EVALUATION OF THE TRYPANOCIDAL ACTION OF COTTONSEED OIL ON *Trypanosoma brucei* AND *Trypanosoma congolense*, IN VITRO

A project report submitted to the
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Zaria in partial fulfilment of the
requirements for the award of
the degree of Master of Science in Biochemistry

BY

MOHAMMED ABDULLAHI JEGA
B.Sc (Hons) A.B.U.

JANUARY, 1992

DECLARATION

I hereby declare that this thesis is a product of my own research work based on real and correctly cited literature. It has not been presented elsewhere for the obtainment of a higher degree. All quotations and sources of information within are indicated and acknowledged by means of references.

MOHAMMED ABDULLAHI JEGA

DATE

CERTIFICATION

This thesis titled 'Evaluation of the Trypanocidal Action of Cottonseed Oil on Trypanosoma Brucei and Trypanosoma Congolense' by MOHAMMED ABDULLAHI JEGA meets the regulation governing the award of the degree of Master of Science in Biochemistry at Ahmadu Bello University, Zaria and it is approved for its contribution to knowledge.

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MEMBER, SUPERVISORY COMMITTEE DATE: 13/4/93

PROF. G.H. OGBADU
HEAD OF DEPARTMENT DATE: 13/4/93

DEAN, POSTGRADUATE SCHOOL DATE: 17/6/97

EXTERNAL EXAMINER

DEDICATION

FOR ALL THE FAMILY OF THE LATE MAGATAKARDA

.....

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ABSTRACT

The trypanocidal action of cottonseed oil was evaluated on T_. bcucei and T. congolense, in vitro. The organisms were maintained in MEM 199 and in coconut fluid medium for periods of up to 72 hours. MEM 199 was supplemented with L.cysteine (ImM), HEPES (25mM or lOmM), 2mM sod,ium pyruvate, 0.2mM 2-mercaptoethanol and 2ug/mL gentamycin. The coconut fluid medium, however, was supplemented only with 2% sodium pyruvate. In addition, horse and rabbit sera were also used in the supplementation of all the media. In all media, a temperature of 37°C and CO_2 atmosphere were used.

Cotton seed oil with and without gossypol elicited trypanocidal activity. This suggests that other trypanocides in the oil, possibly sterculic and malvalic acids elicited trypanocidal activity in the oil containing no gossypol. The oil containing gossypol had greater trypanocidal action. Oil with no gossypol had ED_{50} values of 0.79% and 0.72% for T. brucei and 0.80% and 0.7 1% for T. congolense (in 1 and 3 hours, respectively), respectively. Standard gossypol and salicylhydroxamic acid (SHAM) were also shown to be trypanocidal, using the same protocol. ED_{50} values for gossypol were 82uM (T_. brucei) and 98uM (T_. congolense); the ED_{50} values for SHAM were 222uM (T. brucei) and 224uM (T. congolense).

The test compounds in this study all exhibited trypanocidal activity on T. brucei and T. congolense. Trypanocidal effects of samples I and 2 may be due to the cyclopropene moiety of cyclopropenoid fatty acids and detergent like properties. Gossypol and SHAM elicit their trypanocidal activity via disruption of energy generation.

Samples 1 and 2, thus, could add to the numerous potential trypanocides being investigated.

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LIST OF ABBREVIATIONS

CPFA	Cyclopropenoid fatty acid
DFMO	Difluoromethylornithine
DSMO	Dimethylsulphoxide
FBS	Fetal bovine serum
FCCC OIL	Funtua cotton crushing company oil
HEPES	N z-hydroxyethylpiperazine N'z-ethanesulphonic acid
MEM	Minimum essential medium
ODC	Ornithine decarboxylase
PSG	Phosphate saline glucose
SAM	S-adenosylmethionine
SHAM	Salicylhydroxamic acid

CHAPTER ONE

1.0 INTRODUCTION

The world population was estimated to be 4.2 billion as at 1982 with an increase rate of 2 % annually (ILRAD, 1990). The highest growth rate is in Africa where the population is increasing at a rate of 3.2 % annually. Food production in Africa has not kept pace with the expanding population (Figure 1). Between 1965 and 1982, food production fell by approximately 12 per cent. Compared to Latin America and Asia, which have become almost self-sufficient in cereal production, Africa has become more and more dependent on imports and food aid. In 1963, Africa grew 96 per cent of its cereal requirements; In 1978, it grew only 78 per cent.

Trypanosomiasis, called sleeping sickness in humans and nagana in animals, profoundly affects the pattern of Agriculture in Africa. Trypanosomiasis affects both man and domestic animals in an area of about 4.5 million square miles (Levine, 1973). According to ILRAD (1990), the disease occurs in most of the humid and sub-humid zones, an area totalling approximately 10 million square miles; this is about half the non-desert area of Africa (Figure 2).

Apart from man and cattle, the disease also affects camels, horses, sheep, goats, pigs, and dogs. According to Turner (1982), some 35 million people and 25 million cattle are at risk of infection with the disease. The disease in cattle, is of great

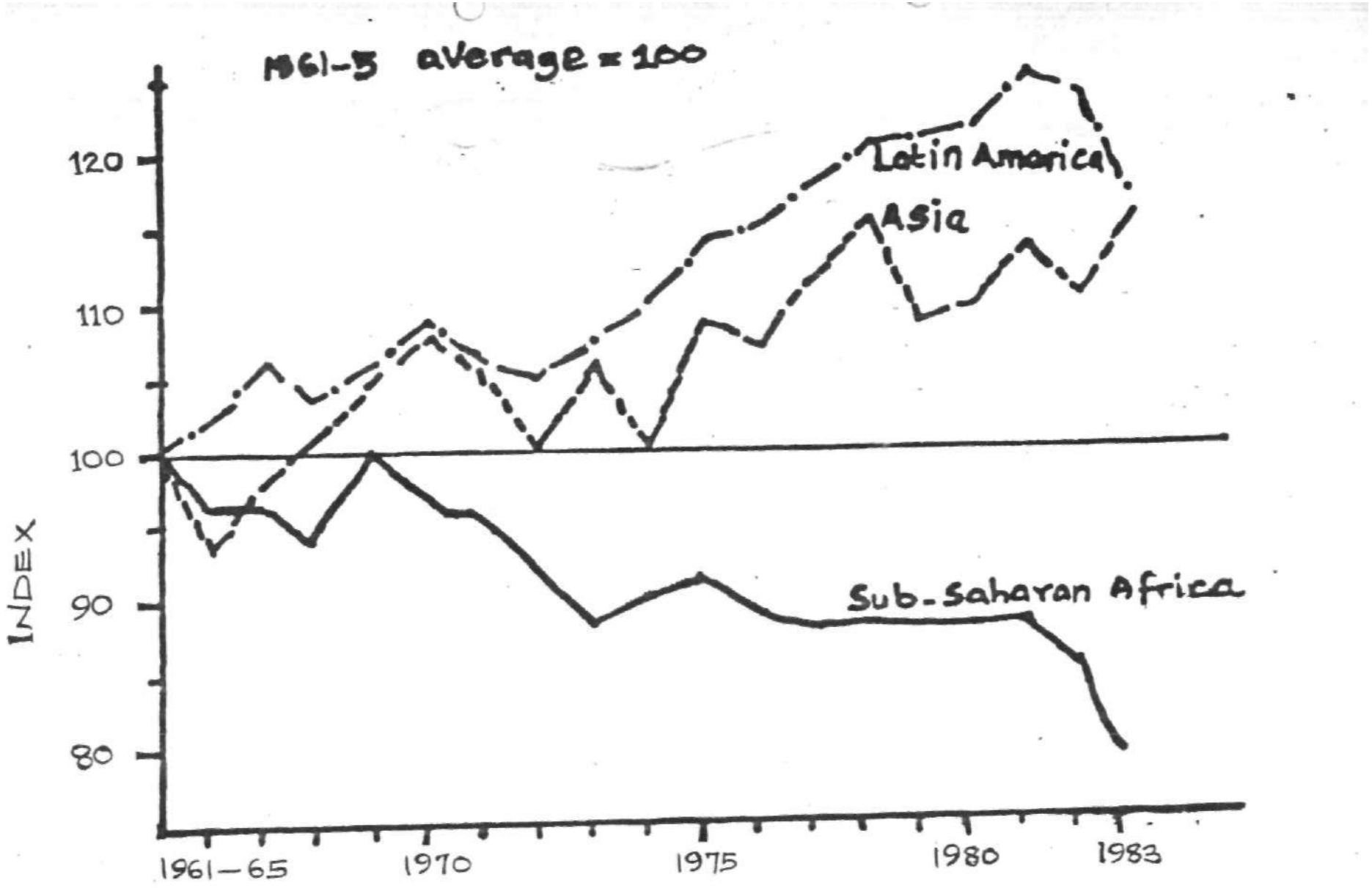


FIGURE 1

GRAPH SHOWING PER CAPITA FOOD PRODUCTION IN LATIN AMERICA, ASIA AND SUB-SAHARAN AFICAN FROM 1961 TO 1983. FROM: Proceedings of a research held at ILRAD (1989).

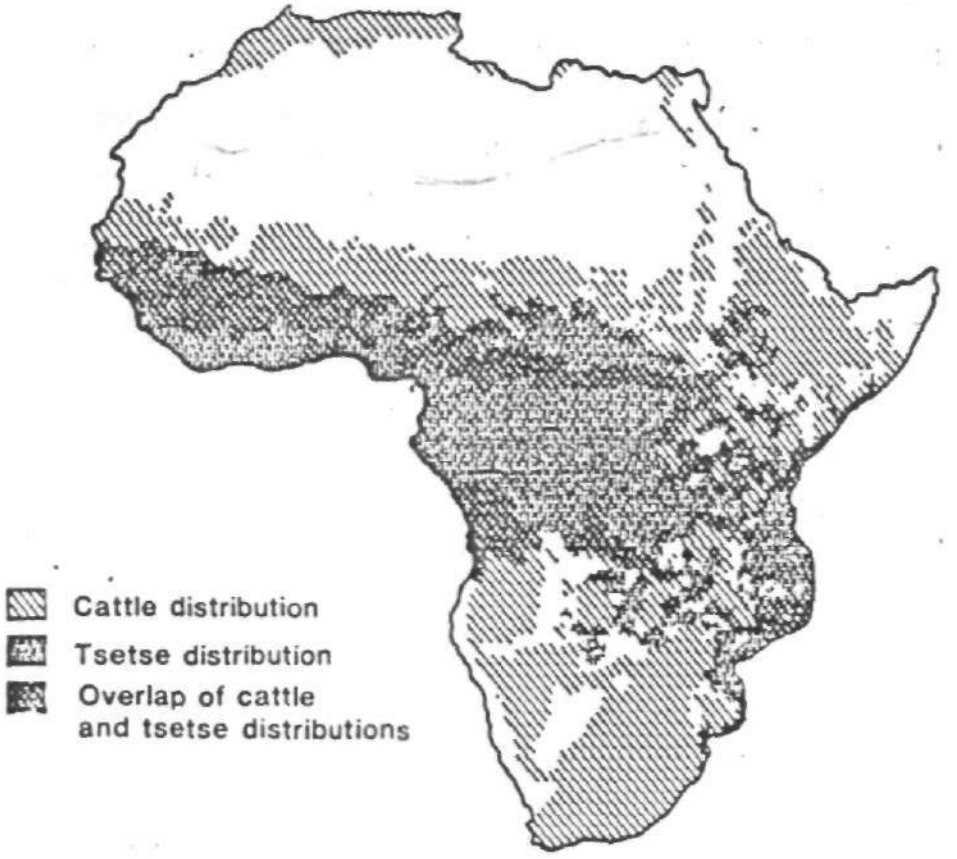


FIGURE 2. Major cattle-production areas and tsetse-infested zones in Africa. There is little overlap except in the regions of West and Central Africa where trypanotolerant livestock are kept.

FROM: PROCEEDINGS OF A WORKSHOP HELD AT ILRAD

economic importance since it causes the deaths of 3 million heads per year and effectively reduces the use of vast areas for ranching (Gutteridge and Coombs, 1977). The disease is transmitted to domestic animals by the bite of a tsetse fly, causing anaemia, reduced productivity, reproductive disorders, and death. In sub-saharan Africa, the disease impedes livestock production directly and crop production indirectly. Livestock are integrated into farming only in the highlands of East Africa which are too cold for tsetse flies, and in a few semi-arid regions which are too dry for the fly. In the sub-humid zones, however, livestock are at greatest risk from trypanosomiasis in the rainy season. During this time stock farmers often entrust their cattle to nomadic pastoralists who move the animals to tsetse-free areas. This makes integration of livestock and crop production difficult. This separation of livestock and arable farming over much of Africa is a major barrier to Agricultural development. Also, of the 10 million square miles now infested with tsetse, some 7 million are thought to be otherwise suitable for mixed agriculture and livestock farming (ILRAD, 1990).

The absence of an effective vaccine and the lack of a coherent strategy for controlling the insect vectors make the livestock industry to be highly dependent on the small number of drugs currently available for treatment (Table2.1) of the disease (Anthony, 1988; Sones, 1988). This curative and preventive chemotherapy of African trypanosomiasis is still in an unsatisfacoty

state (Seebeck and Gehr, 1983). The current WHO list of trypanocides approved for use in animals includes Berenil, Suramin, Homiduim Chloride, Isometamidium, and Quinapyramine (Zweygarth and Rottcher, 1989).

The major problems in the chemotherapy and chemoprophylaxis of trypanosome infections relate to the toxicity of available drugs and the emergence of drug resistance. This necessitates the identification of more potent trypanocides.

Compounds currently being investigated as potential trypanocides include :

(i) Inhibitors of respiration eg. salicylhydroxamic acid (SHAM)

(ii) Purine and pyrimidine analogs such as allopurinol and nucleocidin

(iii) Inhibitors of polyamine synthesis and function, as exemplified by α-difluoromethylornithine (α-DFMO)

(iv) Nitrofurans and nitroimidazoles, typified by metronidazole, and

(v) Inducers of oxidative damage eg. bleomycin, quinones and porphyrins [Campbell, 1986].

Many studies have been directed at the above drugs.

Other studies on trypanosomes have been on their lipid chemistry and biochemistry. Findings about the lipid composition of trypanosomes (Venkatesan and Omerod, 1976), , the metabolism of the lipids (Samad et al; 1988) and their acquisition (Mellors and Samad, 1989) enabled the identification of some lipids that are trypanocidal (Keppie , 1953; Godfrey, 1958; Hiroshi et al; 1988; Ukoha, 1989).

The finding by Godfrey (1958) that cod liver oil depressed the infections of T. congolense, T. cruzi, T. vivax and T. brucei in mice prompted a lot of studies on the lipid metabolism of trypanosomes. Godfrey's study was prompted following the report by Keppie (1953) that T. congolense failed to develop normally in mice given a diet composed of casein, starch, salts, margarine, dried yeast and cod liver oil. Earlier, Godfrey (1957) had indicated that cod liver oil suppresses the multiplication of Plasmodium berghei. The toxic factors in cod liver oil were reported to be the polyunsaturated fatty acids C20:4, C20:5, C22:5 and C22:6 of the family of linolenic acid (C18:3), to which they are degraded in vivo (Godfrey, 1958). These polyunsaturated fatty acids (PUFAs) were reported to be converted to peroxides by the host (Godfrey, 1957). These lipid peroxides may then oxidize sulphydryl groups of some of the parasite enzymes.

Samad et al., (1988) observed that the provision of exogenous fatty acyl-CoA to trypanosomes, along with exogenous lysophospholipds enhances transacylation; the resultant phospholipid being incorporated

into the trypanosomal membrane lipids. The report also indicated the marked preference shown by the acyltransferases to myristoyl-CoA. There occurs a lack of utilization of arachidonoyl-CoA as substrate. This indicates a marked contrast to mammalian acyltransferases where many tissues and organelles possess arachidonoyl-CoA - specific acyltranferases. In another report, Mellors and Samad (1989), observed that bloodstream trypanosomes mimic the blood cells of their host in their dependence on exogenous Lysophospholipids. These nutrients provide essential residues such as choline, ethanolamine, inositol and free fatty acids (FFAs) for the synthesis of membrane phosphoglycerides and membrane protein anchors. In the report, high rates of uptake of Lysophospholipds were observed in trypanosomes when exogenous fatty acyl CoA is provided. This was postulated to be useful to chemotherapy, because a lysophospholipd, into which a trypanocidal fatty acid or analogue has been incorporated could be selectively taken into the parasite by the acyltransferase, and driven by the coadministration of myristate or myristoyl CoA. The host cells which possess arachidonoyl CoA specific acyltransferases, may take up less of the exogenous lysophospholipid and therefore be less susceptible to the toxic effects caused by intracellular accumulation of the toxic fatty acid.

Hiroshi et al., (1988) showed that incorporation of stearylamine into phosphatidylcholine increased the toxicity of stearylamine on T. Gambiense Cells. The preliminary observations suggested that the stearylamine/Phosphatidylcholine (SA/PC) liposomes fuse with the plasma membrane and then act on the membrane.

This finding is consistent with the report of Ukoha (1989)on the trypanocidal action of some polyunsaturated fatty acids [PUFAs] of the W3 and W6 families. Arachidonate and eicosatraynoate had little effects while the eicosapentaenoic acid [C20:5 (n-3)] exhibited a marked effect on trypanosome viability. A similar trypanocidal effect was observed for [C20:3 (n-3)] against [C20:3 (n-6)]. Trypanocidal effects were also reported for the C22 unsaturated fatty acids. In line with Mellors and Samad [1989], Ukoha [1989] reported increase in toxicities of the same PUFAs after they were incorporated into lysophosphatidylcholine.

The present study was conducted to test whether cotton seed oil is trypanocidal to T. brucei and T. congolense, in vitro. Toxic factors in cottonseed oils include gossypol (Figure 2.5.3) and cyclopropenoid fatty acids (Figures 2.5.1, 2.5.2) Gossypol has been demonstrated to lyze T. brucei brucei by interferring with glycolysis in the parasite (Josiane et al., 1988). As African trypanosomes in the host depend solely on glycolysis for energy production (Bowman and Flynn, 1976), gossypol was found to be a powerful inhibitor of their energy metabolism. Biochemical studies have indicated that gossypol inhibits NAD - and NADP - dependent enzymes (Lee and Malling, 1981). The most likely enzyme to be inhibited therefore is the glycosomal glyceraldehyde - 3 - phosphate dehydrogenase and/or glycerophosphate dehydrogenase. A concentration of 20 micromolar gossypol was found to eliminate 70 per cent T. brucei in vitro, after 5 minutes, in serum free medium (Josiane

et al., 1988). However, in the presence of serum, a concentration of 150 micromolar was needed to elicit the same effect. This is because gossypol binds to serum albumin with a dissociation constant of 2 micromolar (Vander Jagt et al.,1982). This, in effect, lowers the concentration of free gossypol available to enter the parasites. Adverse biological effects accompany the ingestion of cyclopropenoid fatty acids in animal diets. These are well known inhibitors of fatty acid desaturation in higher animals [Katherine and Glenn, 1988]. Cyclopropenoid fatty acids also affect insect steroyl-CoA desaturase and interfere with the maturation and reproduction of some insect species thus indicating that, in addition to their traditional role as storage lipids, they also serve as protective agents. Pink colouration of eggs has been observed following the administration of sterculic acid (25 mg/da). The hard lard and sticky butter occuring in some animals are believed to be due to the increased tissue concentrations of stearic acid at the expense of oleic acid, occuring as a result of fatty acid desaturation (Katherine and Glenn, 1988). These CPFAs may be taken up by trypanosomes, and subsequently cause membrane lysis in the parasites.

CHAPTER TWO

REVIEW OF LITERATURE

2.1. EXISTING CHEMOTHERAPY AND ITS LIMITATIONS

Antitrypanosomal drugs can be used in three distinct modes. These include prophylaxis, treatment, and prevention of transmission during blood transfusion. For human African trypanosomiasis, there are drugs for the first two cases : Pentamidine for chemoprophylaxis, suramin for treatment of the early stages of the disease, and Melarsopol for the treatment of late stages when trypanosomes are present in the central nervous system (CNS) (Gutteridge, 1985) However, no drug is in routine use for the prevention of transmission during blood transfusion. In contrast, there are no drugs registered for chemoprophylactic use against chaga's disease, though two, nifortimox and benznidazole, are marketed in some countries in Latin America for treatment of both acute and chronic cases. Gentian violet is, however, used to prevent transmission during blood transfusion. For animal trypanosomiasis, quinapyramine, isometamiduim and pyrithiduim are used for chemoprophylaxis while diminazene aceturate, homiduim, pyrithiduim, isometamiduim and quinapyramine are used for treatment purposes. Resistance to quinapyramine, homiduim and pyrithiduim develops so rapidly that in many African countries, the widely employed trypanocides remain isometamiduim (curative and prophylactic) and diminazene aceturate (curative) (Anthony, 1986).

Limitations of efficacy and/or problems of toxicity impose severe restrictions to the use of trypanocides. The ideal drug for the treatment of either African or American trypanosomiasis would have

the following properties (Gutteridge, 1985):-

(i) active on oral administration in a single dose (or a small number of doses over a short period)

(ii) have minimal side effects, and

(iii) active against all stages of the disease cause by all strains (including innate and drug-induced resistant strains) and species of trypanosomes which cause the disease.

An ideal chemotherapeutic drug would, in addition, require infrequent administration and one used to prevent transmission during blood transfusion would be colourless (Gutteridge, 1985). Presently, none of the drugs in use for either African trypanosomiasis or Chaga's disease meets all these criteria.

There are five basic mechanisms (Gutteridge and Goombs, 1976) that underlie the selectivity of antitrypanosomal drugs:

(i) Drug only permeates to or is concentrated in the parasite eg pentamidine

(ii) Drug attac ks a target present only in the parasite eg suramin

(iii) Drug discriminates between i sofunctional targets in the parasites and the host eg melarsopol

(iv) Drug blocks a target that is of greater importance to the parasites than to the host eg. melarsopol

(v) Drug is metabolized to the active species only in the parasite. eg tryparsamide.

2.1.1 PENTAMIDINE

This is an aromatic diamidine available as two salts, Pentamidine isothionate (M&B 800) and Pentamidine methanesulfonate (Iomidine). It is a colourless powder soluble in water.

A dosage of 4mg per kg body weight is given intramuscularly every 3-6 months as a ten per cent solution for prophylaxis. The treatment dose is 3-4 mg per kg body weight given intramuscularly dai for a period of 7-10 days. It works well as a prophylactic and treatment agent in the Gambian disease.

Pentamidine exerts an effect on the biosynthesis of nucleic acids. The pentamidines are all positively charged at physiological pH and as such bind readily to DNA. The selectivity of this drug appears to be due to differential permeability between the host and the parasite (Gutteridge, 1985) as the trypanosomes contain a pentamidine transport system which raises the intracellular concentration of the drug to many times the plasma concentration.

Side effects include hypotension following histamine release Sometimes, there is a feeling of faintness. These symptons are overcome by injection of epinephrine. The drug also causes pain at the injection site, hypoglycemia and renal damage.

2.1.2 SURAMIN

This is a sulphated napthylamine with trade names sodium suramin, Antrypol, Bayer 205, Belganyl, Fourneau 309, Germanin, Moranyl, Naganol and Naphuride. It is available either as a white powder or an aqueous solution in ampoules. It has a net negative charge at physiological pH.

The usual adult dose is five doses of 1g at weekly intervals. The paediatric dose is based on a value of 20mg/kg of body weight. After intravenous injection, the plasma concentration falls over the first few hours and then more slowly over the next days (Gutteridge, 1985). Some of the drug is bound to serum and remains in circulation. The parasites disappear slowly from blood after injection. Both T. gambiense and T. rhodesiense are equally sensitive to the drug. The cure rates obtained are about 100 per cent (Gutteridge, 1985) when treatment is started before the CNS is involved.

The mechanism of action of suramin is not certain. The drug inhibits a multitude of enzymes in cell-free systems at millimolar concentrations. Suramin markedly inhibits L-x-glycerophosphate oxidase. This enzyme is concerned with the oxidation of NADH in trypanosomes with a Ki of 4.1 μM (Fairlamb and Bowman, 1977). However, the inhibition of L-x-glycerophosphate oxidase alone is not a lethal even in trypanosomes because the high glycerokinase activities present can synthesize ATP under anerobic conditions. Fairlamb and Bowman (1980) have indicated the inhibition of another enzyme involved with the oxidation of NADH in trypanosomes. This enzyme is L-x-glycerophosphate dehydrogenase. This is a pity because

L-α-glycerophosphate Oxidase is an enzyme unique to African trypanosomes which could have enabled one to rationalise the selectivity of suramin on the basis of a target unique to the parasite. Limitations to its use include the inability to pass the blood-brain barrier. This means that it has no curative effect once the disease reaches the CNS.

Suramin exhibits certain side effects. In some persons injection is followed by vomiting, shock and collapse [Cutteride, 1985]. This is usually transient but can be fatal. Less susceptible individuals show vomiting, pruritus, uticaria, paresthesia, peripheral neuropathy, kidney damage and optic atrophy. Most common, however, is some degree of damage to the kidneys resulting in a mild, though often, persistent albuminuria.

2.1.3 MELARSOPOL

This is a trivalent arsenical with trade names Mel B, Melarsen Oxide-BAL, Arsobal.

It is available in ampoules ready for use as a 3.6 per cent (w/v) solution in propylene glycol. The usual recommended dose is a single daily injection of 3.6 mg/kg of body weight for 3-4 days.

Most of the drug is rapidly excreted in the urine but by day 3 a reasonable concentration has accumulated in the plasma. By day 4, small amounts are found in the cerebrospinal fluid. The drug thus cures all stages of both the Gambian and Rhodesian trypanosomiasis

(Gutteridge, 1985). Patients respond rapidly and begin to feel better after 4 days.

Melarsopol exerts its action by disruption of energy generation. The trivalent arsenicals, generally, have a high affinity for sulfhydryl groups which form the active sites of many enzymes eg. the kinases. Flynn and Bowman (1975) have shown that the glycolytic kinases, hexokinase, phosphofructokinase (PFK) and pyruvate kinase (PK) were all sensitive to inhibition by melarsopol. Pyruvate kinase was indicated to be markedly affected. Since it is the reaction catalyzed by pyruvate kinase that produces the net ATP of African trypanosomal energy generation, the effect of melarsopol (and other arsenicals) is thus clear. The apparent specifity for pyruvate kinase is due to its presence in the cytoplasm whereas hexokinase and PFK are sequestrated within a membrane bound organelle, the glycosome.

There are three factors contributing to the selectivity of this drug (Gutteridge, 1985) :

(i) there exists a better binding to trypanosomes than to mammalian host enzymes

(ii) the drug is concentrated within the trypanosomes, and

(iii) the total dependence of trypanosomes on glycolysis for energy generation.

The side effects of melarsopol include fever, pains in the chest,

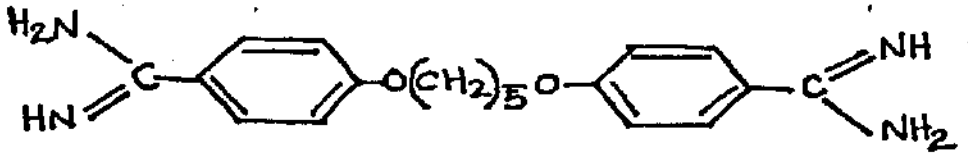


FIGURE 2.1.1. PENTAMIDINE

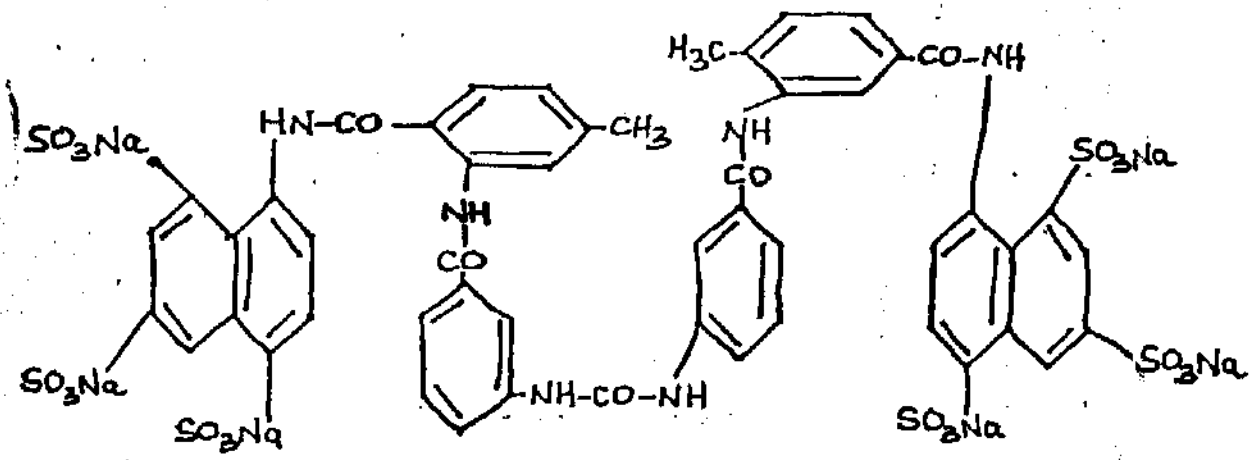
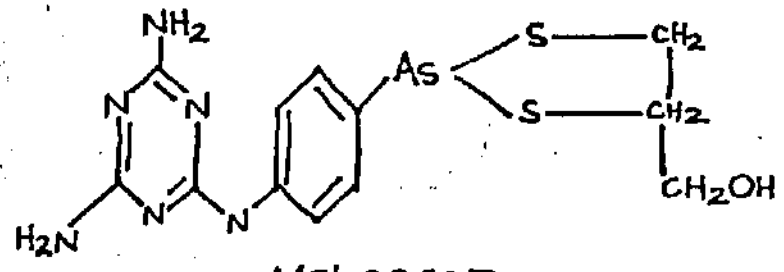


FIGURE 2.1.2. SURAMIN



MELARSOPOL

FIGURE 2.1.3.

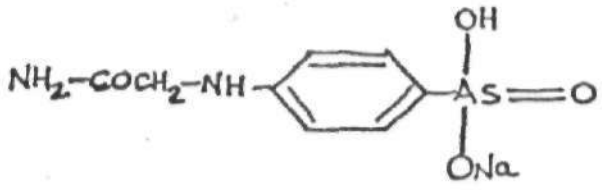


FIGURE 2.1.4. TRYPARSAMIDE

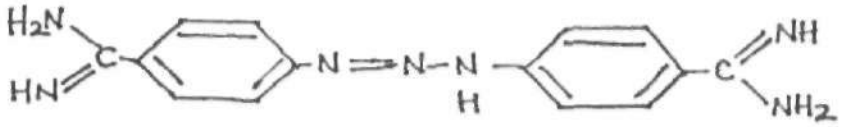


FIGURE 2.1.5. BERENIL

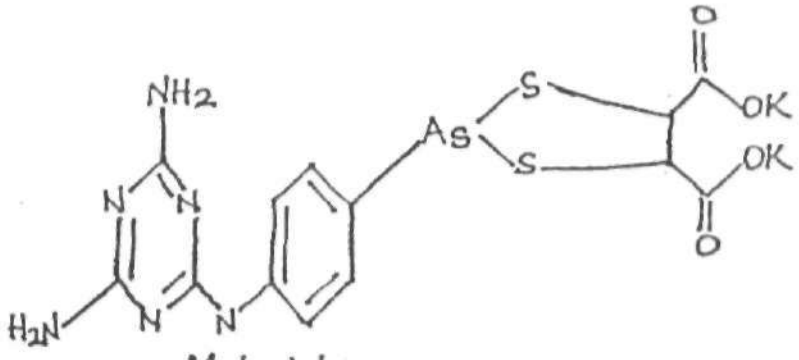
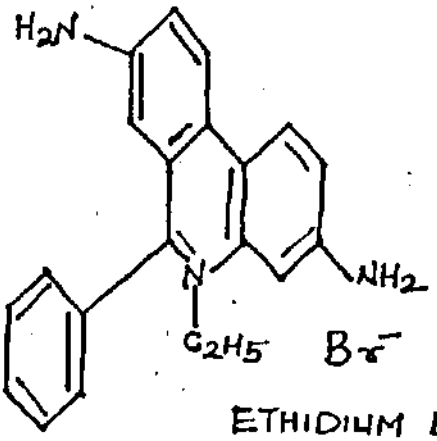


FIGURE 2.1.6. MEL W



ETHIDIUM BROMIDE

FIGURE 2.1.7.

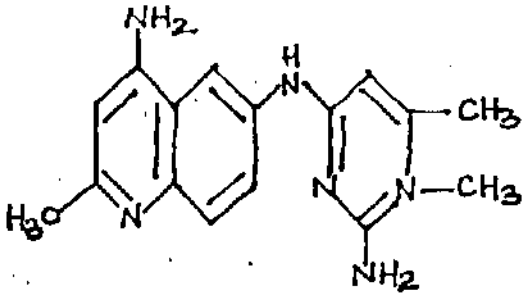


FIGURE 2.1.8. ANTRYCIDE

DRUGS FOR THE TREATMENT OF AFRICAN TRYPANOSOMIASIS IN MAN AND DOMESTIC ANIMALS

DRUG	TRADE NAME	ACTION	SPECIES MAINLY AFFECTED
TRYPARSAMIDE		C	T.b. gambiense
MELARSOPOL	MEL B, ARSOBOL	C	T.b. gambiense T.b. rhodesiense
NITROFURAZONE		C	T.b. gambiense T.b. rhodesiense
PENTAMIDINE	PENTAMIDINE, LOMIDINE	C P	T.b. gambiense T.b. rhodesiense
DIMINAZENE	BERENIL	C	T.congolense T. vivax
HOMIDIUM	ETHIDIUM, NOVIDIUM	C	T. vivax T. congolense
PYRITHIDIUM	PROTHIDIUM	C P	T. vivax T. congolense
ISOMETAMIDIUM	SAMORIUM, TRYPAMIDIUM	C P	T. vivax T. congolense
QUINAPYRAMINE	ANTRYCIDE	P C P	T. congolense T.b. brucei T. vivax T. evansi
SURAMIN	ANTRYPOL, NAGANOL	C P	T. brucei sspp T. evansi
SURAMIN-QUINAPYRAMIN COMPLEX		C P	T. simiae

Table 2.1.0 C- Curative; P- preventive

Source: Anthony (1986).

griping abdominal pains and subjective sensations of heat and disturbance of smell. The most serious side effect is reactive encephalopathy, occuring in more than ten per cent of patients. For these reasons, melarsopol is usually administered to hospitalized patients.

2.1.4 TRYPARSAMIDE

This is a pentavalent arsenical with trade names Tryparsam, Tryparsamidium, Glyphenarsine and Tryparsone. It is available as a white crystalline powder. A stock solution of 20 per cent (w/v) is administered by intravenous injection at a dose of 30mg per kg body weight. Ten to twelve such doses are given at 5-7 day intervals.

Tryparsamide's use is limited to the Gambian disease. It is used occasionally only, as it cures early infections [though with less rapidity than Suramin] and is very toxic. However, since it crosses the blood-brain barrier (Gutteridge, 1985) it is effective in treating late stage Gambian infections; although the side effects are such that melarsopol is preferred.

Tryparsamide is believed to be metabolized to the trivalent form by the host for activity, thus, it is not antitrypanosomal in vitro (Gutteridge, 1985). The mechanism of its action may be the same as for melarsopol. Not surprisingly, therefore, it is also quite toxic.

Frequent side effects include nausea and vomiting, occuring sometimes immediately after injection. The greatest risk from using

this drug, however, is damage to the optic nerve. Usually there is some warning sign (shimmering vision) so that treatment can be stopped and thus the risk of permanent and total optic atrophy is lessened (Gutteride, 1985). Sometimes, however, this may occur suddenly after the first injection. This side effect places severe limitations on the use of tryparsamide.

2.1.5 BERENIL

Berenil (diminazene aceturate) is an aromatic diamidine which is closely related to pentamidine. This drug is usually used for cattle. Berenil is a yellow powder that is dissolved in 5% glucose solution to yield a 2% (w/v) solution.

It is administered intravenously every 7-10 days at a dose of 2mg per kg body weight. It is effective in the early stages of both Gambian and Rhodesian diseases (Gutteridge, 1985). Berenil is also active orally. Three doses of 5mg per kg body weight are given at 2 day intervals. However, some relapses do occur to this treatment. Berenil is well tolerated in man (Gutteridge, 1985). The worst side effects, however, appear to be slight but persistent albuminuria. It has a similar mode of action to pentamidine.

2.1.6 MEL W

This is an arsenical also called melarsonyl potassium, melarsen oxide potassium dimercaptosuccinate. It was developed in the late

1960s as a possible replacement for melarsopol. Mel W is a white powder prepared for use by dissolving in distilled water, saline or saline solution to give a 5 per cent drug solution. The drug is administered by the intramuscular or subcutaneous routes. The dosage is 3 to 4mg per kg body weight for 3 to 4 consecutive days (Gutteridge, 1985). The course is repeated after an interval of 2 weeks.

Mel W readily crosses the blood-brain barrier. It is, therefore, effective in treating late stage Gambian and Rhodesian diseases. It's advantages over melarsopol are due to its solubility in water and its mobilization from muscle and sub-cutaneous sites of injection (Gutteridge, 1985). The mode of action is similar to that of other arsenicals; as such it has similar (or worse) side effects. Nowadays, Mel W is used very occasionally.

2.1.7 HOMIDIUM

Also called homidium bromide or ethidium is 3,8-diamino-5 ethyl-6-phenylphenanthridinium bromide. It is available both as the bromide and chloride [novidium]. The difference between the two is the increased water solubility of the chloride. The dosage of a 1 % aqueous solution is 0.25 - 1mg per kg of body weight. A dosage of 10mg/kg body weight provides freedom from infection for up to five weeks (Franklin and Snow, 1975). The parasites disappear from blood after injection, in less than twenty-four hours.

Homidium provides prophylactic action, with the prophylactic period ranging between 1-4 months.

Homidium is very effective against T. congolense, T. vivax and T. brucei. Homidium binds to DNA of nucleus and the kinetoplast of the flagellated trypanosome-like organism, crithidia fasciculata (Franklin and Snow, 1975). DNA synthesis is immediately suppressed while the synthesis of RNA and proteins continue unaffected (Franklin and Snow, 1975). Gutteridge and Coombs (1976) reported that both DNA and RNA polymerases are sensitive to inhibition as a result of intercalation of the drug with DNA; this causes local unwinding and lengthening of the DNA helix. This interferes with the function of DNA as a primer in nucleic acid synthesis. As the drug also binds to host DNA, the reason for its selective effect on the parasite is unclear. According to Franklin and Snow (1975), bloodstream forms of trypanosomes either carry no net charge or have a slight positive charge while the body's cells are negatively charged. This may enable the drug to penetrate more easily into the parasites than into the host cell. High dosages of up to 10mg/kg body weight can cause transient liver damage. Severe local reactions occur from subcutaneous injections. The deep intramuscular route is advocated for use.

2.1.8 QUINAPYRAMINE

Also called antrycide, is an aminoquinoline compound consisting of quinapyramine sulphate and quinapyramine chloride. Whereas the

sulphate is rapidly absorbed and is mainly trypanocidal with low prophylactic value, the chloride is more slowly absorbed and has a strong prophylactic effect. The two are combined as the 'Antrycide Pro-salt' or 'pro-salt R.F'. Antrycide (Figure 2.1.8) exists as a white to pale yellow crystalline powder that is odourless, with a bitter taste. The chloride is soluble to an extent of 2 per cent in boiling water while the sulphate is readily soluble (1:2); both, however, are insoluble in organic solvents. Antrycide powder consists of 3:2 parts sulphate : chloride having 10 per cent sulphate and 6.67 per cent chloride. The dosage is 0.025 ml per kilogram of body weight administered subcutaneously (Brander and Pugh, 1977).

Quinapyramine sulphate and chloride exert complementary effects against trypanosomiasis (Brander and Pugh, 1977) due to differences in their solubilities. The sulphate provides initial protection and eliminates sub-clinical infections while the chloride continues the protection after the effect of the sulphate has disappeared.

Quinapyramine is active against T. congolense, T. evansi and T. vivax in cattle, horses, camels and dogs. T. equiperdum and T. equinum are also susceptible (Brander and Pugh, 1977]) T. brucei, however, is less affected. Doses repeated every 2 months can prevent deaths from T. congolense infection, or T. vivax, for at least 18 months (Annual Report, N. Rhodesia, 1962) Resistant strains develop in animals as the tissue level of the drug falls. However, resistance is not permanent. Resistance to 5mg per kg doses may be lost after about 110 days (Brander and Pugh, 1977).

Quinapyramine acts by inhibiting cell division and growth. It displaces magnessium ions and polyamines from the cytoplasmic ribosomes of trypanosomes resulting in their aggregation and inactivation thereby blocking protein synthesis. The mechanism of selective toxicity may involve selective permeability (Gutteridge and Coombs, 1977). Side effects usually occur after over-dosage causing trembling, sweating, salivation followed (in severe cases) by an increase in heart rate, respiration, collapse and death. In normal dosages, when symptons occur, they are only of a mild and transitory nature.

2.2.0 DRUG SENSITIVITY TESTS

The search for more effective drugs for the treatment of trypanosomiasis involves the use of *in vitro* screens which possess the advantage of requiring only a small amount of the compound for the test, providing a large through-put of compounds with rapid results at low costs and requiring few animals.

Most early *in vitro* screens employ a short test in which bloodstream stages of trypanosomes were isolated from an infected rodent and maintained for up to 24 hours in a simple drug containing medium. *In vitro* drug screens are confronted by several challenges (Croft, 1986). These include:-

(i) They must provide active or dividing populations of the mammalian stages of the parasite

(ii) They must provide a measure of drug activity that can readily be quantified

(iii) They must accurately show the activity of standard drugs at concentrations close to those achievable in serum or tissues (over a long-time-course if necessary).

Hiroshi *et al* (1988) used simple graphical plots with cell viability plotted against incubation time giving a series of non-intersecting lines. Plots of the reciprocal of the time required to kill fifty per cent of the cells versus moles per cent of drug were linear with

an intercept on the X-axis corresponding to the drug concentration that is theoretically inactive. Reproducible results were obtained with the drugs tested including Mel B, Suramin and Iomidine.

A widely reported and effective sensitivity test involves the suppression of the uptake of radiolabeled macromolecular precursors (Canfield et al; 1970; Irwing and Young, 1977). The method was also used by Chang et al; (1978) on blood form trypanosomes. A similar method was employed by Desjardins et al., (1980). The uptake of two radiolabeled macromolecular precursors (Methyl-^{3}H)-thymidine and L(U-^{14}C) leucine were used as indices of parasite growth in the test system. The incubation time was 3 hours. The method was reported (Desjardins et al.,(1980) to possess several advantages. These include:-

(i) allowing the screening of large number of compounds

(ii) quantitative evaluation of synergistic activity for various cominations of antitrypanosomal agents in vitro

(iii) kinetic analysis that could suggest optimal dosage for initial therapeutic trials when a compound has reached that stage of clinical development.

The disadvantages of the method of Desjardin et al., [1980] stems from the use of automation (a restriction particularly in third world countries, where the disease is endemic. Secondly, Desjardins et al., (1980,) stated that the 3-hour incubation period may not allow sufficient time for the drug to enter the trypanosomes and exert its biological effect. Short incubation period results either in a poor incorporation inhibition curve with an increased IC_{50} (inhibitory concentration) value, or no inhibition at all. It is therefore important to have a long enough test duration, and especially a long enough preincubation period to obtain a stable inhibition curve.

The method of Seebeck and Gehr, (1983) is also not different in principle with the method of Desjardins et al., (1980). Seebeck and Gehr, (1983) tested the effect of phenothiazines on uridine incorporation. Uridine incorporation was reported to be suppressed in the presence of the drugs tested (Dimethyl Sulfoxide, Chlopromazine sulfoxide, promethazine, fluphenazine, chlorpromazine, 2-chloro-10 (3-aminopropyl) phenothiazine and thioridazine. The preincubation time was only 30 minutes and the total incubation period being only 3 hours.

The obvious reason for these short incubation period was due to the inavailability of an axenic (cell-free) culture system that could support the growth of bloodstream trypanosomes. Under these conditions, most trypanocidal drugs may not develop their full trypanocidal action or show no effect at all(eg. Suramin) (Brun et al., 1989)

The discovery by Baltz et al., (1985) that the addition of 2-mercaptoethanol to serum supplemented medium can to some extent replace a feeder layer, allowed incorporation tests with a test duration of 24 to 48 hours in a system with no feeder layer. This system allowed Brun et al., (1989) and Brun and Kunz (1989) to carry out incorporation tests without first adapting trypanosome populations to cell-free culture conditions. According to Brun et al., (1989) triated [^{3}H] hypoxanthine is incorporated by both dividing and non-dividing trypanosome populations.

Brun and Kunz (1989) examined [redacted] inhibition based on incorporation of radiolabeled hypoxanthine. A preincubation period of 16 hours and a total incubation period of 14 hours gave accurate and reproducible tests. This method, like that of Desjardins et al., (1980) could also be used to screen new compounds, though, with selected reference trypanosome populations. All the drugs tested (Berenil, Mel W, Suramin, Samorin and Iomidine) displayed trypanocidal activity [unlike the method of Desjardins et al., (1980)]. The disadvantages of this method include the cost of radiolabeled substrates and the necessity for a cell harvester and liquid Scintillation counter.

The method of Brun et al., (1989) though similar in principle (to the method of Brun and Kunz, 1989)) has certain modifications. Here the preincubation period was 24 hours while the incubation period lasted 15 hours. The advantages and limitations to this method are the same with those of Brun and Kunz (1989)

Zinsstag et al., (1991) reported a method that uses both the eye and a spectrophetometer. This test is dependent on the ability of T. brucei to produce pyruvate. Pyruvate lowers the pH of the culture medium and causes the colour of phenol red to change from red to bright yellow. This change in colour is proportional to the pH change and can be seen by eye and measured spectrophotometrically. The difference in extinction of colour between fresh and used medium is an indirect measurement of the metabolic activity of a growing trypanosome population. A drug inhibited culture produces less pyruvate and the decrease in its colour extinction is lower. Comparison of this extinction with that in control cultures enabled the calculation of an inhibition curve and the determination of IC_{50} and MIC [minimal inhibitory concentration] values. Several indicators were used but phenol red and bromothymol blue were reported to be most suitable. Again 10mM HEPES buffer was found to be effective (25mM for Baltz et al. 1985) Zinsstag et al. (1991) pointed out that strains adapted to culture are necessary.

This method is fast and less tedious with reproducible results and can be used to screen new trypanocidal drugs using reference strains. The photometric test cannot be directly compared with an

incorporation test because the respective principles and kinetics involved are quite different. However, the photometric test of Zinsstag et al., (1991) gave IC_{50} values that were very similar to those obtained in incorporation tests based on the method of Brun et al., (1989).

2.3.0 IN VITRO CULTURE SYSTEMS

The development of *in vitro* culture systems has high priority in trypanosomiasis research as it opens new possibilities for various studies *in vitro* under controlled conditions. This give a large field of applications. The following highlights the wide range of applications and the advantages of trypanosome culture systems. It allows :-

(i) Metabolic studies and the establishment of nutritional requirements

(ii) Screening of compounds to find new therapeutic drugs

(iii) Production of antigens for serodiagnosis

(iv) Mass production of specific parasite stages for biochemical and immunological investigations

(v) Study of differentiation processes : transformation steps are crucial phases in the parasites life cycle. Blocking of such steps would stop further development of the parasite. These studies serve as other methods of control in addition to chemotherapy

(vi) replacement of the use of laboratory animals, or at least a reduction in their numbers

(vii) the production of insect stages in countries where handling the insect vector is not generally allowed (eg. *Glossina* species in U.S.A).

The diverse feeding and living habits of trypanosomes has made difficult the emergence of a single medium that could be called 'defined' and used universally. Instead, what obtains is a collection of 'semi-defined' media with varied compositions.

A culture medium should in addition to supporting the continuous growth of trypanosomes allow the trypanosomes to retain all the characteristics of the bloodstream forms namely morphology, variant surface antigens [VSA], infectivity and metabolic characteristics.

The first continous cultivation of bloodstream form trypanosomes was carried out by Hirumi and Hirumi (1977). These workers cultured T. Brucei using bovine fibroblast culture as a feeder layer in tissue culture medium supplemented with fetal bovine serum(FBS) Ninety-six different culture conditions were tested including 12 different media, 4 different concentrations of FBS and the absence or presence of fibroblast-like cells. A doubling time of 96 hours was observed. Some of the media used include RPMI 1640 and Eagle's minimum essential medium (MEM) with Earle's salts; both media were supplemented with 25mM N2-hydroxyethylpiperazine N'2-ethanesulphonic acid (HEPES) buffer. This buffer has been reported to give active or stable mitochondrial preparations. It also gives a higher rate of protein synthesis in cell-free bacterial preparations than do Tris or phosphate buffers (Norman 1966). This buffer, being zwitterionic(Tris and phosphate buffers have net positive charges) is non-toxic to organisms.

The addition of FBS(20%) or another mammalian serum was found to be obligatory. The co-cultivation of trypanosomes and

mammalian cells proved essential for the cultivation of the vertebrate forms (Brun et al., 1981; Hill et al., 1978). The role of this feeder layer is, however, unclear since in vitro cultivated trypanosomes express antigenic variation indicating that this phenomenon is not induced by host specific factors (Brun and Jenni, 1985).

Brun et al., (1984) evaluated the ability of different cell lines and sera from African wild bovidae in supporting the growth of bloodstream forms of T. b. brucei and T. b. rhodesience. They used Eagles MEM with Earle's salts, 1mM glutamine, 1 per cent MEM non-essential amino acids , 0.001 per cent glucose, 15 percent heat inactivated serum and 10 µg/ml gentamycin. Cultures were made in 24 well tissue culture plates in the presence of feeder layers and incubated at 37°C in 4 per cent CO_2. Most of the isolated cell lines were of the fibroblast cell type. A few, however, were epithelial or a mixture of the two. These were isolated from the embryos of eland (Taurotragus oryx), Impala (Aepyceros melampus) and Grant's gazelle Gazelle granti . These cell lines were reported to influence the growth properties. The influence of cell type was found to be a function of cell type and not the cells origin. Cells of the fibroblast type were found to be superior as compared to epithelial cells. For example, Grant's gazelle epithelial cells were unsuitable for both T. b. brucei and T. b. rhodesiense. Cell lines from eland and Impala supported the growth of T. brucei, whereas, the cell line from Grant's gazelle did not. Brun et al., (1984) reported that Grant gazelle possess innate trypanocidal factors that impede growth; even in T. b. rhodesiense (though a few showed resistance).

Many of the feeder layer/serum combinations used supported the continous growth of T. b. brucei and T. b. rhodesiense. However, Brun et al., (1984) reported that inactivated fetal bovine serum (IFBS) was unsuitablie for the cultivation of bloodstream form trypanosomes (unlike Hirumi and Hirumi, 1977), though the culture could be maintained for a few days. Inactivated rabbit serum, human serum, and the homologeus sera used gave excellent results.

The culture system of Baltz et al., [1985] enabled The cultivation of animal infective forms of T. brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense. Trypanosomes were cultivated for more than 4 months in Eagle's MEM supplemented according to Brun et al., (1984). In addition 2mM pyruvate, 0.2mM 2-mercaptoethanol and 0.1mM hypoxanthine were added as supplements. Peritoneal macrophages were used as feeder layers. These were isolated from swiss mice. Heat inactivated serum from rat, rabbit, horse, fetal calf and human's (AB+) were used. HEPES buffer (25mM) was also added. The presence of a reducing agent eg. mercaptoethanol was reported to be crucial. The role of the reducing agent may be to stabilize the different essential components of the medium such as glutathione and vitamines. A minimum concentration of pyruvate was necessary for adapting parasites to culture, even in T. b. brucei which produces pyruvate. It was noticed that ommission of pyruvate in media containing T. b. brucei arrests growth.

The optimal growth of all the strains tested (except T. gambiense) was obtained with rabbit serum, which also gave the best results with feeder layers. These observations are surprising since most of the trypanosome strains develop a very low parasitemia in rabbits.

The addition of a nucleotide precurssor like hypoxanthine, though essential, was however not obligatory for media supplemented with human or rabbit serum indicating that differences exist between these two sera and the others tested.

The cultivation of T. congolense and T. vivax has witnessed little success [Brun and Jenni, 1985]. For T. congolense, transfer of an infected skin into culture medium retained the infectivity for up to 21 days.

The first continous culture of T. congolense bloodstream forms used a feeder layer of bovine endothelial cells in RPMI 1640 medium supplemented with 16 per cent heat-inactivated adult goat serum (Brun and Jenni, 1985) The bloodstream forms from culture were morphologically indistinguishable from trypanosomes isolated from the mammalian host. They were covered by a surface coat and proved to be infective when inoculated into mice.

Trypanosoma vivax, however, has proven more difficult to cultivate in vitro. Bloodstream forms brought into culture with tsetse tissue, however, were maintained for over one month and were capable of infecting sheep (Trager, 1959). These experiments when repeated sixteen years later failed to demonstrate infectivity.

In 1984, ILRAD developed methods to grow both procyclic and metacyclic forms of T. vivax. Bloodstream forms from mice were introduced into a culture of bovine fibroblasts in MEM, with beads of matrix Green Gel A at 25°C. These transformed to non-infective insect forms and attach to the beads and afterwards to the feeder cells. A small proportion

(<1 per cent) of metacyclics could be detected after 2-3 weeks. Brun and Maloo (1982) have demonstrated the use of microtus embryo fibroblasts as feeder cells in MEM, supplemented with 20 per cent goat serum [heat-inactivated] to cultivate bloodstream forms from a West African T. v ivax stock. However, East African T. vivax stocks have not been grown successfully in this system.

2.4.0 SOME BIOCHEMICAL TARGETS FOR CHEMOTHERAPEUTIC ATTACK IN TRYPANOSOMES

Biochemical studies help in chemotherapy of trypanosomiasis by outlining the unusual structural and functional differences between mammalian and trypanosome cells. The features serve as potential targets because drugs can be directed specifically at them without interferring with the cells of the mammalian host. Some of these differences have received a lot of attention (ILRAD, 1990) over the years and include :

(i) Subcellular localization as exhibited by the glycosome

(ii) differences in rate of enzyme turnover as exemplified by ornithine decarboxylase

(iii) occurence of specific molecules, for example, trypanothione

(iv) microtubules, and,

(v) differences in enzyme structure as in dihydrofolate reductase.

2.4.1 ORNITHINE DECARBOXYLASE AND TRYPANOTHIONE METABOLISM

Ornithine decarboxylase (ODC) together with S-adenosylmethionine (SAM) decarboxylase form the critical steps in the synthesis of polyamines (Figure 2.4.1). The polyamines putrescine, spermidine and spermine have major roles in cell division, differentiation and the maintenance of essential cell functions like conformational

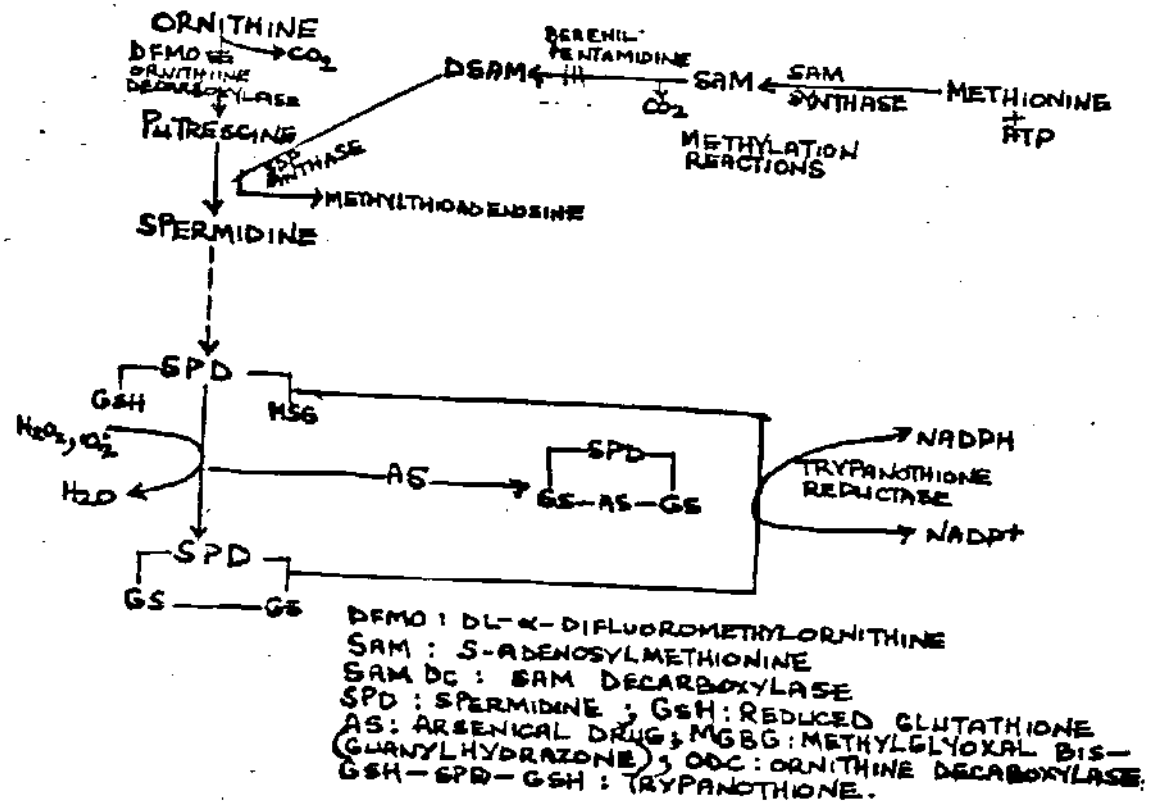


FIGURE 2.4.1. POLYAMINE BIOSYNTHESIS AND RELATED REACTIONS IN AFRICAN TRYPANOSOMES (BACCHI et al., 1989).

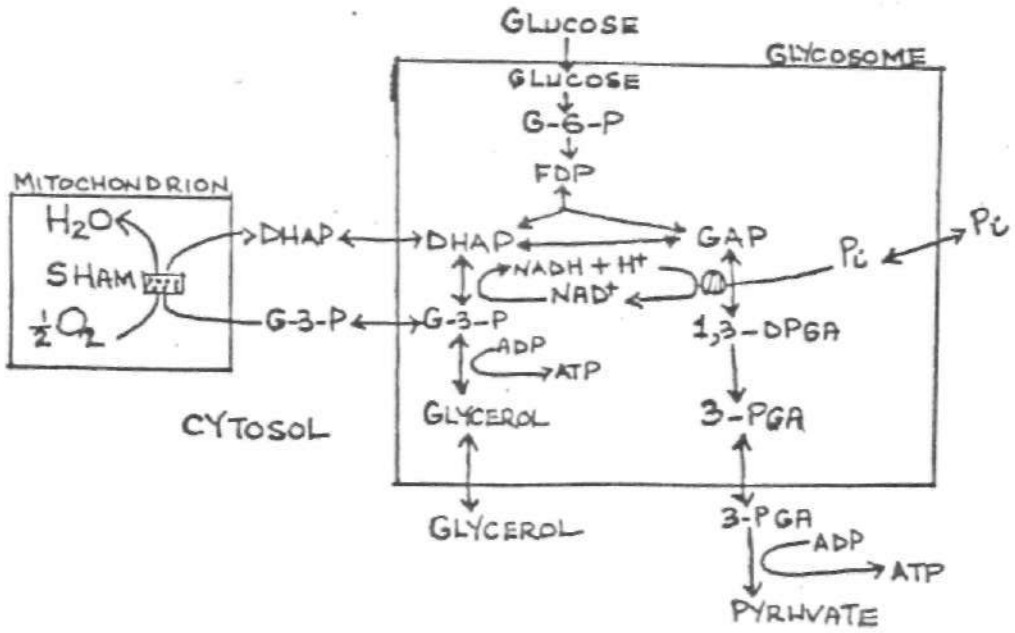


FIGURE 2.4.2. COMPARTMENTATION OF GLYCOLYSIS IN T. brucei (FROM MICHELS, 1988).

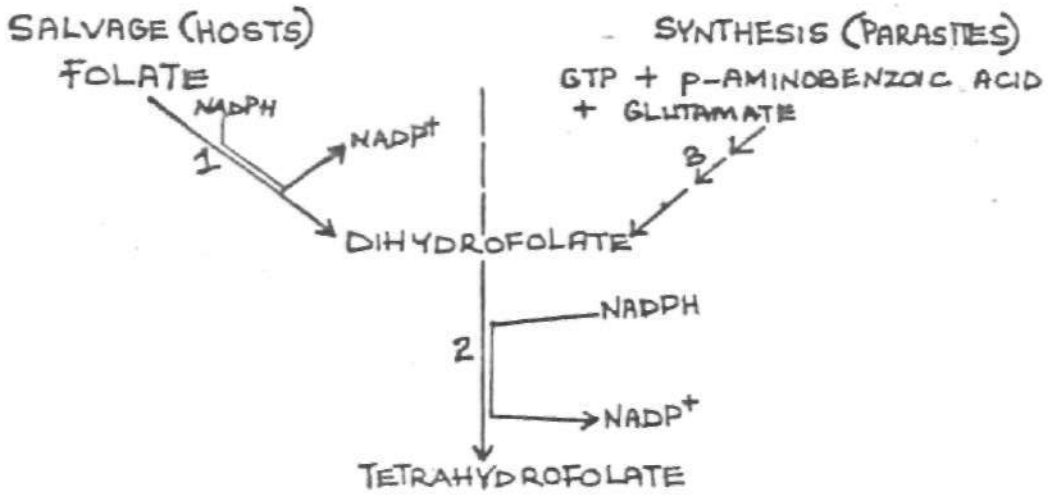


FIGURE 2.4.3.
ALTERNATIVE SOURCES OF TETRAHYDROFOLATE IN PROTOZOAN PARASITES AND THEIR VERTEBRATE HOSTS:
1. FOLATE REDUCTASE
2. DIHYDROFOLATE REDUCTASE.
(GUTTERIDGE AND COOMBS, 1977)

regulation of DNA, elongation and initiation of protein synthesis and the fidelity of translation (Pegg, 1986). Unlike mammalian cells, trypanosomes do not possess classical glutathione reductase or glutathione peroxidase activities (Henderson and Fairlamb, 1987). Instead, these vital enzymes have been replaced by isofunctional trypanothione-dependent counterparts. In trypanosomes, therefore, the trypanothione system has taken the place of the glutathione system for removing peroxides; this being an important function of the molecule in the cell.

The ODC of trypanosomes has a higher half life than that of mammals. The main structural difference between host and parasite ODC being the absence of a 36 amino acid sequence on the C-terminal end of the parasite's ODC (Phillips et al., 1987). This allows a high turnover rate in the mammalian enzyme.

Eflornithine also called DL-α-difluoromethyl ornithine (DL-α-DFMO) inhibits ornithine decarboxylase. The low turnover rate of the parasite ODC implies low synthesis rate allowing for more efficient reduction of enzyme activity by DFMO for a longer time. Also, DFMO treatment reduces levels of trypanothione (which is responsible for maintaining the redox balance of the cell). The resulting stress on the capacity to detoxify free radicals enhances parasite sensitivity to agents that produce free radicals eg. nifurtimox (Fairlamd, 1989).

S-adenosylmethionine decarboscylase is particularly sensitive to the aromatic diamidine berenil and pentamidine. Both block this enzyme thereby

arresting the production of decarboxylated S-adenosylmethionine, and subsequently, spermidine (Figure 2.4.1).

2.4.2 THE GLYCOSOME

Trypanosomes have a unique form of compartmentation of their glycolytic enzymes inside an organelle called the 'glycosome' (Figure 2.4.2). In other eukaryotes glycolysis occurs in the cytosol. African trypanosomes in the hosts bloodstream depend solely on glycolysis for energy production. Some of the glycolytic enzymes are both structurally and kinetically different from their mammalian homologues (Michels, 1988). These features allow the development of drugs that will produce effects only on the parasites. The trypanosomal hexokinase (HK) is an hexamer differing both in subunit composition and in subunit size from the monomeric mammalian enzyme [Michels, 1988].

Phosphofructokinase (PFK), although a tetramer like the mammalian form, has a subunit molecular weight of 50 KDa, this being quite different from that of the enzymes of other eukaryotes (75-120 KDa) and from bacteria (35 KDa) (Michels, 1988).

The arsenicals tryparsamide, Mel W and melarsopol inhibit all the trypanosomal kinases including hexokinase, pyruvate kinase and phosphofructiokinase. Pyruvate kinase is markedly inhibited due to its presence in the cytoplasm (Gutteride, 1985).

Trypanosomes contain a novel enzyme concerned with the reoxidation of NADH. This is the L-glycerol-3-phosphate oxidase (Figure 2.4.2).

This enzyme (unlike that of insect flight muscle and mammalian brain mitochondria) reacts with oxygen without the intervention of pyridine nucleotide coenzymes or of cytochromes (Bowman and Flynn, 1976). Glycerol-3-phosphate oxidase is not sensitive to azide, cyanide, amytal and antimycin A. It has iron, thiol groups and FAD as components. Derivatives of hyrdoxamic acid eg. m-chlorobenzhydroxamic acid (M-CLAM) inhibit glycerolphosphate-supported oxygen uptake in bloodstream forms of T. brucei and T. rhodesiense (Michels, 1988). Other compounds inhibiting the glycerolphosphate oxidase system include Suramin and Salicylhydroxamic acid (SHAM).

2.4.3 DIHYDROFOLATE REDUCTASE

Dihydrofolate reductase (Figure 2.4.3) has been isolated from trypanosomes. It differs from the isofunctional vertebrate enzyme having different cofactor requirements (NADH as well as NADPH) higher molecular weight (about 200,000 daltons compared to 20,000 - 30,000 daltons) (Gutteridge and Coombs, 1977). Also, it has a greater sensitivity to inhibition by 2,4-diaminopyrimidines; eventhough these drugs are not toxic to intact trypanosomes. The enzyme is also inhibited by Suramin (Gutteridge and Coombs, 1977).

2.4.4 THE MICROTUBULES

The trypanosomal cell body contains essentially a single cytoskeletal structure, the cage-like array of subpellicular microtubules that encloses the entire cell body. These microtubules are very closely associated with the overlying cell membrane and serve as a 'membrane Skeleton'. Microtubules are also involved in the structure of the

flagellum which is another important component of the cellular motility system. They represent the mechanochemical motor of the cell, and are structurally and functionally very similar to those of its homologues in the cilia and flagella of most other eukaryotes The microtubules of the trypanosomal membrane skeleton are unusual in several respect. (Seebeck et al, 1990).

They are very stable when the cells are extracted with a variety of buffers, and they are very resistant to depolymerization by low temperatures. This is in contrast to many of their counterparts in higher organisms (Seebeck et al., 1990) which are extremely labile and prone to rapid depolymerization in vitro and at low temperatures. In fact, the marked stability of the trypanosomal microtubules is still a major obstacle to purifying functionally active tubulin from these organisms.

Furthermore, trypanosomal microtubules are resistant to some drugs that have potent anti-microtubular activity in higher eukaryotes eg. colchicine (Seebeck et al., 1990) On the other hand, several drugs that have low activity against mammalian microtubules are potent disruptors of the microtubules of the trypanosomal skeleton (Seebeck and Gehr, 1983).

The primary sequence of the trypanosomal tubulins has been determined (Kimmel , 1985) and compared with those from mammals . No dramatic sequence divergence was detected. However, tubulins from trypanosomes and humans differ

in about 20 per cent of their amino acid residues. Considering that the tubulin is a highly conserved protein, this represents a rather strong divergence. Subtle differences in amino acid sequence are still difficult to interprete since the three dimensional structure of the tubulin protein is not yet known.

Post-translational modification of tubulins investigated (Schneider , 1987; Sherwin , 1987; Sasse and Gull, 1988) revealed a simple isoform pattern suggesting that they are not extensively modified. Two major types of modifications observed include acetylation of α-tubulin and c-terminal tyrosination of α-tubulins. These modifications, however, are common throughout the eukaryotic kingdom (Seebeck et al., 1990).

Electron microscopy of the trypanosomal skeleton indicates that its microtubules are interconnected by a host of microtubule associated proteins (Souto-Padron et al., 1984). Also freeze-fracture experiments have long suggested that microtubules might also be closely linked via microtubule-associated proteins, with the overlying cell membrane. A microtubule associated protein of large molecular mass (320 KDa) was found to consist of essentially strongly conserved repeats of 38 amino acids in length (Schneider , 1988). The protein is located exclusively on microtubules of the membrane skeleton and is absent from those of the flagellar axoneme (Seebeck et al., 1990). Tubulin has long been an important target for the development of anti-cancer drugs. The application of similar concepts to trypanosomal tubulin may be very productive.

2.4.5 PARAFLAGELLAR ROD

This is a conspicuous, highly ordered array of filaments with a function that is yet to be understood. It is found exclusively in the flagella of trypanosomes and does not seem to be involved in flagellar motility (Johnson et al., 1979) . The major components are two biochemically similar proteins with molecular weights of about 68 and 72 KDa. Both proteins may be coded for by the same genes (Schlaeppi et al., 1989).

The paraflagellar rod also contains a host of associate proteins of yet unknown functions and characteristics (Seebeck at al., 1990). One of these has been identified as a possible member of the spectrin/ fodrin family of cytoskeletal proteins, whose known best members are the major components of the erythrocyte membrane skeleton, α-and p-spectrin (Schneider , 1988; Alcina , 1988; Woods , 1989). Multiple structures seem to be involved in connecting the flagellum to the cell body. Further analysis of these structures will be of particular interest since it may improve understanding not only of mechanisms by which the trypanosome attaches to its own flagellum [or vice versa], but also of how it can form contacts with cells of a host organism. However, these connections may be accessible to antibodies from the cell surface, even in the bloodstream form of African trypanosomes, since they do not seem to be covered by the VSG coat (Vickerman and Preston, 1976). This idea is useful in developing trypanosomal vaccines. The paraflagellar rod is a potential target for trypanocidal drugs since it has no homologue in all vetebrate cells.

2.5.0 COMPOSITION OF COTTONSEED OILS

Cottonseed varieties include the American (Gossypuim hirsutum), the Egyptian (Gossypium barbadense), and the Asiatic (Gossypuim herbaceum types.

Cottonseed oil is a by-product arising from the cultivation of cotton. The cotton is attached to the seeds and up to 40 seeds occur in a single cotton boll. The cotton is removed from the seed by ginning the shorter fibres or linter still attached (10-15 per cent of the whole seed). The seed yields approximately 16 per cent oil, 45 per cent meal, 9% linters (depending on type), and 26 per cent hull. Extraction of the oil is usually by pressing or by solvent extraction. The quality of oil obtained depends on the kind of solvent used for the extraction. Whereas hexane extracts oil from cottonseeds with virtually no gossypol. acetone extracts all the available gossypol in cotton seeds (Akanya, 1976). Furthermore, hexane extracts give a yellow colour while a reddish-brown colour is obtained from acetone extracts. Cottonseed oil, generally, has a strong characteristic flavour and odour. The oil is used domestically for cooking, making margarine, salad oil and also as an ingredient of brushless shaving creams.

Cottonseed oil has a wide composition (tables 2.5.1 and 2.5.2). The free fatty acid (FFA) content of the oil depends on the harvesting conditions and the moisture content of the seeds. Good quality oil has FFA content of between 0.5 - 1 per cent (Akanya, 1976). Investigations carried out by Hilditch and Maddison (1940) showed that the oil has a

high percentage of triglyceride Palmito-di-unsaturated constituted 58 per cent, 28 per cent tri-unsaturated and 13 per cent mono-unsaturated triacylglycerides. In another survey carried out on a variety of cottonseed samples by Hilditch and Jones (1934) a 60 per cent triglyceride content was obtained and the oil contained one palmityl and two unsaturated acyl groups. Bailey (1948) in one series of investigations on a selection of varieties of cottonseeds established a range of Iodine values from 90-115 and saponification number of 190-208.

Cottonseed oil is distinguished from other commercial oils by its content of C20, C22 and C24 saturated fatty acids (Gunstone, 1958).

The presence of cyclopropenoid fatty acids (CPFAs) is of even greater importance (0.1 - 3%) (Bailey et al, 1966; Gordon and John, 1983; Randall, 1986b). These acids are responsible for giving a positive Halphen test which is characteristic for cottonseed oils. The major cyclopropenoid fatty acids include sterculic (9, 10-methylene -9-Octadecenoic) and malvalic (8,9-methylene-8-heptadecenoic) acids (figures 2.5.1 and 2.5.2 respectively).

The Cyclopropane fatty acids, dihydromalvalic and dihydrosterculic acids may also be found along with the cyclopropene fatty acids. These, however, occur at very low concentrations (Randall, 1986a).

Although tocopherols exist in crude cottonseed oils, they are partially removed during refining. This, together with removal of

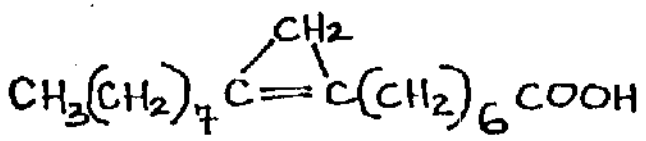


FIGURE 2.51. MALVALIC ACID

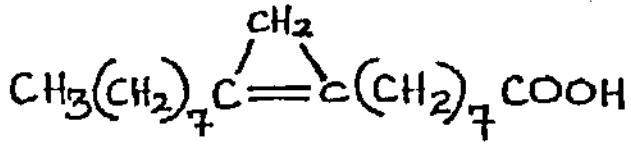


FIGURE 2.5.2. STERCULIC ACID

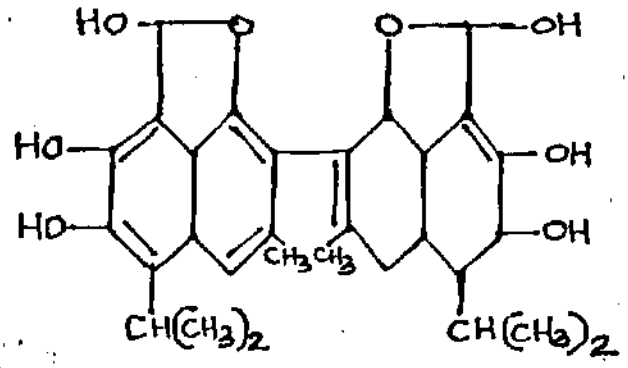


FIGURE 2.5.3. GOSSYPOL

gossypol, is a possible reason for the relatively poor oxidative stability of cottonseed oil as compared to groundnut oil. However, refined cottonseed oils contain 74 o 94 mg per kg tocopherols.

Crude cottonseed oil contains numerous minor non-glyceride components constituting about 2 per cent. These include gossypol, phospholipids, sterols, carbohydrates, pigments, and hydrocarbons (mainly squalene, 0.004 to 0.012 per cent) (Gunstone 1958). Gossypol (figure 2.5.3) is a polyphenolic triterpene of molecular formula $C_{30}H_{30}O_8$ soluble in many organic solvents such as ether and acetone (Akanya, 1976). Gossypol (1,1', 6,6', 7,7' - hexa-hydroxy-5,5' diisopropyl-3,3'-dimethyl - (2,2'-benapthalene)-8,8'-decarboxyaldehyde) is converted to the purple pigment gossypurpurin in seeds stored for prolonged periods. Gossypol occurs in the pigment glands of cottonseeds to an extent of between 0.4-1.2 per cent (Tropical Product Institute, 1962; Akanya, 1976).

CHARACTERISTICS OF FATTY ACIDS OF COTTONSEED OILS

RELATIVE DENSITY ($20^{o}C$/WATER AT $20^{o}C$)	0.918-0.926
REFRACTIVE INDEX	1.458-1.466
SAPONIFICATION VALUE	188 - 198
IODINE VALUE	99 - 119
UNSAPONIFIABLE MATTER (PER CENT)	1.5
HALPHEN TEST	POSITIVE
GLC RANGES OF FATTY ACID COMPOSITION (PER CENT)	
14	0.1
14:0	0.4-2.0
16:0	17 - 31
16:1	0.5-2.0
18:0	1.0-4.0
18:1	13 - 44
18:2	33 - 59
18:3	0.1-2.1
20:0	0.7
20:1	0.5
22:0	0.5
22:1	0.5
24:0	0.5

TABLE 2.5.1

DRAFT CODEX, 1979.

PHOSPHOLIPIDS OF GLANDLESS COTTONSEED OIL

PHOSPHOLIPID	COMPOSITION (PER CENT OF TOTAL PHOSPHORUS
UNCHARACTERIZED, POLAR	4.1
PHOSPHATIDYLINOSITOL	13.4
PHOSPHATIDYLSERINE	2.4
PHOSPHATIDYLCHOLINE	23.2
PHOSPHATIDYLETHANOLAMINE	13.5
PHOSPHATIDYLGLYCEROL	7.6
PHOSPHATIDIC ACID	8.8
LYSOPHOSPHATIDYLCHOLINE	2.6
LYSOPHOSPHATIDYLSERINE	-
LYSOPHOSPHATIDYLETHANOLAMINE	-
UNKNOWN [SUM OF SIX TLC SPOTS]	25.3

TABLE. 2.5.2
(CHERRY, 1983)

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Isoamyl alcohol, starch, tris (hydroxymethyl) aminomethane- HCL, 2-mercaptoethanol, dimethyl-sulfoxide, sodium pynvate, and Giensa stain were products of British Drug House (BDH) Ltd., Poole, England.

Salicylhydroxamic acid (SHAM) was from Aldrich Chemical Co., Inc., Milwaukee, USA. N2-hydroxy-ethylpiperazine N2'-ethanesulphonic acid [HEPES] and Eagles minimum essential medium (Eagles 1999 were from flow laboratories, Irvine, Scotland.

Pipette: E-mil Gold Line BS°748 and Neubauer haemocytometer (depth 0.1mm, 1/400mm² BS 748) were from Hawkslay, London.

Tissue Culture multi-well plate (well capacity: 0.35 ml approx) was product of Linbro Scientific Inc., USA.

3.1.2 TRYPANOSOME SPECIES

Trypanosoma brucei (strain WAMBA) was obtained from the Nigerian Institute for Trypanosomiasis Research (NITR) at Vom, Jos where the parasite has been preserved by passaging in mice. The parasite was brought to Zaria in rats and stored under liquid nitrogen. Trypanosoma congolense (2295) was isolated in 1978 at Makurdi slaughter house and brought to Zaria. It has since then been stored by passaging in mice and liquid nitrogen.

3.1.3 COTTONSEED OIL SAMPLES 1 AND 2

Cottonseeds (Samaru 71, variety) were obtained from the Institute of Agricultural Research (IAR), Samaru, Zaria. The seeds were delinted and genned using concentrated sulphuric acid at IAR. Oil was then extracted using Hexane: acetone (2:1) solvent. This oil is here designated SAMPLE 2. Also cottonseed oil was collected from the Funtua Cotton Crushing Company at Funtua. T e oil was obtained by crushing the seeds at high pressures with presses. This oil we designated as SAMPLE 1.

3.1.4 EXPERIMENTAL ANIMALS

Ra's and mice were purchased from the Animal House at the Faculty of Pharmaceutical Sciences, A.B.U., Zaria. T ese were fed ad libitum with Pfizer animal feeds. Rabbits were purchased locally at Samaru and fed on carrots and tridax. The animals were kept for periods of ten days and screened by taking blood samples from the tail and ear vein for rats, mice and rabbits, respectively.

3.1.5 HORSE BLOOD

Horse blood was obtained from the Department of Veterinary Medicine and Surgery, Faculty of Veterinary Medicine, A.B.U., Zaria.

3.1.6 PREPARATION OF SERUM (Baltz et al., 1985)

Horse and rabbit blood were obtained from the animals from the jugular vein and by cardiac puncture, respectively. The blood was allowed to coagulate at room temperature for two hours, decanted, and stored overnight at 4°C. The serum was then centrifuged at 4000g for 15 minutes, followed by incubation of 30 minutes at 56°C. The serum was then sterilized by filtration under positive pressure using microfilter (millipore 0.45µm) and frozen in 3ml quantities.

3.1.7 PREPARATION OF COCONUT FLUID MEDIUM

This was carried out according to Marquez et al., (1987) with slight modifications. The coconuts were washed thoroughly with water and then with 90 per cent alcohol. The back was broken by hitting gently with a hammer. Using 30 ml syringes and 19 gauge needles, the nut was pierced and the fluid drawn out into sterile flasks. About 250 mls of fluid was obtained from two nuts. The fluid was then sterilized by filtering under positive pressure using millipore (0.22μm). The pH was adjusted to 7.4 using 1N NaOH and stored at 4°C. Before use, the medium was supplemented with 2 per cent serum.

3.1.8 PREPARATION OF EAGLES [199] MINIMUM ESSENTIAL MEDIUM

Into 4 sterile flasks each containing 1000 MLS deonized water was added 9.27g of powdered medium 199 followed by the addition of 2.2g $NaHCO_3$ and 1g glucose. HEPES , 2.383g (Brun and Kunz, 1989) and 5.95g (Baltz et al., 1985) were added into flasks A, B, and C,D to give 10mM and 25mM concentration, respectively. The flasks were then diluted by adding 100ml deionized water. The media were then sterilized by filtering under positive pressure using millipore 0.22 μm. The media were then supplemented according to Baltz et al (1985) by adding 0.22mg sodium pyruvate (2mM), 14 μl of 2-mercoptoethanol [0.22mM], 20mg per litre gentamycin and 10mls amino acid mixture [(10 mg per litre of each amino acid in the mixture)], to all flasks. The pH was adjusted to 7.4 using 1N HCL. The media were stored at 4°C.

3.1.9 COUNTING OF TRYPANOSOMES

The sample to be counted is pipetted into an E-mil GOLD LINE BS 748 pipette to the 1.0 mark. Trypanosomes counting fluid is then drawn in to the 101 mark and the pipette agitated for 2 minutes on a mechanical shaker. A drop of this fluid is then placed on the haemocytometer and counted. The number of trypanosomes counted is multiplied by a factor of 500 in order to express the trypanosomes per ml

3.2.0. MOISTURE CONTENT OF SAMARU 71 COTTONSEED SAMPLE

One gram portions of finely ground cottonseed samples were placed into three pre-weighed, dry sample bottles. The bottles were placed in an oven at 55°C and dried to constant weight. The loss in weight of the sample was assumed to be equal to the moisture content. The percentage moisture content of the sample was calculated from the average of triplicate results.

3.2.1. EXTRACTION OF COTTONSEED OIL

Into a preweighed, clean, dry round-bottomed flask containing some antibumping granules was placed 150 ml of hexane : acetone 2:1 (v/v) solution. The soxhlet extractor unit was then connected. Ten grams of cottonseed flour was placed in dried, pre-weighed extraction thimbles which were then placed in the soxhlet extractor unit. Cold water was circulated through the condenser and the heating mantles were switched on. The extraction was carried out for six hours.

The thimbles, after cooling, were dried and weighed. Also, the flasks were removed from the soxhlet unit and the solvent was evaporated using a rotary evaporator. The flask and its contents were then dried to constant weight. Triplicate analysis were carried out for the extraction. The extractable lipid content of the sample was determined as shown below.

$$\text{Percentage lipid } (\%) = \frac{\text{weight of lipid extracted}}{\text{weight of dried cottonseed sample}} \times 100$$

The weight of lipid extracted is given by :

[1] loss of weight of thimble contents after extraction, and

[2] weight of lipid in the flask after the solvent has been removed.

3.2.2. DETERMINATION OF SAPONIFICATION NUMBER OF SAMPLES 1 AND 2

One gram each of samples 1 and 2 were placed in 25 ml quickfit flasks with antibumping granules. 20 ml of standard alcoholic KOH was added to each flask . The condensers were attached, and the flasks heated gently over a water bath. Refluxing was carried out for one hour. After cooling, 1 ml of alcoholic phenolpthalein was added to each flask. The contents were then titrated with 0.5N HCl. Triplicate analysis were carried out for each sample. The saponification number was calculated using :

Normality of HCl x Volume (ml) of HCl equivalent to the alcoholic KOH used to saponify the lipid x 56.

IODINE VALUE [DAM's METHOD]

3.2.3. DAM's REAGENT

Approximately 41 ml of pyridine was dissolved in 30 ml of concentrated H_2SO_4 in 100 ml of cold glacial acetic acid. To this was added 13 ml bromine in 100 ml glacial acetic acid. Before use, 2.84 ml of this preparation is dissolved in 50 ml. glacial acetic acid.

3.2.4. REAGENT BLANK DETERMINATION

Into a conical flask containing 5 ml of chloroform was added 5 ml of Dam's reagent from a burette. After 15 minutes, 5 ml of 10 per cent (W/v) KI solution and 20 ml distilled water were added with swirling. This solution was then titrated with standard sodium thiosulphate with constant shaking to assure thorough mixing of the two immiscible layers. The end point was attained when both layers were colourless.

3.2.5. IODINE EQUIVALENT OF SAMPLES 1 AND 2

5g each of samples 1 and 2 (dried) were dissolved in 75 ml portions of chloroform. 5ml of these were pipetted into conical flasks and 5 ml portions of Dam's reagent were added from a burette. After 15 minutes, 5 ml of 10 per cent W/v KI solution and 20ml of water were added and titrated with standard sodium thiosulphate. Triplicate analysis were carried out. The iodine equivalent taken up by the lipid was calculated using the relationship :

1 ml of standard 0.025N sodium thiosulphate is equivalent to 0.00377g Iodine.

3.2.6. (a) ESTIMATION OF THE GOSSYPOL CONTENTS OF SAMPLES 1 AND 2 (Akanya, 1976)

PREPARATION OF STANDARD GOSSYPOL SOLUTION

20 mg of gossypol powder was dissolved in absolute ethanol and made up to volume in a 100 ml volumetric flasks.

(b) PREPARATION OF FERRIC CHLORIDE BIPYRIDYL REAGENT

0.5g of ferric chloride and 0.5g of 2,2'-bipyridyl were dissolved in ethanol and made up to volume in a 100 ml volumetric flask.

3.2.7. PREPARATION OF CALIBRATION CURVE

1 ml portions of the ferric bipyridyl reagent were pipetted into a series 25ml columetric flasks containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml portions of the standard gossypol. The flasks were made up to volume with absolute ethanol and kept for 15 minutes for the colour to develop. The absorbance of each solution was measured against a reagent blank at 510nm using a Beckman spectrophotometer. Calibration was carried out three times.

3.2.8. DETERMINATION OF FREE GOSSYPOL CONTENTS OF SAMPLES 1 AND 2

25 mg of the samples, 100 mg of anhydrous Na_2SO_4 and 5ml of 40-60° petroleum ether were mixed and centrifuged for 10 minutes

at speed 5 in an MSE bench centrifuge. The supernatant was discarded and the extraction with petroleum ether repeated twice. The extracts were then evaporated to dryness over a water bath. The resulting residues were dissolved in ethanol, transferred to 25 ml volumetric flasks and made up to volume with absolute ethanol. 2 ml of these extracts and 1 ml portions of ferric chloride bipyridyl reagent were pipetted into 25 ml volumetric flasks and made up to volume with absolute ethanol. After 15 minutes, the absorbance at 510 nm was measured. Three determinations were carried out for each of samples 1 and 2 to correct for any variations in colour yield of the reagent.

3.2.9. HALPHEN TEST

To 30 Ml of CS_2 containing 1 per cent S in solution was added 30 ml amylalcohol. 5 ml of this reagent was mixed with 5 ml each of samples 1 and 2 and heated in a bath of boiling saturated NaCl for 1 hour. The colours formed were recorded.

3.3.0. PREPARATION OF TEST COMPOUNDS/SAMPLES

To 5ml of the n-hexane : acetone extract was added 10 ml of DMSO to give a concentration of 33 per cent drug.

To 4 ml of FCCC oil was added 4 ml DMSO to give a concentration of 50 per cent drug solution.

To 10mls of DMSO was added 577 mg of gossypol acetic acid powder, giving a concentration of 100mM gossypol. To 10mls DMSO was added 138.14mg of sahcylhydroxamic acid(SHAM), giving a concentration of 100mM SHAM.

The drug solutions were stored in the dark at 4°C and used within periods of 30 days.

3.3.1. INOCULATION OF ANIMALS

Stabilates were used to inoculate both mice and rats using 24 gauge needles. About 0.25 ml containing 10^5 parasites were injected into mice and the parasitemia followed by monitoring parasites in tail blood from wet mounts (X40 objective).

3.3.2. HARVESTING OF TRYPANOSOMES FROM BLOOD

Following the method of Baltz et al., (1985) 4 drops of infected blood were added to 4ml of saline and centrifuged for 5 minutes at 300g. The supernatant was decanted and used.

The method of Lanham and Godfrey (1970) was also used. Pre-swollen DEAE-Cellulose (DE 52) was poured into a funnel containing a filter paper [Whatman number 4]. The exchanger was equilibrated with PSG, pH 8.0. Blood was aspirated into a heparin-rinsed syringe by cardiac puncutre of ether-anaesthesized mice. This was added to 3ml of PSG in a test tube and then placed on the exchanger in funnel and eluted with PSG buffer. Trypanosomes were collected in test tubes bathed in ice.

Lastly, 10 drops of infected blood was added to 5 ml of culture medium and centrifuged at 200g for 10 minutes. The supernatant was decanted and used.

3.3.3. PREPARATION OF CULTURE PLATES

200 µl. of culture media containing 10 per cent serum was placed into the wells of the plate and then incubated at 37°C in a dessicator placed in an incubator. Carbon dioxide was generated in the dessicator with a candle flame. After 24 hours, trypanosomes were added (10^4-10^5) to the plates and returned to the incubator. Periodic observation of cultures were done using both the wet mount and an inverted microscope.

3.3.4. PREPARATION OF PLATES CONTAINING DRUGS

Drug solutions were first diluted into serum containing culture media to obtain particular drug concentrations. Different volumes of this were then placed into the wells of the plate and then made up to the same final volume. Trypanosomes were then added in uniform volumes so that all the wells contain the same total volume having same number

of trypanosomes but different drug concentrations. Drug tests were always carried out in duplicate. Plates containing drugs were first preincubated for 15 hours before trypanosomes are added. Example of a plate for drug test is tabulated and presented in Appendix

CHAPTER FOUR

RESULTS

4.1. MOISTURE CONTENT OF SAMARU 71 COTTONSEEDS

Triplicate analysis revealed a moisture content of 4.83 ± 0.03 per cent.

4.2. EXTRACTABLE LIPID CONTENT

A period of 6 hours was required to extract about 90 per cent of extractable lipid. The extractable lipid content obtained from loss in weight of extraction thimbles and also from the lipid collected in the flask after solvent evaporation were identical. This indicates absence of solvent insoluble residues in the extracted sample (table 4.1).

4.3. IODINE VALUES

The iodine value of 79.3 obtained for sample 2 was higher than for sample 1, 78.4. This indicates similar unsaturation in sample 2.

4.4 SAPONIFICATION NUMBERS

Higher values were obtained for sample 1 indicating the presence of lower molecular weight lipids than in sample 1.

4.5 GOSSYPOL CONTENT

Whereas sample 1 was free of gossypol, sample 2 had an appreciable amount (0.384mg/100mg of sample) (appendix 1).

ANALYSIS OF SAMPLES 1 AND 2

	SAMPLE 1	SAMPLE 2
Moisture content (% $w_{/w}$)	-	4.83 ± 0.03
Per cent extractable lipid after 6 hours in hexane: acetone (2:1 $v_{/v}$)	-	24.00 ± 0.03
Visual character of sample	light yellow	Reddish-brown
Saponification number	226.8 ± 0.2	277.3 ± 0.1
Iodine values	78.4 ± 0.2	79.3 ± 0.3
Gossypol content (mg) per 100mg of sample	0.000	0.384 ± 0.01
Halphen test	Light orange	Red

Values are average of triplicate analysis, ±SD

Table 4.1

4.6 HALPHEN TEST

The intensity of colour was deeper for sample 2 indicating the presence of higher amounts of cyclopropenoid fatty acids.

4.7. CULTURE METHODS

Minimum essential medium (Eagles 199) and coconut fluid were tested to find the optimum conditions for growth and/or maintenance. Eagles 199 was supplemented with N,2-hydroxyethylpiperazine N2'-ethanesulphonic acid (HEPES), sodium pyruvate, amino acids and 2-mercaptoethanol. The coconut fluid was supplemented with serum only (figures 4.11 to 4.15) A temperature of 37°C was used for all incubations in the presence of CO_2. All media had a pH of 7.4.

The use of HEPES resulted in stabilisation of pH for more than 30 days. Eagles 199 supported the growth and maintenance of both T brucei and T congolense in the presence of both rabbit and horse serum. Over a 24 hour period, little oscillations in population were produced by horse serum, unlike rabbit serum(figures 4.11 and 4.12). From 24 to 48 hours, however, both sera maintained a constant population. Culture media were improved by the addition of L-cysteine periodically (figure 4.13). A somewhat constant population could be obtained for 70 hours by subculturing every 24 hours.

Coconut fluid media (figures 4.14 and 4.15) was not as effective as the Eagles 199 in the presence of both horse and rabbit serum.

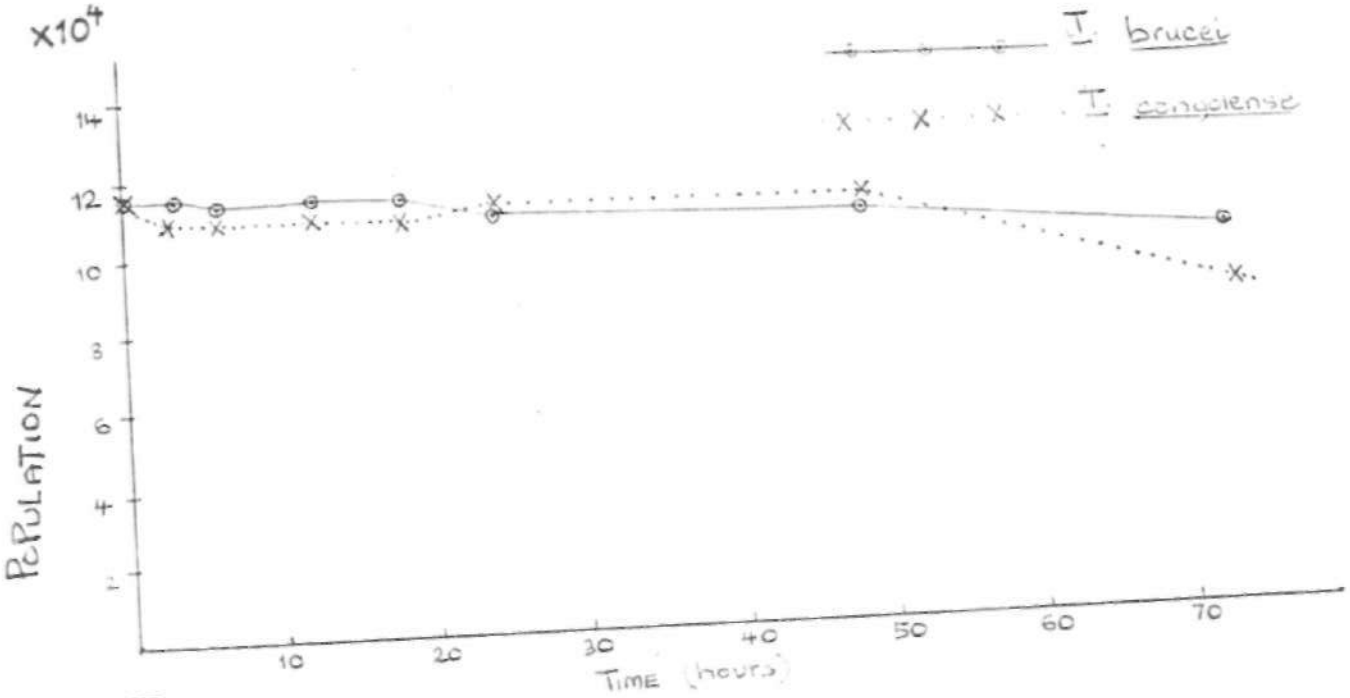


FIGURE 4.1.1
GROWTH CURVE OF *T. brucei* AND *T. congolense* IN MEM 199 SUPPLEMENTED WITH 10 % (v/v) HORSE SERUM

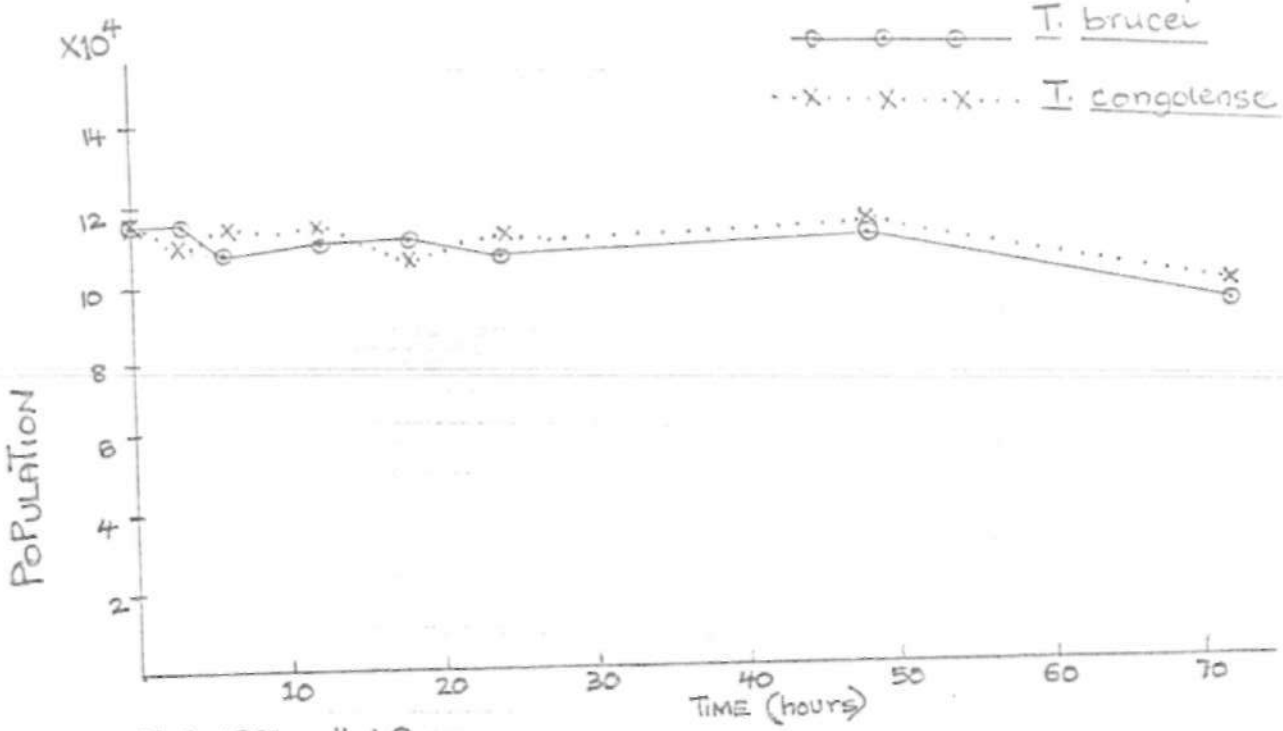


FIGURE 4.1.2.
GROWTH CURVE OF *T. brucei* AND *T. congolense* IN MEM 199 SUPPLEMENTED WITH 10 % (V/V) RABBIT SERUM

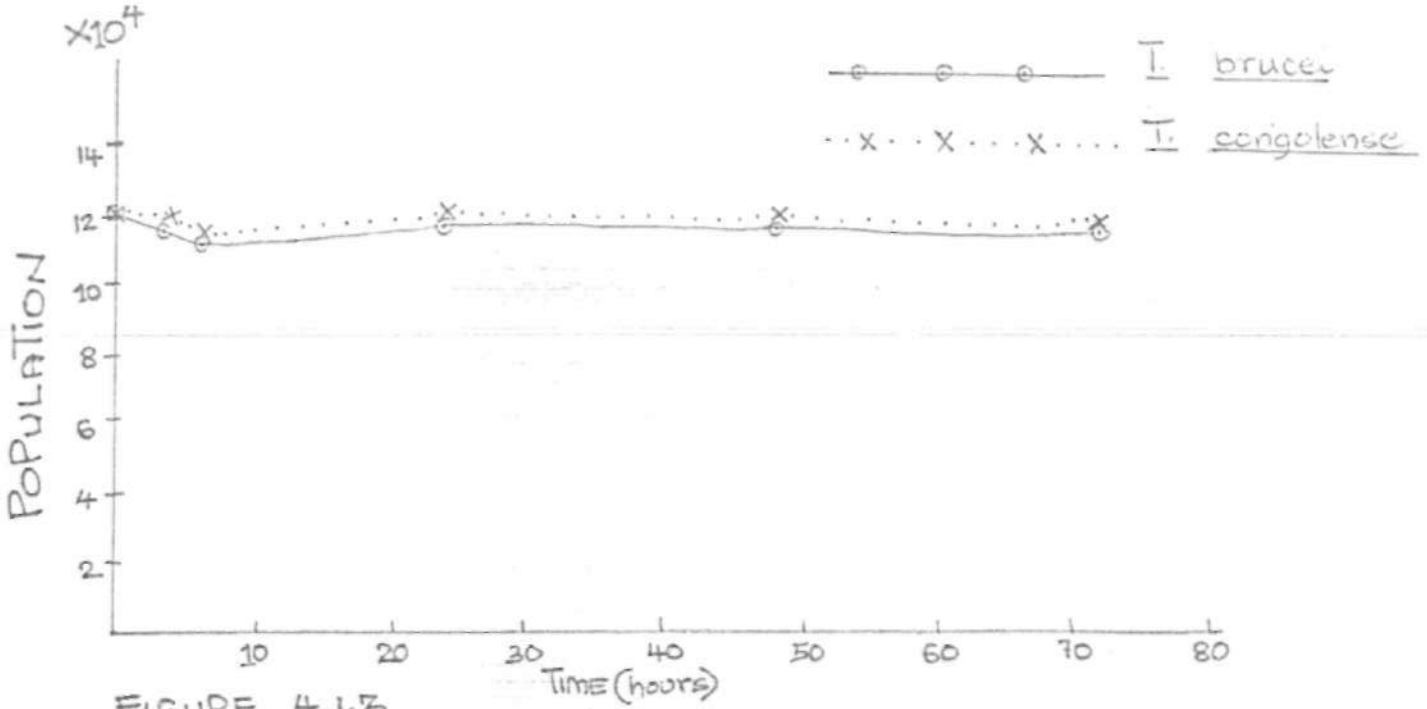


FIGURE 4.1.3.
GROWTH CURVE OF T. *brucei* AND T. *congolense* IN MEM 199 SUPPLEMENTED WITH 10% (V/V) HORSE SERUM AND 1mM L-CYSTEINE.

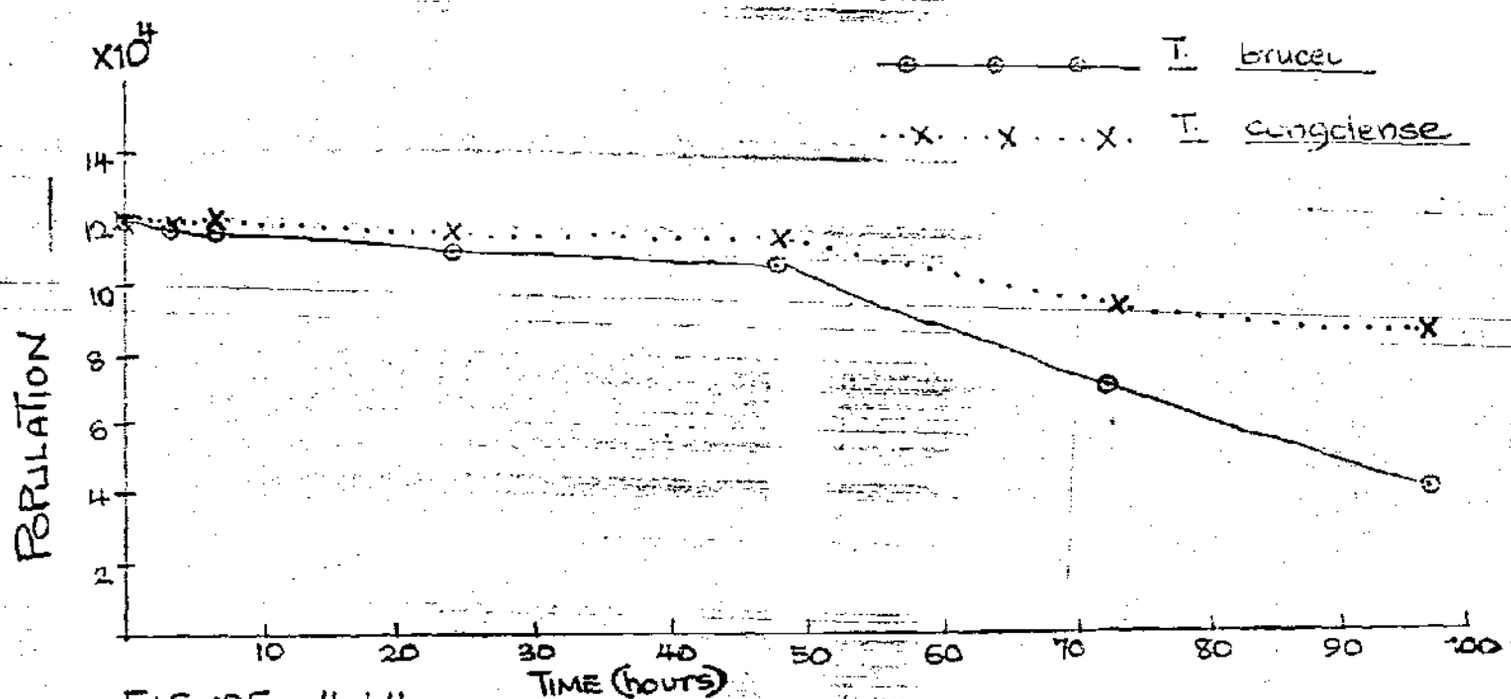


FIGURE 4.1-4.
GROWTH CURVE OF *T. brucei* AND *T. congolense* IN COCONUT FLUID MEDIUM, pH 7.4, SUPPLEMENTED WITH 2mM PYRUVATE AND 2% (V/V) RABBIT SERUM.

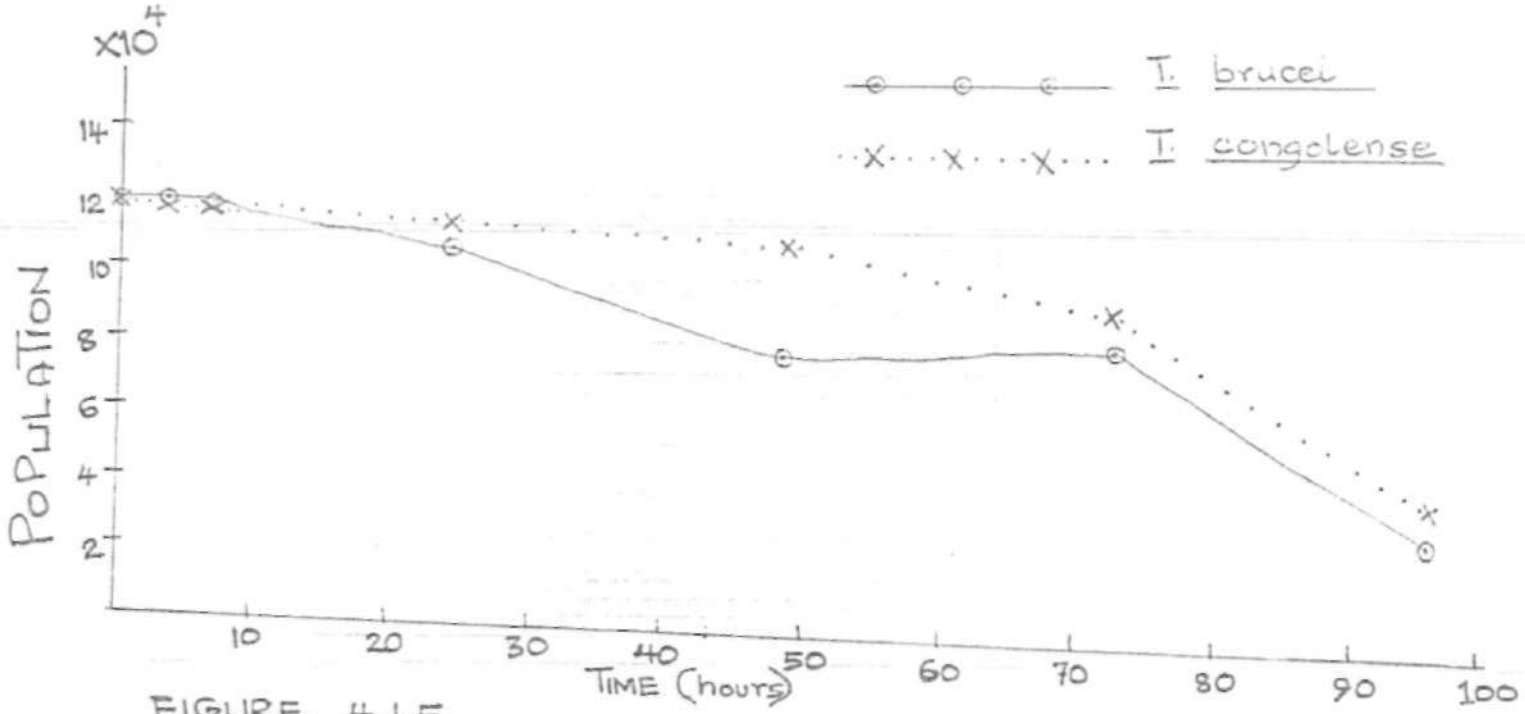


FIGURE 4.1.5.
GROWTH CURVE OF *T. brucei* AND *T. congolense* IN COCONUT FLUID MEDIUM, PH 7.4, SUPPLEMENTED WITH 2mM PYRUVATE AND 2% (V/V) HORSE SERUM.

Rabbit serum could maintain trypanosome populations only for up 30 hours.

4.8. EFFECTS OF SAMPLES 1 AND 2, GOSSYPOL AND SALICYLHYDROXAMIC ACID [SHAM] ON TRYPANOSOMES

Trypanosoma brucei and T. congolense (6.95 x 10^4 cells/well) were incubated with various concentrations of samples 1 and 2, gossypol and SHAM, all dissolved in DMSO (figures 4.16 to 4.21). The ED_{50} values are defined as the concentration of the compound that reduced trypanosome populations by 50 per cent after 1 and 3 hours. Trypanosome viability was assessed by counting non motile parasite in a Neubauer haemocytometer. The effects of samples 1 and 2 are similar for both T. brucei and T. congolense as shown by their ED_{50} values (tables 4.2 and 4.3). Whereas, T. brucei had ED_{50} of 0.79 and 0.72 T. congolense had 0.80 and 0.71 % for 1 and 3 hours, in both. The lowest concentrations used were 0.166%. This concentration elicited trypanocidal effects on more than 10 per cent of trypanosomes. To gain insight into the mechanism of action of samples 1 and 2, the effect of gossypol on the parasites was conducted using the same protocol. Trypanocidal action was indicated for both T. brucei and T. congolense (figure 4.18). More than 60 per cent of trypanosomes were killed by concentrations of about 145 µM gossypol. The ED_{50} values obtained were 82 µM and 98 µM for T. brucei and T. congolense, respectively.

Salicylhydroxamic acid (SHAM) was also found to be trypanocidal (figure 4.19). ED_{50} values of 222 µM and 224 µM were obtained for T. brucei and T. congolense, respectively.

The highest concentration of DMSO used (1.166 %), had no appreciable effect for the time duration of sensitivity tests.

ED_{50} Values of T. brucei at various times

SAMPLE	CONCENTRATION	INCUBATION TIME
Sample 1	0.79 ± 0.13 (%)	1 hour
Sample 2	0.63 ± 0.03(%)	1 hour
Gossypol acetic acid	82 ± 1.13(μM)	1 hour
Salicylhydroxamic acid	222 ± 1.21(μM)	1 hour
Sample 1	0.76 ± 0.05(%)	3 hours
Sample 2	0.56 ± 0.04(%)	3 hours

Results are means of triplicate analysis, ±SD

Table 4.2

ED_{50} Values for T. congolense at various times

SAMPLE	CONCENTRATION	INCUBATION TIME
Sample 1	0.80 ± 0.31	1 hour
Sample 2	0.65 ± 0.11	1 hour
Gossypol acetic acid	98 ± 1.05	1 hour
Salicyl hydroxamic acid	224 ± 1.07	1 hour
Sample 1	0.71 ± 0.16	3 hours
Sample 2	0.59 ± 0.13	3 hours

Results are means of triplicate analysis, ± SD.

Table 4.3

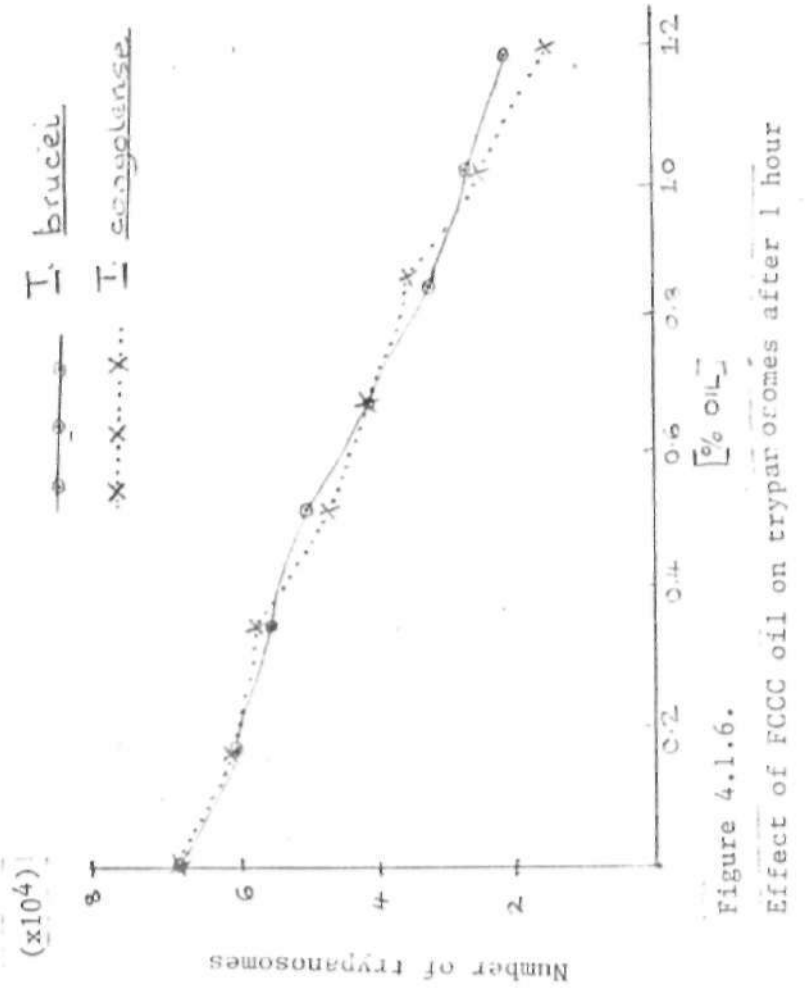


Figure 4.1.6.

Effect of FCCC oil on trypanosomes after 1 hour

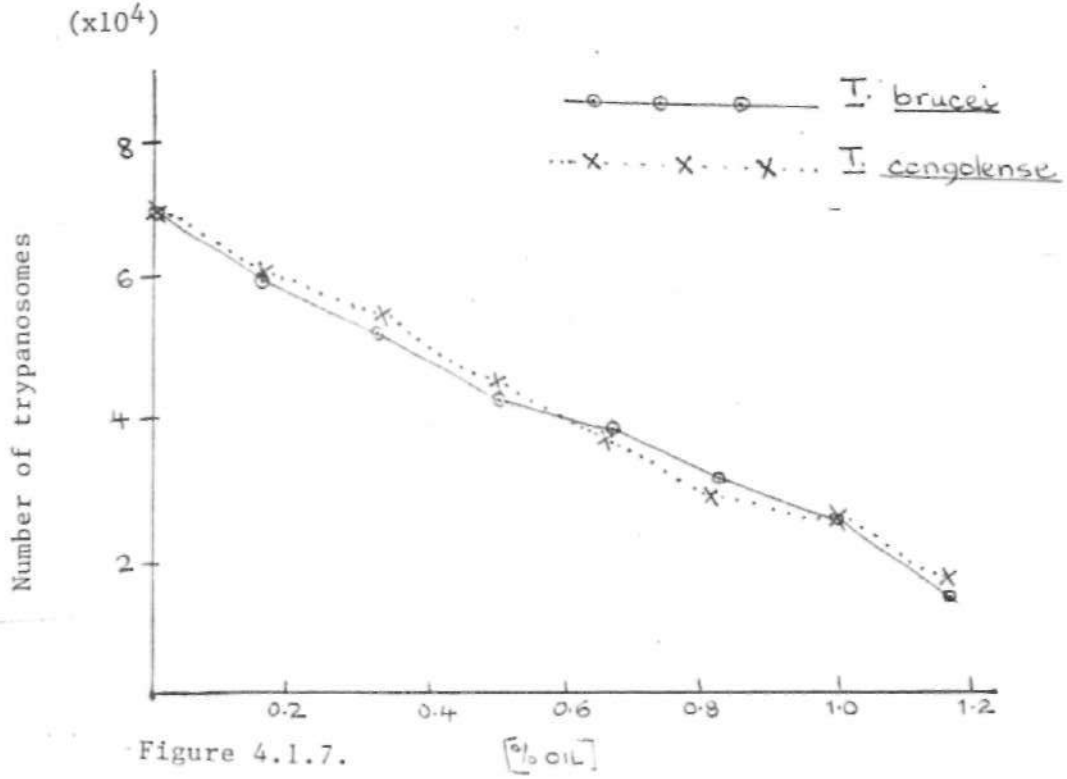


Figure 4.1.7.

Effect of FCCC oil on trypanosomes after 3 hours

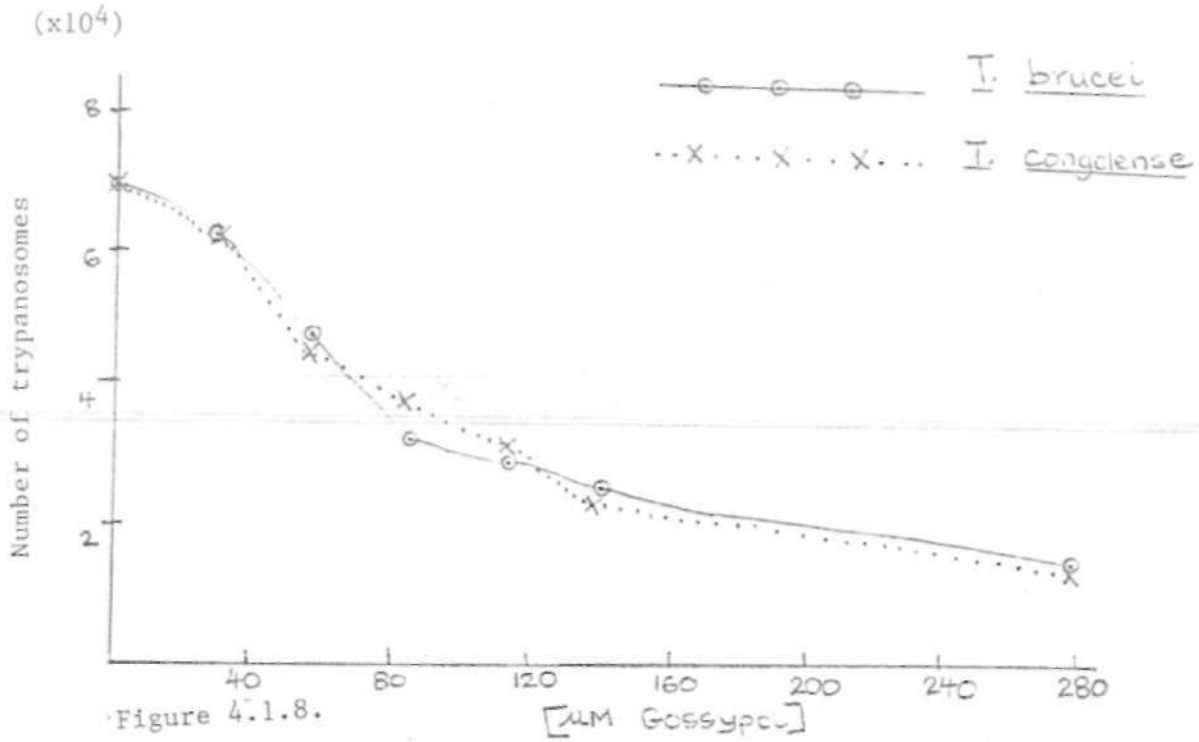


Figure 4.1.8.

Effect of gossypol on trypanosomes after 1 hour

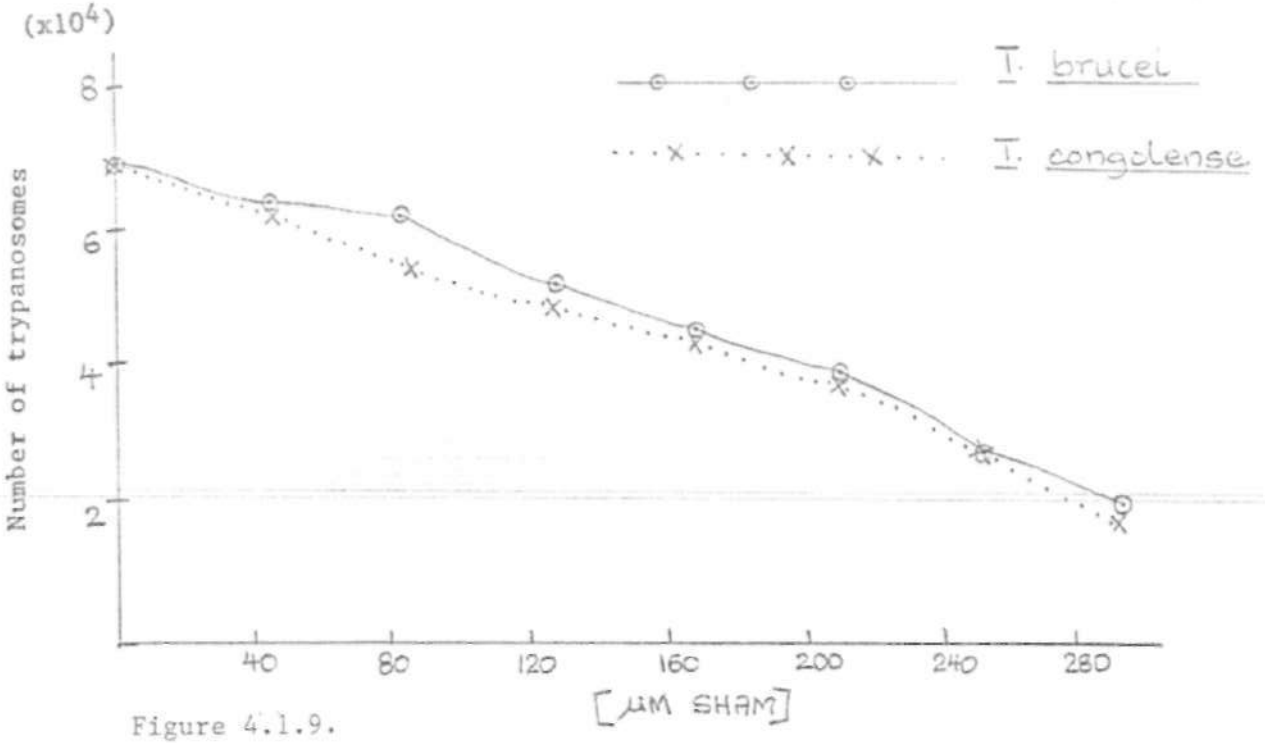


Figure 4.1.9.

Effect of SHAM on trypanosomes after 1 hour

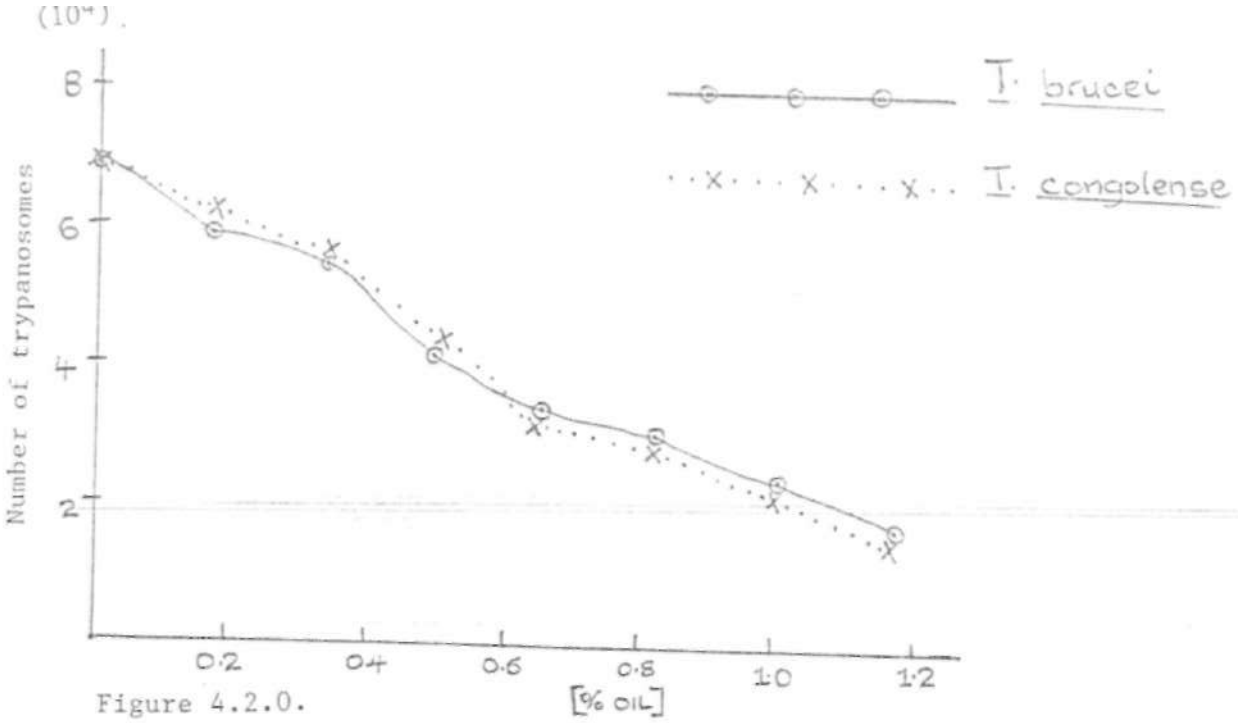


Figure 4.2.0.

Effect of Hexane : acetone extract on trypanosomes after 1 hour

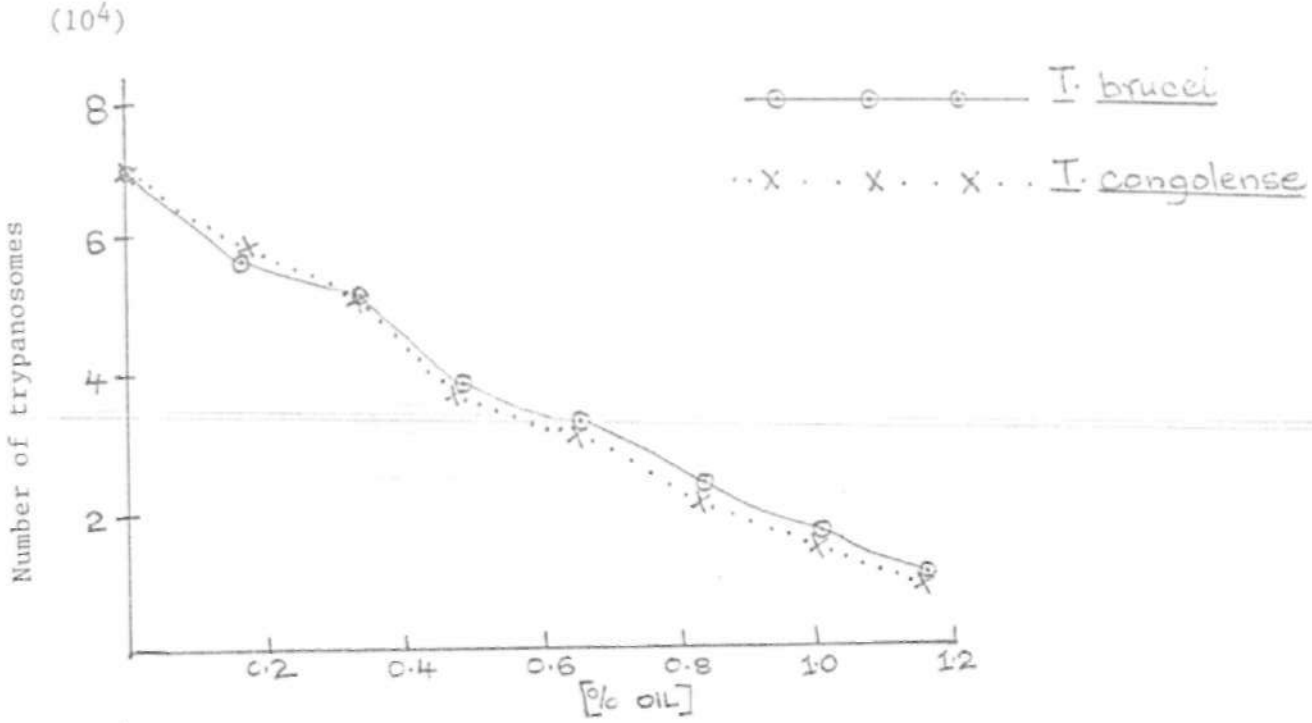


Figure 4.2.1.

Effect of Hexane : acetone extract on trypanosomes after 3 hour

CHAPTER FIVE

5.0.0 DISCUSSION AND CONCLUSIONS

A time duration of 6 hours was used to extract oil from the SAMARU 71 variety of cottonseeds. The oil extracted corresponds to about 90 per cent of extractable lipid. The obtainment of similar values of extractable lipid from the loss in weight of extraction thimbles and the weight of lipid extracted in the flaks after solvent evaporation indicates the absence of solvent insoluble residues like carbohydrates and proteins.

The iodine value of a lipid indicates degree of unsaturation. Sample 2 had an iodine value of 79.3 compared to 78.4 for sample 1, indicating similar degree of unsaturation in the former. Furthermore, sample 2 indicated more cyclopropenoid fatty acids than sample 1; this was confirmed by colour intensities from the Halphen test. The higher unsaturation of sample 2, may be due to the presence of more cyclopropenoid fatty acids (CPFAs).
However, sample 1 had a slightly higher molecular weight lipids than sample 2, as shown (Table 4.1) by the saponification values. This is expected as the presence of unsaturation reduces the molecular weight of a compound.

The iodine and saponification values reported in this study fall outside the range established by Bailey (1948). However, Bailey (1948) himself agrees that climatic conditions and locality variations do influence these values. We will also note that, in

addition to these two, the method of extraction and the duration of extraction can influence both values. (Akanya, 1976).

The gossypol content of oils reflects the colour of an oil. Sample 1, with no detectable gossypol was bright yellow, while sample 2, having appreciable amounts of gossypol (0.304mg per 100mg sample) was orange-red in colour. The gossypol content however falls outside the range (0.74-0.88 per cent) established by the Tropical Product Institute (1962) Akanya (1976) ; having obtained a lower gossypol content also, attributed this to probably lower gossypol content of the cottonseed variety and to the purity of the gossypol used for the estimation (about 89.6 per cent pure). The Samaru 71 variety used in this study was also used by Akanya (1976) However, the gossypol standard used here is slightly purer, about 92.3 per cent.

The use of culture systems has great importance in the screening of compounds for therapeutic effects and other effects as well (Brun and Jenni, 1985). The media used in this study have been reported in use and include Eagles 199 (Hirumi and Hirumi 1977; Brun et al., 1984; Baltz et al., 1985) and coconut fluid (Marquez et al., 1987) Eagles 199 was prepared and supplemented with pyruvate and 2-mercaptoethanol according to Baltz et al., (1985); serum was supplemented as in the methods of Brun and Kunz (1989) and Brun et al., (1989). Trypanosome cultures were carried out with Eagles 199 containing either horse or rabbit serum, and the presence or absence of L-cysteine(Duszenko et al., 1985)

Figures 4.11 and 4.12 indicates that trypanosomes could be maintained in culture using the two sera. The maintenance capability of the media was improved (figure 4.13) by the addition of freshly prepared 1mM L-cysteine. Trypanosome populations could be maintained for periods of about 30 hours with L-cysteine. L-cysteine enhances growth in trypanosome cultures and eliminate feeder cell layer requirements (Hirumi and Hirumi, 1989 and 1990) in both T. brucei and T. congolense. Earlier, Deszenko et al., (1985) demonstrated that L-cysteine is an essential factor for the cultivation of bloodstream forms of T. brucei, axenically; L-cysteine was added freshly-prepared to minimize toxic effects of hydrogen peroxide produced by the autooxidation of L-cysteine. Hirumi and Hirumi (1989 and 1990) used bathocuproine sulphonate to simplify the addition of L-cysteine. This chelating agent had been used earlier by Tegoshi (1988) and reported to be effective. In this study, L-cysteine proved to be an essential supplement; however, the absence of a chelating agent produced no dramatic effects.

The coconut fluid used indicates some potentiality as a culture medium. Marquez et al., (1987) reported excellent results of culture that were comparable with those of Eagles 199. However, unlike the present study, they cultivated some Leshmania and trypanosomes species T. rangeli and T. cruzi). While this study agrees with the 2 per cent serum used by Marquez et al., (1987), the pH of 6.2 used by them could not support the maintenance of the trypanosome species used in this work.

The trypanosomes maintained in culture in this study retained the morphological characteristics of long slender bloodstream forms seen in infected mouse blood. They gave rise to infections in mice which were innoculated intraperitoneally with them. Parasitemia was detected in tail blood after 4 days of inoculation.

This study shows that both T. brucei and T. congolense are susceptible to all the compounds tested (figures 4.16 to 4.21). The trypanosomes became swollen and then die off, leaving cell debris. Sample 1 possess similar ED_{50} values in both T. brucei and T. congolense possibly due to a similar mode of action. This sample, being free of gossypol, contains small amounts of CPFAs (table 4.2). The effect produced on the trypanosomes, therefore, may be due to the CPFAs present, and possibly some unsaturated fatty acids (table 2.5.1). CPFAs are unusual fatty acids to trypanosomes as these have not been reported as part of their lipids (Venkatesan and Omerod, 1976). The toxic effects of sample 1 may be enhanced by both detergent-like properties and the cyclopropene moiety of the CPFAs. Their uptake into trypanosomes may be enhanced, in addition to presence of DMSO, by some phospholipids and myristate present in cottonseed oils (tables 2.5.1 - 2.5.4). These have been shown to act as liposomes and transport other molecules across trypanosome membranes (Mellors and Samad, 1989) that will eventually reach their targets and cause cell lysis (Ukoha, 1989 ; Hiroshi et al., 1988). Lysis of cell may be due to fusing with membranes and formation of peroxides (Godfrey, 1958; Taylor, 1958; Ukoha, 1989).

Sample 2 possessed higher toxic capabilities than sample 1 indicating presence of other factors that are absent in sample 1. Also, similar ED_{50} values of sample 2 on both parasites indicate that the additional toxic factor may be exerting an effect on a target common to the parasites. Table 4.1 shows that gossypol and more CPFAs abound in sample 2. This may account for the lower ED_{50} values of sample 2. Gossypol is trypanocidal (Josiane et al., 1988) to trypanosomes. It blocks NAD-and NADP-dependent enzymes and interferes with glycolysis (Lee and Malling, 1981), upon which trypanosomes obtain their energy (Bowman and Flynn 1976). Furthermore as the presence of these two produced lower ED_{50} values, this indicates the presence of, probably, a synergistic effect.

It is noteworthy, however, that the T. congolense used in this study observed a higher ED_{50} value for gossypol (98 μM) as compared to 82 μM for T. brucei, salicylhydroxamic acid (SHAM) possessed very high ED_{50} values of 222 mM and 224 μM for T. brucei and T. congolense, respectively. SHAM is an inhibitor of L-α-glycerophosphate oxidase (Michels, 1988) that blocks oxygen consumption of washed cell suspensions in trypanosomes. SHAM, however, has been reported as not trypanocidal (Gutteridge and Coombs, 1977).

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APPENDIX A

1. PHOSPHATE BUFFERED SALINE [PBS]

Na_2HPO_4	13.48g
NaH_2PO_4	0.78g
Nacl	4.25g
Distilled Water	1000 ml

The pH is set at 8.0

2. PHOSPHATE BUFFERED SALINE GLUCOSE [PBSG]

PBS	600 ml
Distilled Water	400 ml
Glucose	10 g

The pH is set at 8.0

3. AMINO ACID MIXTURE

Serine	10 mg
Cysteine	"
Proline	"
Cystine	"
Tyrosine	"
Glutamic acid	"
Aspartic acid	"
Alanine	"
Leucine	"
Distilled Water	30 ml

APPENDIX B

1. TRYPANOSOMES COUNTING FLUID

KI	3.2 g
NaCL	1.0 g
Na_2SO_4	5.0 g
I_2	0.29g
Distilled Water	200ml

The solution is filtered before every use.

2. SALINE [Baltz et al., 1985]

Na_2HPO_4	0.568 g
NaH_2PO_4	0.174 g
NaCL	42.5 g
Glucose	5 g
Distilled H_2O	500ml

The pH is set at 7.4 and stored at 4°C.

3. PHYSIOLOGICAL SALINE

NaCL	0.9g
Distilled Water	100ml

4. GIEMSA STAIN

Stock stain was prepared by dissolving o.8g Gimsa stain in 100 mls of a mixture of equal volumes of glycerol and methanol. This was shaken for 48 hours on a mechanical shaker. To 2.5 ml. of the stock solution was added 100 ml of deionized water and shaken for 15 minutes.

APPENDIX C

SHAM TEST ON TRYPANOSOMES

STOCK SOLUTION --- 100mM

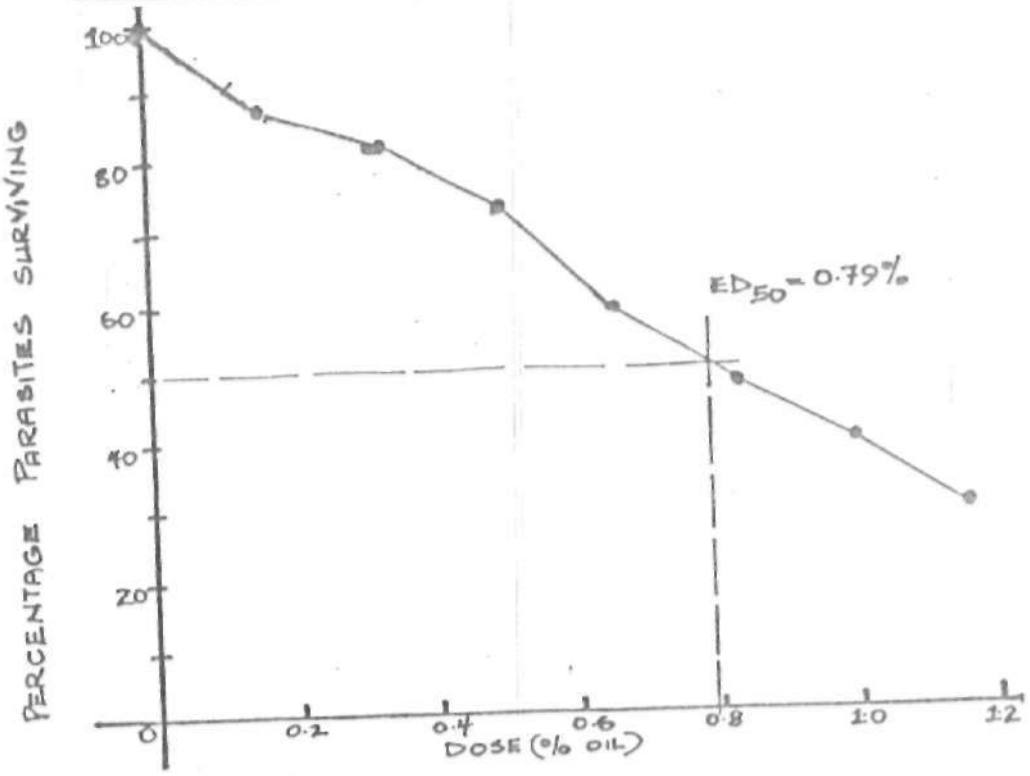
5uL of Stock Solution + 195ml media = 200ml

dilution factor = 40

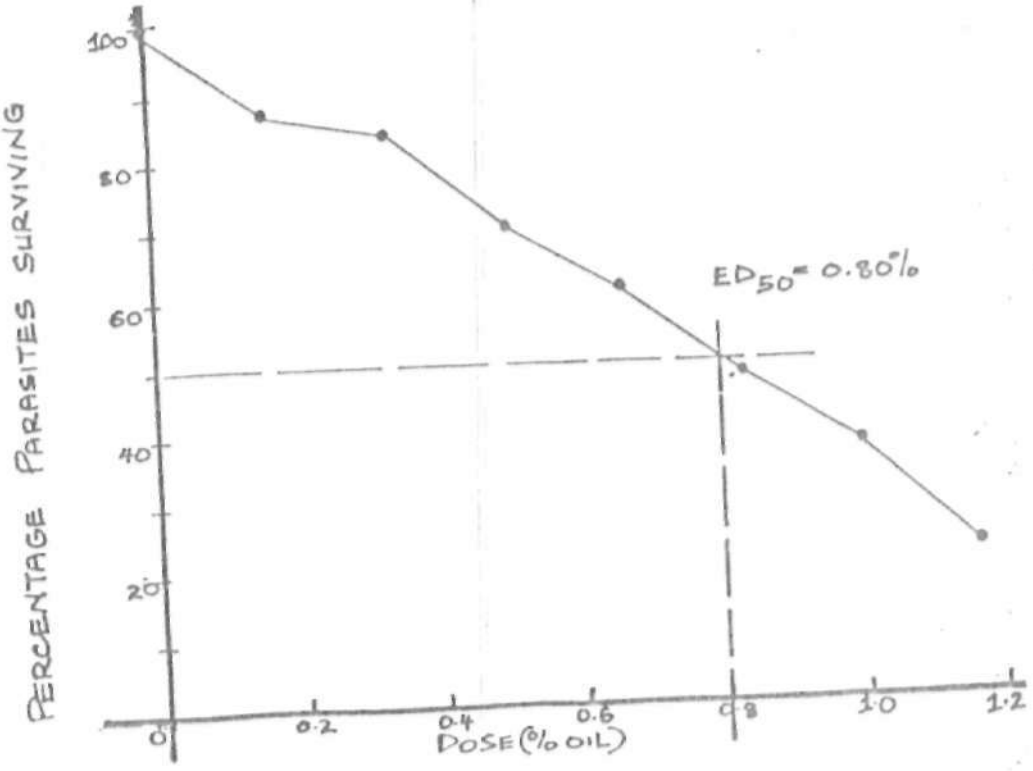
Drug concentration becomes = $\frac{100}{40}$ = 2.5mM

= 2500uM

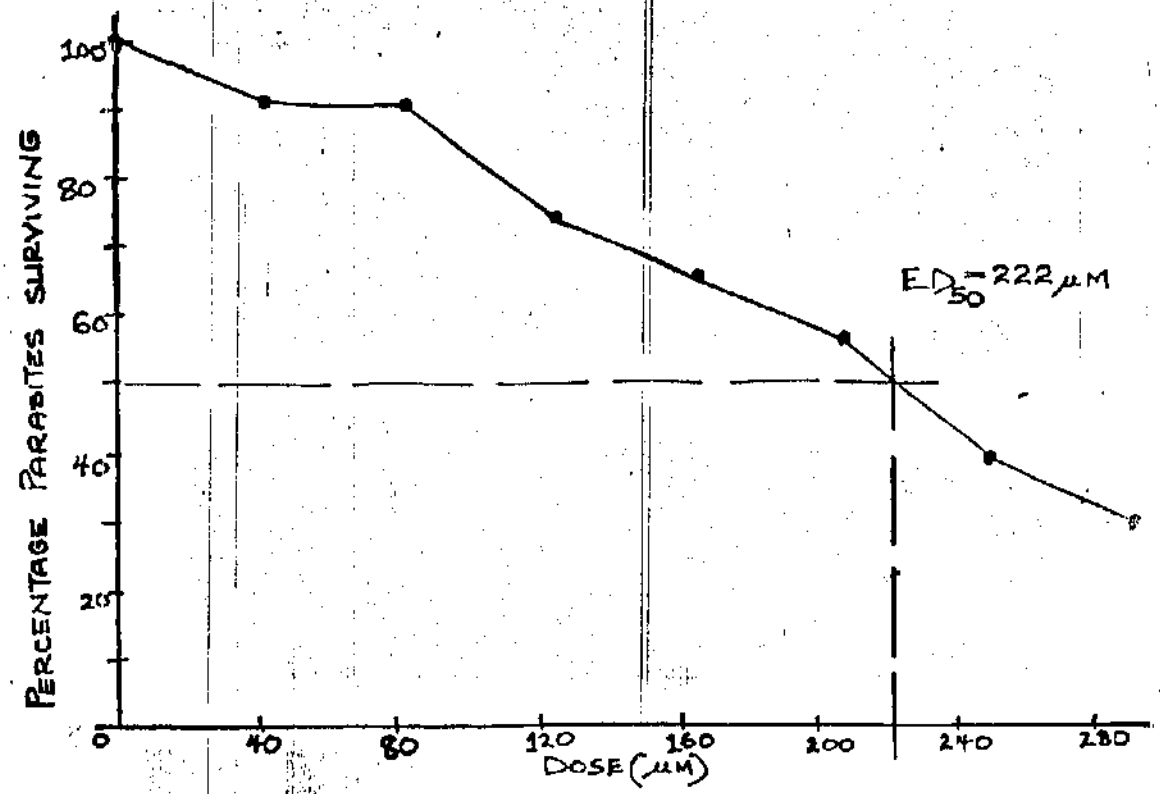
WELLS	1	2	3	4	5	6	7	8	9	10
DRUG (ul)	0	5	10	15	20	25	30	35	40	45
ADDED MEDIA (ul)	100	95	90	85	80	75	70	65	60	55
TRYPS. IN MEDIA (ul)	200	200	200	200	200	200	200	200	200	200
TOTAL VOLUME (ul.)	300	300	300	300	300	300	300	300	300	300
DILUTION FACTOR (x)	-	60	30	20	15	12	10	8.57	7.5	6.66
DRUG CONCENTRATION = $\frac{2500}{X}$ uM	-	41.66	83.33	125	166.66	208.33	250	291.715	333.33	375.03
NUMBER OF TRYPS. AFTER ONE HOUR	-	-	-	-	-	-	-	-	-	-



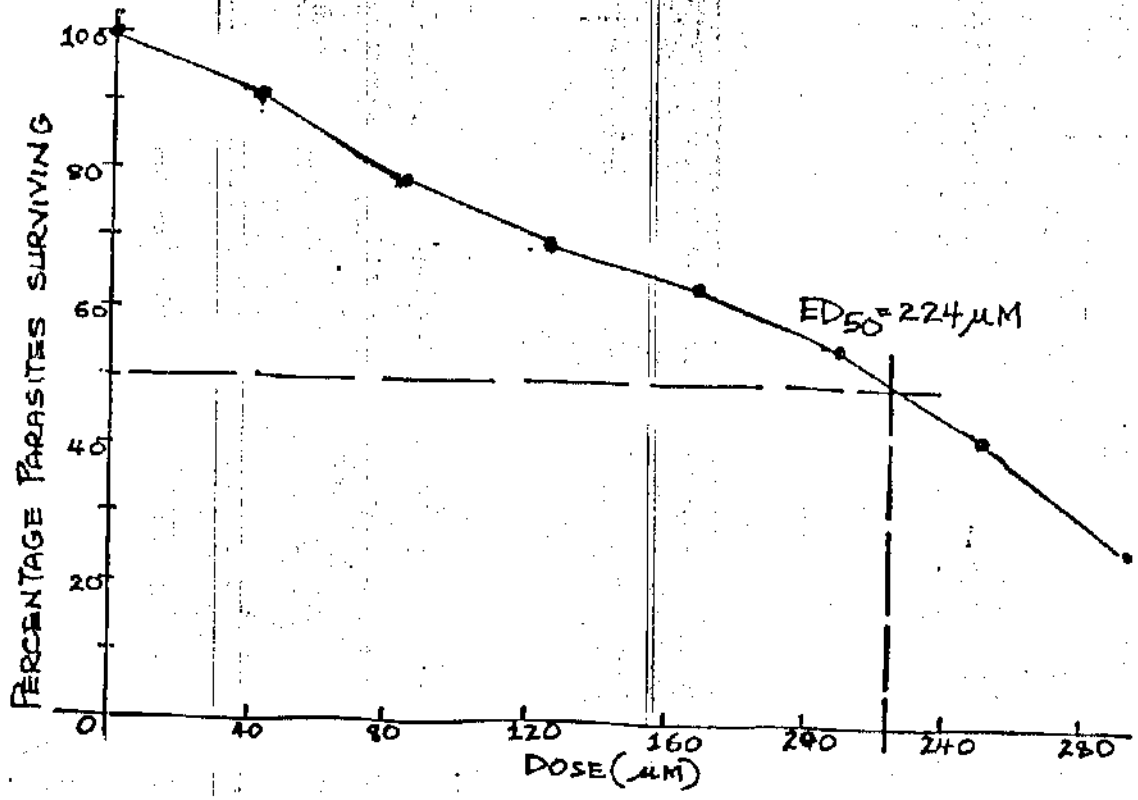
EFFECT OF FCCC OIL ON TRYPANOSOMA BRUCEI : EVALUATION OF ED_{50} (1 HOUR).



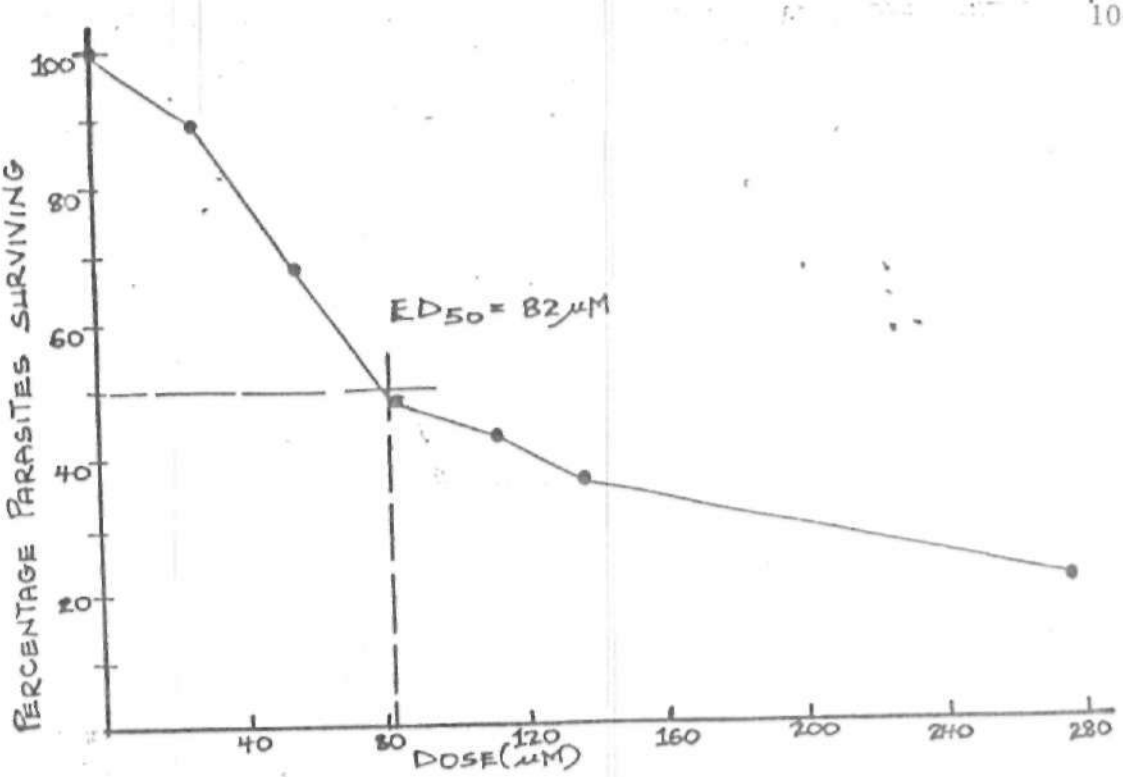
EFFECT OF FCCC OIL ON TRYPANOSOMA CONGOLENSE : EVALUATION OF ED_{50} (1 HOUR)



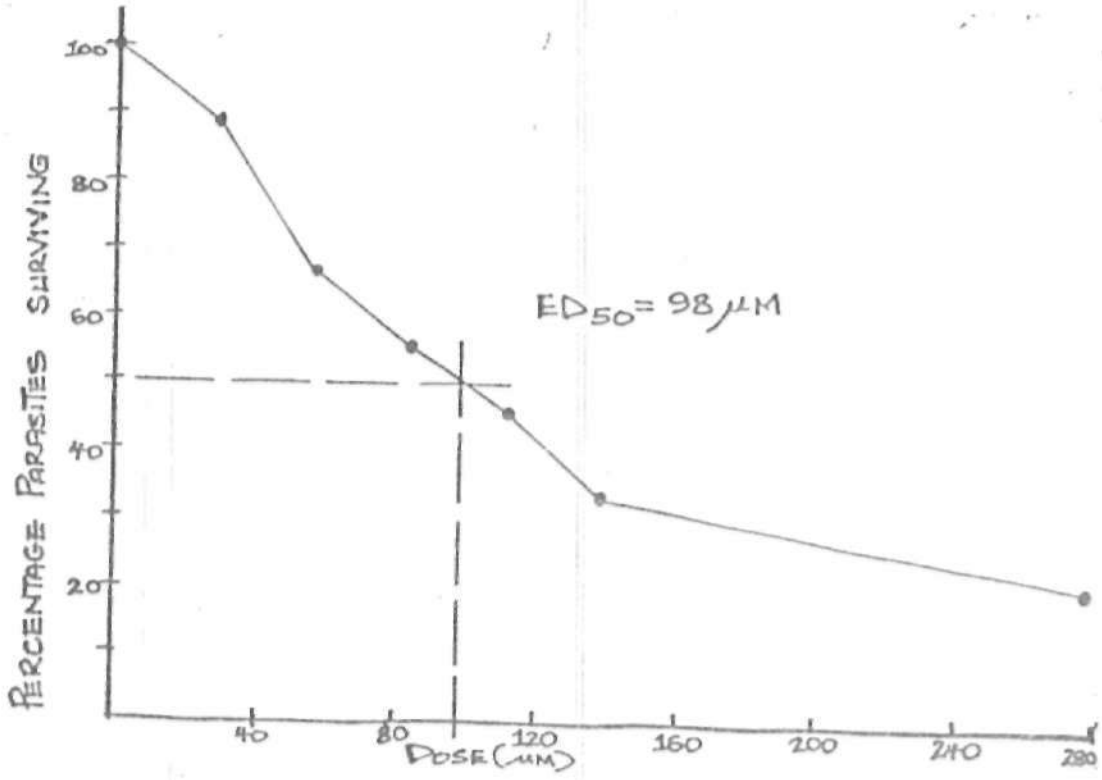
EFFECT OF SHAM ON TRYPANOSOMA BRUCEI: EVALUATION OF ED_{50} (1 HOUR)



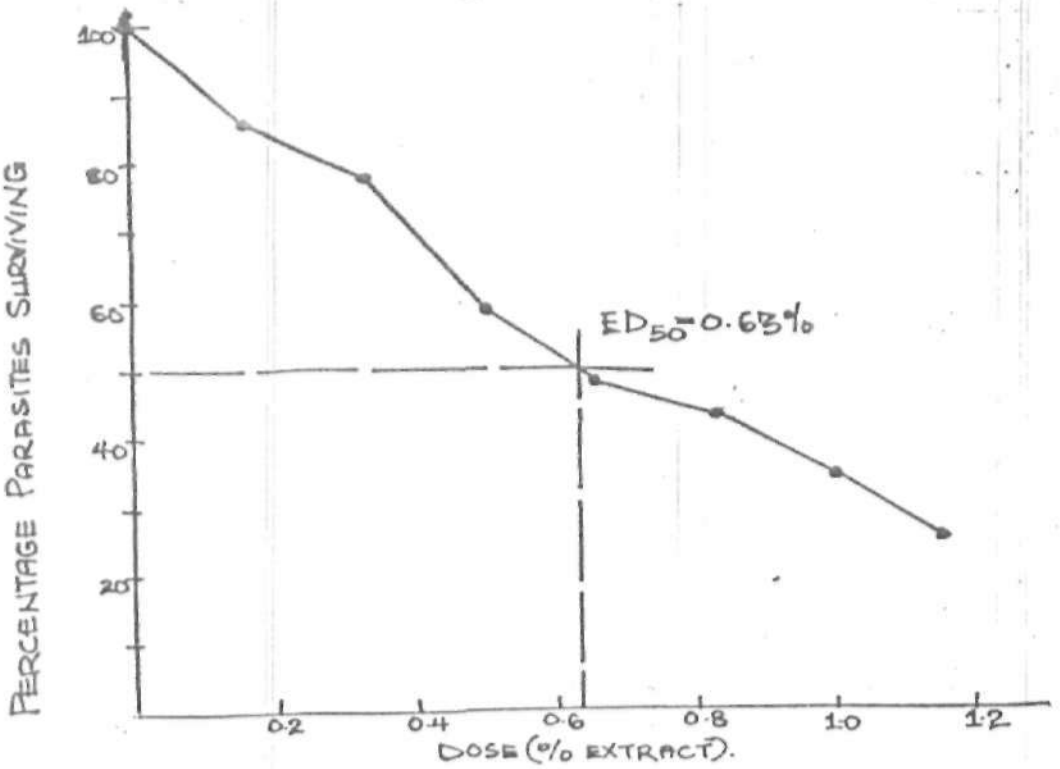
EFFECT OF SHAM ON TRYPANOSOMA CONGOLENSE: EVALUATION OF ED_{50} (1 HOUR).



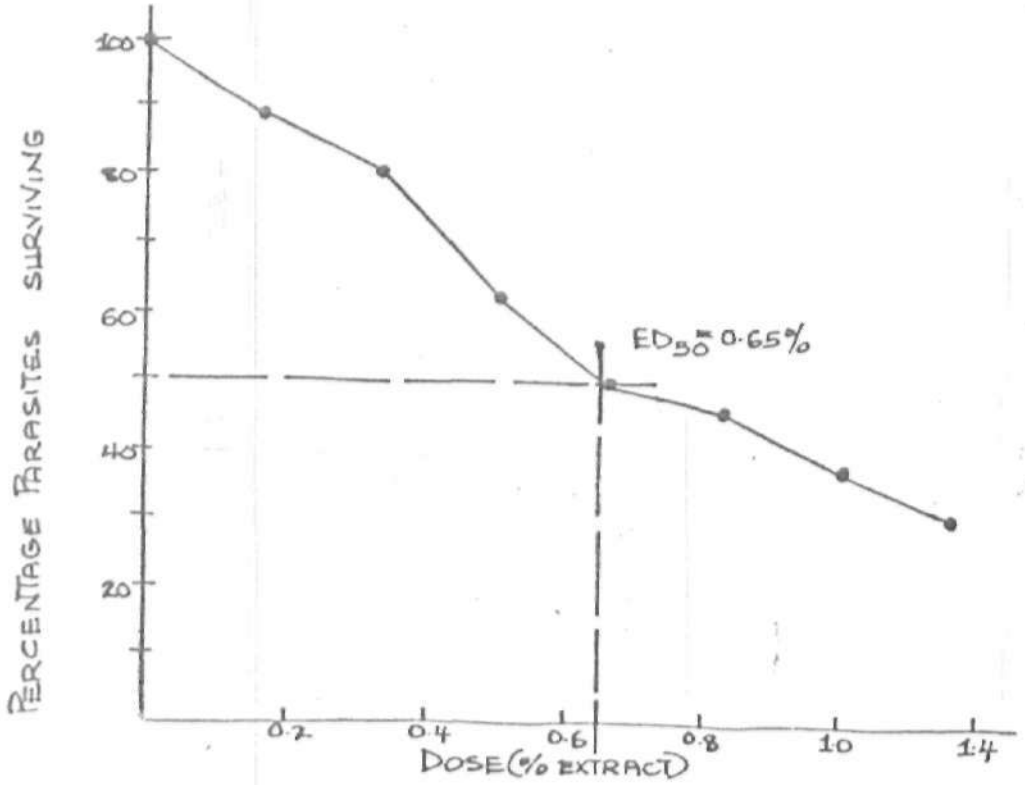
EFFECT OF GOSSYPOL ON TRYPANOSOMA BRUCEI: EVALUATION OF ED_{50} (1 HOUR)



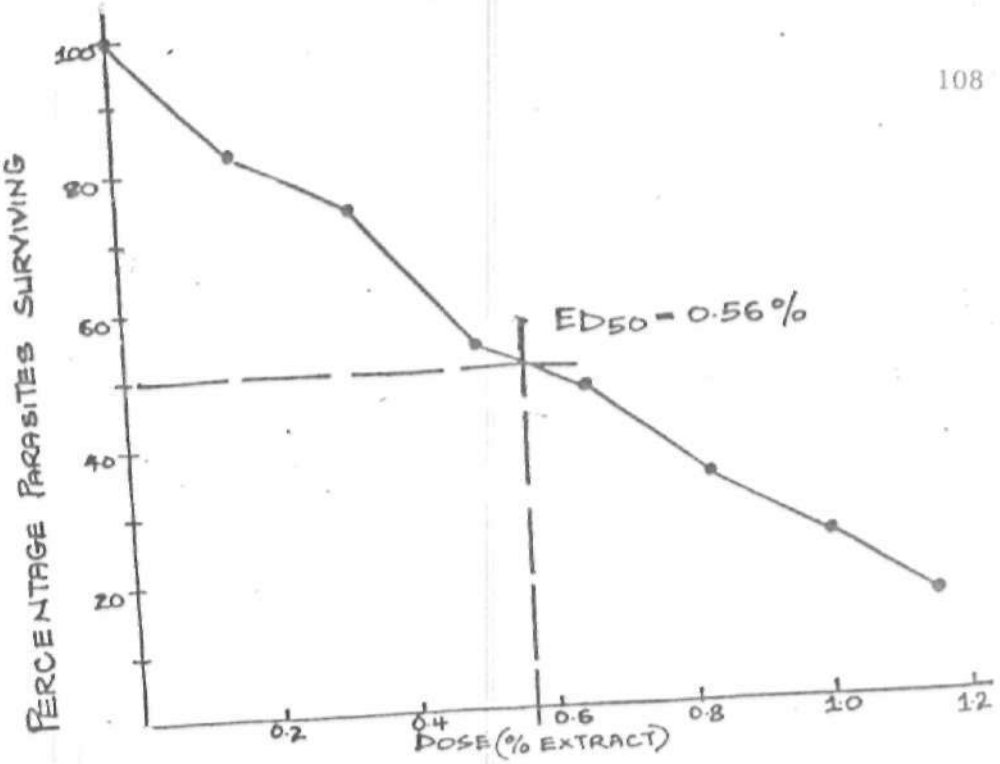
EFFECT OF GOSSYPOL ON TRYPANOSOMA CONGOLENSE : EVALUATION OF ED_{50} (1 HOUR).



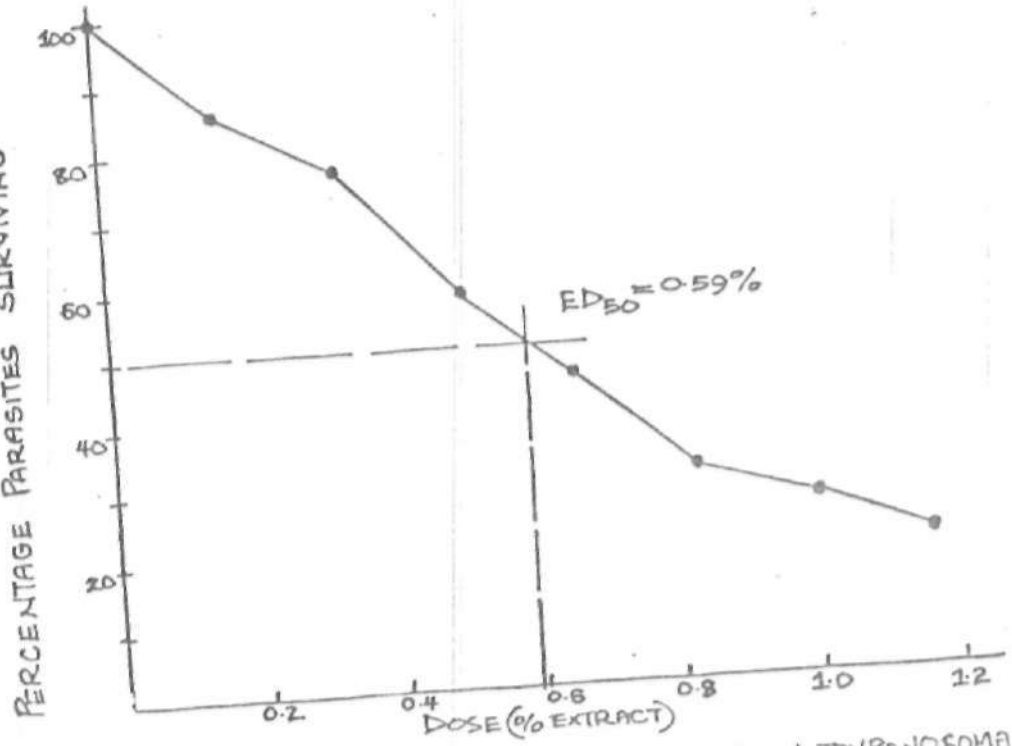
EFFECT OF HEXANE:ACETONE EXTRACT ON TRYPANOSOMA BRUCEI: EVALUATION OF ED_{50} (1 HOUR)



EFFECT OF HEXANE:ACETONE EXTRACT ON TRYPANOSOMA CONGOLENSE: EVALUATION OF ED_{50} (1 HOUR).

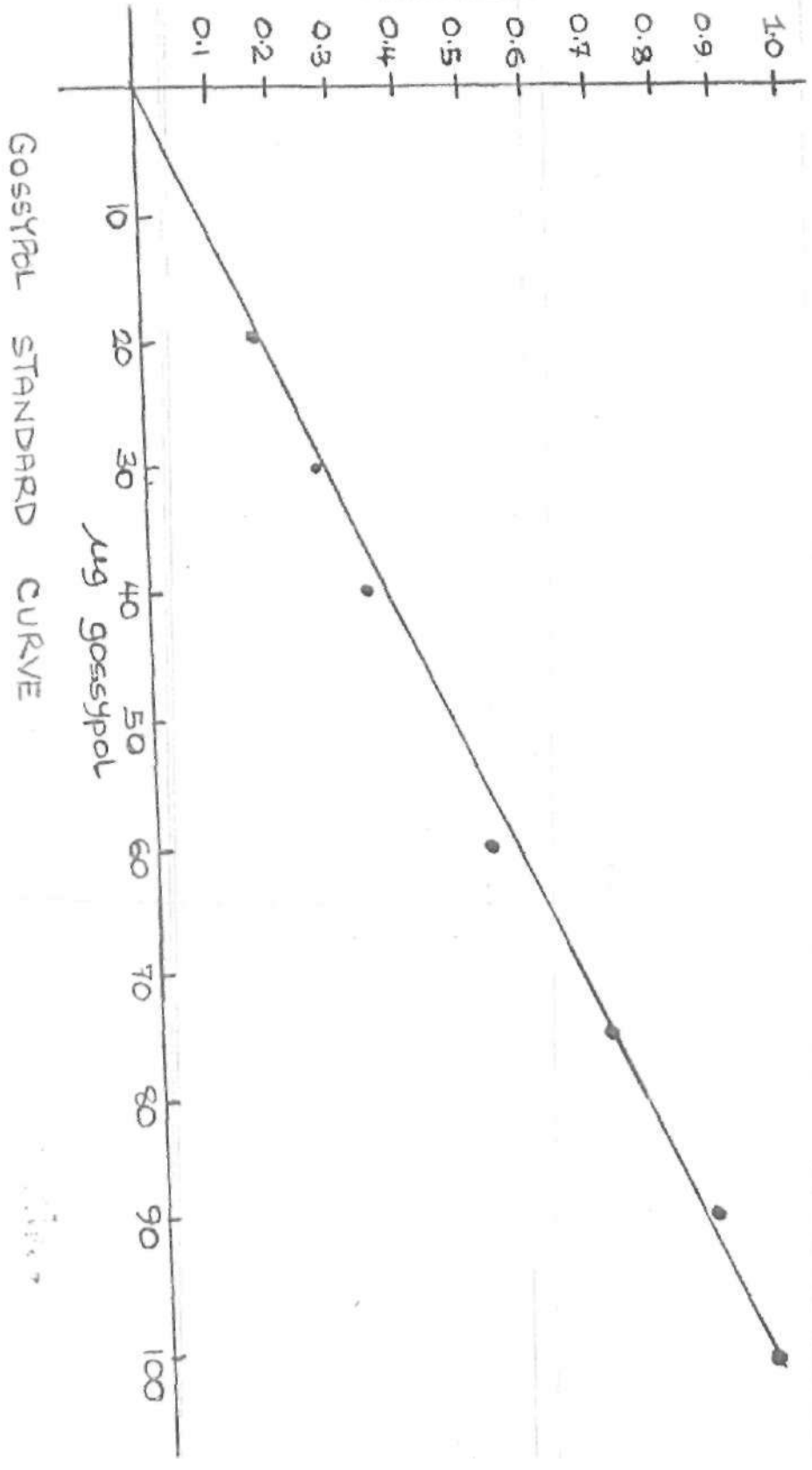


EFFECT OF HEXANE:ACETONE EXTRACT ON TRYPANOSOMA BRUCEI : EVALUATION OF ED_{50} (3 HOURS).



EFFECT OF HEXANE:ACETONE EXTRACT ON TRYPANOSOMA CONGOLENSE : EVALUATION OF ED_{50} (3 HOURS)

APPENDIX J


0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
10
20
30
40
50
60
70
80
90
100
µg gossypol
GOSSYPOL STANDARD CURVE