# STUDIES ON THE EFFECTS OF LEAF EXTRACTS OF *EUCALYPTUS CITRIODORA* (HOOK) AND *VERNONIA AMYGDALINA* (DEL) ON EXPERIMENTAL *SCHISTOSOMA MANSONI* INFECTION IN MICE

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JULY, 2000

## DEDICATION

This work is dedicated to my jewel of inestimable value, my wife Beatrice. I Ogboli and my son Joshua Ogboli.

## DECLARATION

I hereby declare that this thesis is a record of my research work. It has not been presented by me in any previous application for a higher degree. All the sources of information are duly acknowledged by means of references.

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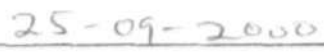

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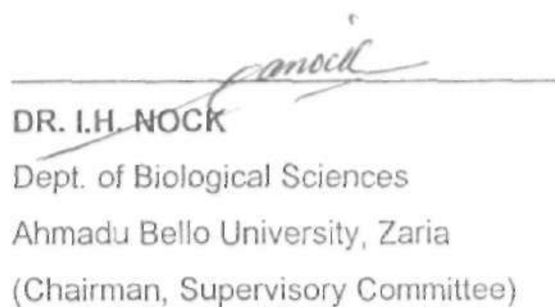
## CERTIFICATION

This thesis entitled "Studies on the Effects of leaf Extracts of *Eucalyptus citriodora* and *Vernonia amygdalina* on Experimental *Schistosoma mansoni* infection in mice" BY OGBOLI AMBROSE UZOMAH meets the regulations governing the award of the Degree of Master of Science (M.Sc) of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.



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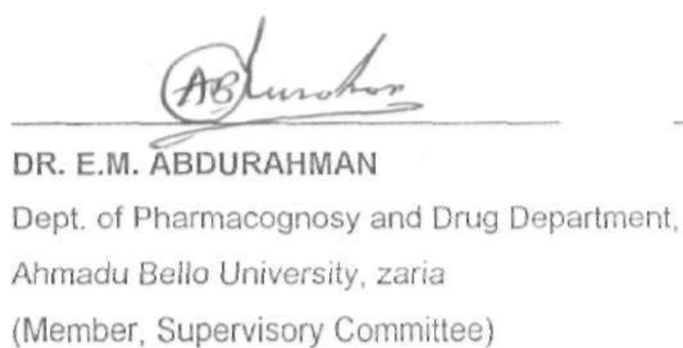

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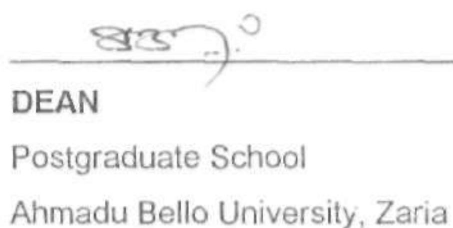

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# ABSTRACT

Nine leaf water extracts were tested for their ability to kill the human type *Schistosoma mansoni* cercariae *in vitro* by exposure of 10 cercariae each to dilutions of each extract at Img/ml, 3mg/ml and 5mg/ml. The time 100% mortality was attained for each extract was noted. The result show that the effect of plant water extracts was dose-dependent. The higher the dose the faster the killing time. The activity of the water extracts of *Vernonia amygdalina* was very potent while that of *Eucalyptus citriodora* was low.

Ethanolic and petroleum ether extracts of both plants were made and their ability at 1mg/kg b.w dose to cure or protect male Swiss albino mice (20-25g; 7-8 weeks) against experimental *Schistosoma mansoni* infection was tested. Reduction in parasite load in mice was 100% and 70.58% for petroleum ether (PEEC) and ethanolic extracts (EEEC) of *E.citriodora* respectively. Petroleum ether (PEVA) and ethanolic (EEVA) extracts of *V. amygdalina* reduced the worm load in mice by 72.30% and 82.61 % respectively. Following the prophylactic application of the extracts, there was zero degree reduction of infection for both PEEC and EEEC while PEVA and EEVA reduced infection by 34.06% and 44.57% respectively.

The difference in worm load between treated and non-treated control was significant ($P<0.05$) whereas differences in worm load was not significant ($P>0.05$) due to prophylactic application of extracts.

Pathologically the infected not treated mice (positive control) had enlarged fatty, friable and mottled liver. The spleen and kidneys were congested and enlarged with severe enteritis. Mice treated with PEEC had normal liver, spleen and kidney but showed mild anaemia. PEVA, EEVA and EEEC treated mice showed enlarged haemorrhagic friable dark liver, enlarged spleen, enlarged kidney and slightly anaemic carcass. Prophylactic

treatment showed similar pathological sign with the positive control. This study has shown that the plants tested have potentials to kill cercariae *in vitro,* cure and protect mice experimentally infected with human schistosome type cercariae.

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## ABBREVIATIONS, DEFINITIONS, SYMBOLS AND GLOSSARIES

| | |
|---|---|
| W.E. | Water Extracts |
| P.E. | Petroleum Ether Extracts |
| E.E. | Ethanolic Extracts |
| *Ad libitum* | without restriction |
| *In vitro* | Outside the living Tissues |
| *In vivo* | Within the living tissues |
| V.A | Vernonia amygdalina |
| AI | *Azadirachta indica* |
| AS | *Allium sativum* |
| MI | *Mangifera indica* |
| CP | *Carica papaya* |
| PG | *Psidium guajava* |
| AO | *Anacardium occidentale* |
| CC | *Cymbopogon citratus* |
| EC | *Eucalyptus citriodora* |
| Conc | Concentration |
| % | Percent |
| Kg | Kilogram(s) |
| b.w | Body weight |
| mg/ml | Milligram per millitre |
| g | gram |
| NS | normal spleen |
| NL | normal liver |
| **ES** | Enlarged spleen |
| **EL** | Enlarged liver |
| **FEL** | Liver fused and enlarged |

# CHAPTER ONE

## INTRODUCTION

Schistosomiasis (Bilharziasis) is one of the major endemic diseases in warm climatic countries affecting over 200 million people (Gustafsson,*et al.,* 1987). The disease is caused by *Schistosoma haematobium, Schistosoma mansoni* and *Schistosoma japonicum*, and is characterised by urinary and other related intestinal symptoms (Ben, 1973).

*Schistosoma haematobium* is found mainly in Africa and the Middle East. *Schistosoma mansoni* is found in Africa, the Arabian peninsula, Eastern and North Eastern South America while *Schistosoma japonicum* is prevalent in South-East Asia, Western Indonesia (Sulawesi), Philippines, Japan and China (Ben, 1973). The transmission of the disease is related to the presence of water and to several species of snails that act as the intermediate host (Brown, 1980).

Schistosomiasis was before now considered to be the second most important parasitic disease after malaria (WHO, 1985). However, the disease has surpassed malaria and is considered the most parasitic disease in the world (Ukoli, 1992). Jordan and Webbe, (1952) stated that inspite of concerted researches on various aspects of the epidemiology and possible control measures, the prevalence of the disease is on the increase in many areas of the world.

Endemic Schistosomiasis is a disease of rural communities, and its recent increase is due to the introduction of large scale irrigation schemes that usually come with water-filled canals which create the breeding sites of snails. This for

instance, happened after the construction of the Aswan Dam in Egypt and the Volta Dam in Ghana (Webbe, 1982).In Nigeria, almost all the regions have schistosome infection caused by *Schistosoma mansoni and Schistosoma haematobium* (WHO, 1985); this is attributed to water impoundment and large scale irrigation projects particularly in the Northern states. For instance Zaria has two large dams and water ponds with accompaying widespread of schistosomiasis (Rollinson and Simpson, 1987).

The treatment and control of schistosomiasis by the use of drugs have been faced with problems of high cost, protracted treatment protocols, drug toxicity and resistance (Madsen, 1986). In addition, there are no effective drugs for the prevention and/or treatment of the disease (Rollinson and Simpson, 1987). Furthermore, drugs in use hardly reach the end users at the rural level (Sofowora, 1993). Therefore, it is only reasonable that new drugs be sought. Hence, natural products from plant materials are increasingly being explored for the extraction of bioactive agents for the development of drugs against diseases e.g. trypanosomosis (Nok, *et al*, 1993 and many others). So far, fewer than 1% of the worlds 265,000 flowering plants have been tested for their curative power (Anonymous,1998). Sofowora (1981) reported that less than 2% of drugs of plant origin are stocked or dispensed in Nigeria. Indeed, the need to protect and sustain our natural plants is being taken seriously, to the extent that Governments are legislating against the destruction of plants (deforestation). In addition, the Government of Nigeria has institutionalized traditional medicine (which relies on treatment of ailments through herbs) into its overall health care programme (Shok, personal comm. 2000). In the light of the need to create and enrich the pool of information on plants with medicinal

value (which will serve as a referral in the future for the development of drugs against several diseases), this study was conceived with the aim of examining the potency of some common available plants to cure experimental schistosomiasis in mice.

Accordingly it has the following objectives:-

1. To Test the potency of the water extracts of the underlisted named plants on *schistosoma mansoni* cercariae *in vitro*:
   a) *Vernonia amygdalina* (Bitter leaf plant)
   b) *Azadirachta indica* (Neem plant)
   c) *Allium sativum* (Garlic)
   d) *Mangifera indica* (Mango)
   e) *Carica papaya* (Pawpaw)
   f) *Psidium guajava* (Guava)
   g) *Anacardium occidentale* (Cashew)
   h) *Cymbopogon citratus* (Lemon grass)
   i) *Eucalyptus citriodora*
2. To determine the *in vivo* effect of the ethanolic and petroleum ether leaf extracts of two selected plants (from 1 above) on experimental *Schistosoma mansoni* infection in mice.

It is hoped that the accomplishment of this study will permit assessment of the curative value of the plants in view of drug development against schistosomiasis in the future.

# CHAPTER TWO

## LITERATURE REVIEW

### 2.1 Schistosomes

#### 2.1. 1 Classification, description and distribution

The genus *Schistosoma* belongs to the family schistosomatidae. Members are dioecious Digenea parasites in the blood vascular system of vertebrates.

The family can be divided into three subfamilies, the schistosomatinae, Bilharziinae and gigantobilharziinae. Only the genus *Schistosoma* is associated with man and has achieved the greatest geographical distribution and diversification in terms of numbers of recognised species and different hosts parasitized (Rollinson and Simpson, 1987). Generally the mature female is more slender than the male and are normally carried in the ventral groove, the gynaecophoric canal ( Rollinson and Simpson, 1987). They also possess oral and ventral suckers which are smaller in diameter than those of the male. The external surface is not papillated but may possess rows of fine spines at the mid/posterior end of the body. In Nigeria *Schistosoma mansoni* infested areas are the North Western States, North Eastern and South Western States as well as the North Central States of Kaduna, Niger, Jigawa amongst others (WHO 1985). Schistosome infection due to *Schistosoma mansoni* also occurs in some other countries of Africa including Egypt, Sierra Leone, Mali, Uganda, Kenya and South Africa etc (Rollinson and Simpson, 1987).

There are also numerous reports of infection caused by *Schistosoma mansoni* in Brazil, Saudi Arabia, the West Indies etc (Rollinson and Simpson 1987), in

addition, *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum* infections have been reported in several species of mammals including Primates, Rodents and Carnivores in Indonesia, Malaysia, Ethiopia and Zimbabwe amongst others (Rollinson and Simpson, 1987).

Experimental studies have shown that *S. mansoni* strains from different geographical areas may display differences in their biological characteristics and in their infectivity or pathogenicity (Rollinson and Simpson, 1987).

### 2. 1.2 Life cycle

The eggs of *Schistosoma mansoni* pass out of the body with urine or faeces and ultimately reach natural fresh water bodies that are polluted by these body wastes (Ukoli, 1992). The eggs hatch in the water into miracidia which then locate and penetrate the tissues of fresh water planorbid snails (*Biomphalaria pfeifferi*) through the cephalopedal area (Rollinson and Simpson, 1987). The hatching of *Schistosoma mansoni* eggs in the outside environment only occurs when certain environmental factors are met, such as temperature, light and osmotic pressure (Erasmus, 1972). In *Schistosoma mansoni*, the eggs hatch both in daylight and in the dark (Rollinson and Simpson, 1987). The rate at which schistosomes produce eggs is correlated positively with uterine egg counts. The number of eggs contained within the uterus of the female worm may vary with the strain of the parasite and the age of the infection but will seem to be reasonably characteristic of the species (Rollinson and Simpson, 1987).

After the miracidium penetrates the snail host, it is fixed in the host. Erasmus (1972) demonstrated that fatty acids are involved in the induction and fixation

processes of the larva. In *Schistosoma mansoni* the miracidium develops near the penetration point, in the subepithelial conjunctive of the cephalopedal zone with a preference for the lateral edges of the anterior third of the foot (Jourdane, 1982).

Inside the snails they multiply and ultimately produce hundreds of microscopic forked tailed larvae called cercariae. These cercariae are then shed into the water by the snails and they swim about till man makes contact with the water and the cercariae penetrate through his skin, thereby producing infection in the absence of a host a sporocyst (metamiracidium) is produced (Ukoli, 1992).

### 2.1.3 Important factors in the spread of infection

Three factors are of great importance in the spread of the infection.

1. Pollution of natural fresh water bodies with human body wastes.
2. Human contact with such water, and
3. The presence of the appropriate snail intermediate host in the water (Ukoli, 1992).

These three factors offer the most significant impetus in the spread of schistosomiasis in endemic areas. In most rural communities in Northern Nigeria and even in some urban centres of Nigeria, the inadequate provision of proper toilet facilities leads to indiscriminate defecating in the open fields, thereby leading to the eventual pollution of fresh water bodies with human body wastes. Consequently due to lack of potable drinking water, people resort to using water from these streams and ponds for domestic purposes thereby exposing them to the snail intermediate host. In Zaria for instance, children

often swim in these polluted shallow ponds and this invariably exposes them to schistosome infection.

With the massive water resource development programmes in recent times through the construction of dams and lakes to boost water supply as well as enhance dry season farming, comfortable breeding sites for snail intermediate host have been created thus creating opportunities for spread of infection (Jordan and Webbe, 1982; Rollinson and Simpson, 1987). Fadama (water-logged areas) farming of rice, sugar cane and other crops has also exposed farmers to the snail intermediate host as well as fishing activities and therefore to schistosome infection (Opaluwa, et al., 1999).

### 2.1.4 Pathology

Pathology of schistosomiasis caused by *S. mansoni* is in two main phases;

**a)** The acute phase

**b)** Chronic phase (Edington and Gilles, 1969).

In the acute phase itching and urticaria fever, abdominal pains, diarhoea, general malaise, weakness, myalgia, headache, diaphoresis, anorexia, cough and weight loss has been reported by Lichtenberg (1987). Edington and Gilles (1969) reported intestinal lesions on the mucosa, particularly the posterior or small intestines is encountered, eggs and elassic granulomas in the submucosa, gradual disintegration of eggs causing infiltration with epitheliod cells, fibroblasts and fibrous tissue formation. During the chronic phase the liver becomes enlarged (fibrosis of the liver) or slightly shrunken. The inner surface of the liver shows the characteristic clay pipestem fibrosis which

causes obstruction of the various blood supply and the rest of the body becomes emaciated (Goez, et al., 1991).

There is also an enlargement of the spleen resulting from the obstruction of the portal venous circulation in the liver (congestive splenomegaly) growth retardation and sexual maturation (Edington and Gilles, 1969; Gerald and Larry, 1981).

### 2.1.5 Control

Ukoli (1992) reported that efforts towards the prevention and control of schistosomiasis are best focused in three main directions;

1. The intermediate host,
2. Provision of safe and potable water and basic sanitation.
3. Chemotherapy and chemoprophylaxis/immunization

**The intermediate host**

One of the most promising approaches in the control of parasitic diseases is through the elimination or at least reduction of the intermediate host population. Measures directed against elimination of the intermediate host include;

**Use of competitor snails**

Competitors have been identified to offer the most promising prospects in the elimination of the snail intermediate host as snail antagonists such as Thiariid Thiara (*Tarebia granifera)* have shown to be effective in displacing *Biomphalaria glabrata* (Prentice, 1983). *Marisa Cornuarietis* eliminates *B. pfeifferi, Bulinus tropicus* and *Lymnaea natalensis* (Nguma et al., 1982) while *Helisoma duryi* displaced *Biomphalaria* spp (Madsen, 1983).

**Use of predators, parasites and pathogens**

Of all putative predators, sciomyzid flies which are obligate predators of molluscs offer the greatest promise. The larvae of this insect are malacophagous; a single larva feeds on the soft tissues of between 8 and 24 snails for its growth and development (Berg, 1953, 1973).

In addition, the fish *Astatoreochromis alluadi* and the Khaki Campbell ducks have been found useful as snail predators in a man-made lake (Ukoli, 1992). Furthermore, the sporocysts of trematodes including schistosomes are known to be capable of exerting negative impact on snail population by reducing their fecundity and causing high mortality (Ukoli, 1992).

Environmental control can be achieved through the reduction of the water

**Ecological modification of snail habitat**

carrying capacity by altering the habitat in such a small population of the target species (Ukoli, 1992). Generally this could be done by increasing the speed of water current, channeling the water, and eliminating pools. Also, aquatic vegetation which serve as food, oviposition sites and shelter for the snails can be removed. Specifically for natural habitats, transmission sites like burrow pits, marshy areas e.t.c. can either be filled or drained while rivers can be canalised to make the edges unsuitable for snails (WHO, 1985).

In addition, the adoption of the overhead sprinkler and trickle irrigation systems rather than the open system not only improves water management but also reduces human contact with water thereby fostering the control of transmission of disease (WHO, 1985).

**Use of chemicals**

The use of chemicals, the so-called pesticides offers the best prospects for control of parasitic diseases. However, the risk of environmental pollution and the harmful effects on non-target organisms, coupled with the phenomenal escalation in cost has limited the continued use of these chemicals in most endemic areas (Ukoli, 1992).

**Provision of safe, potable water and basic sanitation**

Provision of safe, potable water is the most effective method of control. If a good water source could be developed, not only will schistosomiasis be eradicated, but the incidence of other water-borne diseases like gastroenteritis, typhoid fever, hepatitis, poliomyelitis, cholera e.t.c. will be greatly reduced in Africa within a year or two (Ukoli, 1984).

**Use of synthetic drugs**

Chemotherapeutic and chemoprohylactic drugs have been developed against schistosomiasis. Amoscanate, an isothiocyanate ,is an anti-helminthic agent with a broad spectrum activity against schistosomes; however it is affected by its low mutagenic potential (Rollinson and Simpson, 1987) Oltripraz has a low on set of drug action and reduction in glutathione levels which appear to be correlated to the anti-schistosomal activity of a series of congeners (Rollinson and Simpson, 1987). Metrifonate, an organophoshate, was reported by Cerf, et al (1962) as being schistosomicidal. However, it has been found to be useful only for *S. haematobium* (Rollinson and Simpson, 1987).

Furthermore, most of the already developed drugs in use suffer some draw-backs of host - specificity, species- specificity as well as side effects. Rollinson

and Simpson (1987) reported that metrifonate and oxamniquine are species - specific in their efficacy against schistosomes, in contrast to the antimonials and praziquantel which are broad spectrum in action. In addition, host factors may contribute significantly to the susceptibility of schistosomes to therapy e.g. Antimonials are more effective against *S. mansoni* in hamsters than in mice (Berberian and Freele, 1964). Reports have also shown that *S. mansoni* may have developed resistance to oxamniquine and hycanthone (Dias, et al.,1982; Coles, et al.,1986).

Although, praziquantel has been reported to be the most important drug in the chemotherapy of schistosomiasis (Rollinson and Simpson, 1987), it has been reported however by Jaoko, et al., (1996) that pupils infected with *S. mansoni* and treated with praziquantel suffered serious side effects. This among other problems makes it necessary in recent times to shift from tried and tested drugs to natural products of plants.

## 2.2 A search for Natural Cures

Everyone naturally stands to gain from new treatments with natural products that will help people live longer, healthier lives, especially since a growing body of scientific evidence indicates that certain synthetic chemicals, when taken into the body, interfere with the body metabolism (Anonymous, 1998). Presently, the use of herbs as medicine is growing worldwide because of its relative level of safety. Herbal medicines are made from parts of plants such as roots, flowers, barks, stem or roots (Anonymous, 1999). Many drug companies and individuals have made huge investments into research and development of drugs from medicinal plants from which several plant materials

with antimicrobial, antiparasitic and other healing properties have been identified (Okpanyi and Ezeukwu, 1981, Obiora, 1986, Gbile, 1986, Nok *et al,* 1993; 1994; 1996, and Mann, 1998 Anonymous, 1999).

The success of these investigations prompted this study which seeks to test the potency of some local plant materials against experimental schistosomiasis.

## CHAPTER THREE

## MATERIALS AND METHODS

### 3.1 Leaf Materials

#### 3.1.1 Collection of leaves of plants

The leaves of nine plants (listed below) were collected from Ahmadu Bello University, Main Campus, Zaria. They were identified and assigned voucher numbers (as indicated) by the herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria.

Voucher No. Scientific Name Family Common name/Hausa name

No.675 *Vernonia amygdalina Del (Compositae): Bitter leaf plant/Shuwaka*

No.900151 *Azadirachta indica (Meliaceae): Neem plant/Dogon Yaro*

No.2196 *Allium sativum L (Liliaceae): Garlic/Tafarnuwa*

No.3253 *Mangifera indica (Anacardaceae): Mango/Mangoro*

No. 1944 *Carica papaya Linn (Caricaceae): Pawpaw/gwanda*

No. 015 *Psidium guajava (Myrtaceae): Guava/Gwaiva*

No.991 *Anacardium occidentale Linn (Anacardaceae): Cashew,/ Yazawa*

No.1882 *Cymbopogon citratus (DC) Stapf (Graminae): Lemon grass*

No.2767 *Eucalyptus citriodora Hook (Myrtaceae): Eucalyptus/itche-turare*

#### 3.1.2 Preparation of leaf material

250g of each of the leaves were air-dried at ambient temperature. The dried leaves were powdered using pistle and mortar and later sieved and stored separately in cellophane bags at room temperature (Brain and Turner, 1975).

### 3.1.3 Water extracts

When required, 0.5g of each stored leaf powder in 3.1.2 above was soaked in 100ml distilled water in a 200ml beaker and allowed to stand for five hours after which the content was stirred with a glass rod and filtered through a sieve and the water extract (WE) filtrate collected in a 200ml beaker. Portions of the 5mg/ml filtrate were diluted to give concentrations of 3mg/ml and 1mg/ml (Gennar, 1985), which were used in 3.2.3 below.

### 3.1.4. Petroleum ether and ethanolic extracts

The solvent extraction technique by Butterworth and Morgan (1986) and Sofowora (1993), was used for extraction of *Eucalyptus citriodora and Vernonia amygdalina*. The choice of these plant from the nine lots followed observations of their effect on cercariae *in vitro in* 4.1 below. 100g of each powdered plant leaf material was defatted with 300ml of petroleum ether at $60^0$C-$80^0$C in a soxhlet extractor for 36h until the draining solvent was clear. The petroleum ether extract (PE) collected was evaporated to dryness in vacuum in a rotatory evaporator at $45^0$C and the resultant residue of fats, waxes and steroids (Gennar, 1985) were collected and stored in specimen bottles in a refrigerator at 4°C until required in 3.3. below.

The marc (residue) collected from the petroleum ether extract above was extracted exhaustively with 300ml 95% ethanol for 36h in a soxhlet extractor to obtain the ethanolic extract (EE). The EE was evaporated to dryness in rotatory evaporator at $45^0$C and the resultant residue of alkaloids, tannins,

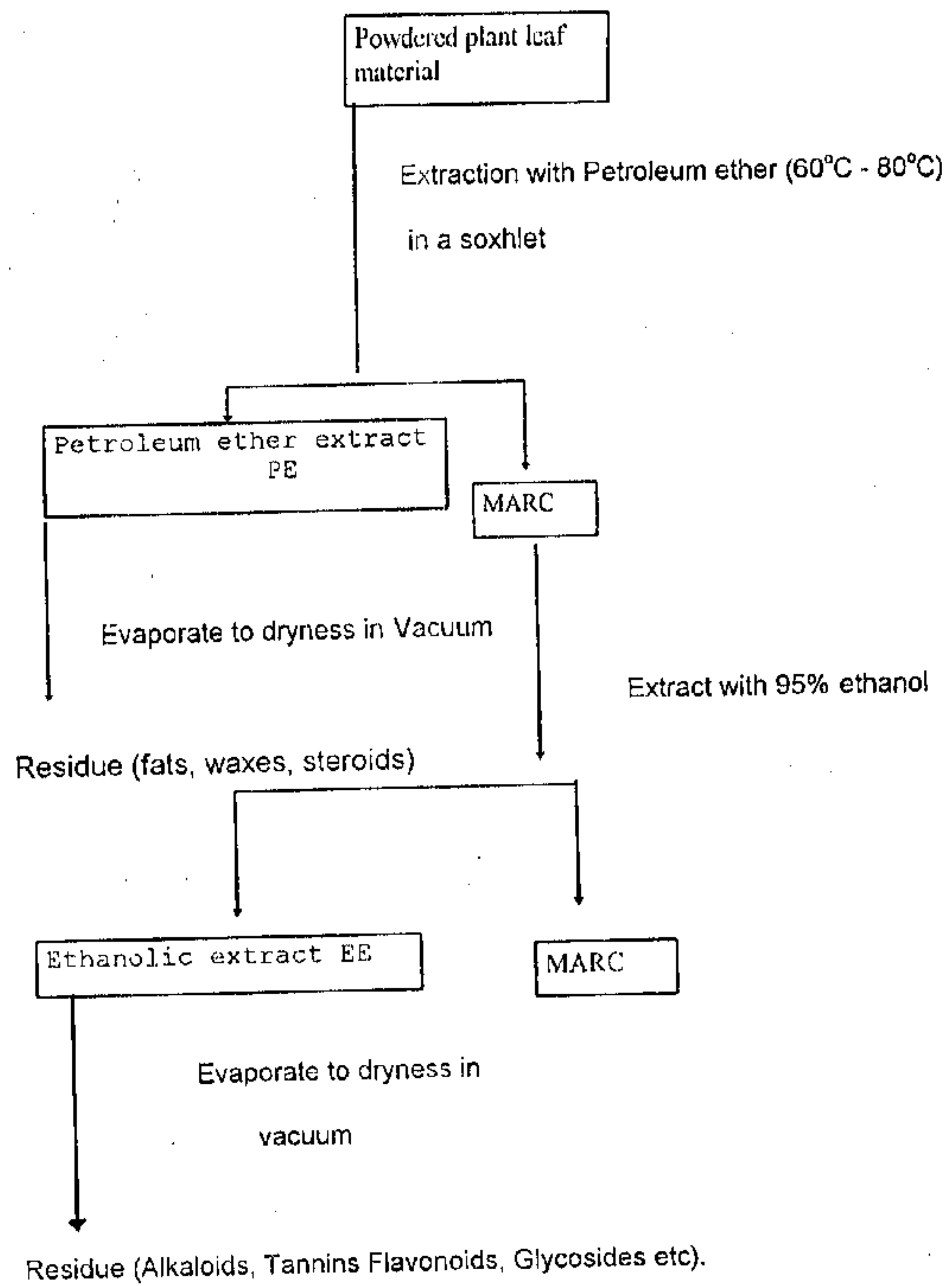


Fig 1: **Extraction Flow Chart for Petroleum Ether and Ethanolic Extracts.**

flavonoids, glycosides etc (Gennar, 1985) were collected and stored in specimen bottles in refrigerator at 4°C until required in 3.3 below.

### 3.2 ***Schistosoma mansoni* cercariae**

#### 3.2.1 **Collection of snail intermediate host and observation for infection**

The snail intermediate host of *Shistosoma mansoni* in Nigeria, *Biomphalaria pfeifferi*, (Rollinson and Simpson, 1987) were collected from Ahmadu Bello University Dam and the stream behind Danfodio Hall both on the Main Campus, Samaru-Zaria, and put in an intransmissible container and taken to the laboratory for identification (Rollinson and Simpson, 1987). Snails were put singly in tubes and exposed to light (100 Watt electric bulb) for 3 h and only those shedding pure populations of human schistosome type brevifurcate cercariae were selected for use in 3.2.2 below:

#### 3.2.2 **Collection and enumeration of cercariae from snails**

The method described by Christensen, *et al* (1984) was used with modification to observe and count the emerging cercariae. Five snails, in 3.2.1 above, were put in a 100ml beaker containing 20ml dechlorinated water and exposed to artificial illumination using a bench lamp with a 100Watt electric bulb for 3 hours. The emerging cercariae were identified under the microscope. Two separate drops of the cercariae were taken and placed on a glass slide using a dropper and a drop of Lugol's iodine was added to kill and stain the cercariae. The number of cercariae per drop was recorded. The mean number of cercariae in the two representative drops was computed, from which the total number of drops required to give 200 cercariae (pre-determined infective dose) was calculated as described by Christensen, et al. (1984) . Volume

adjustment where necessary, was made to give 200 cercariae per 0.1ml for inoculation into mice as in 3.2.5 below.

### 3.2.3 Sensitivity of cercariae to crude leaf water extract of selected plants.

For the purpose of selecting plants for the main study, (on the effect of extracts on experimental infection in mice), the water extracts of nine the selected plants in 3.1.1 were made and tested for their ability to kill cercariae *in vitro* as follows:

0.2ml of the crude leaf water extract (WE) of each plant at 1mg/ml, 3mg/ml and 5mg/ml (3.1.3) was put into wells of the multi-well plate after which 10 active moving cercariae were added with a dropper. The content was examined under the microscope and the time taken to kill (absence of movement on touch with a probe) 100% of the schistosome cercariae was noted (Brain and Turner, 1975).

### 3.2.4 Toxicity of leaf extracts to mice

PE and EE leaf extracts of *Vernonia amygdalina (VA)* and *Eucalyptus citriodora (EC) (3.1.4)* were administered intraperitoneally to a group of five mice each 20 - 25g weight (7-8weeks old) at 5mg/kg, 4mg/kg, 3mg/kg, 2mg/kg and 1mg/kg body weight at 0.2ml each in order to test for toxicity of the leaf extracts in normal mice. 0.2ml of 2% Tween 20 was inoculated in a group of five mice to ascertain its safety as an emulsifying agent in the dissolution of petroleum ether extract preparation. The number of deaths in each group was recorded after 24 hours (Gennar, 1985). The probit of kill and log dose were computed

(Gennar, 1985). Based on this, the dose safe for administration to mice was chosen for experiment 3.3.1 below.

### 3.2.5 Inoculation of mice with human schistosome - type cercariae

Laboratory bred Swiss albino male mice (20-25g) 7-8 weeks old were inoculated intrapritoneally with 200 cercariae, (obtained from snails in 3.2.2. above) per o.1ml using a 1ml syringe (Price and Reed, 1970).

### 3.2.6 Examination of cercariae - inoculated mice for infection

Faecal examination for eggs from the inoculated mice started as from week 3 post inoculation of mice with cercariae, by the direct microscopic examination of wet faecal preparation for lateral spined eggs (Price and Reed, 1970). Three pellets of fresh mouse faeces were collected in a test tube and dissolved in 20ml distilled water using a glass rod. This was allowed to stand for 20 minutes. The supernatant was discarded and 20ml distilled water added to the sample and kept for 10 minutes. The supernatant was again discarded and the sediment was stained with 2 drops of Lugol's iodine. Two drops of the suspension were taken onto a glass slide using a dropper and examined under the microscope for eggs (Price and Reed ,1970).

## 3.3 Administration of Plant Extracts to infected mice

Prior to the administration of the extracts into the mice, 0.5mg/ml of the ethanolic extracts (3.1.4) was dissolved in 100ml sterile distilled water to give a concentration of 5mg/ml. The petroleum ether extracts were first dissolved in 10ml of 2% Tween 20 solution (emulsifying concentrate) and later to a final

volume of 100ml with sterile distilled water to give a concentration of 5mg/ml. Each solution was further diluted to a concentration of 1mg/ml (Burger,1988).

### 3.3.1 Therapeutic application of the extracts to infected mice

At the onset of detection of infection (week 10), mice were grouped into six of five mice each designated A,B,C,D,E and F. The PE and EE of *Eucalyptus citriodora (PEEC and EEEC)* were administered to the infected mice in groups A and B respectively at 1mg/kg b.w. Infected mice in groups C and D were treated with the PE and EE respectively of *Vernonia amygdalina* (PEVA and EEVA) at 1mg/kg b.w. Mice in group E were infected but not treated (positive control) while those in group F were not infected and not treated (negative control). Animals were treated for 5 consecutive days and infection was continuously monitored for 2 weeks post treatment by wet film examination of faeces. For necropsy, all mice were sacrificed by cervical dislocation, the abdominal cavity was carefully opened to expose the viscera. Mice that died before treatment were opened up and organs examined. Necropsy was conducted on the organs and adult worm load was determined. The whole gastro intestinal tract was removed and weighed and crushed using spatula on a glass slide after which 5% KOH was added in the ratio of 10ml KOH/g of tissue to digest the tissues; the worms liberated from the tissues were counted under the microscope (Price and Reed, 1970; Christensen et al, 1984). This was expressed as worm load per gram of tissue.

Based on the result of the above treatment, a group of five infected mice group G, were allowed to develop full blown infection (emaciated, losing hair and almost dying) before they were treated at week 13 with PEEC at 1kg/bwt for 5



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consecutive days. Animals were thereafter put on observation for 30 days after which they were sacrificed and necropsy done as described above.

### 3.3.2 Prophylactic application of extracts

Plant extracts namely: PEEC, EEEC, PEVA and EEVA were each administered once by intraperitoneal injection at 1mg/kg b.w to four groups of five mice each designated P, Q, R and S respectively and kept for 24hr (Gennar, 1985), after which they were then challenged by inoculation with 200 cercariae as in 3.2.5 above. Five mice in group T served as control without inoculation of cercariae. Examination of eggs commenced 3 weeks post inoculation and continued until week 12. Infected mice were then sacrificed by cervical dislocation and carefully opened to expose the viscera. Necropsy was conducted and adult worm load determined (Price and Reed, 1970; Christensen et al., 1984).

### 3.4 Statistical Analysis

The analysis of variance (ANOVA), Duncan Multiple range test, student 't' test and coefficient of correlation was used to test whether the difference in the observed variables were significant.



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# CHAPTER FOUR

## RESULTS

### 4.1 Sensitivity of Cercariae to Water Extracts

The results obtained are shown in Table 1. A varying degree of cercaricidal activity was observed. The killing time was inversely dependent on the dose of the extracts used; the higher the dose of extract the shorter the killing time of cercariae. Water extract of *Allium sativum* (WEAS) was consistent as the most effective extract with shortest killing time at all concentrations. This was followed by *Vernonia amygdalina* (WEVA) and thirdly by *Azadirachta indica* (WEAI).

The water extract of *Cymbopogon citratus* (WECC), *Eucalyptus citriodora* (WEEC) and *Mangifera indica* (WEMI) exhibited very low cercaricidal activity.

Based on this result, the plant *Vernonia amydalina and Eucalyptus citridora* were chosen (as one with high and low activity against the cercariae respectively) for further studies as earlier stated above (3.1.4; 3.2.4; 3.3.1; 3.3.2).

### 4.2 Toxicity of Petroleum Ether and Ethanolic Extracts on Normal Mice.

Table 2 shows the different extract doses used and the number of death recorded in each group. Higher concentration of extracts recorded higher number of death of mice and consequently higher probit values. The ethanolic extract of *Vernonia amygdalina* (EEVA) and ethanolic extract of *Eucalyptus citriodora* (EEEC) and petroleum ether extract of *Vernonia amygdalina* (PEVA) (Figures 2,3 and 4) respectively had an LD50 of 3.6 mg/kg body weight while

Table 1: Effect of crude water extracts of some selected plants on cercariae

| Water Exract (WE) | No. Cercariae | Time (Min.) of killing 100% of cercariae at: | | |
|---|---|---|---|---|
| | | 5mg/ml of extract | 3mg/mg of extract | 1mg/ml of extract |
| WEVA | 10 | 30 | 40 | 60 |
| WEAI | " | 50 | 60 | 90 |
| WEAS | " | 13 | 30 | 38 |
| WEMI | " | 120 | 180 | 120 |
| WECP | " | 120 | 90 | 120 |
| WEPG | " | 90 | 150 | 150 |
| WEAO | " | 30 | 40 | 150 |
| WECC | " | 150 | 180 | 180 |
| WEEC | " | 60 | 100 | 180 |
| Water (Control)* | " | - | - | - |

* Control group showed no death over 24hr period.

Table 2: Toxicity of Ethanolic and Petroleum Ether leaf extracts of *Vernonia amygdalina and Eucalyptus citriodora* on normal mice after 24h.

| Group | Extract | Dose mg/kg | No. of dead mice (%) | Probit of kill | Log dose Q |
|---|---|---|---|---|---|
| 1 | EEVA<br>EEEC<br>PEVA<br>PEEC | 5<br>"<br>"<br>" | 4 (80)<br>4 (80)<br>4 (80)<br>4 (80) | 5.842<br>5.842<br>5.842<br>5.842 | 0.689<br>0.689<br>0.689<br>0.689 |
| 2 | EEVA<br>EEEC<br>PEVA<br>PEEC | 4<br>"<br>"<br>" | 4 (80)<br>3 (60)<br>3 (60)<br>4 (80) | 5.842<br>5.253<br>5.253<br>5.842 | 0.689<br>0.602<br>0.602<br>0.698 |
| 3 | EEVA<br>EEEC<br>PEVA<br>PEEC | 3<br>"<br>"<br>" | 3 (60)<br>2 (40)<br>3 (60)<br>3 (60) | 5.253<br>4.747<br>5.253<br>5.253 | 0.602<br>0.477<br>0.602<br>0.602 |
| 4 | EEVA<br>EEEC<br>PEVA<br>PEEC | 2<br>"<br>"<br>" | 1 (20)<br>1 (20)<br>1 (20)<br>1 (20) | 4.158<br>4.158<br>4.158<br>4.158 | 0.301<br>0.301<br>0.301<br>0.301 |
| 5 | EEVA<br>EEEC<br>PEVA<br>PEEC | 1<br>"<br>"<br>" | 0<br>0<br>0<br>0 | 0<br>0<br>0<br>0 | 0<br>0<br>0<br>0 |
| 6 | 2% Tween 20 | | 0 | 0 | 0 |

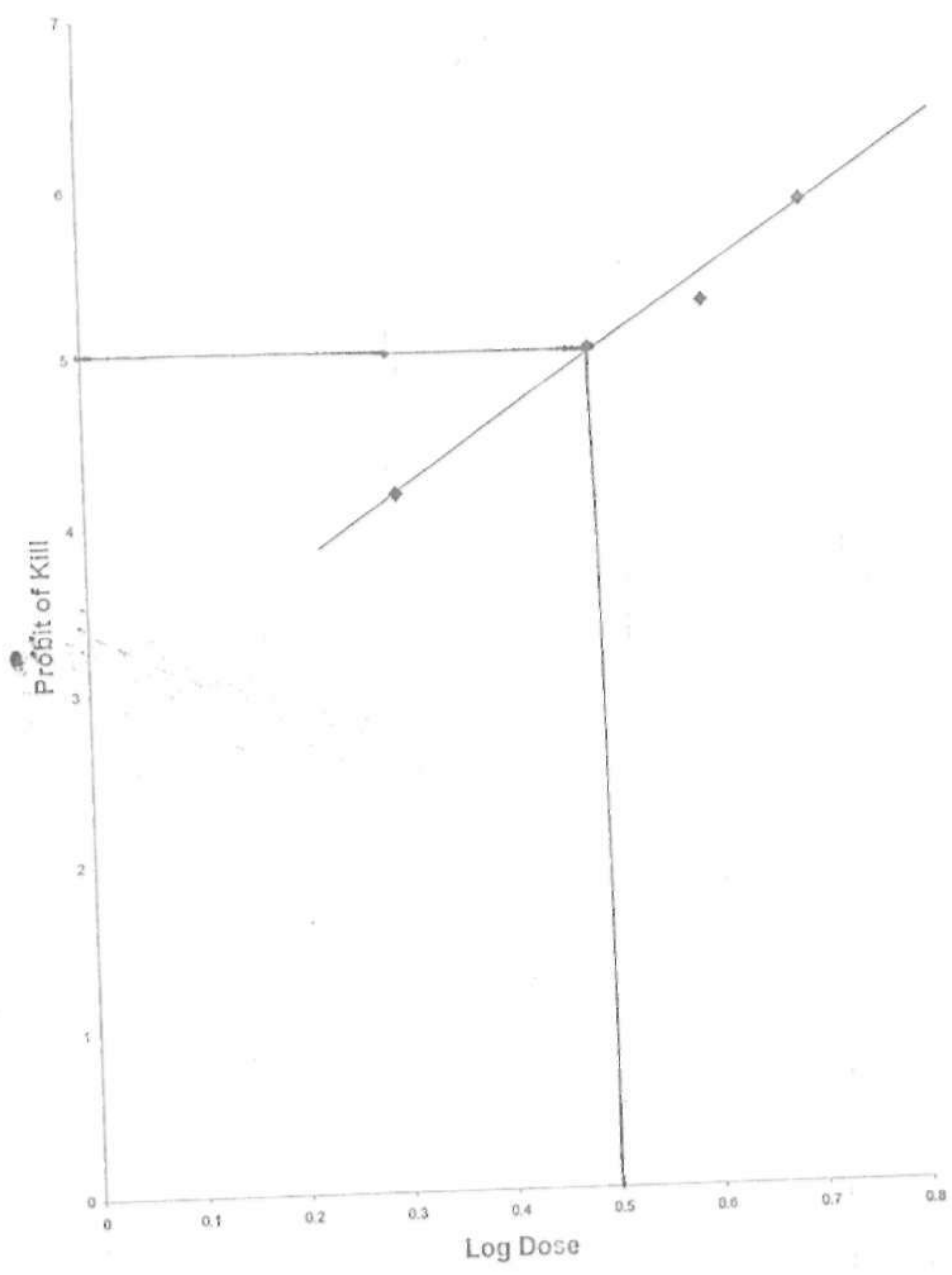


Figure 2: Probit of Kill Against Log Dose for EEVA in normal mice

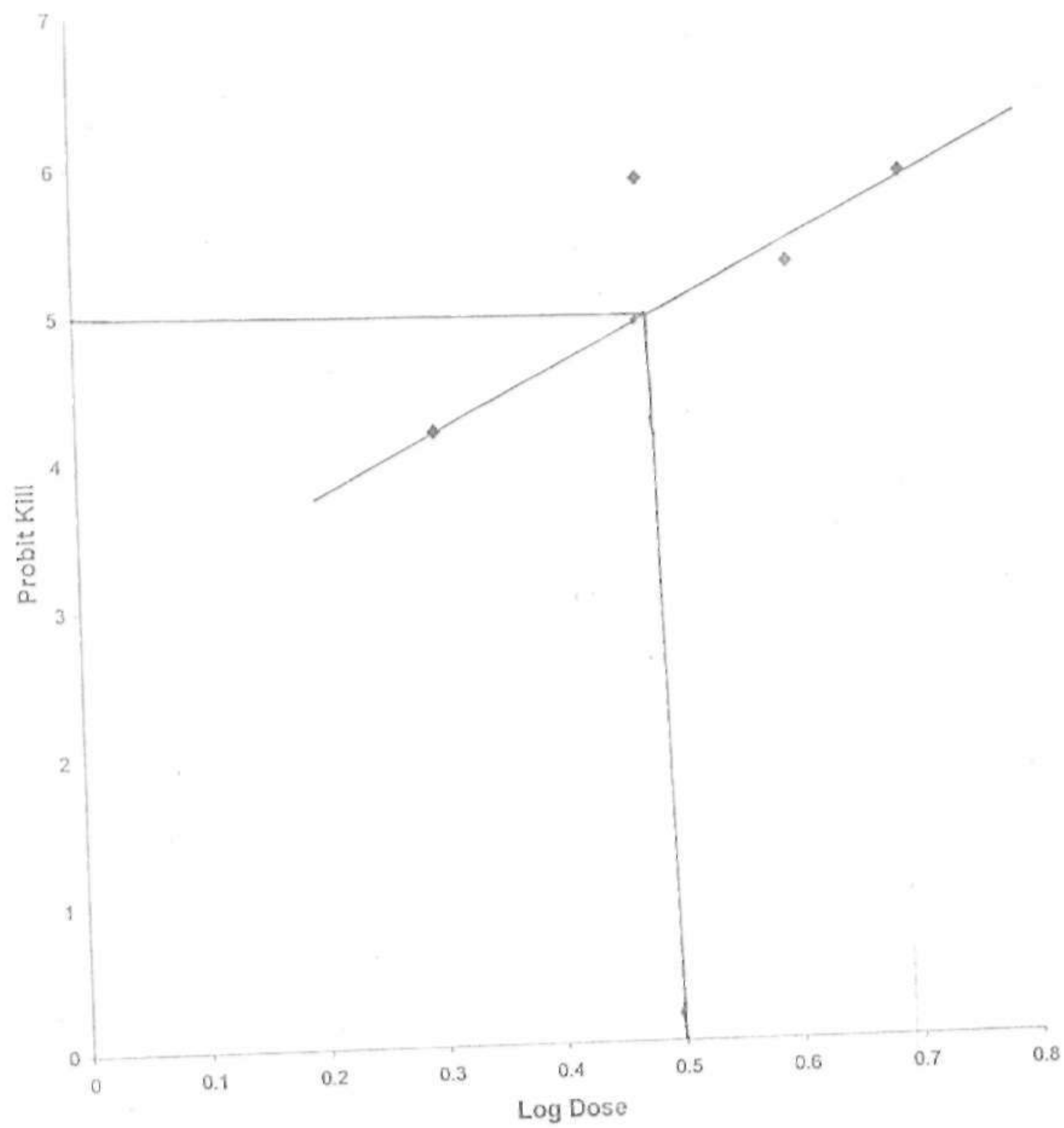


Figure 3: Probit of Kill Against Log Dose for EEEC in normal mice

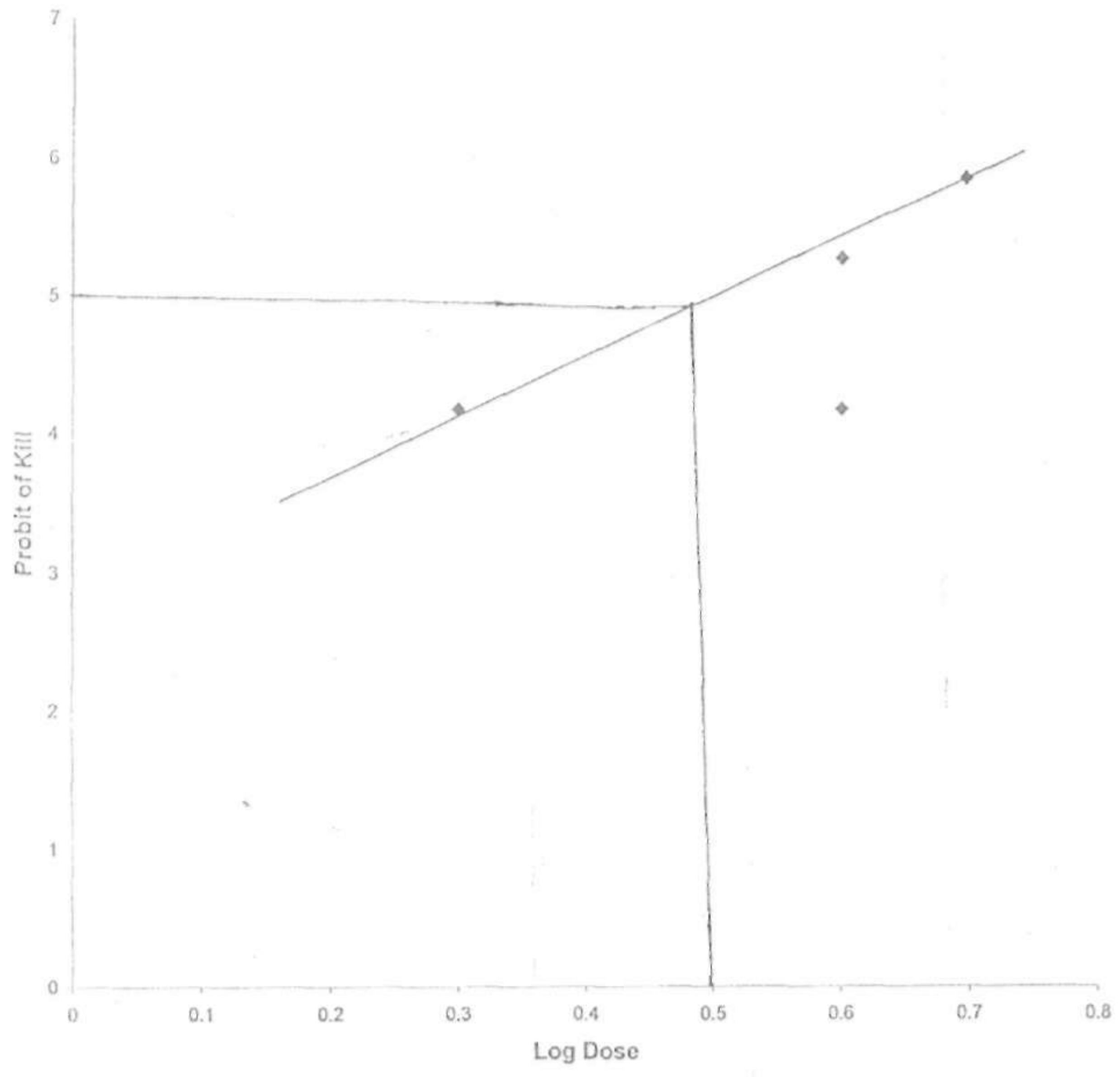


Figure 4: Probit of Kill Against Log Dose for PEVA in normal mice

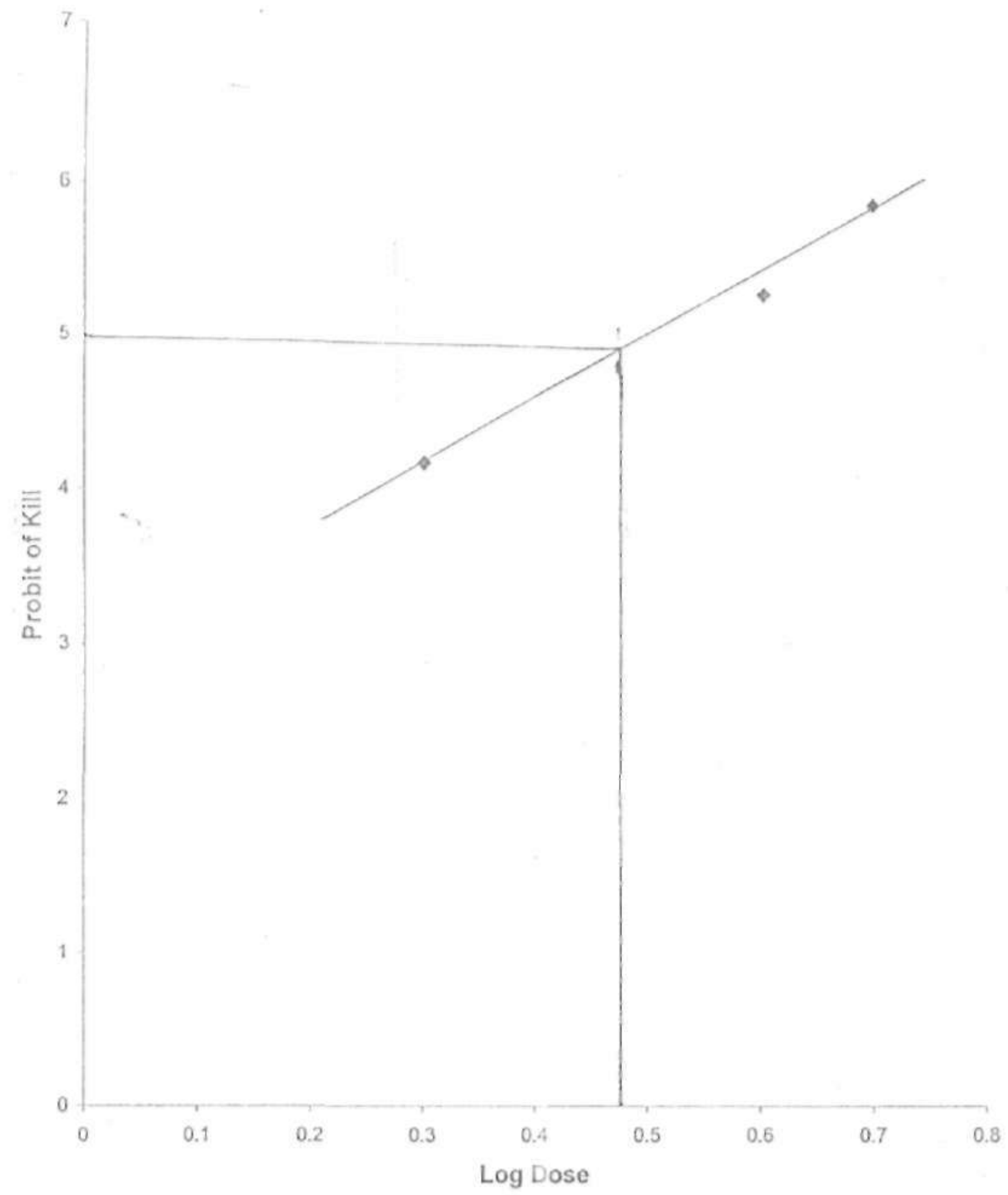


Figure 5: Probit of Kill Against Log Dose for PEEC in normal mice

petroleum ether extract of *Eucalyptus citridora* (PEEC) (Figure 5) had an LD50 of 3.4 mg/kg body weight.

## 4.3 Therapeutic Effect of Extracts

The results obtained (Table 3) show that treatment of infected mice with PEEC was the most potent; it caused a 100% reduction of parasite load (compared with the positive control) representing a total clearance of the parasite with the lowest mean liver weight of 1.10g and a mean worm load of 0.00/g of tissue. Treatment with EEEC caused 70.58% reduction of parasite load with a mean liver weight of 1.84g and mean worm load of 1.5/g. Both PEVA and EEVA reduced the worm load by 72.3% and 82.61% resulting in a worm load of 2.14/g. and 1.5/g of tissue and mean liver weight of 1.15g and 1.60g respectively.

Mice in the positive control (group E, infected but not treated) had 100% infection and a mean liver weight of 2.43g and mean worm load of 5.52/g. The negative control mice Group F, (not infected not treated,) had a mean liver weight of 1.14g. Mice in Group G that were emaciated with lost hair, and almost dying (Plate I) but received therapeutic treatment with PEEC showed 100% recovery, with a mean liver weight of 1.46g and a zero worm load at day 30 post-treatment (Plate II).

Pathologically, the infected not treated mice (positive control) had enlarged fatty, friable and mottled liver. The spleen and kidneys were congested and enlarged with severe enteritis coupled with severe anaemia. Mice treated with PEEC had normal liver, spleen and kidney but showed mild anaemia. Mice treated with PEVA, EEVA, and EEEC showed enlarged heamorrhagic and

**Table 3:** The effect of therapeutic treatment of *Schistosoma mansoni*-infected mice with petroleum ether extract and ethanolic of *Vernonia amygdalina and Eucalyptus citriodora.*

| Mice group | Treatment | Liver Weight (x ± SE) | Mean worm load/g tissue ± SE | % Reduction in parasite load | Pathological sign |
|---|---|---|---|---|---|
| A | PEEC | 1.10 ± 0.00 | 0.00 | 100 | Anaemia normal liver, kidney and spleen |
| B | EEEC | 1.84 ± 0.17 | 1.5 ± 0.32 | 70.58 | Liver dark, friable slightly congested |
| C | PEVA | 1.15 ± 0.37 | 2.14 ± 0.25 | 72.3 | Enlarged haemorrhagic liver, kidney slightly enlarged (bilateral) |
| D | EEVA | 1.60 ± 0.26 | 1.5 ± 1.36 | 82.61 | Liver dark and slight enlarged |
| E | Postitive control (infected not treated | 2.43 ± 0. 18 | 5.52 ± 1.21 | Nil | Enlarged fatty, friable and mottled liver, spleen and kidneys congested and enlarged. Anaemic carcass intestine with enteritis, heart normal. |
| F | Negative control (not infected not treated | 1.14 ± 0.05 | Nil | Nil | All organs normal, No gross lesions. |
| G | PEEC | 1.46 ± 0.00 | 0.00 | 100 | All organs normal, No gross lesions |

Plate 1: *Schistosoma mansoni* infected mouse emaciated and showing loss of hair (alopecia).

Plate II: Mouse treated with PEEC recovering from infection and loss of hair.

Plate III:Pathological effects of *Schistosoma mansoni* infection in mice

- Normal liver (NL) and spleen (NS) (Group 1)
- Fused, fatty enlarged Liver (FEL) (Group 2)
- Enlarged dark spleen (ES) and liver (EL) (Group 3)

friable dark liver, enlarged spleen, enlarged kidney and slightly anaemic carcass (Plate III). The negative control (Group F) had normal organs with no gross lessions (Plate III ;Table 3).

When the results of the treatments and control were subjected to analysis of variance (ANOVA), there was significant difference between them ($P < 0.05$). There was however no significant difference between the treatments ($P > 0.05$).

## 4.4 Prophylactic Effects of Extracts

Mice prophylactically treated with extracts showed different degrees of infection. Treatment with PEEC and EEEC did not inhibit infection as all the treated mice developed infection showing a mean worm load of 5.60/g and 7.0/g, mean liver weight of $2.5 \pm 0.18$ and $1.45 \pm 0.03$ with a zero degree reduction of parasite load respectively (Table 4). Pathologically, they had enlarged mottled and eutrophied liver with enlarged spleen fused with the stomach, kidney congested and enlarged, intestine with interitis ( (Plate III).

Prophylactic treatment with PEVA reduced infection by 34.06%. The infected mice showed mean liver weight of $1.89 \pm 0.13$ and mean worm load of 5.0/g of tissue. Treatment with EEVA protected 44.57% of the mice against developing infection. Infection in the rest of the mice was accompanied by a mean liver weight of $1.89 \pm 0.1$ and a mean worm load of $4.7/g \pm 1.71$. Mice in group T (control) had all organs normal with no gross lesions.

There was no statistical significant difference between the liver weights of the treated and control ($P > 0.05$). There was also no significant difference between the treatment ($P > 0.05$). Reduction of worm load by PEVA and EEVA was significant ($P<0.05$) in comparism to the worm load of the positive control.

Table 4: The effect of prophylactic treatment with petroleum ether extract and ethanolic extract of *Vernonia amygdalina* and *Eucalyptus citriodora* on *Schistosoma mansoni* infected mice.

| Mice group | Treatment | Mean liver weight (g) ± SE | Mean worm load (g/tissue ± SE) | % Reduction of parasite load | Pathological sign |
|---|---|---|---|---|---|
| P | PEEC | 1.45 ± 0.03 | 7.0 ± 0.01 | 0.0 | Liver grossly mottled enlarged spleen fused with stomach |
| Q | EEEC | 2.5 ± 0.18 | 5.60 ± 1.36 | 0.0 | Liver is firm and eutrophied with marked diffused necrotic areas and fibrosis with adhesion to stomach spleen enlarged, kidneys congested and enlarged intestine has enteritis. Lung and heart were normal. |
| R | PEVA | 1.89 ± 0.13 | 5.0 ± 1.46 | 34.06 | Anaemia, enlarged liver with diffused necrotic areas, spleen enlarged and congested, kidneys congested, intestine with enteritis. |
| S | EEVA | 1.89 ± 0.01 | 4.7 ± 1.71 | 44.57 | Anaemic, enlarged liver, spleen kidneys with severe lesions. |
| T | (Control) | 1.14 ± 0.05 | Nil | Nil | All organs normal, No gross lesions. |

## CHAPTER FIVE
## DISCUSSION

In this study, the nine leaf water extracts exhibited some degree of cercaricidal effects on the *Schistosoma mansoni* cercariae with the water extract of *Allium sativum* (WEAS) having the highest degree of killing activity.

This result showed that there is an inverse relationship between the time for 100% mortality of the schistosome cercariae and the concentration of the leaf water extracts. This means that the more the concentration, the faster the killing time. This result agrees with Akubue (1997) that noted that there is a relationship between the effect of a chemical and dose or quantity administered: the greater the quantity (dose) the greater the effect.

In the light of the recorded cercaricidal effects of these crude water extracts at the various concentrations, the plant extracts could be explored for further possible use in the control of the cercariae in water (ponds) with a view of eliminating or reducing the prevalence of schistosomiasis in endemic areas; the effects of the extracts on non-target organisms will need to be ascertained first.

The therapeutic experiment showed that both PE and EE of the plants can halt Schistosome infection significantly in mice. The PEEC showed greatest potency against the disease having eliminated by 100% the schistosome load in mice. The mean worm load in the treated mice was significantly less than in the infected non-treated mice. The novelty of PEEC that confers the strong schistosomicidal activity would require investigation in order to determine the

bioactive components of the plant. However, it is worth noting that the water extracts of the Eucalyptus plant exhibited low cidal action against cercariae.

It was also observed that the chronic phase of infection in both positive control (infected not treated) mice were similar to the infected but treated mice and the observable symptoms were loss of appetite, loss of weight (emaciation), anaemia, dullness and loss of hair (alopecia), (Plates I and II) similar to that documented by Litchtenberg (1987).

Similarly, they showed various pathological symptoms of enlarged, dark and friable and fatty liver with areas of necrosis as well as enlarged spleen and congested kidneys. This agrees with that documented by Goez, *et al.*,(1991). Gerald and Larry (1981) reported that enlargement of the spleen could cause growth retardation. According to the reports of the quantitative autopsy studies of Smith and Christie (1986), there is a consistent correlation of high organ egg loads with severe organ pathology. The observed loss of weight in the infected mice and emaciation observed could be as result of impairment of feed conversion efficiency as noted in trypanosomosis (Audu, et al.,1995). This situation may have been different if enough recovery time was given for the drug to act so that the mice regain a stable condition before terminating the experiment.

The prophylactic activities of the PE and EE of all leaf crude extracts used showed that the administration of a single dose of the extracts before inoculation with cercariae was not sufficient to protect the mice against the infection. However, the level of infection varied between the various treatments. A significant reduction of infection was noticed in mice administered EEVA (44.57% reduction) and PEVA (34.06% reduction). While the administration of

PEEC and EEEC caused no reduction. The mean liver weights of the treatment groups and control were not significant, however there was significance in the mean worm load of the mice groups. It was also observed that the pathological signs among the groups were similar and the symptoms were enlarged mottled liver and spleen, intestine with interitis. This apparent low or lack of prophylactic action could be because the single dose administered was inadequate to protect the mice.

Taken together, there may be pharmacokinetic and pharmacodynamic reasons for the activity of the crude drugs. According to Gustafsson, *et al.* (1987), variablity in drug response can be of four types: variation in absorption, distribution, metabolism, excretion (biliary or renal) of the drug. The gastro intestinal uptake of drugs may be decreased by rapid intestinal transit time, as in diarrhoea. Lipophilic drugs like praziquantel and ozamniquine do undergo metabolic transformation to more polar compounds before renal and or biliary excretion is possible. Since the crude extracts used in this work have not yet been refined, to identify their bioactive components, definite statements can not be made on the results observed. Seymour and Walton (1988) reported that drug-drug interaction effect may modify the effects of another drug at the time of absorption, as the drug is distributed throughout the body, or as the drug is metabolized. A classic example of such an interaction is the increased absorption of sympathomimetic amines, which occurs in the presence of monoamine oxidase inhibitors (Seymour and Walton, 1988). In addition, Eisenach (1999) reported that combination therapy is effective for the treatment of pain. The rational for such combination is that the drugs may work by acting at different sites or through different mechanisms, consequently resulting in the

use of smaller doses of each component, with fewer side effects. Traditional health practitioners in Nigeria treat with herbs in a concocted form. They too believe that such a combination may potentiate the other or they may act in synergy. Therefore, it is possible that a combination of these extracts with one another or other extracts may promote their therapeutic and prophylactic action against the human *Schistosoma mansoni* type infection in mice.

In conclusion, this study has shown that the plants tested have the potentials to kill cercariae *in vitro* and to cure mice experimentally infected with human schistosome type cercariae. It is worth carrying out further studies on their bioactive components towards understanding the kinetics of their action. This should aid further work on the development of drugs for the control of schistosomiasis particularly here in Nigeria, given too, that these plants are in abundance in our environment.

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