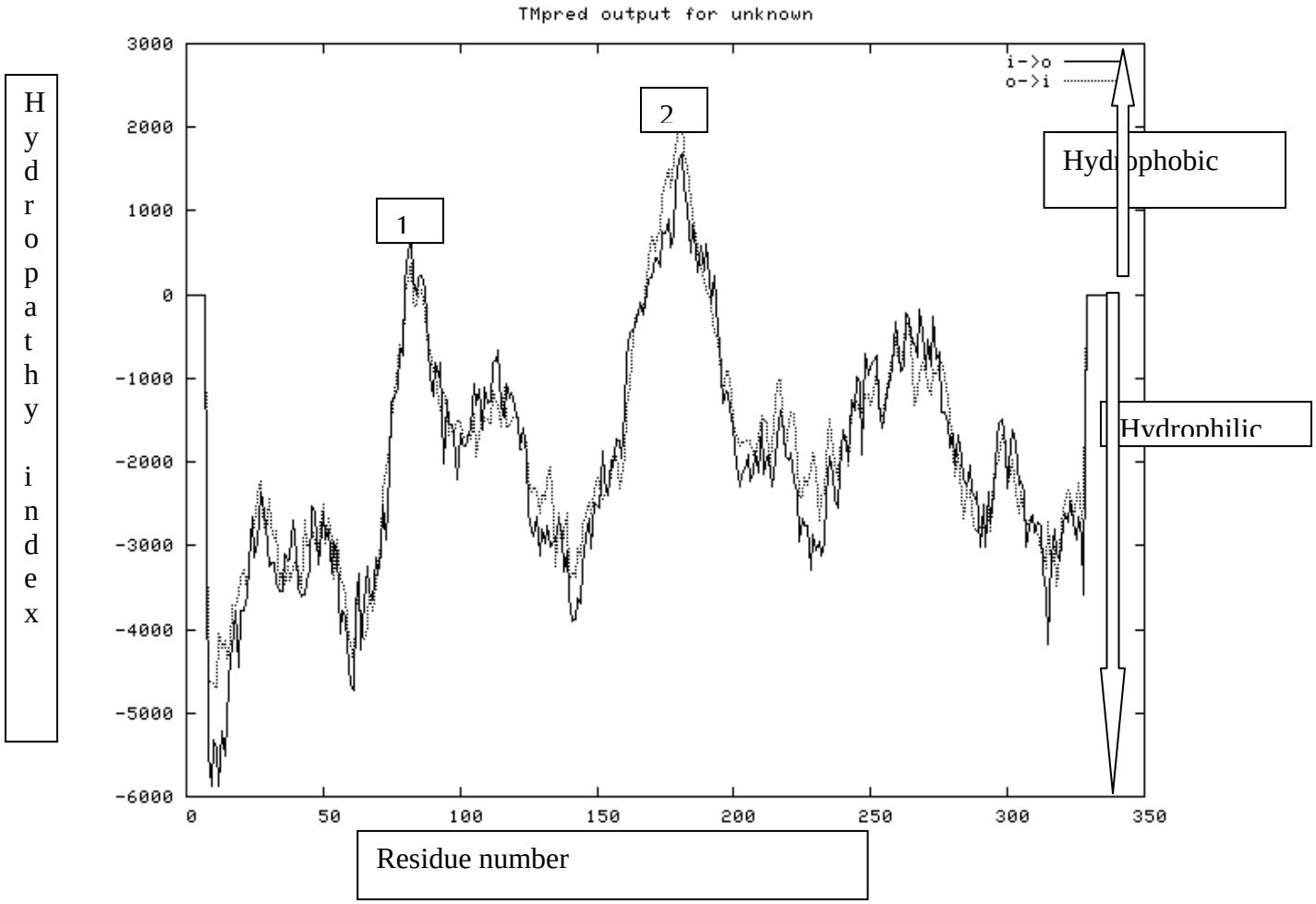

Fig 13: TMpred program analysis of R2 subunit

Protein families' domain

The description of protein families' domain of R 1 and R2 subunit were done using Pfam program. Domain is a region of a protein that can adopt a particular 3-dimensional structure. The following domains are retrieved from domain database (Table 10) at NCBI:

- ATP- cone domain was identified within sequence 6 – 94,
- Rinucleotide reductase– alpha domain within 146 – 223 sequence
- Rinucleotide reductase barrel domain at 225 – 765 sequence with active site for R1 subunit at N437, E441, Y759 and Y760

Scan Prosite results together with ProRule – based predicted intra-domain feature of R1 and R2 subunit (Table 11) as follows:

- ATP- cone domain was identified within sequence 6 – 97
- Multicopper Oxidases signature1 domain was identified within sequence 599- 619
- Rinucleotide reductase large subunit signature at 603 – 625sequence
- Rinucleotide reductase, small subunit domain sequence at 115 – 131
- G- protein coupled receptors family 1 signature domain sequence at

314 – 330

Table 10: The result of Pfam program analysis of R1 and R2 subunits

Pfam	**Description**	**Entry type**	**Sequence Start- End**	**Predicted active site**
R1 subunit				
ATP-cone	ATP cone domain	Domain	6 - 94	n/a
Ribonuc red lgN	Rinucleotide reductase, all – alpha domain	Domain	146 - 223	n/a
Ribonuc red lgC	Rinucleotide reductase, barrel domain	Family	225 - 765	N437,E441,Y759,Y760
R2 subunit				
Ribonuc red sm	Rinucleotide reductase, small chain	Domain	7 - 298	n/a

Table 11: The result of Prosite program analysis of R1 and R2 subunits

Prosite	Description	Start-End	Sequence
R1 subunit			
ATP-cone	ATP cone domain	6 – 97	KLVTKRDGSVEPYDEKVVRSRIVNLMSGI VGEGVRE----GMSTSML DELLAETAAYCvtkhpDYGLLAGRLAVTAL
Multicopper_ Oxidase1	Multicopper Oxidases signature1	599- 619	GlWdWdSlKErvVkVGMrnsL
Ribored_ large	Rinucleotide reductase large subunit signature	603 – 625	WdsLkervvkvGMRNsllIApmP
R2 subunit			
Ribored_ small	Rinucleotide reductase, small subunit	115 – 131	MEn.IHSeTYsvLidtYV
G_Protein_Recep_F1_1	G- protein coupled receptors family 1 signature	314 – 330	GEYqKAGVMSSERSSkV

The PSORT II results of Protein localization by the K – NN Prediction

R1 Subunit

Signal peptide prediction

N-region: length 11; pos.chg 3; neg.chg 1

H-region: length 0; peak value -12.17

cleavage site: between 36 and 37

65.2 %: cytoplasmic

30.4 %: nuclear

4.3 %: mitochondrial

R2 Subunit

Signal peptide prediction

N-region: length 11; pos.chg 4; neg.chg 0

H-region: length 0; peak value -13.90

cleavage site: between 39 and 40

R-2 motif at 20 SRK|EG

Peroxisomal targeting signal: RLIGMNSQL at 258

43.5 %: cytoplasmic
30.4 %: nuclear
17.4 %: mitochondrial
3 %: cytoskeletal
4.3 %: plasma membrane

CHAPTER FOUR

4.0 DISCUSSION

Many studies have been carried out in recent years on the pharmacological effects of wheat and garlic crude extracts (Suttle *et al.*, 2000). Fermented wheat extract has been reported to have anti-proliferative action that target nucleic acid synthesis enzymes (Tian *et al.*, 1999). The extract also has analgesic, antimicrobial, anti-inflammatory and immunological effects (Tsen, 1985). Garlic has been used as a remedy for infection (Koch, and Lawson, 1996). It has been claimed to help in preventing heart disease, high cholesterol, high blood pressure and cancer (Mader, F. H. 1990; Block, 1992; Silagy and Neil, 1994; Gardner *et al.*, 2007).

Upon invasion of the mammalian system trypanosomes proliferate rapidly to establish its population in infected host (Poltera, 1985; Pentreath and Kennedy, 2004). Toxins are released into the mammalian system (Nwagwu *et al*, 1987; Boutignon *et al*, 1990; Ekanem, 1989; Ekanem *et al*, 1994, 1996).The antibodies produced by the host against the parasite are not

effective because of the ability of the parasite to produce a large repertoire of antigens. The host defense mechanism is only partially specific and often lagging behind the progress of the disease in terms of antigen-antibody interaction (Sternberg, 2004). Eventually, there is a breakdown of the host immune system coupled with parasite invasion of the central nervous system leading to coma and death. Removal of the parasite from the system and simultaneously boosting the host immune system could be very relevant in the control of African sleeping sickness (Hoet *et al*, 2004; Chibale, 2005).

Fermented wheat and garlic bulbs extract has anti-trypanosomal properties as well as the ability to extend the life span of *T.brucei*-infected rats (Fig 1). This may be as a result of phytochemical constituent of the extracts. Phytochemical analysis result showed that the extracts have appreciable amount of alkaloids , glycosides and saponins (Table 1). The presence of glycoside can explained the antioxidant properties of the extract. Antioxidants neutralize highly unstable and extremely reactive molecules, called free radicals, which attack the cells of human body (Stauth, 2007). Free radical damage is believed to contribute to a variety of health problems,

including cancer, heart disease and aging (Stauth, 2007). Also another reason for the medicinal properties of the extract may be due to cleanse and purify blood properties of saponins (Kenner and Requena,1996). Alkaloids are known to act probably by causing structural changes to internal compartment, resulting in destruction of organelles such as mitochondria.

Analysis of serum enzymes have been reported to be of value and are early warning signs for certain diseased conditions. Wilknison (1962) reported also that changes in enzymes levels are a good marker of soft tissue damage; he also noted that damage to body Cells result in the alteration of membrane permeability and consequent release of enzymes into the extracellular fluid (ECF). Elevated enzyme levels may also result form effect of trypanosomes lyses resulting from effect of the host defense mechanism (Kennedy, 2004). The enzyme consider in this study are useful marker enzymes of liver cytolysis, damage to the plasma membrane of liver cells and oxidative stress (Xu *et al.,* 2002; Dobbs *et al.,* 2003; Oyewole and Malomo, 2009; Elstner and Osswald, 1994).

The increase in serum Aspartate transaminase activities of infected

untreated group when compared with infected treated wheat and infected treated garlic groups (Fig 2 and 7) may be an indication of earlier report that infection could gradually affect enzyme level with the extracts ameliorating the effect (Kennedy, 2004). Similarly, the increase in serum Alanine transaminase activities with concomitant decrease in liver activity of infected untreated group when compared with infected treated garlic group (Fig 8), suggested that there was a leakage of the enzyme from tissues as a result of damage to the cell membrane (Hanley *et al*., 1986). However, the decrease in serum alkaline phosphatase activities of uninfected treated and infected with wheat in comparison with the infected untreated group (Fig 4) maybe as a result of infective condition and interaction effect between the constituents of the extract. The increase in liver ALP activity of infected treated garlic compared with the infected untreated group(Fig 9) maybe as a result of increase in enzyme synthesis.

Antioxidant systems are in living aerobic organisms to counter the effect of oxidative stress. Oxidative stress occurs when the rate of cellular antioxidant removal and its rate of replacement is not balance. Catalase and superoxide

dismutase are enzyme, involved in antioxidation (Ledig and Doffoel, 1988). The specific activities of catalase were significantly reduced in both serum and liver infected untreated, infected treated wheat and infected garlic treated groups when compared with uninfected untreated (normal) group (Fig 5 and10). While SOD activities increased significantly in the serum and liver of infected treated wheat and infected garlic treated groups when compared with the infected untreated group (Fig 6 and11). This could be an indication increase in enzyme synthesis as a result of continuous utilization as a scavenger since reactive oxygen species are generated in so many ways such as infection and drug metabolism (Ledig and Doffoel, 1988).

African trypanosomiasis is characterised by haematological changes, which drastically influence the pathogenesis of the disease (Stephen, 1986; Neiger *et al.*, 2002). The disease is associated with decline in the red blood cell (RBC) counts, haemoglobin (Hb) concentration, and haematocrit or packed cell volume (PCV) in the infected hosts, confirming that anaemia is a critical feature in the pathogenesis of African trypanosomiasis. The increase in PCV of infected treated with the extracts when compared with infected untreated (Table 2) is an indication of the

effect of the extract in preventing anaemic condition as well as reduction in severity of *T.brucei* infection as measurement of anaemia gives an indication of the severity of the disease (Anosa, 1988; Suliman and Feldman,1989). Hb and RBC also increased in infected treated groups, this is added proof to the ameliorative effect of the extracts. Increased WBC in infected untreated groups indicates the level of infections compared to infected treated groups (Table 2).

Electrolyte in the clinical laboratory is taken to refer to only the inorganic ions, but in practice, a request for electrolytes determination usually means the determination of sodium, calcium, potassium, bicarbonate and chloride ions (Bectel, 1970). Change in electro-neutrality affect the whole body physiology especially conduction of nerve impulse (Datta and Ottaway, 1979).

Na^+ is essential to the electrical activity of cellular membranes. Their concentration is mainly maintained by the fact that the excessive ingestion of these ions in the diet is eliminated by the kidney or the intestine (Carlson, 1989), bearing in mind that hydro-electrolytic disorders could be associated to food deprivation due to sleepiness and brain injury that affect the central nervous system. The findings in this study show significant increase in Na^+ concentration of infected untreated

group compared with infected treated groups (Table 3) which may be as a result food deprivation due to sleep disorder and damage to central nervous system (WHO, 1998 ; WHO, 2001). There were no significant difference in other electrolyte parameters (K^+, Cl^- , HCO_3^- ,P) monitor, which was in agreement with the earlier reports of normal values recorded in experimental *T.brucei* infections in mice (Moun, 1968; Awobode, 2006).

The result of GC/MS and ^{1}H-NMR analysis of the isolated fractions of fermented wheat and garlic bulbs extract showed that the most prominent compound to be oxygenated hydrocarbon and n- hydrocarbon which represented Oleoic and ursolic acid, an essential oil. The chemical shifts observed for the fractions corresponded to those reported in the literature for the glycosides and saponins (Aldrich *et al.,* 1984a; Aldrich *et al.,* 1994; Sagar *et al.,* 2000; Allan *et al*., 2002; Audino *et al.,* 2007).

Membrane proteins play important roles in many physiological processes, constituting approximately 20-30% of all open reading frames (ORFs) in fully sequenced genomes. Membrane proteins are involved in various functions, such as intercellular communication, molecular transport and biogenesis, and are

associated with various human diseases, such as Alzheimer's disease, diabetes, Hodgkin's disease and liver cirrhosis. Many membrane proteins are primary drug targets because their localization is easy for drug delivery and their functions are mainly involved in converting extracellular signals into intracellular processes. Currently, about 60% of approved drugs target membrane proteins (Hopkins *et al.*, 2002). For example, G protein-coupled receptors (GPCRs) account for 25% of all drug targets in the market, Therefore, elucidating the structure and function of membrane proteins is essential for the identification of drug targets and development of therapeutic applications.Purine and pyrimidine metabolism are target for antitryoanosomal by the enzyme, ribonucleotide reductase. The enzyme consist of two subunit R1 and R2, which are each inactive alone. The R1 and R2 subunit sequence were analyzed for transmembrane domains and sub-cellular location using the PSORT II and TMpred algorithsms. TMpred identified internal transmembrane domains, predicting possible transmembrane helices location at five (5) different position for R1 (215-231; 267-286; 511-527; 613-633 and 732-748)and two(2) for R2 (73-91 and 173-190).(fig 12 &13). PSORT also identified N-terminal signal on length 11 for R1 and R2. and recognized the cleavage sites

36 and 37 for R1 and 39 and 40 for R2. The two subunits are predicted by both PSORT and TMpred to be cytoplasmic sub cellular localization.

The description of protein families' domain of R1 and R2 subunit was done using Pfam and Prosite. Domain is a region of a protein that can adopt 3-dimensional structure. ATP- cone domain was identified within sequence 6 - 94 by Pfam and 6-97 by Prosite., which support the earlier report of ATP as positive effectors for enzyme activity.(Hofer *et al.*,1998). The predicted active site for R1 subunit was predicted by pfam program as N437, E441, Y759 and Y760 which was supported by earlier report that the R1 subunit has active site cysteines (Cys-225, Cys-439, Cys-462), electron transport pathway (Tyr-730, Tyr-731) and the two cysteines that transfer electrons between thioredoxin or glutaredoxin and sulfhydryl groups (Cys-754, Cys-759)(Hofer *et al.*,1998).

Therefore, the results suggest that the extracts probably has antitrypanocidal properties as well as the ability to reduce parasitaemia and the severity of the disease. Antioxidation is probably one of the ways by which the extracts achieves the results (Adsule *et al.*,1986; Paul *et al.*,1987; Holland *et al.*, 1991; Andorfer *et al.,* 2003; Lee *et al.,* 2003).Methoxy – substituted

benzoquinone, which are present as glycosides implicated active component of fermented wheat (Tian *et al.*, 1999; Suttle *et al.*, 2000) and sulphur containing compounds of garlic, *allicin* (Lee *et al.*, 2003) might be the cytotoxic constituent conferring trypanocidal properties. These can be explore along with the possibility of inactivating possible toxins extra-cellularly released by trypanosomes. However, it can be suggest at this point that the extracts could be a useful cheap agent for the management of African sleeping sickness .

4.1 CONCLUSION

This study is significant because about 300 – 500,000 new cases of trypanosomisis are reported every year, with many remaining undetected. The old drugs are becoming limited due to drug resistance , cost and toxic side effect. Therefore,there is urgent need for new and cheaper drugs.

In this study, it has been established that:

- Fermented ethylacetate wheat extract has significant effect against *Trypanosoma brucei* in vivo by extending the life span from 8days of the control to 14days and reducing parasite replication of the infected rat.
- Fermented methanolic garlic extract has significant effect against *Trypanosoma brucei* in vivo by extending the life span from 8days of the control to 17days and reducing parasite replication of the infected rat.
- The extracts (Fermented wheat and garlic) show ameliorative effect on enzyme activities with confirmed safer dose level of 300 mg/kg body weight.
- The extracts also prevent anaemic condition caused by the parasite in infected rats.

- The GC/MS and ^{1}H – NMR analysis shows that the active constituents of extracts are major n- hydrocarbon and oxygenated hydrocarbon.
- TMpred identified R1 subunit of ribonucleotide reductase to have possible transmembrane domains helices location at five (5) different position and two (2) position for R2.
- PSORT identified R1 and R2 subunit of ribonucleotide reductase to be cytoplasmic sub cellular localization and also, identified N-terminal signal on length 11 for R1 and R2 and recognized the cleavage sites 36 and 37 for R1 and 39 and 40 for R2.
- Pfam and Prosite identified ATP- cone domain within sequence 6 – 97.
- Active site for R1 subunit was predicted by Pfam program as N437, E441, Y759 and Y760.

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APPENDIX 1

PREPARATION OF SOLUTIONS

(i) 0.4 N Sodium hydroxide

16g of sodium hydroxide was dissolved in a little quantity of distilled water. It was then transferred into standard flask and made up to 1 litre with more distilled water.

(ii) Sucrose solution (0.25M)

86.575g of sucrose was dissolved in a little quantity of distilled water and made up to 1 litre in a volumetric flask with distilled water.

(iii) BSA Standard

1g BSA (Bovine Serum Albumin) was dissolved in a little quantity of distilled water and made up to 100ml in a volumetric flask with distilled water.

(iv) Biuret reagent

Cupric sulphate ($CUSO_4.5H_20$) of 1.5g and 6.0g of sodium potassium tartarate were dissolved in 500ml of distilled water and transferred to 1 litre flask. 1g of potassium iodide (KI) and 300ml of 10% NaOH solution were

added. The resulting solution was made up to the 1 litre mark with more distilled water.

(v) 10% NaOH solution

10g of NaOH was weighed into beaker and then dissolved with a little quantity of distilled water. The solution was then transferred into 100ml flask and made up to the mark with more distilled water.

(vi) 0.3nM Adrenaline

0.01g of Adrenaline was dissolved in 17ml of distilled water.

(vii) 0.05M Phosphate buffer (pH 10.2)

6.97g of K_2HPO4 and 1.36g KH_2PO4 was dissolved in 200ml of distilled water. The pH was then adjusted to 10.2 and made up to 250ml with distilled water.

APPENDIX 2

STATISTICAL ANALYSIS

Duncan Multiple Range Test (DMRT)

For any significant analysis of variance test, it is important to perform a further test in order to identify the source of the significant difference among treatment means. To achieve this, the Duncan Multiple Range Test was employed for the analysis of the results.

Procedure for Duncan Multiple Range Test

Step 1: Arrange the treatment means in ascending of descending order of magnitude

Sep 2: Any two treatment means that are next to each other are regarded to be two step apart.

Example

Treatment	1	2	3	4
Means	15.5	22.3	16.7	27.8
Arrange means	15.5	16.7	22.3	27.8
	X_1	X_2	X_3	X_4

Treatment 1 and 3 are 2 steps apart

Treatment 1 and 2 are 3 steps apart

The test statistics is carried out as follows

$$W_r = D_{r,v,\alpha} \sqrt{\frac{MSE}{P}}$$

r denote the number of steps between any pair of treatments

v denote the degree of freedom for error

α is obtained from Duncan's table.

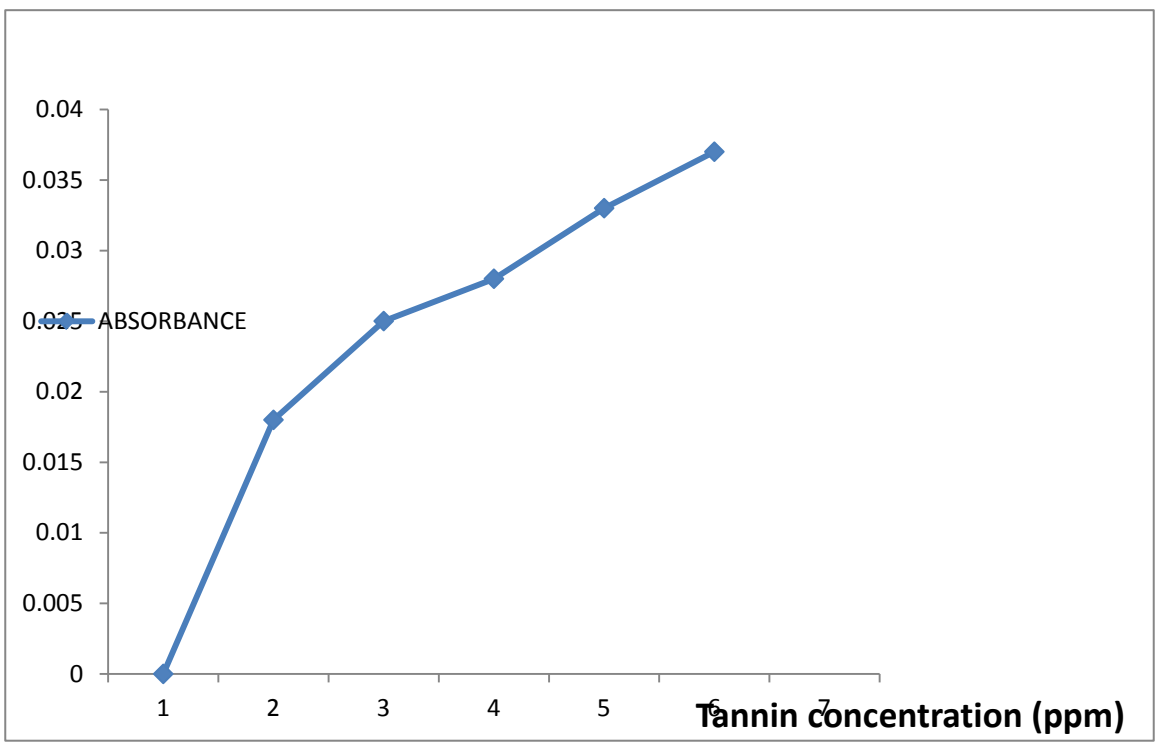
MSE is the mean square error from ANOVA table

P is the average number of replicate for any pair of treatments

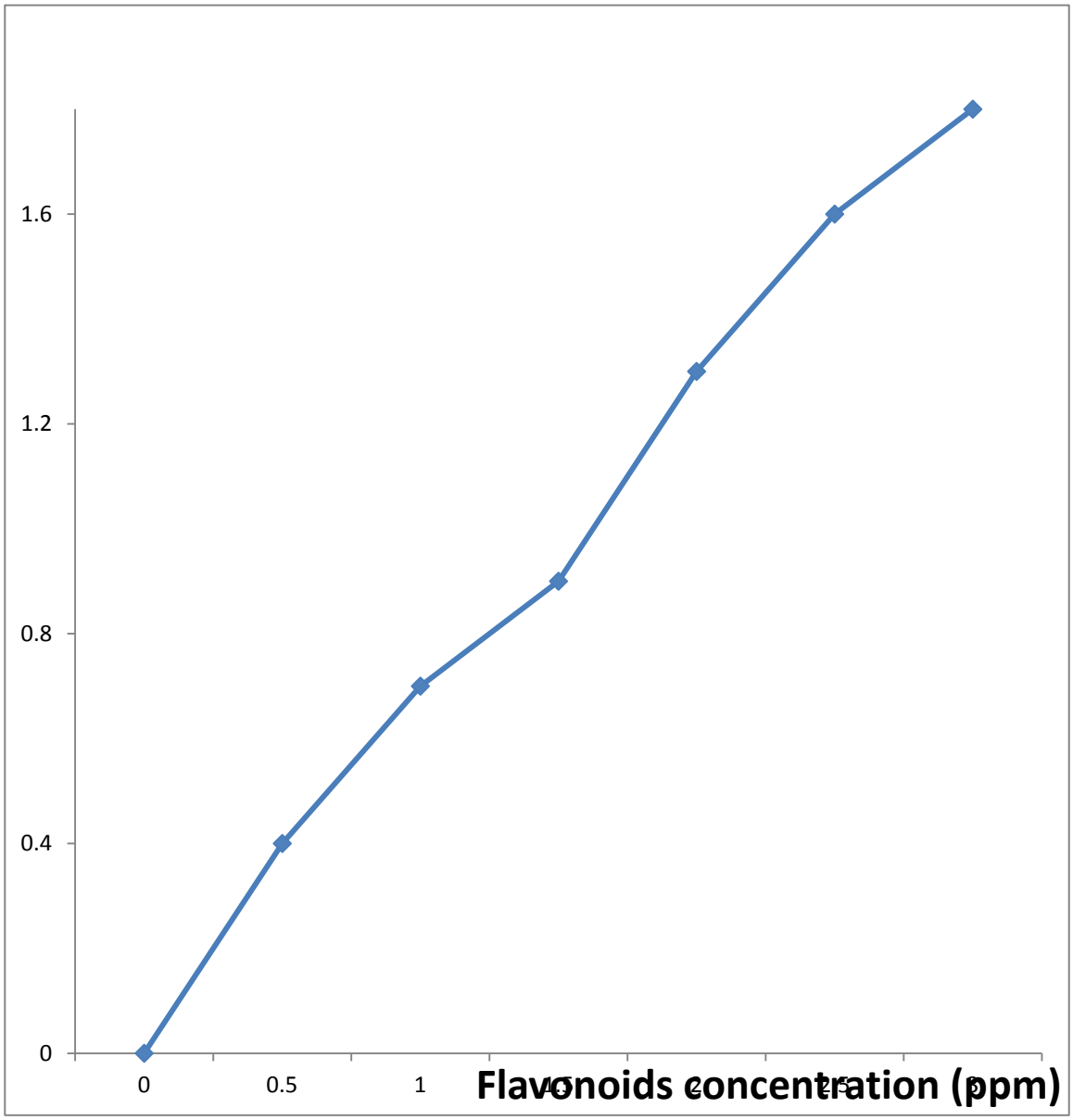
Decision rule

If $/ X_1 - X_3 /$ is greater than W_2, the difference between Treatment 1 and Treatment 3 is significant, otherwise the difference is not significant

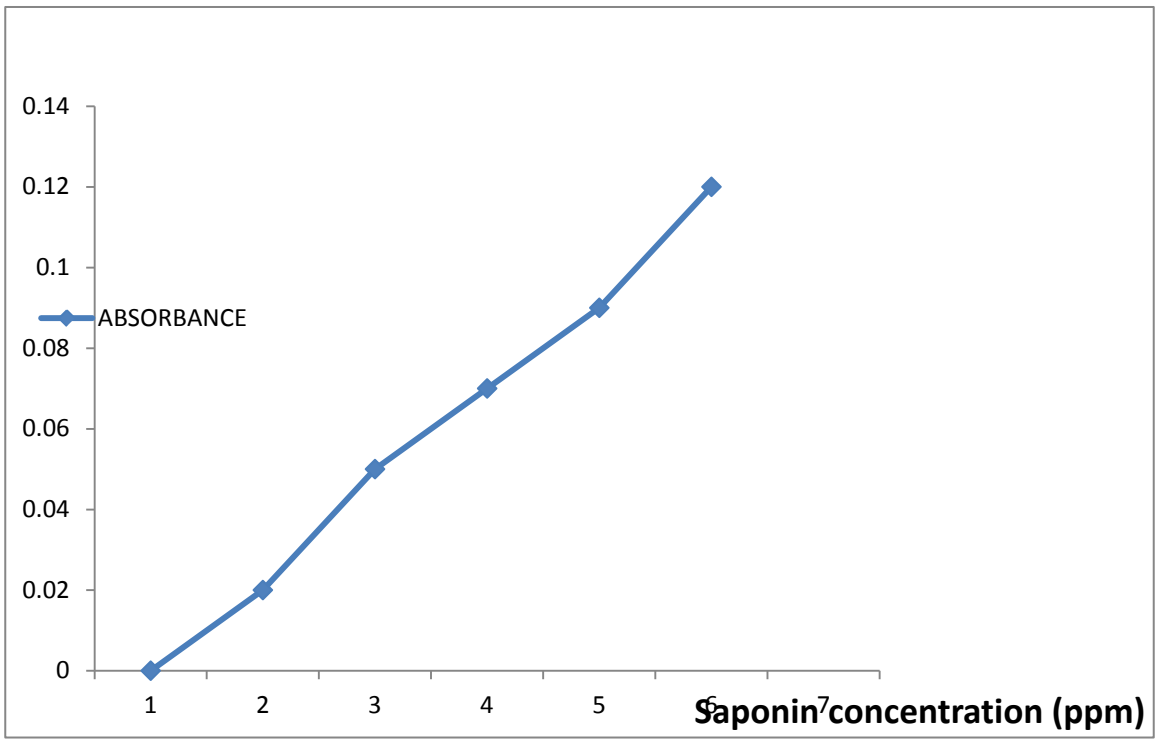
APPENDIX 3



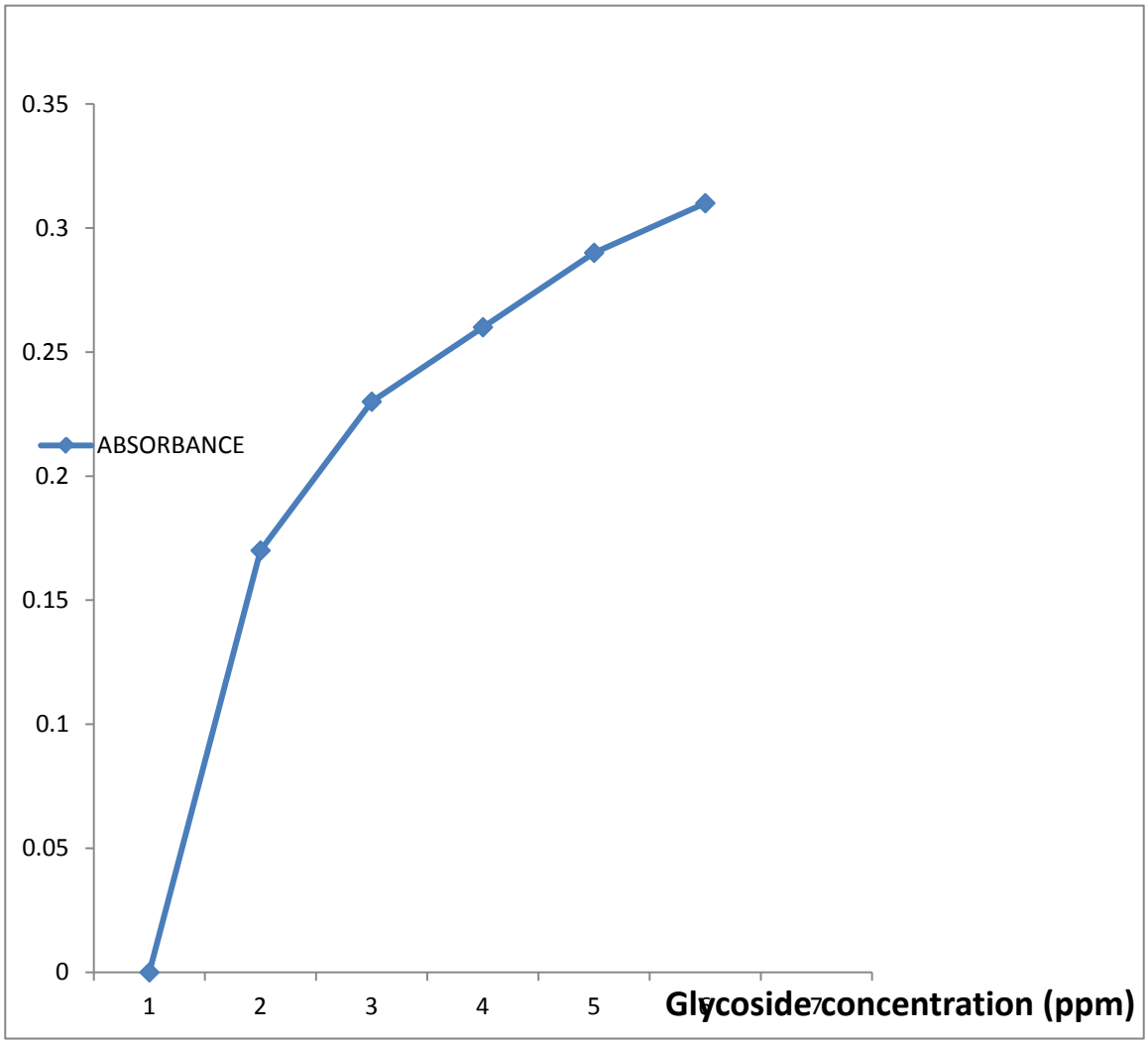
Appendix 3a: STANDARD CURVE FOR TANNIN



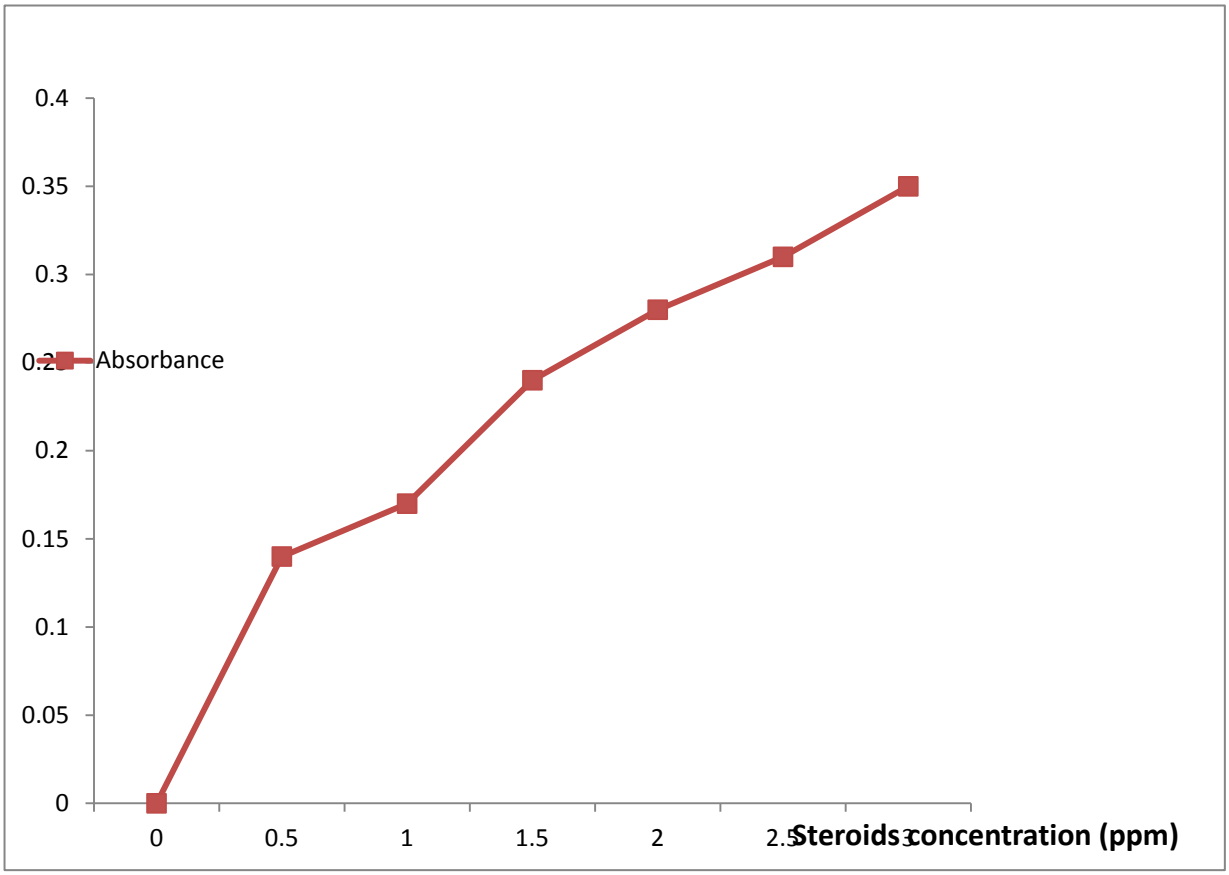
Appendix 3b: STANDARD CURVE FOR FLAVONOID



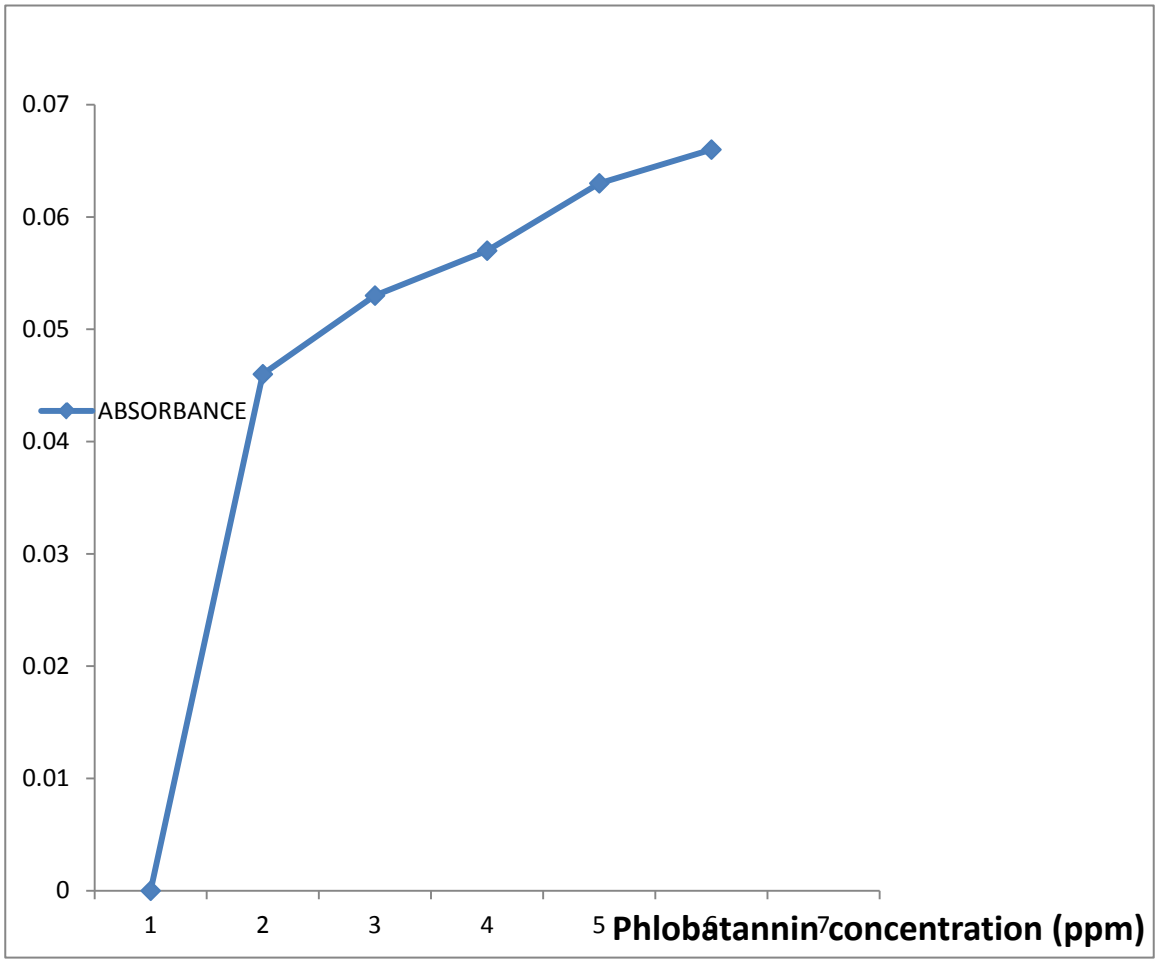
Appendix 3c: STANDARD CURVE FOR SAPONIN



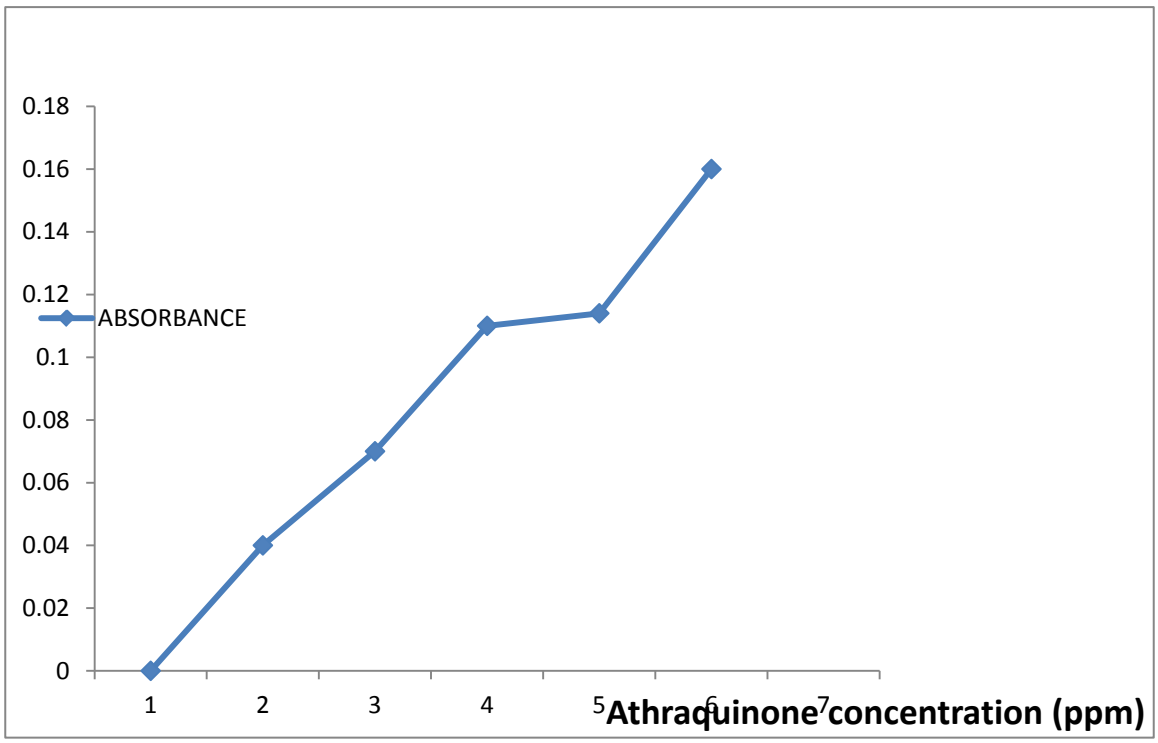
Appendix 3d: STANDARD CURVE FOR GLYCOSIDE



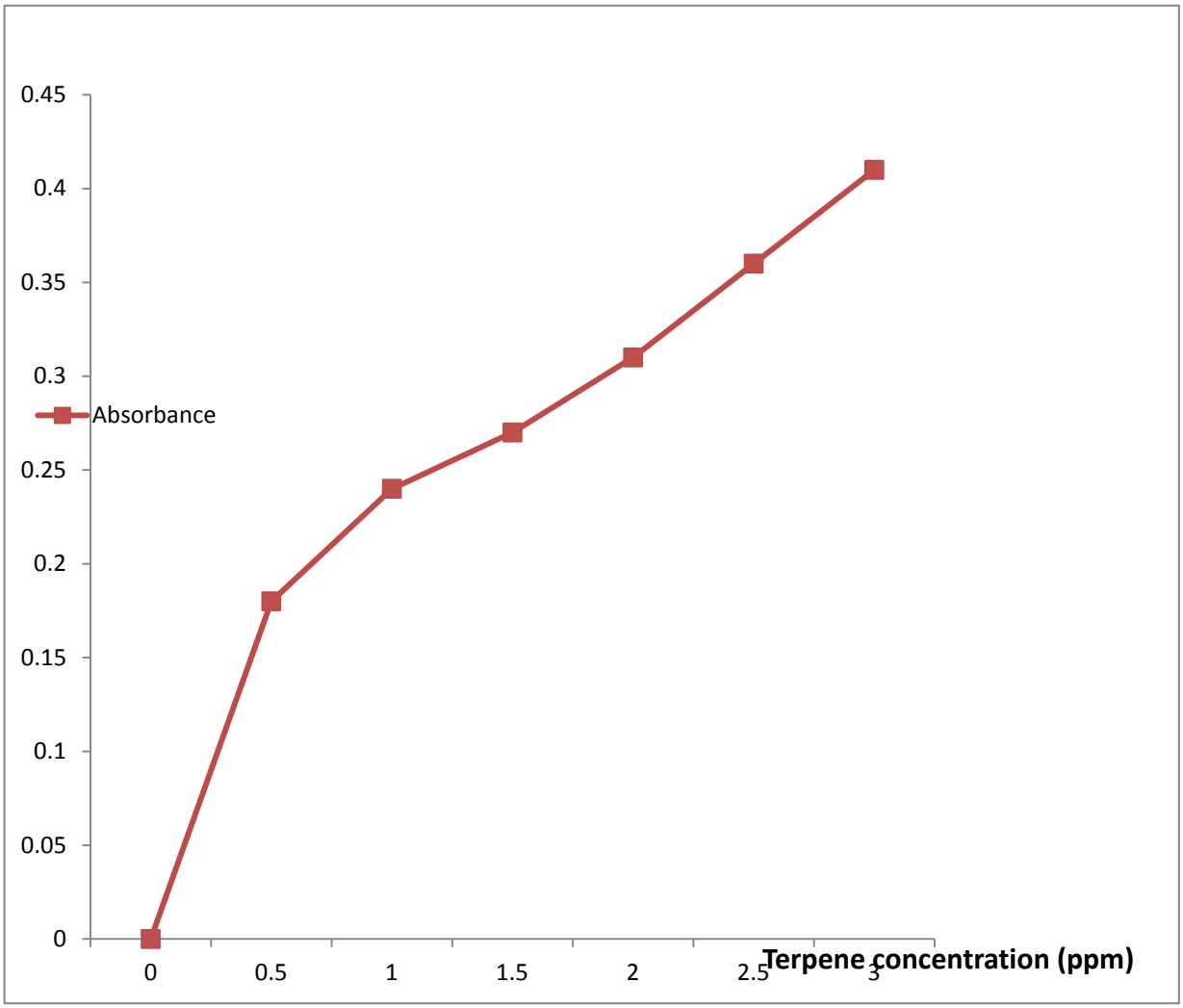
Appendix 3e: STANDARD CURVE FOR STEROID



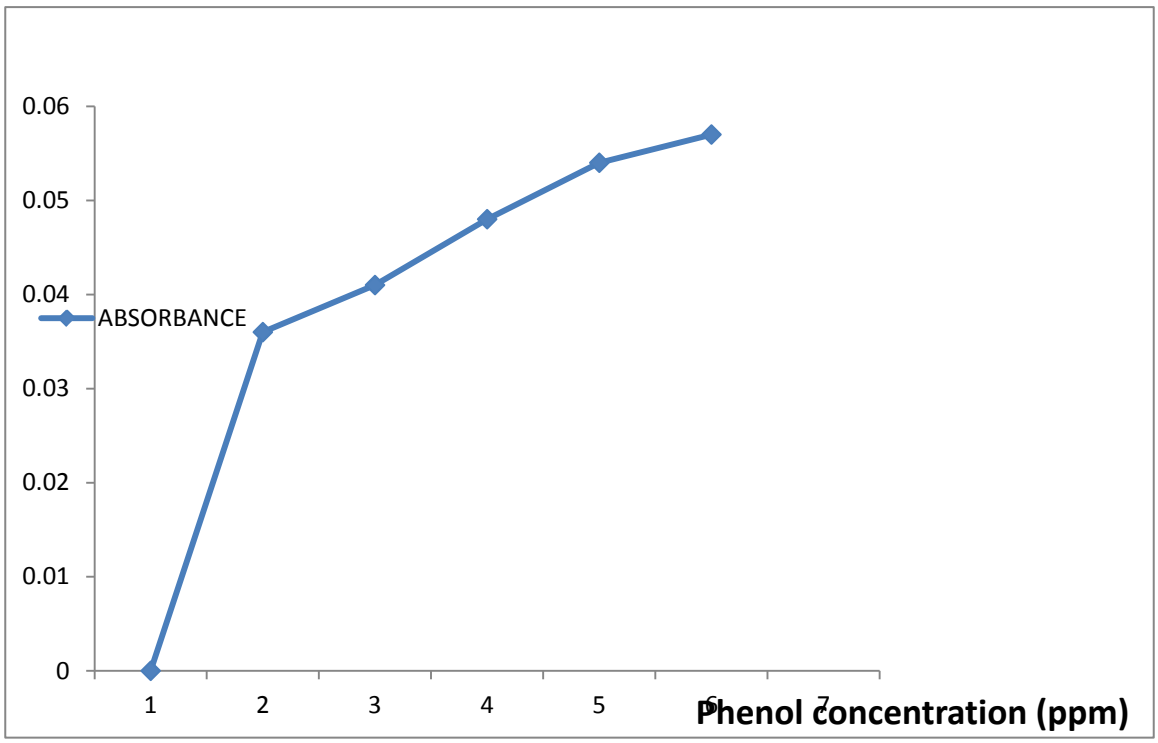
Appendix 3f: STANDARD CURVE FOR PHLOBATANNIN



Appendix 3g: STANDARD CURVE FOR ATHRAQUINONE



Appendix 3g: STANDARD CURVE FOR TERPENE



Appendix 3i: STANDARD CURVE FOR PHENOL