PLANT MOLLUSCICIDES FOR SNAIL CONTROL IN THE SOUTH AFRICAN CONTEXT

By

TANZA E. CLARK

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FRONTISPIECE

Haematuria, the classical symptom of infection for *Schistosoma haematobium* (a seven year old child, Mtwalume, KwaZulu-Natal, South Africa, July 1993).

" George Bernard Shaw wrote in *Man and Superman*, 'The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore, all progress depends on the unreasonable man.' Rural health needs to be 'unreasonable'. We need to step outside the facility or provider-driven models to search for new solutions."

Robert T. Van Hook
Foreword to *Rural Health Care*
(Straub and Walzer, 1992)

PREFACE

The experimental work described in this thesis was carried out in the Department of Zoology and Entomology, University of Natal, Pietermaritzburg, from January 1992 to December 1994, under the supervision of Professor C.C. Appleton and Professor S.E. Drewes.

These studies represent original work by the author and have not been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it has been duly acknowledged.

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ABSTRACT

Despite more than half a century of international research on schistosomiasis control, this disease remains a public health concern in many Third World countries. Four to five percent of the world's population is estimated to be infected. In South Africa, bilharzia is prevalent in rural communities which lack piped water and adequate sanitation. Transport and treatment costs limit access to Western medication.

In the last decade, plant molluscicides have received considerable attention in the ongoing search for cheaper alternatives to chemotherapy and synthetic molluscicides. Plant molluscicides may be locally harvested, extracted and applied for the control of the snail host. This approach is based on a philosophy of self-reliance and self-determination. However, such involvement presupposes recognition of the infection as a real problem. Before selecting and evaluating plants for molluscicidal activity, it was first necessary to establish the status of schistosomiasis in rural communities, and the willingness of community members to become involved in control efforts.

Field surveys showed that rural South African communities do not share the indifference of the past health-care system towards schistosomiasis in this country. Concern for the disease was matched by a prevalence of 75.14% for *S. haematobium* in the study area.

The potential of the South African flora to provide suitable molluscicidal candidates has never been systematically assessed. As random screening is costly and is historically of limited success, a need has existed for the development of an objective selection procedure. A simple scoring system was devised, based on the criteria for "good" plant molluscicides as defined by the World Health Organization (WHO). Three plant species were subsequently selected for further intensive evaluation: *Warburgia salutaris*, *Gardenia thunbergia* and *Apodytes dimidiata*. Initially, the activity of crude aqueous suspensions of all three candidates was investigated, since this application form is the most practical for use in a rural situation. Further, the stability of extracts under changing physical and chemical conditions was assessed, as were the effects of molluscicides on a wide range of

non-target fauna and flora. *Gardenia thunbergia* ranked highest overall with respect to the toxicity of aqueous suspensions, cultivation potential, medicinal value, stability, and toxicity to non-target organisms.

Studies on the mode of action of the three plant extracts revealed that death occurred via distinct physiological pathways; this despite the similarities in clinical symptoms. Such is the complexity of snail physiology that until more intensive neurological investigations are undertaken, the mechanisms of molluscicidal action can only be inferred.

Chemical compounds responsible for activity were identified from *Apodytes dimidiata*; genipin, a monoterpene, was one such active constituent. Although not previously recorded as molluscicidal, earlier workers have isolated genipin from *Genipa americana* and *Gardenia jasminoides*. Notably, this compound has not yet been isolated from *Gardenia thunbergia*. Further activity in *A. dimidiata* extracts appeared to be the result of a synergistic effect, potentially involving (S)(-) ethyllactate.

Research on mammalian toxicity, and registration procedures for plant molluscicides in South Africa should be prioritized for further investigation, before pilot field trials are initiated. Almost thirty years have passed since the first reports on the activity of *Phytolacca dodecandra*. Although more than 1000 plant species have since been tested world-wide, it appears that no plant molluscicide has ever been endorsed by the WHO. More concerted efforts are necessary to ensure that appropriate molluscicidal technologies are provided to infected communities.

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GENERAL INTRODUCTION

Schistosomiasis (bilharziasis) is one of the most important public health issues for rural and agricultural communities living near slow-moving water in the tropics and sub-tropics. Frequently quoted estimates place the overall prevalence of infection at 200 million people in 73 countries (McCullough and Mott, 1983), or approximately 4 - 5% of the world's population (Basch, 1991). Five to six hundred million people are at risk of infection (Bergquist, 1990). In most endemic areas, population growth has resulted in an increase in the 5 - 15 year old age group which is also known to have the highest prevalence of infection, and to be most responsible for transmission through indiscriminate urination (Rollinson and Simpson, 1987; Webbe, 1987a; Chandiwana *et al.*, 1988; Nelson, 1990; Mqoqi, 1991). In 1987, 40% of the Black South African population was under the age of 15 years (Evans *et al.*, 1987).

Africa currently has the highest human population growth rate (Hunter *et al.*, 1993). A result of growing population numbers is an increased demand for water, and hence the intensification of water resources development programmes. Along with the development of impoundments and irrigation schemes has come an expansion of habitats favourable for numerous parasite infections, including the aquatic intermediate snail hosts of schistosomiasis. Human disease-risks are known to increase with an intensification of water-related activities, such as fishing, irrigation and domestic tasks (Hunter *et al.*, 1993). In this way schistosomiasis is spread into new areas.

There are 11 species of schistosomes of the genus *Schistosoma* in Africa (Basch, 1991), three of which infect man in South Africa (Pitchford, 1980; Van Wyk, 1983):

Schistosoma haematobium (Bilharz)

Urinary schistosomiasis occurs exclusively in man, although one naturally infected rodent and one buffalo have been reported (Pitchford, 1980). Its intermediate snail hosts are *Bulinus africanus* (Krauss) and *B. globosus* (Morelet).

S. mattheei Veglia and Le Roux

An intestinal parasite of game species, domestic stock and some rodents and primates. It occurs quite commonly in man in either the urinary or intestinal tracts. Infection is invariably mixed with *S. haematobium* and/or *S. mansoni* (Pitchford, 1980). *S. mattheei* shares the same intermediate snail hosts as *S. haematobium*.

S. mansoni (Sambon)

Intestinal schistosomiasis, occurring primarily in man. It has been found in other primate species, and quite commonly in rodents (Pitchford, 1980). Intermediate snail host: *Biomphalaria pfeifferi* (Krauss).

Morbidity due to infection is reputedly outranked only by malaria, respiratory infections, diarrhoea and measles (Stephenson and Holland, 1987). The relationship between morbidity and infection is however, extremely complex. Parasite virulence varies geographically, and appears to be linked to the genetic and immunological characteristics of the local population (Gryseels, 1992; Huang and Manderson, 1992). Details of this complex relationship are discussed later. Generally speaking, acute infection with *S. haematobium* is characterized by haematuria, and frequent and painful urination. These symptoms are not exclusive to schistosomiasis; haematuria occurs for, among others, bladder infections, kidney disorders and infective hepatitis (Department of National Health and Population Development, 1991). Chronic infection may result in bladder cancer, urinary tract infection and subsequent kidney failure. Acute infection with *S. mansoni* is not easily distinguishable from other dysenteric infections, since diarrhoea with or without blood is characteristic of infection. Chronic infection may result in ascites, liver damage and jaundice (Bello and Edungbola, 1992). The effect of *S. mattheei* is difficult to define since it usually co-occurs with the abovementioned schistosomes in the urinary or intestinal tract, but may be presumed to be similar.

PREVALENCE AND INTENSITY OF INFECTION IN SOUTH AFRICA

Schistosomiasis is an ancient infection. Historical reviews include references to the disease in Egyptian papyri, Babylonian inscriptions, medieval medical literature and to the Chinese "water poison disease" of the seventh century (Ansari, 1973; Malek, 1980). In 1851 a young German pathologist, Theodor Bilharz, discovered the helminth responsible for transmission whilst performing an autopsy in Cairo. The contributions of Manson, Sambon and Leiper during the late 19th and early 20th century led to the identification of distinct urinary and intestinal forms. In 1954, the generic name *Schistosoma* was approved by the International Commission of Zoological Nomenclature (Malek, 1980).

The history of the discovery of schistosomiasis in South Africa has been reviewed by Bhagwandeen (1968) and Gear and Pitchford (1977). The first discovery of *S. haematobium* was made in 1864 by Harley. Eggs of the parasite were found in the urine of patients living in Uitenhage and Port Elizabeth on the Eastern Cape coast. Numerous

reports of infection followed and by 1872 schistosomiasis was recognised as endemic to the regions known then as the eastern Cape and Natal. By 1890, widespread records had been received from the western Transvaal. The first records of *S. mansoni* by Turner in 1908 came from the eastern Transvaal and Natal. Veglia and Le Roux were then responsible for the discovery and description of *S. mattheei* from sheep, again in the Eastern Cape. Porter (1938) later defined the geographical distributions of the intermediate snail hosts for schistosomiasis in South Africa.

On the 10th of March 1967, a parliamentary report estimated three million people to be infected in South Africa (Pitchford, 1980). Recent estimates of the prevalence of *S. haematobium* and *S. mansoni* in man are shown in Figure 1. The distribution of *S. mattheei* follows that of *S. haematobium*. The data used for constructing these figures were derived from Gear *et al.* (1980) and Van Eeden *et al.* (1982). The "Bilharzia Atlas" is the only comprehensive compilation of prevalence data for South Africa.

Estimates for neighbouring states are 150 000 infected people in Swaziland (Webbe, 1987a) and two million in Zimbabwe (Ndamba *et al.*, 1989). In the latter country, more than 50% of the population who live in highly endemic zones are infected with *S. haematobium* (Chandiwana *et al.*, 1991).

In this investigation, particular attention has been given to the province of KwaZulu-Natal on the east coast of South Africa. For this region, the first assessment of estimates of infection with *S. haematobium* were made by Bhagwandeen (1968). He estimated the prevalence of infection to be 30% for the Black and Indian populations of Durban. More recent estimates have placed prevalence at between 25 and 50% inland, and in excess of 70% along the coast (Gear *et al.*, 1980; Schutte *et al.*, 1981; Cooppan *et al.*, 1986; Mqoqi, 1991; Mqoqi and Dye, 1992).

Cooppan *et al.* (1986) observed that within endemic areas in South Africa, prevalence rates of less than 5% occur where there are piped water supplies, and good recreation and sanitary facilities. Prevalences of greater than 60% have been found in children living in vast rural areas which are ill-provided with piped water, recreation facilities and sanitation. Schistosomiasis in South Africa is therefore largely a problem for rural Black communities.

The scale of the "problem" has been heavily debated since considerable geographical variation in morbidity has been recorded, even at a local level (Chen and Mott, 1989; Tanner, 1989a). In a given community, the intensity of the majority of infections are low and the clinical effects are said to be "mild". Few have high schistosome burdens and hence suffer "serious" morbidity (Hoffman *et al.*, 1979).

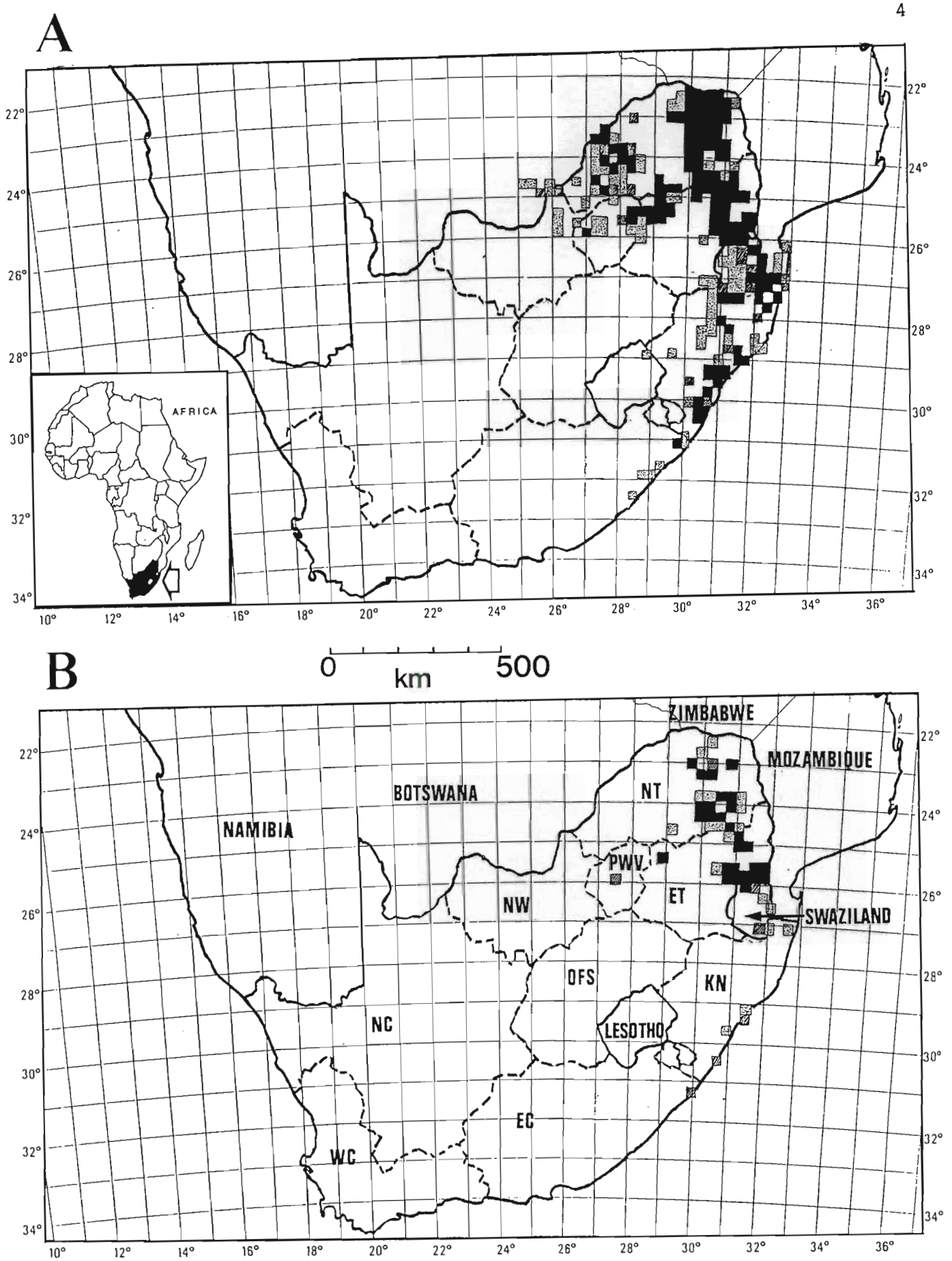


Figure 1 Prevalence of infection with *Schistosoma haematobium* (A) and *S. mansoni* (B) in South Africa (25 - 50% positive, 51 - 70% positive, > 70% positive). Dashed lines indicate provincial boundaries. Provinces are abbreviated as follows: NC = Northern Cape, WC = Western Cape, EC = Eastern Cape, OFS = Orange Free State, NW = North-West, NT = Northern Transvaal, ET = Eastern

Attempts to define the scale of the problem in terms of economic loss have been unsuccessful. This stems from difficulties in measuring lost productivity due, for example, to lethargy (Herrin, 1986).

MORBIDITY ASSOCIATED WITH SCHISTOSOMIASIS

Clinical reports from Egypt and east Africa have described high morbidity and considerable mortality due to schistosomiasis, whereas those from south and west Africa have not (Chen and Mott, 1989; Gryseels, 1989; 1992). In northern Brazil, Egypt, and Sudan, the rural inhabitants claim that their ability to work is severely reduced by weakness and lethargy caused by the disease (WHO, 1990). Since approximately only half of the eggs laid by mature female schistosomes are released from the host, the remainder become embedded in body tissues. One reason for the apparent tolerance to infection is the slow process of tissue damage, for the infected individuals often do not fully recognize the imposed abnormality until irreversible destruction of the liver and kidneys has occurred (WHO, 1989). In Africa, schistosomiasis *haematobium*-linked bladder cancer is 32 times more prevalent than the incidence of simple bladder cancer in the USA (WHO, 1990).

Although the degree of pathology encountered in this country has been highly variable, it largely contrasts with the morbidity described for other countries (Cooppan *et al.*, 1986). Pijper (1934), Cooppan *et al.* (1986), Mqoqi (1991) and Mqoqi and Dye (1992) all gave the impression that morbidity due to infection is low. This, coupled with low mortality, was considered to have little effect on the quality of life of those living in endemic regions. Haematuria was reported to be accepted as a normal part of life. It is significant that these observations were not supported by accompanying knowledge, attitudes and perception studies.

Quite in contrast to their suggestions are the clinical and pathological observations of Bhagwandeen (1968) who highlighted the threat of severe uretic and hepatic damage as a result of infection.

Further debate centres on the impact of infection on both scholastic achievement and activity in children (Haycock and Schutte, 1983). Kvalsvig (1981a; 1981b), Haycock and Schutte (1983), Kvalsvig *et al.* (1991a), Huang and Manderson (1992) and Kvalsvig and Connolly (1993) reviewed these effects. Opinions vary widely; this may be due to the confounding effects of other parasitic infections and social factors (e.g. nutrition) on measures of scholastic achievement or energy. Differences in experimental design may also account for discrepancies in findings.

The current state of ambivalence in South Africa towards the significance of the effects of schistosomiasis is reflected in the following observations:

- The closure of the Medical Research Council's schistosomiasis research effort (within its own institutes) in 1992, and the absence of a national control programme for the disease.
- The non-availability of Bayluscide® in South Africa (C.M. Fourie, Bayer, Johannesburg, *pers. comm.*, 1992). The wettable powder formulation can be imported on request but was sold in 1990 for R66/kg (± US$18.86/kg) (A.C. Evans, Medical Research Council, Nelspruit, *pers. comm.*, 1992).
- Biltricide®, a safe oral antischistosomal drug, is available on prescription from pharmacies. Dosage is dependant on the body mass of the patient. In 1993, treatment costs amounted to R2.67/kg body mass (± US$0.77/kg body mass) when bought from an urban pharmacy.

In the absence of a national control programme which could fund or initiate control measures, the costs of control and treatment are prohibitively expensive. Political change in South Africa over the last five years has harnessed considerable social energy. Together with changes in health priorities, the effects of schistosomiasis have been largely unmonitored.

Factors such as malnutrition, schistosomiasis and the presence of other parasites should be viewed in terms of their cumulative effects on children and adults. Despite dissent over the effects of schistosomiasis, these factors in combination must be expected to have some social and economic impact. Curing man of illness, and preventing suffering and death, is considered a basic moral imperative (Ukoli, 1984; Stephenson and Holland, 1987).

DISEASE CONTROL

Control options available for schistosomiasis include: chemotherapy and mollusc control using molluscicides, and ecological and biological control methods. Chemotherapy, followed by molluscicide use, is in many cases the most cost-effective control method available. Ecological control of the intermediate snail hosts, achieved through habitat alteration (Bradley and Webbe, 1978), is labour intensive and usually applied in combination with either or both chemotherapy and molluscicide use (Malek and Cheng, 1974). Biological control, exploiting complex ecological relationships between snails and various predators, parasites and competitors (Michelson, 1957; Malek and Cheng, 1974;

Jordan, 1985; Van Schayck, 1986; Madsen, 1990), has not been successful in producing widely applicable solutions. The search for a vaccine is ongoing but will likely remain an elusive prospect in the next decade (Bergquist, 1990).

Oral antischistosomal drugs

Antimonials were introduced in the early twentieth century as one of the first treatments for schistosomiasis (Shekhar, 1991). Administered intravenously, they produced severe side-effects and were replaced by hycanthone mesylate (ETRENOL®, Winthrop) and lucanthone. Hepatotoxicity and gastrointestinal disturbances led to the withdrawal of these drugs. A generation of orally administered drugs which succeeded them included: oxamniquine (VANSIL®, Pfizer Inc.), metrifonate (BILHARCIL®, Bayer A.G), praziquantel (BILTRICIDE®, Bayer A.G) (Lemma *et al.*, 1979; Chandiwana and Taylor, 1990; Shekhar, 1991). Although praziquantel is currently the treatment of choice for most trematode and cestode infections, its high cost limits its use in developing countries (King and Mahmoud, 1989). One of the major advantages of chemotherapy is that it reduces the number of eggs entering the environment, although reinfection is a reality (Webbe, 1987b). Drug therapy is likely to remain a major means of control (Duncan, 1985). However, in most African countries only 20% of inhabitants have access to modern health care facilities and drugs. This coverage is expected to decrease in accordance with the upward trend in population growth and declining economic conditions (Abebe, 1987).

Traditional anthelminthic prescriptions

Traditional plant use is an alternative to western medication (Nyazema, 1987) and more than 75% of the world's population, most of them in developing countries, rely mainly on traditional remedies in health-care (Sofowora, 1982; Balick, 1990; Farnsworth, 1990; Nigg and Siegler, 1992). These remedies are popular because they are readily available compared to western drugs. In addition, the raw materials are often easily obtained, and the products are culturally acceptable.

The potential of higher plants as sources of new drugs is still largely unexplored (Hamburger and Hostettmann, 1991). There are 119 compounds of known structure which are extracted from higher plants and used in western medicine (Farnsworth, 1990). These compounds are produced commercially from only 90 plant species, from a potential of more than 250 000 taxa (Farnsworth, 1990). Only limited research into indigenous plants used as anthelminthics has been conducted. However, lists of African

plants traditionally used in the treatment of schistosomiasis have been published (Kokwaro, 1976; Iwu, 1993; Ndamba *et al.*, 1994). This apparent lack of interest in drug development is discussed further in Chapter 2 (Section 2.4.5). One of the problems with traditional anthelminthic prescriptions is that they frequently treat symptoms (notably haematuria) and not the cause (Marston and Hostettmann, 1987). *Diospyros* species (EBENACEAE), for example, are used as a cure for schistosomiasis in central Africa. They have been shown to reduce blood-loss from the patient but not to affect schistosomes or their eggs (Marston and Hostettmann, 1987). Considerable potential exists for further investigation.

Synthetic molluscicides

The intermediate snail host has been considered to be one of the weakest links in the life cycle of the parasite (Ritchie, 1973; Malek and Cheng, 1974). During the 1960s, molluscicides were regarded as the only reliable approach to the control of schistosomiasis (Ritchie, 1973). It now is generally recognised that the successful control of the disease should be based on an integrated approach, including the control of the snail (Mott, 1987a). Molluscicide is, however, still considered the most important means of control where the volume of water *per caput* at risk of infection is small, i.e. well suited to arid areas where transmission is confined to small seasonal habitats (Webbe, 1987a; Madsen, 1990). Molluscicides may additionally contribute to the control of aquatic and amphibious snail hosts of *Fasciola* (liver fluke) and *Paramphistomum* (conical fluke) (McCullough and Mott, 1983; WHO, 1983; Combes and Cheng, 1986).

Numerous synthetic molluscicides have been used in the control of the intermediate hosts of schistosomiasis. These include: organotins, copper sulphate ($CuSO_4$), sodium pentachlorophenate (NaPCP), N-tritylmorpholine (Trifenmorph or FRESCON®) and niclosamide (BAYLUSCIDE®). Webbe (1987a; 1987b) reviewed the early use of molluscicides. Further discussion of their properties has been covered by Malek and Cheng (1974), Malek (1980), WHO (1983) and Webbe (1987a; 1987b).

Niclosamide is the only commercially available molluscicide still recommended for schistosomiasis control. It was synthesized in 1958 (Andrews *et al.*, 1987) and registered for use in South Africa in 1982 (Kvalsvig, 1986). Although highly effective, toxicity to non-target organisms and high importation costs have led to concern regarding its use, particularly in developing countries (WHO, 1982; 1983; Kloos and McCullough, 1987; Lambert *et al.*, 1991).

Thomas *et al.* (1986) have investigated the potential for the incorporation of short-chain carboxylic acids, which serve as potent attractants, into slow-release molluscicide formulations. Consumer costs are again likely to be inhibitive, and there has been no further development of these ideas.

No new outstanding, novel, synthetic molluscicide has been developed in the past decade. Industry has claimed that this is due to high development costs and the lack of an assured market (Jordan and Webbe, 1982; Webbe, 1987a). The high cost of control measures has simply deterred control operations and made more urgent the need for development of less costly technology (Webbe, 1987a). No drug or chemical molluscicide will be successful, no matter how safe or effective, unless it is cheaply available. Finances are one of the most fundamental factors in controlling disease in third world countries (Hostettmann, 1984). The need for less costly control operations and the pioneering research efforts of Akililu Lemma on endod from *Phytolacca dodecandra*, has resulted in a surge of interest in plant molluscicides - plant secondary compounds which on extraction from plants kill the freshwater intermediate snail hosts of schistosomiasis.

Plant molluscicides

A review of plant molluscicide research is given in Chapter 2 so that the intention here is simply to discuss the philosophy behind their use.

Bioactive molecules occur in plants primarily as secondary metabolites. Their production may be linked to chemical defences against herbivory, fungal attack, and microbial and viral infection (Cox, 1990). In short, plants offer a wide array of compounds, which on extraction from the plant may show insecticidal, piscicidal, molluscicidal, medicinal and other biological activities (Watt and Breyer-Brandwijk, 1962; Cheeke, 1990). Plant compounds can either be extracted directly and used in their natural state, or serve as models for pharmaceutical chemists to synthesize identical compounds or analogs (Cavé, 1986). One of the possible benefits of the use of indigenous plant compounds in their natural state is that they may provide economic advantages which outweigh the relatively higher activity of better characterized but more costly synthetics (Hoffman *et al.*, 1979). Infected communities are more likely to accept the use of local indigenous plants since they are familiar with their properties and growth characteristics. There is likely to be greater incentive to use and promote the cultivation of plants which have more than one local application (e.g. as medicine, food, fuel, for construction and in spiritual healing).

If molluscicidal compounds which occur in indigenous plants can be extracted using local labour and simple technology, and if these metabolites are sufficiently toxic, as well as ecologically sound, then it should be possible to develop culturally acceptable and inexpensive molluscicides (Kloos and McCullough, 1987). This supposition forms the basis of this thesis. Although not a new idea, the concept of the use of plant molluscicides is particularly pertinent to the South African context where schistosomiasis control is excluded from the National Health Budget. There are numerous other health issues equally deserving of such attention; nutrition and other helminth infections are not the least among these. Befidi-Mengue *et al.* (1992) have shown that the inter-relationships between these factors may be complex. Growth impairment of children, due to malnutrition and inadequate food supplies, is significantly exacerbated by parasite infections such as schistosomiasis.

Health solutions which harness the energies of the community and promote a philosophy of self-reliance whilst reducing the impact of the infection deserve urgent promotion to ensure that all communities have a fair chance of a healthy existence. This should occur without unduly pressurizing an already overburdened health system.

OBJECTIVES

The following factors were the foci of attention in this thesis, and are among many which require investigation before plant molluscicides can be promoted for use in South Africa:

1. **Would rural South Africans use plant molluscicides?**

   Control operations cannot be implemented in a social vacuum. Cultural attitudes and other health priorities will have a major impact on the success of these programmes. Plant molluscicides require intensive community involvement in growing and harvesting plants, and preparing and applying plant extracts. If schistosomiasis is not a primary community concern, then little effort will understandably be spared in such intensive control measures. It is therefore necessary to first understand how communities perceive schistosomiasis and compare these perceptions with actual levels of prevalence and intensity in the community. Secondly, on the basis of these results, to assess the likelihood of the community becoming actively involved in the use of plant molluscicides for snail control. The results of an investigation of this kind in a rural South African community are presented in **CHAPTER 1**.

2. **What potential does the South African flora offer for the use of plant molluscicides?**

Little is known of South African molluscicidal plants, other than indirectly through the research efforts of other African nations. Endod from *Phytolacca dodecandra* in Ethiopia is the most noteworthy example. Mass screening and evaluation in search of South African alternatives is both costly and time consuming. Random selection as an alternative does not guarantee success. Data already available in the literature need to be utilised and incorporated into an objective selection process. In this way, taxa known to possess some molluscicidal activity, as well as potentially active species, can be identified. The selection criteria used in the assessment of South African plants, and resultant lists of priority species are given in **CHAPTER 2.**

3. **How active are the aqueous suspensions of selected candidate plants?**

If plants are to be used in rural community situations they should ideally be cultivated (if insufficient material is available in local plant populations), harvested, and applied using simple technology. Application in the form of crude aqueous suspensions is most desirable. The activity of crude aqueous suspensions can be assessed relative to guidelines presented by the WHO (1965). Select active plants can then be prioritized for comprehensive evaluation. **CHAPTER 3.**

4. **How stable are crude aqueous suspensions under changing physical and chemical conditions?**

Numerous physical and chemical factors are known to affect the activity of both synthetic and plant molluscicides. Among the most important are the effects of temperature, sunlight, biodegradation, long-term storage and the use of fresh, as opposed to dry material. **CHAPTER 4.**

5. **What environmental impacts can be expected from the application of plant molluscicides?**

The effect of aqueous suspensions on non-target fauna and flora is a serious consideration. One of the major concerns of the use of synthetic molluscicides is the disruption of freshwater ecosystems through detrimental effects on fish and other aquatic fauna and flora. The broader toxicology of aqueous suspensions is presented in **CHAPTER 5.**

6. What is the mode of action of aqueous suspensions?

Newly identified molluscicidal compounds may reveal new paths of activity which cause death in freshwater snails. Such information, while important to the development of new synthetics, also satisfies academic curiosity relating to how some compounds and not others are able to induce mortality in snails. **CHAPTER 6.**

7. What compounds are responsible for molluscicidal activity?

The identity of molluscicidal compounds is extremely useful in understanding extract stability and toxicicity to non-target organisms. Possibilities for synthesis and subsequent commercial production can also be assessed. An investigation of the chemistry of molluscicidally active crude aqueous suspensions of *A. dimidiata* is presented in **CHAPTER 7.**

CHAPTER 1

SCHISTOSOMIASIS AND THE USE OF INDIGENOUS PLANT MOLLUSCICIDES: A RURAL SOUTH AFRICAN PERSPECTIVE

The content of this chapter has been presented as papers at the Conference for the South African Association for Botanists (University of the Witwatersrand, Johannesburg, January 1994) and at the Conference of the Parasitological Society of Southern Africa (University of Pretoria, Pretoria, July 1994). It has also been submitted to *Social Science and Medicine* (November 1994) under the same title and authored by T.E. Clark[1], C.C. Appleton[1] and J.D. Kvalsvig[2]

1 Department of Zoology and Entomology, University of Natal, Pietermaritzburg.

2 Human Sciences Research Council, Congella, Durban.

HYPOTHESES

i. Schistosomiasis is recognised as a public health problem in rural South African communities

ii. Perceived levels of infection are correlated to actual levels of prevalence and intensity of infection in the community.

iii. Western medication is inaccessible.

iv. Plant molluscicides are culturally acceptable and an attractive alternative to western medication and synthetic molluscicides.

ABSTRACT

In the last decade plant molluscicides have received considerable attention in the search for cheaper alternatives to chemotherapy and synthetic molluscicides in schistosomiasis control. The attraction of a locally grown molluscicidal plant is based on the development of a philosophy of self-reliance and community involvement. This approach is dependant on community recognition of the infection as a public health problem, and their acceptance of proposed control measures. The objectives of this study were firstly, to assess the knowledge of schistosomiasis in a rural community, their attitude to the use of indigenous plant molluscicides and their knowledge of suitable candidates. Secondly, to assess the prevalence and intensity of infection in relation to its severity as perceived within the community.

Study sites were located at Mtwalume (KwaZulu-Natal, South Africa). Sixty-nine

community members were interviewed during four focus-group interviews and two depth interviews. Urine and stool samples (354 and 306 respectively) from children and young adults (2 - 25 years old) were analyzed for parasite infections. Results indicated that despite a poor understanding of schistosomiasis, it is a primary health concern for those dependant on river-water for their water requirements. Concern for schistosomiasis was matched by a prevalence of 75.14% for *Schistosoma haematobium*. Oral antischistosomal drugs are inaccessible primarily due to the cost of transport and secondly, due to the cost of treatment. The concept of molluscicidal control, as an alternative, was enthusiastically received by all respondents. *Warburgia salutaris* and *Apodytes dimidiata,* both potentially suitable candidate molluscicidal plants (see Chapter 3), were known to the community.

1.1 INTRODUCTION

Schistosomiasis remains one of the most important public health issues of the tropics and sub-tropics. A decade ago prevalence was estimated at 200 million people in 73 countries; an additional 500 to 600 million people are exposed to the risk of infection (McCullough and Mott, 1983; Bergquist, 1990). This is likely an underestimation of current infection, given the rising population growth rate and an increase in the five to 15 year old age class which carries and transmits the bulk of the infection (Rollinson and Simpson, 1987; Nelson, 1990).

In South Africa schistosomiasis is restricted to the Eastern and Northern Transvaal and KwaZulu-Natal. The infection is characteristically associated with an absence of piped water, good recreation and sanitary facilities (Cooppan *et al.*, 1986). The foci of endemic areas are therefore expected to be in rural areas. Despite records of prevalence in excess of 70% for large areas of the endemic region (Figure 1, General Introduction) schistosomiasis is not a notifiable disease. Under these circumstances there is no National Control Programme to fund or initiate any control measures. Hence, the extensive use of chemotherapy and/or synthetic molluscicides is cost prohibitive.

Plant molluscicides have received considerable attention in the last decade in the search for cheap, effective and environmentally acceptable alternatives (Kloos and McCullough, 1987). This is particularly true for Africa where the adoption of Western health-care has resulted in an expensive, hospital-based, doctor-dependant, urban-biased system which remains beyond the reach of the rural mass (Good, 1987). The attraction of the use of indigenous molluscicidal plants lies not only in economic benefit but also in the development of a philosophy of self-reliance in rural communities (Hoffman *et al.*,

1979). Plant material would be cultivated, harvested, processed and applied focally to human water contact points using an appropriate form of technology (Ndamba *et al.*, 1989). This is not an unrealistic goal and numerous plant species have undergone systematic field evaluation in other endemic areas in Africa. The most notable work has been done in Ethiopia on *Phytolacca dodecandra* L' Hérit (PHYTOLACCEAE). Other species include: *Ambrosia maritima* L. (ASTERACEAE) (Egypt), *Swartzia madagascariensis* Desv. (FABACEAE) (Tanzania), *Tetrapleura tetraptera* Taub. (FABACEAE) (Nigeria) and *Jatropha curcas* L. (EUPHORBIACEAE) (Sudan) (McCullough and Mott, 1983; Hostettmann, 1984; Duncan and Sturrock, 1987).

The success of such self-help programmes is, however, dependant upon the support of the affected communities. Community willingness to become involved, in turn, presupposes recognition of infection and its effects as a public health problem (Farooq *et al.*, 1966; Kvalsvig *et al.*, 1991b; Huang and Manderson, 1992). The study of community beliefs and habits is vital because these intrinsic elements influence their health and determine their involvement in intervention programmes (Kark and Steuart, 1962; Cohen, 1989). There have been surprisingly few studies (none in South Africa) of local knowledge, attitudes and perceptions of schistosomiasis (Kloos *et al.*, 1986; Huang and Manderson, 1992). Stopforth (1976) recorded that 47 families at Adams Mission (KwaZulu-Natal) considered schistosomiasis to be the most important illness prevailing among children between 6 - 16 years, and Cooppan *et al.* (1986) used a questionnaire to briefly assess its morbidity in Natal. The work of Kvalsvig *et al.* (1991b) on perceptions of cestode and nematode infections and Ndamba *et al.* (1989) on the knowledge of schistosomiasis and the use of *Phytolacca dodecandra* in Zimbabwe, have provided the basis for this investigation into the South African situation.

The following objectives were set:

i. To assess rural peoples' knowledge, attitudes and perceptions of schistosomiasis, its transmission, and current control measures (including the concept of molluscicidal control).

ii. To assess prevalence and intensity of infection in relation to its perceived severity.

1.2 MATERIALS AND METHODS

1.2.1 STUDY AREA

The study was conducted at Mtwalume (3030BC, KwaZulu) during May - August 1993 (Figure 2). At this time the provinces of Natal and KwaZulu were under separate administrations.

Mtwalume is a rural area with no formal sanitation provisions. Fines can theoretically be levied by tribal authorities for those households not having pit latrines but many do not have the financial or logistical means to comply (Kvalsvig and Connolly, 1993). Few have access to borehole water and most households are dependant on rivers to meet their water requirements. All have access to local clinics or mobile clinics which are able to treat minor ailments. Treatment for schistosomiasis can only be obtained from a hospital, the nearest of which is between 40 and 50km away at either Port Shepstone or Scottburgh.

1.2.2 KNOWLEDGE, ATTITUDES AND PERCEPTIONS SURVEY

Various techniques for attitude measurement are applied in sociology and psychology (Weisz, 1972); among these are the use of questionnaires, focus-group interviews and depth interviews. Their relative merits have been discussed by Katzenellenbogen and Joubert (1991), Katzenellenbogen *et al.* (1991), Skinner (1991) and Cohen and Manion (1992). Self-administered questionnaires, while allowing for a large sample size, are expensive to distribute and require literacy. Interviews are sensitive to researcher bias. Depth interviews allow for a more thorough investigation of the ideas of an individual, but are time consuming. Respondents may also feel both threatened, and removed from their own context. Focus-group interviews can be arranged in a short space of time, are cost effective and the views of a greater number of people can be assessed. However, peer pressure may limit responses.

Since only 53% of the adult population has a minimum of 6 years of schooling (Kvalsvig and Connolly, 1993) questionnaires were not practical. Focus-group interviews were most suitable and depth interviews were used where possible. Interviews were held at community-established crèches, and the mothers of local crèche or schoolchildren were invited. Since the mothers are traditionally responsible for the health and education of children in this community, men were not included unless they so wished. Interviews included 10 - 20 respondents, an interpreter (Mrs O. Mbambo, a community worker) and a group coordinator (Clark). The objectives of the investigation were carefully explained to Mbambo prior to interviews. Interviews lasted for 60 - 90 minutes and a total of 65 people (59 women, 6 men) were interviewed during 4 focus-group interviews and 2 depth interviews.

Data were recorded by hand following consent from respondents. No recording equipment was used as it is unfamiliar technology and a potential threat to the informality of the occasion. Attempts were made to be interested, neither approving or

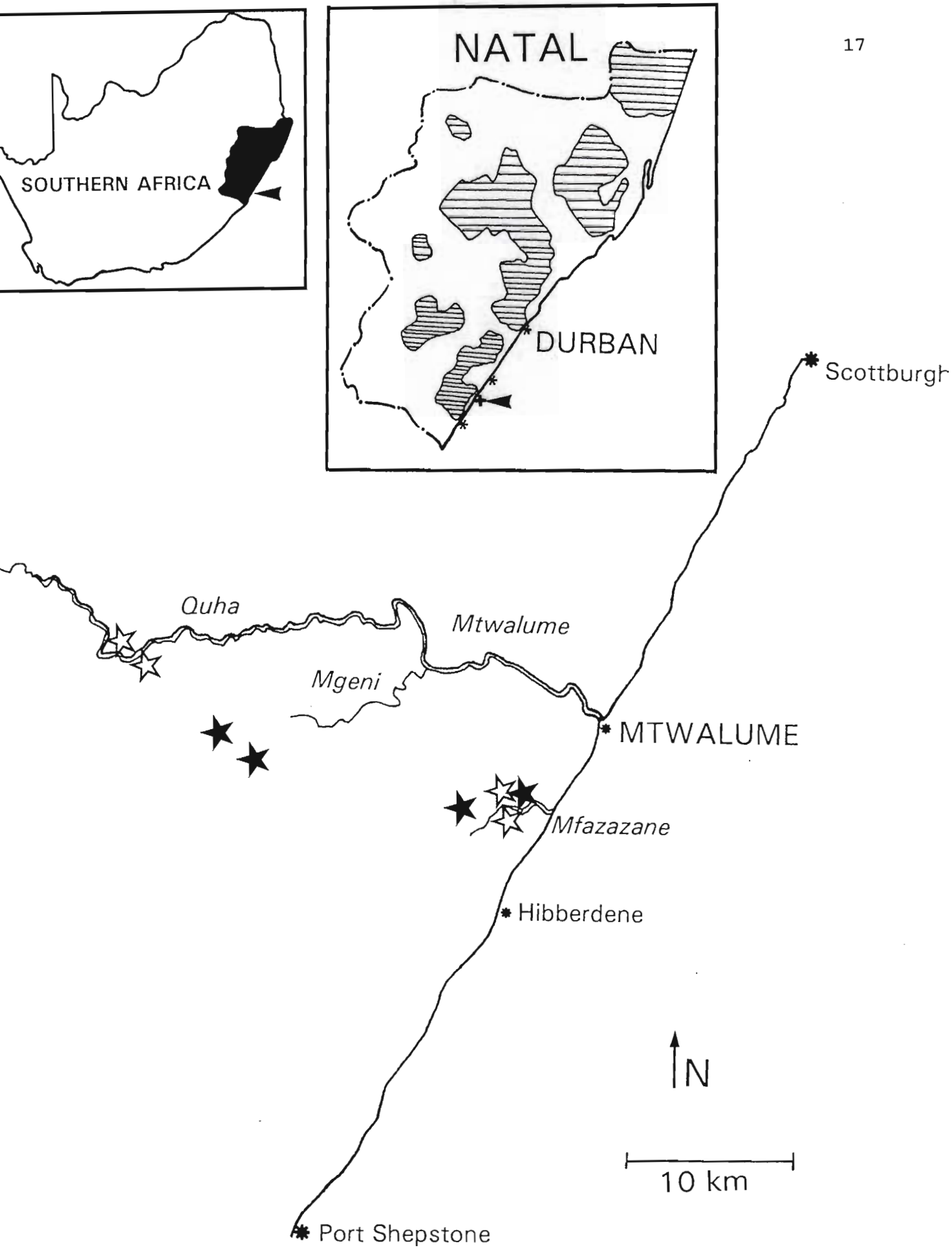


Figure 2 Map of the study area (urine and stool sample collection sites ☆, interview sites ★, KwaZulu ▤).

disapproving. Although interviews followed predefined questions or themes, respondents were encouraged to express their own views. The following issues were addressed:

a. Community understanding of schistosomiasis

i. What is schistosomiasis?

ii. How is it transmitted?

iii. What are the symptoms?

iv. What precautions are taken to prevent infection?

v. What treatment is used?

vi. What is the primary health concern of the community?

b. Molluscicidal and piscicidal plant use

i. What is their attitude to the use of medicinally valuable plants, locally grown, extracted and applied focally for molluscicidal control?

ii. What plants are used as fish poisons?

iii. Do members of the community recognize or utilize the three plants identified for comprehensive evaluation as plant molluscicides (see Chapter 3): *Apodytes dimidiata* E. Meyer ex Arn. subsp. *dimidiata* (ICACINACEAE), *Warburgia salutaris* (Bertol. F.) Chiov. (CANNELLACEAE) and *Gardenia thunbergia* L.f. (RUBIACEAE)?

Content analysis was used in exploring the data for common themes (Skinner, 1991). These themes were coded and where possible percentage response was calculated. The interpretation of these percentages is difficult since a negative or a low response does not necessarily reflect ignorance, but may rather be a measure of reluctance to answer (Bradley and Gilles, 1981).

1.2.3 PARASITOLOGICAL STUDY

Urine (n = 354) and stool (n = 306) samples were collected from junior and senior primary schoolchildren between the age of 2 - 25 years. Samples were collected between 10h00 and 14h00 and transported back to the laboratory for subsampling (10mℓ urine, 1g stool) (Pugh, 1979). Samples were stored in 10% buffered formalin until analyzed. Urine samples were examined by sedimentation-centrifugation (Cheesbrough, 1981). *Schistosoma* ova were counted individually. Red and white blood cells were classed as:

occasional 1 - 5 cells per field of view (40x objective)

(+) 5 - 20 cells per field of view

\+ 21- 40 cells per field of view

\+ + 41 - 60 cells per field of view

+ + + 61 - almost entire field of view

+ + + + cells packed throughout the field of view

The eggs of other parasites were also individually counted. In many samples the presence of thick crystalline deposits made egg counting extremely difficult. In these cases, egg distribution was assumed to be random, aliquots were taken and the number of eggs extrapolated to 10mℓ.

Stool samples were examined by means of the formol-ether concentration technique (Cheesbrough, 1981). The presence of *Schistosoma* ova, all other helminths and protozoa were quantified. Categories of intensity of infection followed Chandiwana (1988), i.e. light <200 eggs/10mℓ urine, moderate 201 - 1200 eggs/10mℓ urine, heavy infection >1200mℓ urine. Heavy infections with *S. mansoni* included samples having >100 eggs/g of faeces.

Statistical analysis was done using STATGRAPHICS (STSC Inc., 1989-1991). Since the frequency distribution of egg counts was strongly positively skewed, a log transformation was used. Under these conditions the geometric mean egg count for infected individuals provide a better measure of central tendency than the arithmetic mean (Chandiwana, 1988). T-tests, one-way analysis of variance (ANOVA) and least significant differences were used to identify significant differences in prevalence and intensity of infection between sex and age. Spearman's Rank Correlation Coefficient (SRCC) was used to identify correlations between red and white blood cell counts and intensity of infection. Regression analysis and the Product-Moment Correlation Coefficient (r) was then used to identify the form and strength of the relationship.

1.3 RESULTS

1.3.1 KNOWLEDGE, ATTITUDES AND PERCEPTIONS SURVEY

1.3.1.1 Schistosomiasis

Group interviews were set in an informal atmosphere although respondents regarded the apparent importance of the exercise with some seriousness. The atmosphere improved dramatically once the group discovered the co-ordinator's interest in traditional plant use, despite coming from a Western background. Discussion of the treatment of schistosomiasis and the use of plant molluscicides was lively. Respondents were unafraid to express divergent opinions, or to recognize that their knowledge of the disease was limited.

Responses have been categorized and summarized in Table 1. All respondents had heard of schistosomiasis and understood it to be some kind of human infection although

their understanding of its transmission, symptoms of infection and approach to treatment was variable. Most respondents could make some association between the infection and contact with water, either by drinking or contact with the skin when wading or swimming.

Table 1. Summary of consensus points relating to schistosomiasis.

CATEGORY OF RESPONSE	% RESPONSE
1.1 Identification	
- human infection or ailment	100
1.2 Transmission	*
- do not know	25
- life cycle involves water	75
- life cycle involves snails	4
- life cycle involves *Zkowe*/"insect"/"insect-like creature"	49
1.3 Symptoms of infection	
- do not know	38
- blood in urine	62
1.4 Precautions	
- children advised not to swim in rivers	13
1.5 Treatment of infection	*
- home remedies/treatment from traditional doctors	
- the success of the latter treatment	95
- Western medicine from hospitals	42
- the success of the latter treatment	5
	0
1.6 Primary health concern	
- indifferent/do not know	12
- schistosomiasis	46
- diarrhoea and/or "dirty water"	39
- dry cough (tuberculosis?)	3

* Total percentage is not equal to 100 since some respondents provided more than one suggestion

Few understood that snails were involved in the life cycle, but not as the intermediate host; rather as the agent responsible for the infection. The snail was understood to enter the body via the genitalia when swimming, or be ingested with the water. Others thought the agent was a small insect (*Zkowe*) or "insect-like creature" living in water, but sometimes also along river banks. Again it was thought to enter the body via the genitalia. During its feeding

and drinking of the "body water" blood was expelled from the body via the genitalia.

The presence of blood in urine was seen as the only characteristic symptom of infection. Associated symptoms mentioned included: pain on urination, frequent urination, pains in the lower abdomen, blood in stools, tiredness and redness in the eyes. Although one respondent suggested that the brain could be affected, all other respondents disagreed. One respondent also expressed the belief that a woman could miscarry if the father of the child was infected with schistosomiasis.

Children were seen to be the most severely affected because they frequently swim in the rivers. Boys and girls are equally at risk of infection, although a suggestion was made that boys contracted it more than girls because they spent more time swimming while herding family animals.

Home remedies and treatments from traditional doctors were the first resort in treating the infection. Traditional doctors prescribe strong diuretics in the belief that they will be ridding the body of infection. One interesting home-remedy mentioned by all groups was the use of *Imfinyezi*. These are described as small brown "snails" or "creatures" collected in kraals. One of these organisms is orally administered to children having persistent bed-wetting problems, as well as those infected with schistosomiasis. Although no specimen of this organism was available, specimens could be easily found by young boys herding goats and cattle, since these creatures were often found in association with dung. Further investigation revealed that reference was being made to pillbugs (Diplopoda).

Less than half of the respondents had any faith in the success of these remedies and suggested that bilharzia is one of a number of diseases best cured by Western medicine. This is surprising since all respondents recognized that Western medication was unavailable from local clinics. The expressed confidence in Western antischistosomal medicine was not substantiated by further questioning. Few had been able to travel to hospitals to obtain medication because they could not afford the transport costs. One respondent claimed to have travelled to hospital but was then unable to afford the cost of medication. Those who had finally succeeded in receiving treatment said the treatment failed to cure the infection.

The primary health concern of this community was for schistosomiasis, followed closely by diarrhoea. Extreme concern was displayed by a local school headmaster and headmistress whose attention had been brought to the seriousness of the disease because of the presence of blood on school toilet-seats. Indifference towards the disease was characteristically shown by those having access to borehole water. Only 12 % had immediate access to borehole water.

1.3.1.2 Molluscicidal and piscicidal plant use

Responses have been summarized and categorized in Table 2. All respondents, regardless of their level of understanding of the disease and its importance as a health concern in the community, enthusiastically welcomed the concept of the treatment of local water bodies with extracts of medicinally valuable plants. All showed great willingness to grow plant material at their homes, particularly if these plants were of medicinal value.

Table 2. Summary of consensus points relating to the use of molluscicidal and piscicidal plants.

CATEGORY OF RESPONSE	% RESPONSE
2.1 Concept of the use of plant molluscicides in a community situation	
- positive	100
2.2 Plants used as piscicides	
- sisal/fibre (*Agave sisalana* Perr. (AGAVACEAE)	100
- *Mahedeni* (*Phytolacca dodecandra*, Doke *et al.*, 1990)	100
- *Umthongwane* (*Chrysophyllum natalense* Sond. (SAPOTACEAE) or *Oncoba spinosa* Forssk. (FLACOURTIACEAE), Doke *et al.*, 1990)	35
- *Dakha* (*Apodytes dimidiata*, Doke *et al.*, 1990)	35
2.3 Recognition and utilization of potentially suitable plants	
- *Apodytes dimidiata*	48
- *Warburgia salutaris*	48
- *Gardenia thunbergia*	0

It is significant that *Phytolacca dodecandra*, the most extensively studied molluscicidal plant (Chapter 2, Section 2.1.1) and *Apodytes dimidiata* (not previously recorded as being piscicidal), are both being used as piscicides in this community.

Of the three priority plants identified in a survey of potentially suitable plant molluscicides for South Africa (Chapter 2), *Warburgia salutaris* and *Apodytes dimidiata* were known to the community by their Zulu names, *isiBaha* and *umDakane* respectively (Pooley, 1993). Both species were identified as being medicinally valuable (*Gardenia thunbergia* was not recognized by its Zulu name, *umValasangweni* or *umKwakhwane* (Pooley, 1993)).

1.3.2 PARASITOLOGICAL SURVEY

1.3.2.1 Urine analysis

The overall prevalence of infection was 75.14% and the geometric mean egg count per 10mℓ urine (GMEC) was 205.17. There were no significant differences in prevalence ($t = -0.629$, $P = 0.529$) or intensity ($t = -0.254$, $P = 0.797$) of infection between females and males (Figure 3). In some individuals more than 100 000 eggs/10mℓ were detected, indicating the severity of infection. The mean intensity of infection may be classed as moderate (201 - 1200 eggs/10mℓ urine) (Figure 4).

There were no significant differences in prevalence (F-ratio = 0.658, $P = 0.6559$) and intensity (F-ratio = 0.146, $P = 0.9812$) of infection between age classes (Figure 5). The unusual occurrence of high infections in the youngest age class (1 - 3 years old) can be attributed to the small sample size for this group ($n = 5$).

Haematuria was common (Figure 6) and there was a characteristically strong positive correlation between haematuria and intensity of infection (SRCC = 0.7732, $P < 0.001$). This relationship was not linear but is best described by a multiplicative model ($y = ax^b$) ($r = 0.818$, $r^2 = 66.92\%$), i.e. haematuria increased rapidly with an initial increase in intensity of infection (Figure 7). White blood cell counts were also strongly positively correlated to intensity of infection (SRCC = 0.7758, $P < 0.001$). Again this relationship is best described by a multiplicative model ($r = 0.7932$, $r^2 = 62.92\%$). It is noteworthy that 43% of those not infected with *Schistosoma haematobium* also showed evidence of haematuria. Haematuria is not exclusive to schistosomiasis and also occurs for, among other disorders, bladder infections, kidney disorders and infective hepatitis. The presence of any other parasites in urine samples was also recorded (Table 3).

Table 3. Prevalence of other parasites in urine samples due to faecal contamination.

PARASITE	% PREVALENCE
Schistosoma mattheei	3.67
Ascaris lumbricoides L.	3.1
Trichuris trichiura L.	0.84
Taenia solium L.	0.56
Hookworm species	0.28

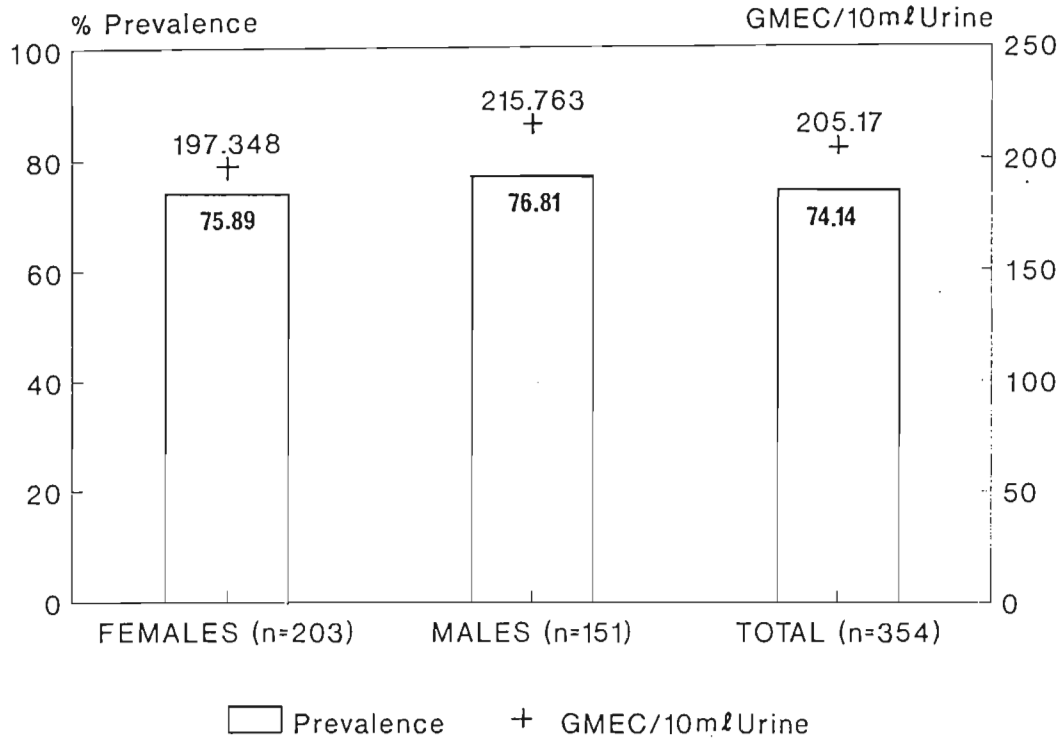


Figure 3 The prevalence and intensity of infection with *Schistosoma haematobium* in relation to sex.

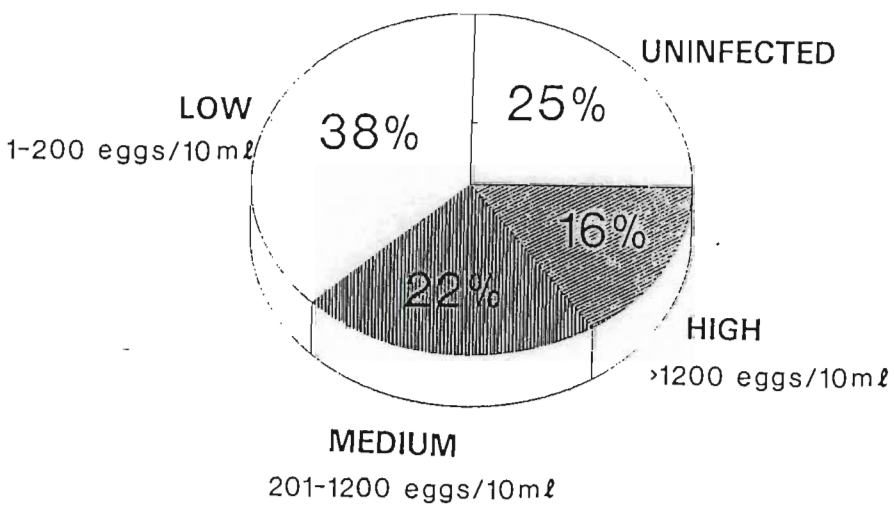


Figure 4 Relative intensity of infection with *Schistosoma haematobium* in children and young adults at Mtwalume.

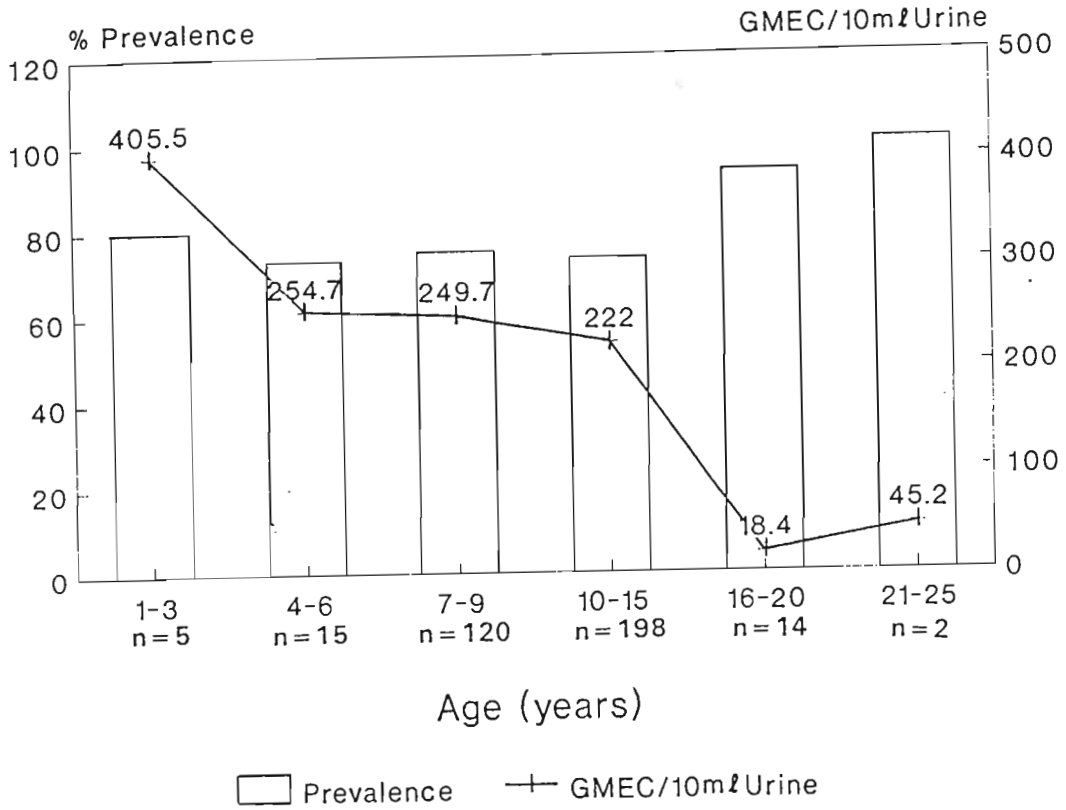


Figure 5 The prevalence and intensity of infection with *Schistosoma haematobium* in relation to age.

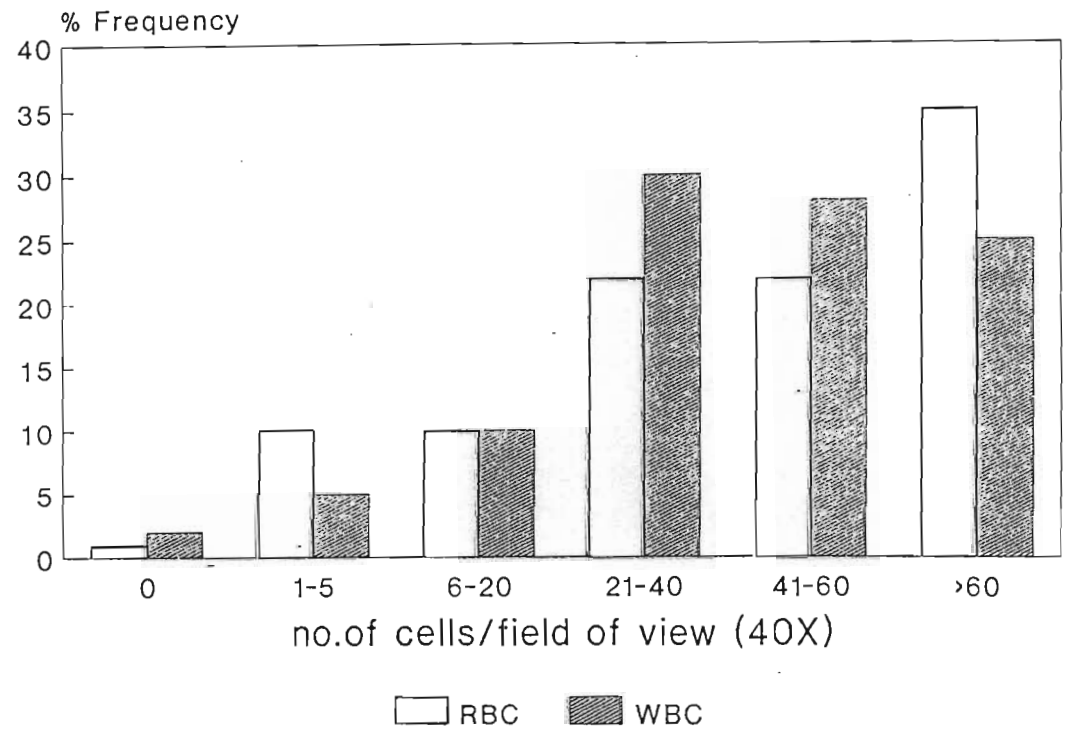


Figure 6 The frequency of red (RBC) and white blood cells (WBC) present in individuals infected with *Schistosoma haematobium*.

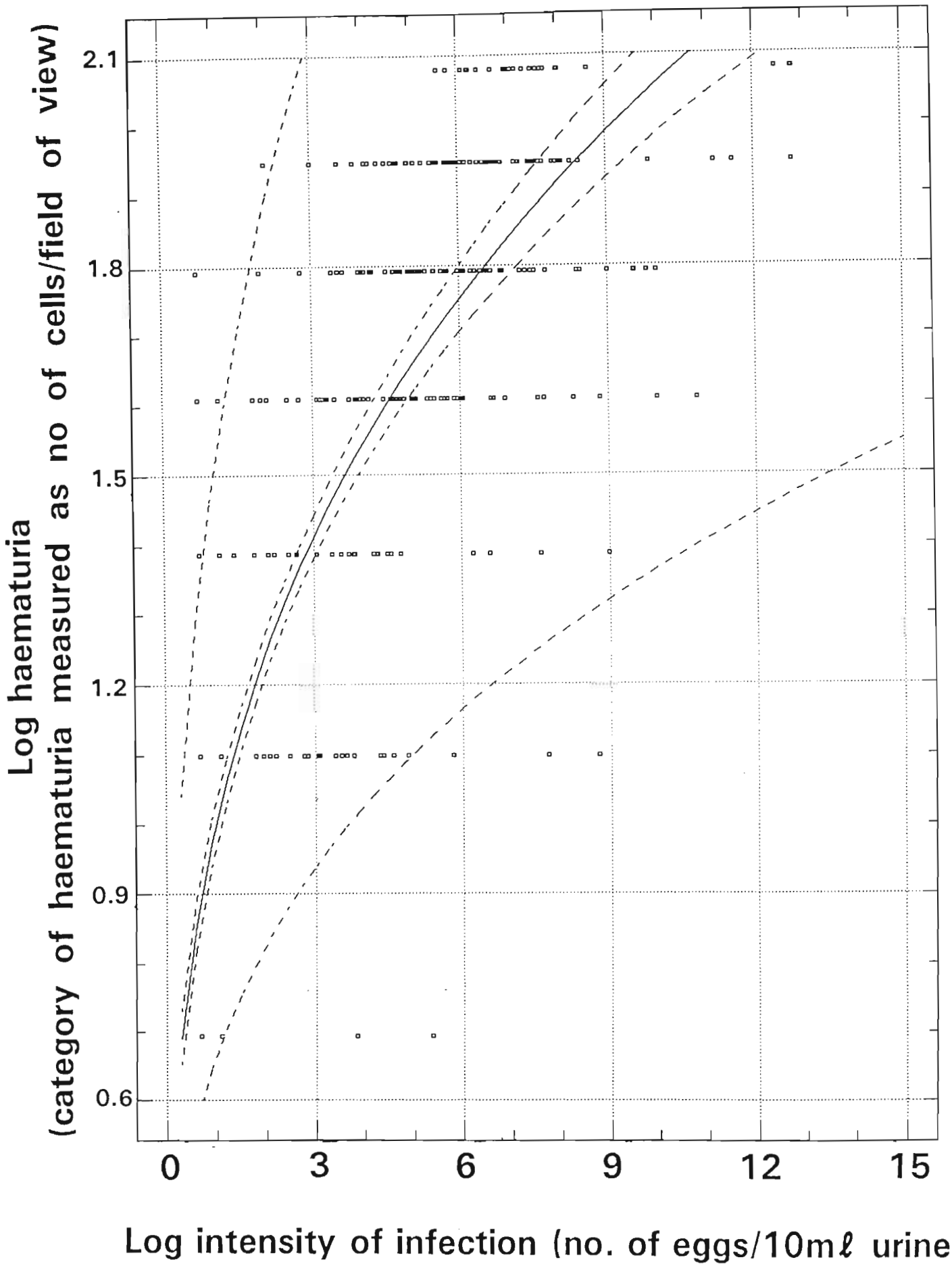


Figure 7 The relationship between blood cell counts and intensity of infection with *Schistosoma haematobium*. Dotted lines show 95% and 99% confidence limits.

1.3.2.2 Stool analysis

Parasites present in stools were recorded (Table 4). *Trichuris trichiura* (GMEC/1g of stool = 122.357) was the most prevalent of parasitic infections in both urine and stool samples in this community. This was followed by *Ascaris lumbricoides* (GMEC/1g of stool = 116.8) in stool samples. There was very little *Schistosoma mansoni*. Of the protozoan infections, only *Giardia duodenalis* and *Entamoeba histolytica* are pathogenic and their occurrence was found to be low in the community.

Table 4. Prevalence of parasites in stool samples.

PARASITE	% PREVALENCE
Helminths	
Trichuris trichiura	92.48
Ascaris lumbricoides	62.74
Schistosoma haematobium	34.64
Hookworm species	30.4
Trichuris vulpis Rud.	12.09
Schistosoma mansoni	3.92
Taenia solium	1.63
Schistosoma mattheei	1.3
Enterobius vermicularis L.	0.65
Fasciola hepatica L.	0.65
Strongyloides stercoralis (Bavay)	0.65
Protozoa	
Entamoeba coli (Loesch)	42.11
Endolimax nana (Wenyon and O'Connor)	15.04
Giardia duodenalis Davaine	7.85
Entamoeba hartmanni Prowazek	7.48
Entamoeba histolytica Schaudinn	5.56
Iodamoeba bütschlii (Prowazek)	3.26
Chilomastix mesnili (Wenyon)	1.63

1.4 DISCUSSION

1.4.1 SCHISTOSOMIASIS AS A PUBLIC HEALTH PROBLEM

Schistosomiasis was recognized by this rural community as a public health problem. Their perception was indeed matched by a high prevalence (75.14%) and intensity (205.17 eggs/10mℓ urine) of infection for *Schistosoma haematobium*.

Concern for the disease is based largely on the presence of blood in the urine and this is not considered healthy, or part of menstruation and puberty, as has been found in Nigeria (Patwari and Aneja, 1988; Akogun, 1989; Bello and Edungbola, 1992). Haematuria is characteristically correlated to intensity of infection (Feldmeier and Poggensee, 1993; Lengeler *et al.*, 1993), and the high prevalence of haematuria (43%) and white blood cells (50%) in uninfected individuals is suggestive of urinary tract disorders.

The poor understanding of schistosomiasis was highlighted by a complete misunderstanding of the life cycle. Respondents in similar studies in Egypt (Kloos *et al.*, 1982b) and Zimbabwe (Chandiwana *et al.*, 1991) suggested very similar modes of transmission. In the former country many could correctly describe the whole life cycle because it is taught at primary and secondary school levels (Kloos *et al.,* 1982b). In this study, apart from suggestions of the threat of miscarriage and potential brain damage to infected individuals, most suggestions were conservative. Modes of transmission recorded elsewhere are remarkably imaginative, e.g. eating unripe sugar cane, mosquitoes, walking barefoot (Kloos *et al.*, 1986) and jumping over fires (Woolhouse, 1987). As was found in Zimbabwe, none could make the association between poor hygiene and the infection (Taylor *et al.*, 1987).

Secondary to schistosomiasis in terms of a community health concern was diarrhoea. High helminth infections (*Trichuris trichiura* - 92.4%, *Ascaris lumbricoides* - 62.7%) may be responsible. This highlights the need for education in basic sanitation and hygiene.

1.4.2 WESTERN MEDICATION

An estimated 80% of Black South Africans consult traditional healers as a first resort when ill (Hennig, 1992) and an estimated 75% of the world's population relies on traditional remedies (Sofowora, 1982; Balick, 1990; Farnsworth, 1990; Nigg and Siegler, 1992). The results of this study reiterate this. Home remedies and treatments from traditional medical practitioners are affordable and readily accessible, although there is doubt as to its efficacy in curing schistosomiasis.

As has been found by Nyamwaya (1987) for the Kenyan Pokot, Zulus do not regard indigenous and Western medicine as conflicting. Some illnesses are regarded as uniquely African and therefore not treatable by Western medicine. Green and Makuba (1984) found that Swazi traditional healers believed that schistosomiasis is best cured by Western doctors. Although a similar attitude was expressed by respondents during this study, Western medicine is mainly inaccessible. This is due to the cost of transport necessary to travel to the nearest hospital for treatment. Without the personal resources to do so, Mtwalume and other rural communities cannot access the available health-care facilities (Straub and Walzer, 1992). Additionally, the poor understanding of disease transmission means that precautions are not taken and reinfection soon results. Consequently there is poor credibility in this community of available antischistosomal drugs. A need is highlighted for basic health education through the Primary Health Care already offered by clinics.

1.4.3 INDIGENOUS MOLLUSCICIDAL PLANT USE

The concept of the use of indigenous plant molluscicides (cultivation, harvesting and extraction) was extremely well received, regardless of their level of understanding of the disease. The use of plant material is culturally acceptable and certainly more obtainable than Western antischistosomal drugs whose accessibility is limited primarily by the cost of transport and secondarily by the cost of drug treatments. Of the three plants selected as potentially suitable for further investigation in Chapter 2, *Warburgia salutaris* and *Apodytes dimidiata* were known to the community and sought after for their medicinal properties. Additionally, *Apodytes dimidiata* was identified as piscicidal. Piscicidal and molluscicidal activity are often closely related, thus providing circumstantial evidence of the potential activity of *Apodytes*.

This study has identified plant molluscicides as warranting exploitation in South Africa, as well as further investigation. Use at community level is imperative. This philosophy is in line with Straub and Walzer (1992) who suggested that rural health needs to step beyond the facility- or provider-driven models in search of new solutions. This is especially so since an expanding demand for health care is going to place increased pressure on limited health service resources.

CHAPTER 2

THE IDENTIFICATION AND SELECTION OF INDIGENOUS MOLLUSCICIDAL PLANTS

The contents of Chapters 2 and 3 have been presented as a paper and poster titled "Surveying South African plants for Molluscicidal Activity: a Simple Scoring System" at the 1993 Schistosomiasis Research Programme International Conference on Schistosomiasis (Egypt, Cairo, March 1993). It has also been presented as a poster titled "A New Approach to Identifying Molluscicidal Plants for use in the Control of Schistosomiasis" at IV Congreso Cubano de Microbiologia y Parasitologia y I Congreso Cubano de Medicina Tropical (Havana, Cuba, October 1993). These presentations included T.E. Clark[1], C.C. Appleton[1] and S.E. Drewes[2] as authors.

[1]Department of Zoology and Entomology,

[2]Department of Chemistry and Chemical Technology, University of Natal, Pietermaritzburg.

HYPOTHESES

i. The South African flora offers potential molluscicidal candidates.

ii. Candidate plants can be objectively selected from the literature based on pertinent characteristics identified by the World Health Organization (WHO).

ABSTRACT

The high cost of synthetic molluscicides for schistosomiasis control has resulted in growing interest in plant molluscicides. During the last decade emphasis in research changed from large random surveys for activity to more focused searches for compounds responsible for activity. Definitive and comprehensive evaluations of toxicity ultimately led to field evaluations. Screening programmes conducted in African countries have covered some plants indigenous to South Africa. However, South African flora potentially suitable for local use have never been assessed. Random screening is costly and generally unrewarding, hence the need for an objective selection procedure. Apart from revealing new potential, data already available in the literature should be utilized. In this study, the objective assessment of indigenous plants was achieved through a simple scoring system.

Six hundred and fifty-five molluscicidal species were identified from the literature. These included those with some record of activity, closely related taxa, and those plants showing piscicidal activity. Of these, 150 occur in southern Africa. Twenty six were active according to standards set by WHO. Thirty seven species, although untested, were

considered potentially molluscicidal. Each of this set of 63 species was ranked on cumulative scores for the following three criteria: (a) coincidence of the endemic areas of the plant, snail host and disease; (b) plants with additional ethnomedicinal value provide greater incentive for cultivation; (c) if molluscicidal activity is known then the LD_{90} should be $\leq$ 100ppm. Two lists of species resulted, those with recorded and those with potential activity. Time and financial constraints limited further investigation to plants from the former list. The list of potential species identified is, however, important in prioritizing future research on molluscicidal plants in South Africa. From the 17 species with scores $\geq$ 70% of the total score possible, material for six was available locally. Preliminary screening of South African plant material of these species is described in Chapter 3.

2.1 INTRODUCTION

2.1.1 PLANT MOLLUSCICIDES REVIEWED

Since the first reports on the molluscicidal activity of *Balanites aegyptiaca* (L.) Delile (BALANITACEAE) in the Sudan (Archibald, 1933) and *B. maughamii* Sprague in South Africa (Wager, 1936), more than 1100 species have been tested (Kloos and McCullough, 1987). China was responsible for the largest survey of plants in the search for molluscicidal activity. Their programme, the details of which are unclear, involved the screening of approximately 600 herbs (Maegraith, 1958). Although Archibald was the pioneer of molluscicide research, Akililu Lemma's work on endod from *Phytolacca dodecandra* L' Hérit (PHYTOLACCACEAE) has been another important contribution. Lemma (1965) discovered the activity through the observation that *Phytolacca* berries used in the production of soap caused high snail mortalities immediately downstream of sites where clothing was being washed. Over the 14 years of intensive investigation that followed, more than 40 scientific publications on endod emerged from laboratories in Ethiopia, U.S.A. and the United Kingdom. These studies focused on endod chemistry and extraction procedures, definitive and comprehensive evaluation of molluscicidal activity and ultimately field evaluation. Lemma *et al.* (1979) thoroughly reviewed this early literature on endod.

Lemma's (1965) findings and the subsequent publication of WHO guidelines for the screening and evaluation of molluscicides (WHO, 1965) stimulated searches for new and greater molluscicidal activity from plants.

Kloos and McCullough (1982) provided the first comprehensive review of molluscicidal plants, covering some 61 species. Within five years Farnsworth *et al.* (1987) and Kloos and McCullough (1987) had expanded it to include 640 taxa. This is an indication of the extraordinary interest in plant molluscicides during the 1980s. Chemical compounds for use

as non-plant derived molluscicides were also receiving considerable attention during this period. Major contributions to plant molluscicide research were made in the following areas:

- classes of natural products with molluscicidal activity (Hostettmann *et al.*, 1982; Hostettmann, 1984; Marston and Hostettmann, 1985; Hostettmann, 1986; Hostettmann and Marston, 1987).
- the screening and evaluation of numerous plants (Adewunmi and Sofowora, 1980; Kloos and McCullough, 1981; 1982; Webbe and Lambert, 1983; Kloos and McCullough, 1985; 1987).
- the toxicology of plant molluscicides (Duncan, 1985; Koeman, 1987).
- the biochemistry of plant molluscicides (Duncan, 1987a; Henderson *et al.*, 1987).

An essential text on plant molluscicide research until 1987 was produced by Mott (1987a). At this stage field testing had been largely confined to *P. dodecandra* (Ethiopia), *Ambrosia maritima* L. (ASTERACEAE) (Egypt) and *Tetrapleura tetraptera* Taub. (FABACEAE) (Sudan) (Sturrock and Duncan, 1987). The most promising plant molluscicides recorded to date include the above, as well as those from *Jatropha curcas* L. (EUPHORBIACEAE) (Philippines) (McCullough and Mott, 1983; Hostettmann, 1984) and *Swartzia madagascariensis* Desv. (FABACEAE) (Tanzania) (Tanner, 1989c; WHO, 1989).

Over the last six years (1988-1993) mass screening and evaluation programmes have given way to more intensive assessments of plants already known to be active. Work on *P. dodecandra* focused on mass cultivation and propagation techniques (Demeke *et al.*, 1992), problems of geographical variation in morphology (Adams *et al.*, 1989) and variability in molluscicidal activity (Ndamba and Chandiwana, 1988). Other countries also assessed their potential (Brazil) (De Souza *et al.*, 1987).

In the interim, Vassiliades *et al.* (1986) found *A. maritima* (and *Annona senegalensis* Pers. (ANNONACEAE)) to be less active than previously recorded and their introduction into Senegal was therefore halted. Belot *et al.* (1993) confirmed that the large amounts of crude material required to achieve snail number reductions in the field, compromised its use in control programmes. *T. tetraptera* underwent further evaluation in Nigeria. Toxicological screening and a 24-month field trial showed that snail populations could be significantly reduced (Adewunmi, 1989). Subsequent to these trials, Gebremedhin *et al.* (1994) have investigated the effects of aridan from *T. tetraptera* on non-target organisms.

This noteworthy change in direction and pace of research is discussed further in Section 2.1.2. Remarkably, despite considerable research efforts on potentially valuable plants (e.g *P. dodecandra*), countries endemic for schistosomiasis have not yet incorporated

plant molluscicides into wide-scale control programmes. Likely reasons for this are discussed in Section 2.4.5.

2.1.2 THE CHANGE IN PACE AND DIRECTION OF MOLLUSCICIDAL RESEARCH

The large expense of available synthetic molluscicides and oral antischistosomal drugs prompted the search for plant molluscicides. The lack of an assured market for new synthetic molluscicides also assisted this shift (Jordan and Webbe, 1982). Random mass screening was the standard approach prior to the early 1980s. Moderate to high investments in time, money and effort did not pay off since serendipitous discoveries of biologically active compounds are uncommon (Farnsworth and Morris, 1976; Cox, 1990; Nigg and Siegler, 1992). The mass screening of herbs in China serves as a good example (Maegraith, 1958). None of the 600 plants tested were considered cost-effective for large scale use as molluscicides, and less than 20 were mildly toxic at less than 10 000ppm (Maegraith, 1958). The result of such unprofitable effort has been a lack of further investment in plant compound discovery programmes (Farnsworth, 1990; Kinghorn, 1992). Ultimately, approaches must alter in response to changes in financial investment. Accordingly, researchers have been forced to consider the available potential. Decisions had to be made as to which species warrant further investigation since the development of biological activity, from identification to isolation, requires considerable investment (Svendson and Scheffer, 1982).

Discussions with students of the Department of Chemistry and Chemical Technology at the University of Natal (South Africa) have also revealed a shift in interest over the last decade from chemical sources of bioactivity to the synthesis of new compounds. Nigg and Siegler (1992) confirmed this observation. The decline in new plant-derived drugs in the U.S.A. could be linked to a stronger emphasis on synthetic chemistry and structure-activity relationships. So apart from changes in plant molluscicide research due to the low productivity and expense of mass screens, there has been a natural shift in research interests away from investigations on the sources of activity to the synthesis of active compounds.

2.1.3 PLANT MOLLUSCICIDE RESEARCH IN SOUTH AFRICA

It is ironic that one of the first reports of plant molluscicides was from South Africa (Wager, 1936). Very little research followed. No mass screening efforts were ever conducted (Appleton, 1985; Pretorius, 1988; Pretorius *et al.*, 1988). The only notable activity recorded for individual species was that for *Apodytes dimidiata* (Pretorius *et al.*, 1991) and *Combretum* spp. (Lawton *et al.*, 1991). The most recent publication from this country is that

of Appleton *et al.* (1994) who isolated warburganal from South African *Warburgia salutaris* (Bertol. F.) Chiov. (CANNELLACEAE). Plants indigenous to South Africa have been tested during the screening programmes of other African countries, where these plants are also known to occur (Adewunmi and Sofowora, 1980). Locally acceptable alternatives to *P. dodecandra* have, however, never been investigated in this country.

2.1.4 THE SELECTION OF SOUTH AFRICAN CANDIDATES

The selection of plants to be studied is a crucial factor for the ultimate success of any investigation of this kind (Hamburger and Hostettmann, 1991). Given the lack of research in South Africa, greater productivity can be achieved by using what data are already available in the literature. Databases such as NAPRALERT (NAtural PRoducts ALERT) (Farnsworth *et al.*, 1979), are important sources of information on plants previously tested for molluscicidal activity. Potential activity can also be inferred from the presence of molluscicidal compounds. Some 80 such compounds, primarily saponins, flavonoids, alkaloids and terpenoids, have been isolated from plants (WHO, 1983; Henderson *et al.*, 1987; Hostettmann and Marston, 1987). Additional candidates may be added from plant families known to have numerous molluscicidal members, e.g. Fabaceae, Euphorbiaceae, Rubiaceae, Polygonaceae, Phytolaccaceae and Asteraceae (Marston and Hostettmann, 1987). Ethnomedicinal information on insecticidal or piscicidal activity may also prove valuable (WHO, 1983; Kloos and McCullough, 1987).

Selection is further aided by documented characteristics for good plant molluscicides. These criteria have been outlined in numerous publications (WHO, 1965; Kloos and McCullough, 1982; Hostettmann, 1984; Marston and Hostettmann, 1985; Koeman, 1987; Lugt, 1987; Mott, 1987b; WHO, 1992). Characters which received priority in this investigation are given in Table 5.

It may not be practical to screen all potential candidates; further selection of priority species is normally undertaken. A process for objectively prioritizing plants has not previously been described. This inadequacy led to the development of the scoring system devised in this study. Kloos and McCullough (1985) went as far as identifying important criteria but never assigned these values. The system described below is well suited to the processing of large numbers of potential plant species. Desirable characteristics have been assigned weighted scores. Cumulative scores were ranked and plants having the highest scores were then selected for further evaluation.

Table 5. Desirable characteristics for the selection of candidate molluscicidal plants (adapted from Kloos and McCullough, 1982).

TOXICITY	High toxicity to target organisms but low, or no, toxicity to non-target organisms at molluscicidal concentrations.
AVAILABILITY	The plant should be abundant in the endemic area or should be available through larger-scale agricultural production (Hostettmann, 1984). The use of a plant common in the area has the additional advantage(s) of being well-known and/or well-adapted (Duncan, 1985). If the plant is to be cultivated locally then there should be a high yield of molluscicidal material per plant, and per unit area of cultivated land.
GROWTH CHARACTERISTICS	Perennial, rather than annual growth. High propagation and growth rates with minimum capital and labour input. High adaptability to differing local environmental conditions, such as drought or frost. Resistance to attack by diseases or pests.
LOCALIZATION OF ACTIVITY	Activity should occur in regenerative parts: berries, fruits, flowers, nuts and leaves or vegetatively planted tubers. Leaves from evergreen plants are most suitable since seasonal availability of material is avoided.
STORAGE	If molluscicidal material is produced seasonally and requires storage, potency should be stable for at least one year. McCullough and Mott (1983) recommend 2 years.
PHYSICAL AND CHEMICAL STABILITY	Retention of molluscicidal potency under physical and chemical influences: pH, sunlight, temperature, and biodegradation or uptake by organic matter.
ETHNOBOTANICAL VALUE	The plant should serve more than one purpose (Combes and Cheng, 1986). Rural communities are likely to be more agreeable to growing and utilising material which possesses other uses (Duncan and Sturrock, 1987).
EXTRACTION	If preparation of the plant material is necessary, it should ideally require only crushing or grinding using the simplest of equipment. Extraction of the active ingredient should not require expensive solvents and/or complex apparatus. Water is the most practical solvent.
APPLICATION	Application of the extract should be as simple as possible. The plant should not cause dermal or other toxic effects upon contact with those involved in handling and processing it.

2.2 METHODS

2.2.1 A SURVEY OF SOUTH AFRICAN PLANTS

Data up to 1987 were available for 640 molluscicidal plants, world-wide. Farnsworth *et al.* (1987) listed 571 of these species. Their data was derived from references given in Kloos and McCullough (1987), the NAPRALERT database, and major reviews on the occurrence of saponins and glycoalkaloids in plants. The 69 remaining species originated from Kloos and McCullough (1987).

More recent literature, as well as ethnomedicinal information on South African piscicidal and saponin-containing plants (Watt and Breyer-Brandwijk, 1962; Von Reis and Lipp, 1982) expanded this number by a further 15 species, to 655. All species were identified as being positively, negatively, or (in the case of some South African plants) potentially molluscicidal.

Time and financial constraints limited preliminary trials to six plants. Comprehensive evaluations were later limited to the three most suitable candidates for use in a rural South African situation.

2.2.2 THE SELECTION PROCEDURE

The selection procedure employed has been summarized (Figure 8). Since the only information available for all 655 plant species included distribution and some record of toxicity, these two criteria were used to reduce the total number of species to a more manageable size. Of the 655 species, only 150 occur in southern Africa (including Namibia, Botswana, South Africa, Lesotho and Swaziland) (Gibbs Russel *et al.*, 1985; Gibbs Russel *et al.*, 1987; Arnold and De Wet, 1993). Further selection considered molluscicidal activity, relative to the suggested levels of activity defined by WHO (1983, 1984). Plants selected included those active at $\leq$ 100ppm (WHO, 1983; Marston and Hostettmann, 1985) and those identified as potentially active, although untested. The latter species were included since no comprehensive list has been compiled of South African molluscicidal plants, or potentially active species.

The reduced total of 63 species was still beyond practical screening capabilities and required further objective selection to ensure that the final choices would yield species with the greatest potential.

2.2.2.1 Choice of characters

Data for only three desirable characters (Table 5) were available for all 63 taxa. They were:

(a) distribution - the endemic areas of the plant, snail host and disease must coincide,

(b) ethnobotany - the plant should be of local medicinal or other utilitarian value. Additional value improves incentive to cultivate the plant for snail control,

(c) molluscicidal activity - if the plant is active, the WHO (1983) suggested a $LD_{90} \leq$ 100mg/ℓ (ppm) for crude suspensions and ≤ 20mg/ℓ (ppm) for aqueous and solvent extracts. Guideline activity levels are the subject of debate in Section 2.4.3. Since the plants under investigation were not being evaluated for commercial development, activity of aqueous extracts of ≤ 100mg/ℓ was considered to be of practical use.

2.2.2.2 Scoring system

Plant species distribution and toxicity were considered of primary importance; ethnomedicinal value was secondary. Distribution and toxicity therefore received equal weighting and together accounted for 80% of the total score possible. The remaining 20% was allocated to ethnomedicinal worth. Score ratings were:

i. Distribution

Distribution data for all species were obtained from the National Botanical Institute (Pretoria) and Coates Palgrave (1990). Scores were weighted in favour of species occurring in both the Eastern Transvaal and KwaZulu-Natal provinces in which schistosomiasis is endemic (40 points). Plants occurring in the endemic areas of either of these provinces received 30 points. Plants which occur outside the endemic areas received a negative score (-40). The latter species could simply have been excluded but, as already indicated, there was no list of molluscicidal plants for South Africa, and were therefore retained.

ii. Toxicity

Species active ≤ 50ppm received 40 points; ≤ 100ppm, 30 points. Toxicity recorded from any plant part (roots, leaves, fruits, etc.) was considered. Untested species received no score. Again, the latter were not excluded from the list but were later ranked separately to those species for which toxicity data were available.

iii. Medicinal value

Information on ethnomedicinal value were gathered from Watt and Breyer-Brandwijk (1962), Von Reis and Lipp (1982), Coates Palgrave (1990) and A.B. Cunningham (Max Plank Institute, Namibia, *pers. comm.* with C.C. Appleton, 1992). Scores assigned were as follows:

Extensively used (> 5 local uses), 20 points e.g. *W. salutaris*,

Having **several** uses (2-5 local uses), 15 points e.g. *Solanum nodiflorum* Jacq. (SOLANACEAE),

Having **few** uses (1 or 2 local uses), 10 points e.g., *Lonchocarpus capassa* Rolfe

(FABACEAE),

No medicinal value recorded, 0 points e.g. *Glinus lotoides* (AIZOACEAE).

2.3 RESULTS

Table 6 summarizes the resultant scores for the 63 species. Table 7 gives the final list of candidate plant species. Plant material was not readily available for all species having scores ≥ 90% of the total score possible. Species with scores ≥ 70% of the total score were therefore included. These two lists of species represent priority areas for further research in South Africa.

Naturalized species, which are often invasive, have been included in these lists, but the use of indigenous plants is preferable. Members of the Euphorbiaceae, also included, are not recommended due to the presence of irritant phorbol esters (WHO, 1983; Kloos and McCullough, 1987; Freitas *et al.*, 1991). Potential danger to handlers precludes their use.

In this investigation priority was given to species that had some previous record of activity. From the 17 "previously tested" species listed in Table 7 sufficient plant material was available for the following 6 species for preliminary trials: *W. salutaris, A. dimidiata* subsp. *dimidiata, G. thunbergia, R. caffra, A. nilotica* subsp. *kraussiana* (Benth.) Brenan and *K. africana*. Previous reports of the toxicity of *A. nilotica* did not indicate which subspecies had been used. *Acacia nilotica* subsp. *kraussiana* is the only subspecies occurring in this country (Coates Palgrave, 1990) and was therefore used in the evaluation.

2.4 DISCUSSION

Time and financial constraints do not permit large indiscriminate surveys for molluscicidal plants. Since little work has been done in South Africa, profit can be made of data already existing in the literature. Research conducted in neighbouring African countries is of particular value. Ethnomedicinal, phytochemical and systematic information are also good indicators of potential activity.

The scoring system described above facilitates the objective selection of candidates for comprehensive evaluation. Characters or character weights can easily be modified to suit the requirements of the researcher. However, despite its simplicity and flexibility, the system is not without limitations.

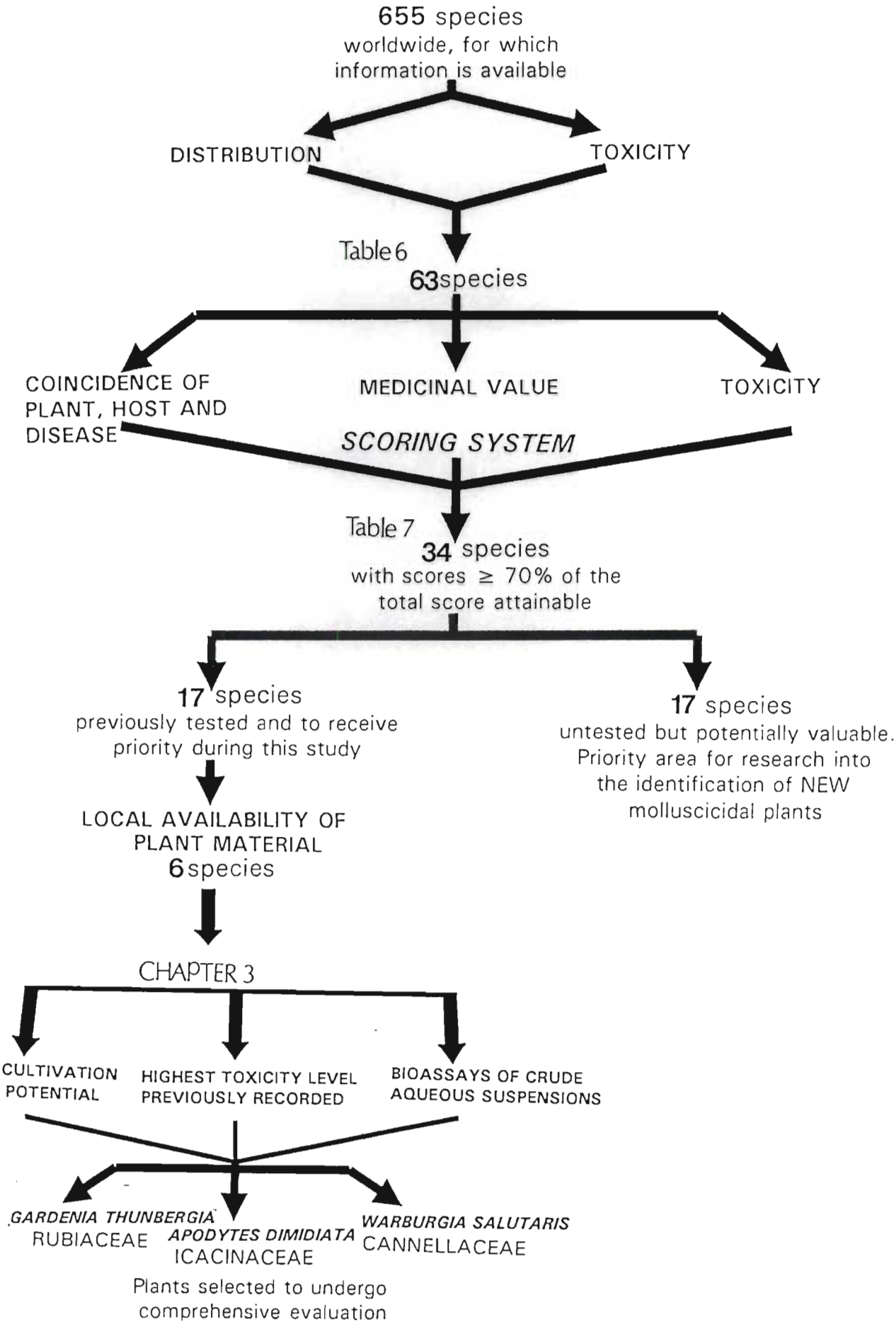


Figure 8 Summary of the procedure followed in selecting candidate South African molluscicidal plants.

Table 6. Scores for 63 southern African candidate molluscicidal plants. Unless otherwise indicated, original references to molluscicidal activity, piscicidal activity and the presence of molluscicidal compounds are given in Kloos and McCullough (1987) and Farnsworth *et al.* (1987) (* = naturalized species; E. Tvl = Eastern Transvaal; N = KwaZulu-Natal).

FAMILY/GENUS/ SPECIES	DISTRIBUTION max 40 points	MEDICINAL VALUE max 20 points	TOXICITY TO SNAILS max 40 points	TOTAL SCORE max 100 points
AIZOACEAE				
Glinus lotoides L. var. *lotoides*	E. Tvl 30	None 0	Untested 0	30
AMARANTHACEAE				
Amaranthus spinosus L.	E. Tvl & N 40	Several 15	Untested 0	55
ANACARDIACEAE				
Smodingium argutum E. Meyer ex Sonder	E. Tvl & N 40	None 0	Untested 0	40
ANNONACEAE				
Annona senegalensis Pers.	E. Tvl & N 40	Wide 20	<100 ppm 30	90
APOCYNACEAE				
Rauvolfia caffra Sonder	E. Tvl & N 40	Wide 20	<100 ppm 30	90
BIGNONIACEAE				
Kigelia africana (Lamk.) Benth.	E. Tvl & N 40	Wide 20	<100 ppm 30	90
CANNELLACEAE				
Warburgia stuhlmannia Engl. (= *W. ugandensis*)	E. Tvl & N 40	Wide 20	<50 ppm 40[1]	100
Warburgia ugandensis Sprague (= *W. salutaris*)				
Warburgia salutaris (Bertol. F.) Chiov.				
COMBRETACEAE				
Combretum fragrans F. Hoffm. (= *C. ghasalense* Engl. & Diels)	Neither -40	Few 10	<100 ppm 30	0
C. imberbe Wawra	E. Tvl 30	Wide 20	<50 ppm 40[8]	90

FAMILY/GENUS/ SPECIES	DISTRIBUTION max 40 points	MEDICINAL VALUE max 20 points	TOXICITY TO SNAILS max 40 points	TOTAL SCORE max 100 points
C. molle R. Br. ex G. Don	E. Tvl & N 40	Wide 20	<50 ppm 40[8]	100
C. padoides Engl. & Diels	E. Tvl 30	None 0	Untested 0[2]	30
ASTERACEAE				
**Chrysanthellum procumbrens* A. Richard (=*C. americanum* (L.) Vakke)	Neither -40	None 0	Untested 0	-40
**Chromalaena odorata* (L.) R.M. King & H. Robinson	E. Tvl & N 40	None 0	<100 ppm 30	70 Declared weed
CUCURBITACEAE				
Acanthosicyos horrida Welw.	Neither -40	Wide 20	Untested 0	-20
Coccinia hirtella Cogn.	N 30	Few 10	Untested 0	40
Peponium mackenii Engl.	E. Tvl & N 40	None 0	Untested 0	40
EBENACEAE				
Diospyros usambarensis F. White	Neither -40	None 0	<100 ppm 30	-10
EUPHORBIACEAE				
Bridelia atroviridis Muell. - Arg.	Neither -40	None 0	<100 ppm 30	-10
Croton gubouga S. Moore (=*C. megalobotrys* Muell. - Arg.)	E. Tvl 30	Few 10	Untested 0[3]	40
Euphorbia cooperi N.E. Brown ex A. Berger	E. Tvl & N 40	Few 10	Untested 0[3]	50
E. ingens E. Meyer ex Boiss.	E. Tvl & N 40	Few 10	Untested 0[3]	50
E. tirucalli L.	E. Tvl & N 40	Several 15	Untested 0	55
**Jatropha curcas* L.	E. Tvl & N 40	Several 15	<50 ppm 40	95
Spirostachys africana Sond.	E. Tvl & N 40	Several 15	Untested 0	55

FAMILY/GENUS/ SPECIES	DISTRIBUTION max 40 points	MEDICINAL VALUE max 20 points	TOXICITY TO SNAILS max 40 points	TOTAL SCORE max 100 points
ICACINACEAE				
Apodytes dimidiata E. Meyer ex Arn. subsp. *dimidiata*	E. Tvl & N 40	Few 10	<100 ppm 30[4]	80
LECYTHIDACEAE				
Barringtonia racemosa (L.) Roxb.	N 30	Few 10	Untested 0[3]	40
LILIACEAE				
Asparagus racemosus Willd. (=*Protasparagus racemosus* (Willd.) Oberm.)	E. Tvl & N 40	Few 10	Untested 0	50
Eriospermum abyssinicum Bak.	Neither -40	Several 15	<100 ppm 30	5
LEGUMINOSAE				
Acacia arabica (Lamk.) Willd (=*A. nilotica* (L.) Delile).	E. Tvl & N 40	Few 10	<100 ppm	80
Albizia anthelmintica Brongn.	Tvl 30	Few 10	Untested 0	40
**A. procera* (Roxb.) Benth.	E. Tvl & N 40	Few 10	Untested 0	50
Derris uliginosa (Willd.) Benth. (=*D. trifoliata* Lour.)	N 30	Several 15	Untested 0[3]	45
Dichrostachys cinerea Wight & Arnott (=*D. glomerata* (Forsk.) Chiov.)	E. Tvl & N 40	Few 10	<100 ppm 30	80
Entada spicata (Meyer) Druce (=*E. natalensis* E. Benth.)	E. Tvl & N 40	None 0	Untested 0[3]	40
**Glycyrrhiza glabrata* L.	Neither -40	Several 15	Untested 0	-25
Indigofera suffruticosa Mill.	Neither -40	None 0	<100 ppm 30	-10
Lonchocarpus capassa Rolfe	N 30	Few 10	Untested 0[3]	40
**Medicago sativa* L.	E. Tvl 30	Few 10	Untested 0	40

FAMILY/GENUS/ SPECIES	DISTRIBUTION max 40 points	MEDICINAL VALUE max 20 points	TOXICITY TO SNAILS max 40 points	TOTAL SCORE max 100 points
Mundulea sericea (Willd.) A. Cheval. (=*M. suberosa* Benth.)	E. Tvl & N 40	Few 10	Untested 0[3]	50
Neorautanenia ficifolius (Benth.) C.A. Smith	E. Tvl 30	Few 10	Untested 0	40
Sesbania sesban (L.) Merr.	E. Tvl & N 40	None 0	<100 ppm 30	70
Tephrosia macropoda Harv. (=*T. diffusa*)	N 30	Wide 20	Untested 0[3]	50
T. purpurea (L.) Pers. subsp. *leptostachya* (DC.) Brummitt var. *delagoensis* (H.M. Forbes) Brummitt			Untested 0[3]	
var. *leptostachya*	N 30	few 10		40
var. *pubescens* Bak.	E. Tvl & N 40	few 10		50
*subsp. *purpurea*	E. Tvl 30	few 10		40
subsp. *canescens* (E. Mey.) Brummitt	N 30	few 10		40
	N 30	few 10		40
MYRSINACEAE				
Myrsine africana L.	E. Tvl & N 40	None 0	Untested 0	40
OLACACEAE				
Ximenia americana L.	E. Tvl & N 40	Several 15	<100 ppm 30[5]	85
Ximenia caffra Sond.	E. Tvl & N 40	Several 15	Untested 0	55
PHYTOLACCACEAE				
Phytolacca americana L.	E. Tvl & N 40	Wide 20	<100 ppm 30	90
**P. dioca* L.	E. Tvl & N 40	Several 15	Untested 0	55
P. heptandra Retz.	E. Tvl & N 40	Several 15	Untested 0[3]	55
P. octandra L.	E. Tvl & N 40	Few 10	<50 ppm 40	90

FAMILY/GENUS/ SPECIES	DISTRIBUTION max 40 points	MEDICINAL VALUE max 20 points	TOXICITY TO SNAILS max 40 points	TOTAL SCORE max 100 points
PRIMULACEAE				
**Anagallis arvensis* L.	E. Tvl & N 40	None 0	Untested 0	40
RUBIACEAE				
Gardenia lutea Fresen. (=*G. thunbergia* L.f.)	N 30	Few 10	<100 ppm 30	70
RUTACEAE				
**Ruta graveolens* L.	E. Tvl 30	Wide 20	Untested 0	50
SOLANACEAE				
**Solanum nigrum* L.	E. Tvl & N 40	Wide 20	Untested 0	50
S. nodiflorum Jacq.	E. Tvl & N 40	Several 15	<50 ppm 40	95
XYRIDACEAE				
Xyris anceps Lamk.	No records -40	None 0	<100 ppm 30	-10
ZYGOPHYLLACEAE				
Balanites aegyptiaca (L.) Delile	Neither -40	Several 15	<50 ppm	15
B. maughamii Sprague	E. Tvl & N 40	Several 15	<50 ppm 40[6,7]	95
F. isotricha Murb.	Neither -40	None 0	<50 ppm 40	0

[1] Appleton *et al.* (1994)
[2] Marston and Hostettmann (1985)
[3] Watt and Breyer-Brandwijk (1962)
[4] Pretorius *et al.* (1991)
[5] Coates Palgrave (1990)
[6] Pretorius *et al.* (1988)
[7] Lewis and Reavall (1990)
[8] Lawton *et al.* (1991)

Table 7. Candidate molluscicidal plants with scores ≥ 70 % of the maximum score possible (* = naturalized species, ☼ = species known to contain irritants to man).

Species NEVER previously tested but potentially suitable based on their taxonomic relationship to molluscicidal species, the presence of molluscicidal compounds or their use as a piscicide Maximum score = 70 points	Species PREVIOUSLY tested and active at < 100 ppm Maximum score = 100 points
Solanum nigrum 60	*Warburgia salutaris* 100
Abrus precatorius 60	*Combretum molle* 100
Spirostachys africana 55	*Solanum nodiflorum* 95
Ximenia caffra 55	*☼*Jatropha curcas* 95
☼*Euphorbia tirucalli* 55	*Combretum imberbe* 90
**Amaranthus spinosus* 55	*Kigelia africana* 90
Phytolacca heptandra 55	*Phytolacca octandra* 90
**Phytolacca dioca* 55	*Phytolacca americana* 90
☼*Euphorbia cooperi* 50	*Annona senegalensis* 90
☼*Euphorbia ingens* 50	*Rauvolfia caffra* 90
**Albizia procera* 50	*Balanites maughamii* 85
**Ruta graveolens* 50	*Ximenia americana* 85
Mundulea sericea 50	*Dichrostachys cinerea* 80
Protasparagus racemosus 50	*Acacia nilotica* 80
Tephrosia macropoda var. *diffusa* 50	*Apodytes dimidiata* 80
Tephrosia purpurea var. *leptostachya* 50	*Sesbania sesban* 70
Derris trifoliata 45	*Gardenia thunbergia* 70

2.4.1 INADEQUATE DOCUMENTATION

The problems of inadequate documentation in phytochemical literature are well recognised (Farnsworth and Morris, 1976; Kingsbury, 1979). In this study the choice of South African plants was based on data provided in the literature. Any such survey depends on the accurate and conscientious presentation of results in articles and reviews. Examples of incorrect citations and incomplete information were found; errors resulted in either artificially high or low scores. *Kigelia africana* provides a good example of this. In Kloos and McCullough (1987, p.77) *K. africana* was recorded as producing 100% mortality at 100ppm. Data in the original reference (Adewunmi and Sofowora, 1980) record 10%, and not 100% mortality at 100ppm. The result of this error was a spuriously high score. During preliminary screening for toxicity (Chapter 3) *K. africana*'s curious inactivity prompted confirmation of the toxicity levels as cited in the review. *Kigelia africana* was subsequently removed from the list of priority species.

Documentation of any new phytochemical information also requires that a voucher specimen be deposited in a public herbarium. This allows verification of a plant's identity because errors are not uncommon (Hedberg, 1987). Only 160 of 2399 novel compounds isolated from plants up to 1977 had voucher specimens (Hedberg, 1987). In this study discrepancies in results for the activity of *A. dimidiata* (Chapter 3) and those found by Pretorius *et al.* (1991) could not have been investigated in terms of possible geographical variation because of the absence of a voucher specimen for their study.

2.4.2 CHARACTER SCORING

The allocation of quantitative values (scores) to qualitative attributes (medicinal value) is not an easy task. Ethnomedicinal information is poorly documented for some plants. This information is passed down verbally through generations and few written records are kept. Watt and Breyer-Brandwijk (1962) provided the only comprehensive coverage of South Africa's medicinal plants. Potentially valuable species could still, however, easily be overlooked due to the availability of limited information.

2.4.3 TOXICITY DEFINED BY THE WHO

Suggested levels of toxicity for various extracts were initially presented in WHO (1965) and appeared unchanged in WHO (1983; 1984). Levels of toxicity were suggested for various solvents. Any activity below prescribed levels would mean disqualification from comprehensive evaluation. Whether levels of activity refer to suitability for use in rural communities, or for the development of alternative synthetic compounds, is unclear. If a

plant molluscicide is to be used by local communities as a crude aqueous suspension, and it is toxic (although at levels lower than required by WHO), readily available, and non-toxic to non-target organisms, then surely it should be considered sufficiently valuable for the purposes for which it is required?

In their natural state phytotoxins are generally less active than commercial equivalents (Duke, 1990). They also tend to have a lower half-life, a lower residual and hence a lower environmental impact (Duke, 1990). The relative merits of activity versus other factors such as reduced impact on non-target organisms has to be weighed-up before a plant is either accepted or rejected for use.

Researchers are advised to pursue several different extraction procedures before choosing to ignore potential candidates. Guidelines for extraction procedures are also sorely needed; the activity of an extract can be affected by the duration of extraction, degree of homogenization and the type of solvent used. Plants active according to WHO standards as crude aqueous suspensions may not be adequately active as alcoholic extracts and vice versa.

2.4.4 BIOLOGICAL VARIATION

Biological variation, both seasonal and geographical, is an additional consideration when studying plants for pharmacological activity (Sofowora, 1982; Farnsworth *et al.*, 1985). Failure to duplicate observed biological activity in a second collection of plant material is a common problem (Farnsworth, 1984). Activity may be seasonal such as in *Piper guiniense* L. (PIPERACEAE) or dependant on the age of plant parts e.g. *Mentha piperata* L. (LAMIACEAE) (Sofowora, 1982). In the experience of Farnsworth (1984), approximately one quarter of the plants found to show promising activity did not show similar activity when further collections of the same plant were similarly evaluated. Hence, despite careful selection of plants from data in the literature, there is no guarantee that similar levels of activity will be seen in local plant material following screening. An obvious solution would be to undertake systematic screening of plants from different climatic zones (Anand and Nityanand, 1984). Here again such screening can only really be deemed necessary if the species is to be cultivated for wide-scale use, or is being considered for synthetic development. Where small-scale community efforts are being sought, control strategies may be quite different. This again raises the issue of why, despite exhaustive evaluation of some plants for community use, countries do not appear to be actively promoting their use locally.

2.4.5 TAKING PLANT MOLLUSCICIDES FURTHER FOR COMMUNITY USE

Numerous factors may have contributed to slowing the implementation of the results of comprehensive evaluations. One is that, until recently, little attention had been paid to the sociological consequences of this technology were it to become available (see Chapter 1). Developed countries doing research on behalf of those nations affected by schistosomiasis, and even the countries of origin, have appeared to be finding solutions for communities without prior consideration of the acceptability of the "technology" offered. Since community involvement is fundamental to the principle of mollusc control using indigenous plants, the lack of this research may be viewed as having a negative effect on further development.

Another frequently raised issue is that of the lack of reproducibility in activity due to geographical and seasonal variation (Farnsworth *et al.,* 1985). This may be a very real problem especially where control needs to be initiated over large geographical areas. The use of these plants could nevertheless be promoted in restricted areas. In some cases, the problems of seasonal changes in molluscicidal activity could be addressed through the storage of plant material.

The next and probably the most fundamental problem which emerges is linked to the difficulty of endorsing a natural product which falls outside the registration regulations for synthetic compounds. Inadequate legislation for the registration of plant extracts is a significant obstacle for *S. haematobium* control, where most of the infection is endemic to Africa. As for synthetic pesticides, natural compounds also require that a patent search be done (Duke, 1990). Any previous publication of pesticidal activity may result in patenting problems. Literature relating to the bioactivity of natural compounds is extensive when compared to synthetic compounds. The result is that it becomes less complicated to patent synthetic analogues which do not require the identification of the natural source of the chemical family (Duke, 1990). Health regulations may also pose problems in clearing a complex mixture of many biologically active compounds for use by the public (Duke, 1990). Cooper Weil *et al.* (1990) have highlighted the fact that there are few global or regional surveys of pesticide related legislation, or studies analysing the problems of enforcing pesticide legislation and regulation. The result is that the final transfer of "technology" for the control of schistosomiasis to the communities for whom it was created, is impeded.

McCullough *et al.* (1980) suggested that there is no reason for public health and legislative authorities to not use the same regulations with respect to acute or chronic toxicology as for synthetic products. Evidently, plant molluscicide researchers have been hesitant to follow their advice. Lambert *et al.* (1991), after following Tier 1 studies for *P. dodecandra* in accordance with OECD guidelines, concluded that field testing of endod was

now justifiable. After 15 years research! The desperate need for local alternatives to synthetics is being crippled by indecision on how to manage ownership of information relating to natural products, and poorly defined requirements for the registration of natural products in developing countries, in which many molluscicidal plants are found. Serious efforts need to be made in transferring this technology to the communities for which it was originally intended.

2.5 CONCLUSION

The search for local alternatives to *P. dodecandra* required a survey of South African molluscicidal plants. Little research has been done in South Africa, hence our dependence on knowledge of indigenous plants from other countries. A literature survey identified taxa with previously recorded molluscicidal activity, and those with potential activity based on their relationship to molluscicidal or piscicidal species. The simple scoring system devised allowed for the objective selection of six candidates for preliminary screening (Chapter 3). A list of potentially suitable candidates, although untested, provides direction for future research on South African molluscicidal plants.

Inadequate documentation, obscurity of minimum activity levels defined by the WHO, and biological variation are inherent limitations of the scoring system. Objective selection is, nevertheless, of greater value in revealing potential than any random screening procedure.

Whilst South africa improves its knowledge of potential molluscicidal plants, efforts are going to have to be made to address the problems of registering plant molluscicides for use in rural communities. Without registration legislation, the product cannot be formally endorsed for use in target communities.

CHAPTER 3

MOLLUSCICIDAL ACTIVITY OF AQUEOUS SUSPENSIONS OF CANDIDATE PLANTS: SELECTION FOR COMPREHENSIVE EVALUATION

HYPOTHESES

i. Leaf material of selected plants are not equally molluscicidal as crude aqueous suspensions

ii. The activity of crude aqueous suspensions, the lowest previous record of toxicity, and cultivation potential are factors which facilitate the objective selection of three from six species for comprehensive evaluation.

ABSTRACT

Time and financial constraints limited comprehensive evaluation to three of the six molluscicidal candidates selected in Chapter 2 (*Warburgia salutaris, Gardenia thunbergia, Apodytes dimidiata, Rauvolfia caffra, Acacia nilotica, Kigelia africana*). Since it was necessary to avoid complex extraction procedures when developing techniques for rural communities, crude aqueous suspensions of leaf material were bioassayed for activity using *Bulinus africanus*. Probit analysis was used to calculate LD_{50} and LD_{90} values. Species were ranked on toxicity as aqueous suspensions, the highest toxicity level previously recorded in the literature, and on their cultivation potential. Ranks for each plant were summed and the three plants with the lowest cumulative rankings (i.e. the highest molluscicidal activity and greatest cultivation potential) were prioritized. In this manner, *Gardenia thunbergia*, *Apodytes dimidiata*, and *Warburgia salutaris* were selected for comprehensive evaluation.

3.1 INTRODUCTION

Practically, comprehensive evaluation of molluscicidal activity could only be made for three of the six species selected earlier (Chapter 2). The activity of crude aqueous suspensions serves as a good indicator of development potential since this is the form most suitable for use in rural communities. Complex extraction procedures or the use of organic solvents may be cost prohibitive or impractical in the absence of adequate facilities. Crude aqueous suspensions are ideal because of the use of water as a solvent. Dried, ground plant material can easily be soaked in water for a prescribed length of time and the supernatant then applied to focal transmission sites. The plant material used should ideally be regenerative, unseasonal in its availability and activity, and should not require destructive harvesting, such as would be required for bark and roots. Sustainable harvesting of molluscicidal material is

an important consideration for the long-term control of snails at transmission sites. Accordingly, cultivation potential and growth characteristics of subjects should be assessed. Useful indicators of this potential include germination success, growth rate and drought tolerance. Previous records of toxicity are only an indication of potential toxicity, given the noted geographical variation in toxicity of single taxa (Ndamba and Chandiwana, 1988).

Ranking of plants on the basis of these factors permits the selection of suitable candidates for comprehensive evaluation for rural community use.

3.2 METHODS

3.2.1 TOXICITY OF AQUEOUS SUSPENSIONS

3.2.1.1 Bioassays

The molluscicidal activities of crude aqueous suspensions of the six plants under investigation were bioassayed using the intermediate host of *Schistosoma haematobium*, *Bulinus africanus* (Krauss). The standard bioassay methods are described in Appendix 1. All plant material was collected from the field and the following voucher specimens deposited in the Herbarium of the University of Natal, Pietermaritzburg (NU):

Warburgia salutaris (Bertol. F.) Chiov., HL & H Mining Timber, Tree Improvement Centre, White River, EASTERN TRANSVAAL, (2531 AC), *T.E. Clark 6* (NU).

Gardenia thunbergia L.f., Natal National Botanical Garden, Pietermaritzburg, KWAZULU-NATAL, (2930 CB), *T. E. Clark 1* (NU).

Apodytes dimidiata E. Meyer ex Arn. subsp. *dimidiata*, Natal National Botanical Garden, Pietermaritzburg, KWAZULU-NATAL, (2930 CB), *T. E. Clark 5* (NU).

Rauvolfia caffra Sonder, Queen Elizabeth Park, Pietermaritzburg, KWAZULU-NATAL, (2930 CB), *T. E. Clark 4* (NU).

Acacia nilotica (L.) Delile subsp. *kraussiana*, Bisley Valley, Pietermaritzburg, KWAZULU-NATAL, (2930 CB), *T. E. Clark 2* (NU).

Kigelia africana (Lamk.) Benth., Natal National Botanical Garden, Pietermaritzburg, KWAZULU-NATAL, (2930 CB), *T. E. Clark 3* (NU).

Plant extracts were prepared according to the standard procedure given in Appendix 2. Endod and Bayluscide® were used as reference molluscicides. The following concentrations of dry leaf material were tested (concentrations are given in g/ℓ; the number of replicates follows in parentheses).

W. salutaris:	8 (2), 5 (8), 1 (6), 0.5 (4), 0.05 (2)
G. thunbergia:	10 (2), 5 (6), 1 (3), 0.8 (2), 0.5 (6), 0.1 (2), 0.05 (2), 0.01 (2)
A. dimidiata:	10, (4), 5 (8), 2 (2), 1 (4), 0.1 (4), 0.05 (4), 0.01 (4)
R. caffra:	10 (2), 5 (9), 1 (9), 0.5 (4), 0.1 (9), 0.05 (2)
A. nilotica:	10 (1), 5 (2), 1 (4), 0.5 (2), 0.1 (2), 0.05 (1), 0.01 (1)
K. africana:	5 (2), 1 (2), 0.5 (2), 0.1 (2), 0.05 (2), 0.01 (2)
Bayluscide®:	0.006 (2), 0.004 (2), 0.002 (2), 0.001 (2)
Endod:	0.01 (2), 0.008 (2), 0.006 (2), 0.004 (2), 0.002 (2)

3.2.1.2 Data analysis

Graphical methods for solving dose-response curves described by Litchfield and Wilcoxon (1949) are no longer acceptable (Baudo, 1987). It is now considered necessary to calculate a regression line relating response (i.e. mortality) to the logarithm of the concentration. Three standard transformations for response data are: probit (Finney, 1971), logistic, and complementary log-log (Collett, 1991). Chi-squared is used to test the goodness of fit of the data to these models. LD_{50}s and LD_{90}s may then be accurately calculated from the fitted line.

In this study LD_{50}s and LD_{90}s with 95% confidence limits were estimated using Fieller's method as implemented in the GENSTAT 5.0 program (Payne, 1987). This method allowed for either a probit, logit or complementary log-log link. Fieller's theorem was originally described in 1940 (Collett, 1991). In the current study, a probit model was fitted to all dose-response data. When this model showed a significant lack of fit, the complementary log-log model was used. The probit model when plotted as the proportion of snails dying versus log dose was a sigmoid curve which was symmetrical about the point of inflection. The complementary log-log model does not suffer from this constraint. In two cases, the latter model was found to give a better fit than a probit model, and hence improved estimations of LD_{50}s and LD_{90}s.

The toxicity of aqueous suspensions was finally assessed in terms of LD_{50} values. This was because with sigmoid dose-response curves the LD_{50} lies on the straightest section of the curve, and hence exhibits the least variability. LD values for the extremities of the curve have larger confidence intervals and hence require larger numbers of replicates to make accurate estimations (Greenberg, *et al.*, 1992).

Abbot's correction for control mortality was not used since control mortality did not exceed 10%-20% (Busvine, 1971; Greenberg, *et al.*, 1992).

3.2.2 PREVIOUS RECORDS OF TOXICITY AND CULTIVATION POTENTIAL

Previous records of toxicity were identified from the literature. Data on habit, habitat, seasonality, germination, growth rates and favourable growing conditions were also identified.

3.2.3 PRIORITY RANKING

Plants were ranked in decreasing order of importance based on the molluscicidal activity of aqueous suspensions, lowest previous records of toxicity, seasonality in production of the necessary plant part, and cultivation potential (i.e. germination, growth rate and drought resistance). Ranks were summed and the three plant species having the lowest scores (i.e. the highest activity, cultivation potential, etc) were selected for comprehensive evaluation.

3.3 RESULTS

3.3.1 TOXICITY OF AQUEOUS SUSPENSIONS

Results of toxicity trials are presented in Table 8; the transformed data are plotted in Figures 9.1 - 9.6. The data for *A. dimidiata* and endod were best described by a complementary log-log model. Analysis of deviance tables which show the fit of dose-response data to probit and complementary log-log models are shown in Appendix 3. From Table 8 it appears that only *G. thunbergia* approximates the WHO (1965; 1983) standard toxicity of 100ppm (i.e. 0.1g/ℓ) for crude aqueous suspensions. It should be emphasized that these suggested levels are only guidelines. Problems with the interpretation of the WHO guidelines have been discussed (Chapter 2, Section 2.4.3). Bayluscide® and endod were clearly more active than the crude aqueous suspensions tested (Table 8, Figure 9.6).

3.3.2 PREVIOUS RECORDS OF TOXICITY

3.3.2.1 *Warburgia salutaris*

Previously documented levels of toxicity are given in Table 9. Three sesquiterpene dialdehydes (warburganal, polygodial, ugandensidol) have been isolated from this species (Blaney *et al.*, 1987). Warburganal has been successfully isolated by numerous groups (Nakanishi, 1984) and most recently in South Africa by Appleton *et al.* (1994). All extraction procedures required one or more organic solvents and the expertise of an organic chemist. Their use in a rural context in a purified form is therefore impractical.

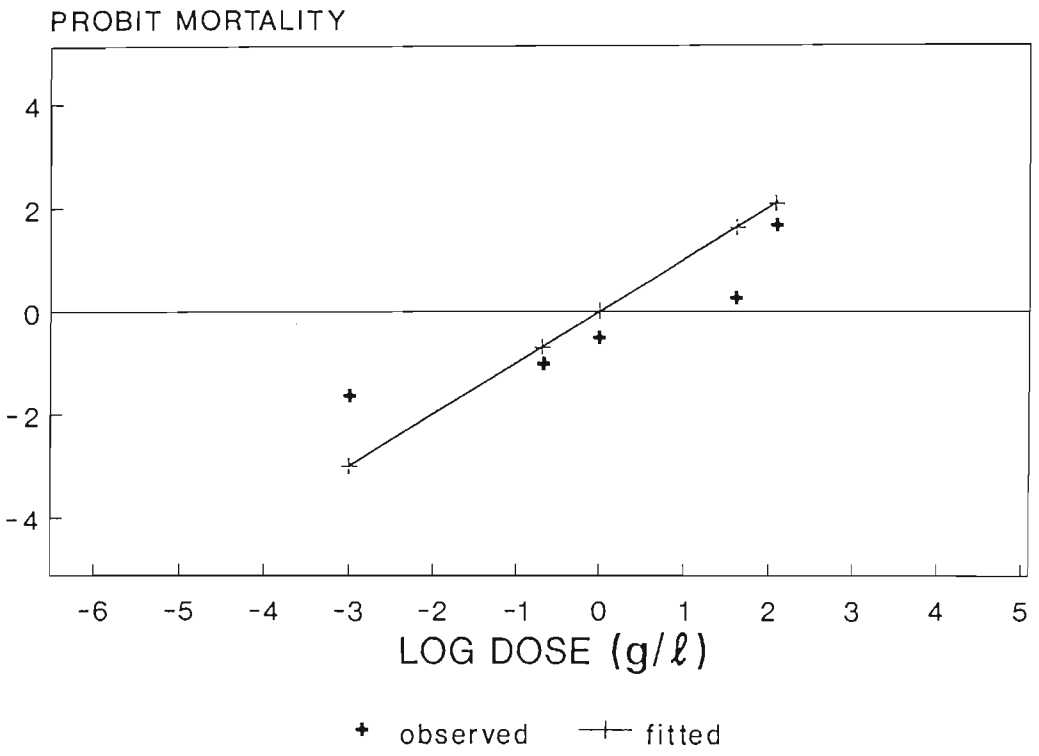


Figure 9.1 The observed and fitted empirical probits for aqueous suspensions of the leaves of *Warburgia salutaris*.

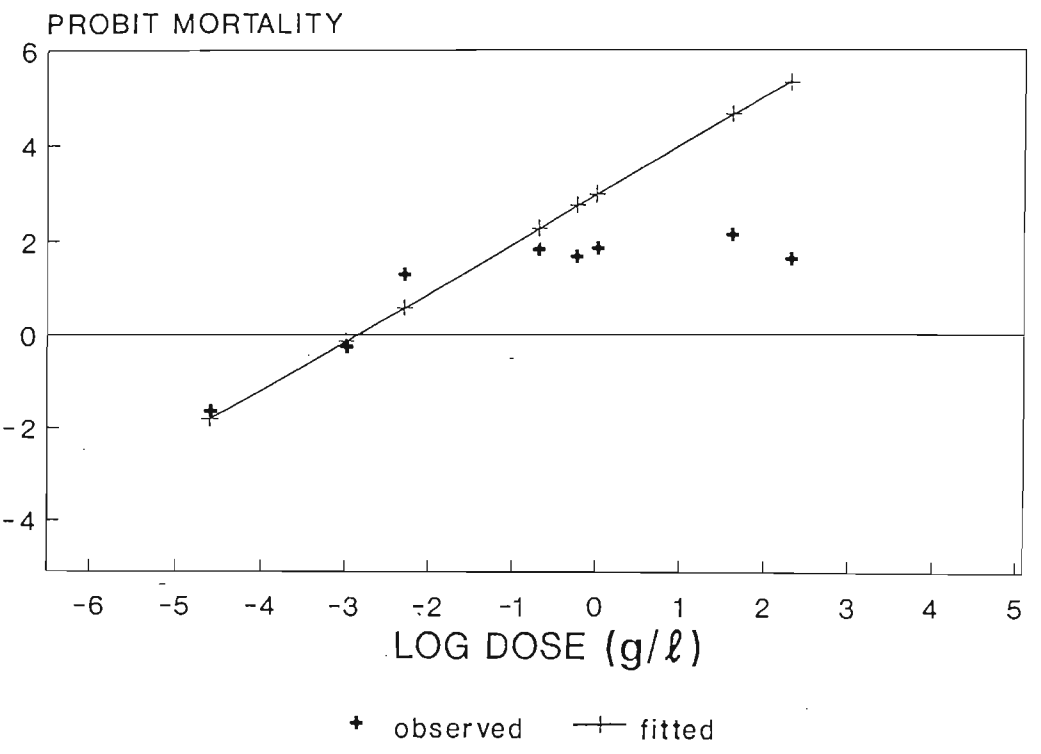


Figure 9.2 The observed and fitted empirical probits for aqueous suspensions of the leaves of *Gardenia thunbergia*.

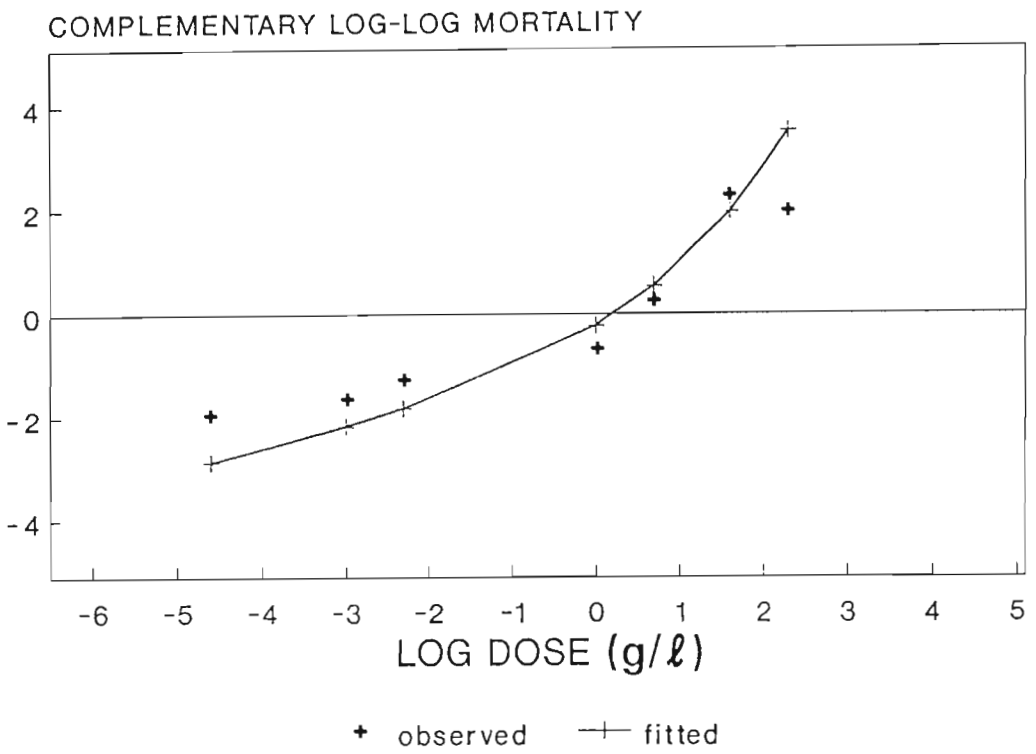


Figure 9.3 The observed and fitted empirical log-logs for aqueous suspensions of the leaves of *Apodytes dimidiata*. A probit model showed a significant lack of fit; hence the use of a complementary log-log model.

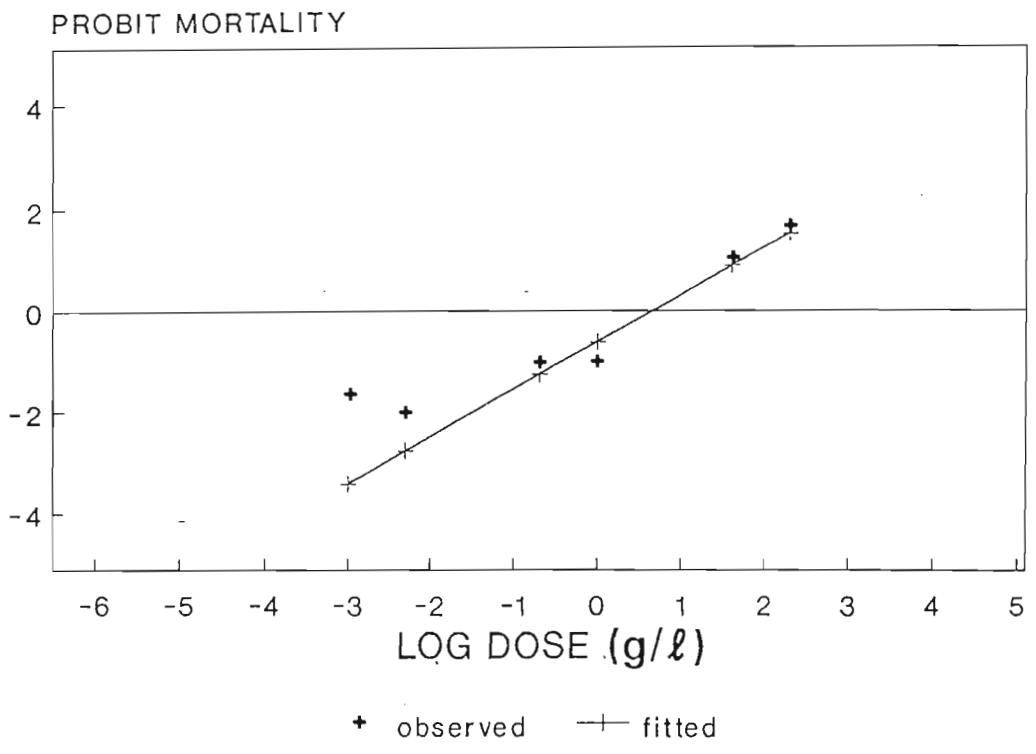


Figure 9.4 The observed and fitted empirical probits for aqueous suspensions of the leaves of *Rauvolfia caffra*.

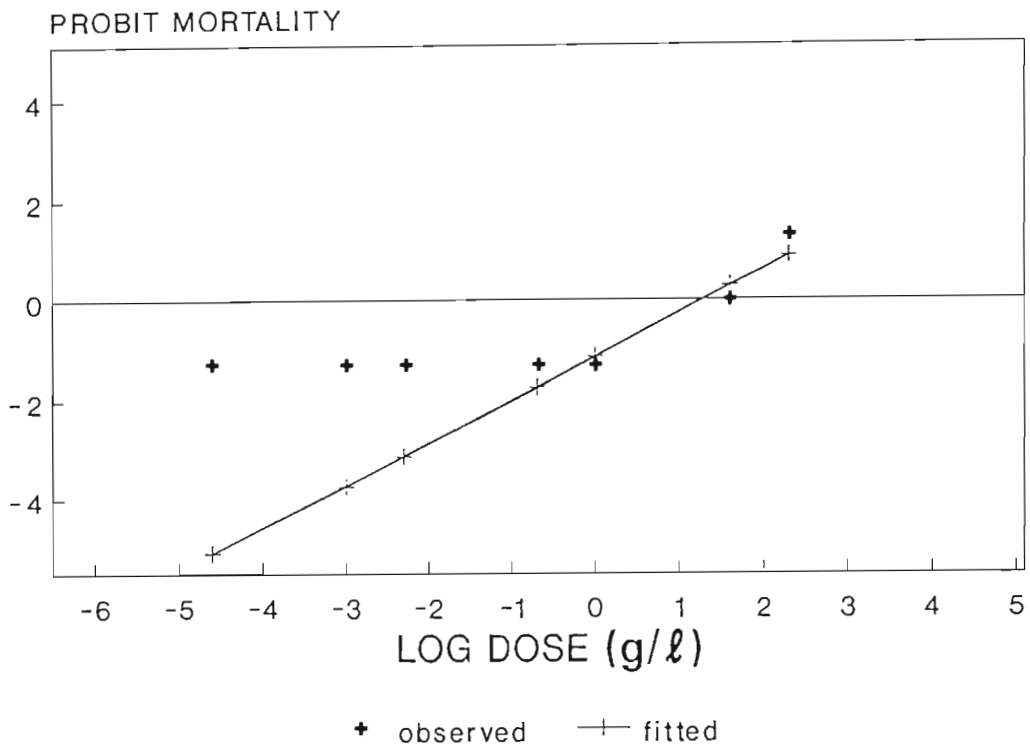


Figure 9.5 The observed and fitted empirical probits for aqueous suspensions of the leaves of *Acacia nilotica*.

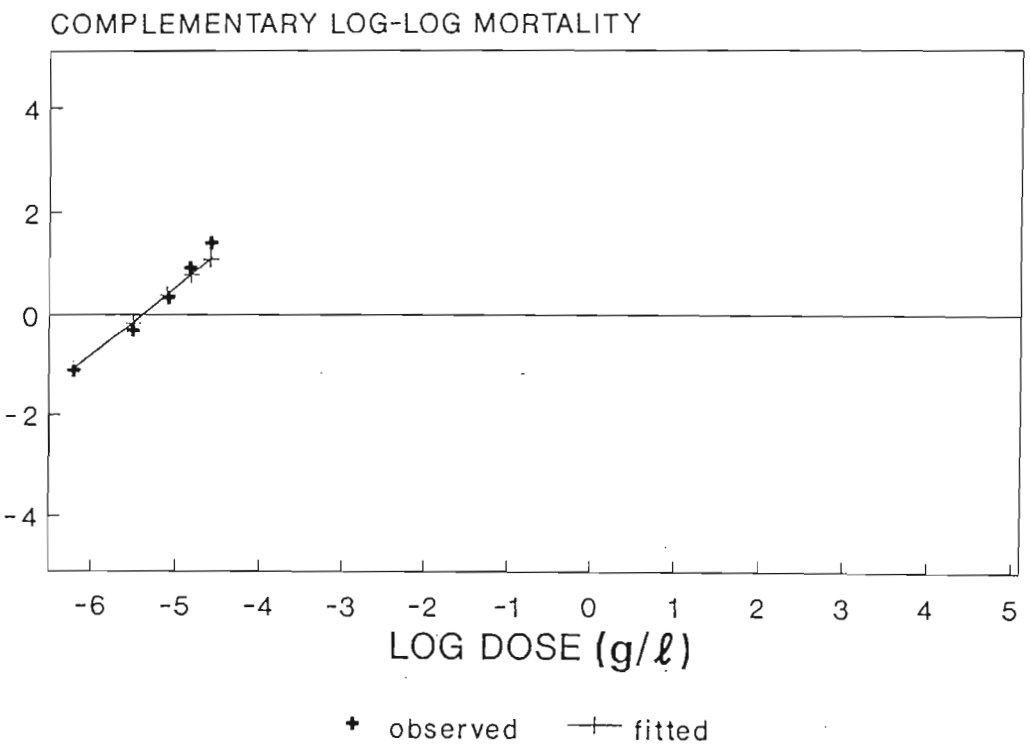


Figure 9.6 The observed and fitted empirical log-logs for endod. A probit model showed a significant lack of fit; hence the use of a complementary log-log model.

Aqueous suspensions of leaf material, as tested in this study, have not been assessed either in the laboratory or in the field.

Additional information on the bioactivity (such as insect antifeedant activity) and toxicology of warburganal gives some indication of potential toxicity to non-target organisms (Kubo *et al.*, 1976; Ma, 1977; Nakanishi, 1980; 1984). Without structural modification, the broad spectrum activity of warburganal may prove to be hazardous (Kubo and Nakanishi, 1979). Before resorting to structural modification it has been necessary to investigate the activity of aqueous suspensions and material from other geographical areas.

Table 8. LD_{50} and LD_{90} values for crude aqueous suspensions of leaf material applied to ***Bulinus africanus***.

PLANT SPECIES	LD_{50} g/ℓ (95% confidence limits)	LD_{90} g/ℓ (95% confidence limits)
Warburgia salutaris	2.483 (1.636 - 3.965)	17.350 (8.805 - 71.10)
Gardenia thunbergia	0.0571 (0.0311 - 0.0906)	0.196 (0.120 - 0.451)
Apodytes dimidiata	1.251 (0.795 - 1.713)	3.404 (2.523 - 4.983)
Rauvolfia caffra	1.943 (1.464 - 2.616)	7.697 (5.158 - 14.47)
Acacia nilotica	3.612 (2.148 - 8.783)	15.57 (7.07 - 137.7)
*Kigelia africana**	no mortality at ≤ 5g/ℓ of dry leaf material, or ≤ 100g/ℓ of fresh leaf material	
REFERENCE MOLLUSCICIDES		
Bayluscide® EC 250	Too few partial mortalities to accurately calculate LD_{50}s and LD_{90}s. The following mortality was observed: 100% at 0.002g/ℓ, 50% at 0.001g/ℓ.	
Endod-S	0.0049 (0.0033 - 0.0060)	0.0095 (0.0076 - 0.0151)

* The inactivity of this taxon was initally thought to be due to the use of leaf material rather than fruit pulp. However, although Kloos and McCullough (1987) recorded 100% mortality at 100ppm, the original source of this data (Adewunmi and Sofowora, 1980) showed only 10% mortality at this concentration. The misinterpretation of activity in *Kigelia africana* by Kloos and McCullough (1987) resulted in the plant receiving a spuriously high score when using the described scoring system (Chapter 2). The inactivity of the leaf material resulted in its exclusion from further evaluations.

Table 9. Previous documentation of molluscicidal activity for *Warburgia salutaris.*

REFERENCE	1	2	3	4	5
SNAILS USED IN BIOASSAY	*Biomphalaria glabrata* (Say)	*B. glabrata*	*B. glabrata*	*Lymnaea natalensis* (L.)	*Bulinus africanus* and *Biomphalaria pfeifferi* (Krauss)
PLANT PARTS TESTED	bark	bark	bark	bark	bark
SOLVENTS USED	crude extract in deionized water	successive organic solvents	successive organic solvents	water	successive organic solvents
CONCN. TESTED (ppm)	5-10	2	20	10	3
MORTALITY (%)	100 (in 2 hours)	50	50	100 (in 2 hours)	90
COMPOUND/S IDENTIFIED	muzigadial and warburganal	warburganal	mukadiaal	warburganal	warburganal

1 Results cited in Marston and Hostettmann (1985), original work presented in Nakanishi and Kubo (1977)
2 Results cited in Marston and Hostettmann (1985), original work presented in Kubo *et al.* (1983)
3 Results cited in Marston and Hostettmann (1985), original work presented in Kubo *et al.* (1983)
4 Nakanishi and Kubo (1977)
5 Appleton *et al.* (1994)

3.3.2.2 *Gardenia thunbergia*

Results, previously documented in the literature, are given in Table 10. No data on toxicity to non-target organisms are available.

3.3.2.3 *Apodytes dimidiata*

Results, previously documented in the literature, are given in Table 11. Toxicity tests have earlier indicated some piscicidal activity (*Oreochromis mossambicus*) (Pretorius *et al.*, 1991). Their results suggested the suitability of this plant for rural community use, but their investigation was limited to molluscicidal and piscicidal activity. No further research on the toxicology of *A. dimidiata* has subsequently been reported on. There is a notable lack of information on the compounds responsible for molluscicidal activity in this species (Table 11). This point is again addressed in Chapter 7.

3.3.2.4 *Rauvolfia caffra*

Results, previously documented in the literature, are given in Table 12. Although the leaves were inactive at 100ppm, Adewunmi and Sofowora (1980) classified this plant as a promising molluscicide. The absence of work on South African *R. caffra* prompted further investigation on leaf material.

3.3.2.5 *Acacia nilotica*

Results, previously documented in the literature, are given in Table 13. Hussein Ayoub (1982) suggested that molluscicidal activity may be due to the presence of tannins. Since tannins may prove to be less toxic to non-target organisms than saponins, compounds from this class have been considered worthy of further investigation (Marston and Hostettmann, 1985). Further molluscicidal activity was attributed to a compound containing two gallates. It is too unstable for practical use when isolated, but is protected from oxidation in the fruit (Nakanishi, 1984). This bodes well for its use in a crude aqueous suspension.

The species involved in previous research included *Acacia nilotica* subspecies *nilotica, tomentosa* and *astringens* (Hussein Ayoub, 1983). No work had previously been done on *A. nilotica* subsp. *kraussiana,* the only subspecies occurring in South Africa.

3.3.3 OCCURRENCE, ETHNOMEDICAL VALUE AND CULTIVATION POTENTIAL

Information relating to occurrence, habit, cultivation, and medicinal and other uses of the six plant species has been summarized (Table 14).

Table 10. Previous documentation of molluscicidal activity for *Gardenia thunbergia*.

REFERENCE	1
SNAILS USED IN BIOASSAYS	*Biomphalaria pfeifferi* and *Bulinus truncatus* (Audouin)
PLANT PARTS TESTED	fruit palp
SOLVENTS USED	alcohol
CONCENTRATION TESTED (ppm)	100
MORTALITY (%)	100
COMPOUND/S IDENTIFIED	alkaloids, saponins, sterols and/or triterpenes

1 Ahmed *et al.* (1984)

Table 11. Previous documentation of molluscicidal activity for *Apodytes dimidiata*.

REFERENCE	1	1	1
SNAILS USED IN BIOASSAYS	*B. africanus* and *B. pfeifferi*	*B. africanus* and *B. pfeifferi*	*B. africanus* and *B. pfeifferi*
PLANT PARTS TESTED	leaves	root bark	root bark and stem bark
SOLVENTS USED	water	successive organic solvents	water
CONCENTRATION TESTED (ppm)	≥100	45 (for *B. africanus*) and 35 (for *B. pfeifferi*)	100-200
MORTALITY (%)	100	100	10
COMPOUND/S IDENTIFIED	none	none	none

1 Pretorius *et al.* (1991)

Table 12. Previous documentation of molluscicidal activity for *Rauvolfia caffra*.

REFERENCE	1	1	1
SNAILS USED IN BIOASSAY	*Bulinus globosus* (Morelet)	*B. globosus*	*B. globosus*
PLANT PARTS TESTED	root	stem	leaves
SOLVENTS USED	methanol	methanol	methanol
CONCENTRATION TESTED (ppm)	100	100	100
MORTALITY (%)	100	10	0
COMPOUND/S IDENTIFIED	alkaloid and saponins	saponins	none

1 Adewunmi and Sofowora (1980)

3.3.4 CUMULATIVE RANKING

The results of the cumulative rankings are given in Table 15. *Apodytes dimidiata, G. thunbergia* and *W. salutaris* had the lowest cumulative rankings with 12, 12, and 13 points respectively. *Rauvolfia caffra* and *A. nilotica* followed with 14 and 17 points respectively. On the basis of these scores the former three species were selected for comprehensive evaluation.

3.4 DISCUSSION

Crude aqueous suspensions of all except *K. africana* were found to be molluscicidal. The identification of *K. africana* as a source of molluscicidal activity was later found to be due to erroneous reporting in a review (Kloos and McCullough, 1987). *Warburgia salutaris, G. thunbergia* and *A. dimidiata* ranked most highly, based on the toxicity of crude aqueous suspensions, previous records of toxicity, and cultivation potential. *Rauvolfia caffra* and *A. nilotica* ranked lower than the above three species. Since limited resources necessitated the selection of only three species for further evaluation, *R. caffra* and *A. nilotica* were excluded. A significant discrepancy was observed in the activity recorded for *A. dimidiata* with that of an earlier study. Pretorius *et al.* (1991) recorded 100% mortality at 0.1g/ℓ. However, in the current study, the lowest concentration producing 100% mortality was 4g/ℓ. Geographical or seasonal variation in activity may account for this difference. Without further research and reference to voucher specimens deposited in herbaria, it is not possible to test this hypothesis.

Table 13. Previous documentation of molluscicidal activity for *Acacia nilotica*.

REFERENCE	1	2	3	4	4	4	5	6
SNAILS USED IN BIOASSAY	*B. truncatus*	*B. truncatus*	*B. glabrata*	*B. pfeifferi*	*B. pfeifferi*	*B. pfeifferi*	*B. truncatus*	*B. pfeifferi*
PLANT PARTS TESTED	fruit	fruit pulp	stem	leaves	seeds	pods	not given	not given
SOLVENTS USED	water	water	water	water	water	water	water	not given
CONCN. TESTED (ppm)	120	75	500	1000	1000	1000	75	75
MORTALITY (%)	100	100	100	20	100	40	100	10
COMPOUND/S IDENTIFIED	none	none	none	none	none	none	none	epigallo-catechin-7-gallate (I) (phenol)

1 El-Kheir and El-Tohami (1979a; 1979b)
2 Hussein Ayoub (1982)
3 Kloos *et al.* (1982a)
4 Kloos *et al.* (1987)
5 Marston and Hostettmann (1985)
6 Hussein Ayoub (1984a; 1984b)

Table 14. The occurrence, ethnobotanical value and cultivation potential of the six species investigated for selection for comprehensive evaluation.

PLANT SPECIES	OCCURRENCE	ETHNOBOTANICAL VALUE	CULTIVATION POTENTIAL
Warburgia salutaris Pepperbark isiBaha (Zulu) (Pooley, 1993).	Northern KwaZulu-Natal and Eastern Transvaal. Small evergreen tree 5-10m high (Coates Palgrave, 1990). Status in KwaZulu-Natal is vulnerable (Hall *et al.*, 1980).	"*salutaris*" = health giving (Coates Palgrave, 1990). Used as a chest remedy, for urethral infections, stomach aches, constipation, fevers, toothache, rheumatism and general muscular pains (Gerstener, 1938; Watt and Breyer-Brandwijk, 1962). Used as a snuff and as a spice in food (Kubo *et al.*, 1976). Also used to treat headaches (Nyazema, 1987). The wood saws, planes and polishes well and the resin has been used to fix handles (Watt and Breyer-Brandwijk, 1962).	It grows from seed but this source is susceptible to parasitic infections. It is more practical to grow from root suckers. Growth rate is moderate (50cm/year) with growth being optimal in warm summers with moderate to good rainfall. Frost sensitive (Johnson and Johnson, 1993).
Rauvolfia caffra Quinine tree umHlambamanzi (Zulu) (Pooley, 1993).	Eastern KwaZulu-Natal and Transvaal. Medium to tall evergreen tree (6-20m high) (Coates Palgrave, 1990).	Latex used in the treatment of malaria, infant diarrhoea, skin disorders and stomach complaints (Coates Palgrave, 1990). Treats physical weakness by expelling impurities and making the patient strong and healthy. The bark is used to treat rashes, urticaria and measles (Bryant, 1966). The wood is used for furniture and bowls (Pooley, 1993).	Can be grown in a container and transplants well. It germinates readily from seed and is fast growing, although frost sensitive when young (Coates Palgrave, 1990). Growth rate is estimated at 1.2m/year (Johnson and Johnson, 1993). It is usually associated with groundwater and grows naturally on stream banks and forest margins (Pooley, 1983).
Acacia nilotica Scented thorn umNgawe (Zulu) (Pooley, 1993).	KwaZulu-Natal and Eastern Transvaal. A small deciduous tree (<10m high) (Coates Palgrave, 1990).	Pods nutritious and browsed by game and stock. The gum is edible and makes a good glue. Dye is made from the pods and a decoction from the bark is used for respiratory complaints. The wood is hard and used for fence posts and furniture (Coates Palgrave, 1990; Pooley, 1993).	Growth rate is estimated at 60cm/year. Suitable for dry climates (Johnson and Johnson, 1993).

PLANT SPECIES	OCCURRENCE	ETHNOBOTANICAL VALUE	CULTIVATION POTENTIAL
Gardenia thunbergia White Gardenia umValasangweni or umKhwakhwane (Zulu) (Pooley, 1993).	East coast of KwaZulu-Natal. A shrub or small evergreen tree (2-5m high).	Fruits used for gastric complaints (Pooley, 1993). Root used as an emetic for biliousness and skin eruptions in leprosy. The leaf is thought to be a cure for syphilis. The wood is hard and therefore good for tool handles (Coates Palgrave, 1990).	Easily grown from seed (Pooley, 1993) or truncheons. Slow growing but hardy (Coates Palgrave, 1990). Growth rate is estimated at 30cm/year. This species enjoys a temperate to warm summer with moderate rainfall although it is able to survive considerable drought (Johnson and Johnson, 1993).
Apodytes dimidiata White Pear umDakane (Zulu) (Pooley,1993).	KwaZulu-Natal and Eastern Transvaal. A small evergreen tree (3-5m high) (Coates Palgrave, 1990).	An infusion of the roots is used as an enema for intestinal parasites. The leaf is used in the treatment of ear inflammations (Watt and Breyer-Brandwijk, 1962). The wood is hard and suitable for implements and furniture (Coates Palgrave, 1990).	Germination may be slow, taking 4-12 months (Coates Palgrave, 1990). Growth rate is fairly rapid following germination (70cm/year). Ideal for cultivation because it survives under a wide range of conditions (Johnson and Johnson, 1993).
Kigelia africana Sausage tree UmBongothi, umVunguta or iBelendlovu (Zulu) (Pooley, 1993).	Northern KwaZulu-Natal and Eastern Transvaal. A medium to large evergreen tree up to 18m high (Coates Palgrave, 1990).	The fruit reputedly cures acne (Pooley, 1993). Unripe fruits are used in remedies for syphilis and rheumatism. Ripe fruits are baked and added to beer to aid fermentation. Powdered fruit is made into a dressing for ulcers and sores. It is also used to increase lactation and when rubbed on babies supposedly makes them fat. The seeds can be roasted and eaten. Ground and boiled fruit is used as an enema for treating children with stomach ailments (Coates Palgrave, 1990).	Quick growing from seed or truncheons (Pooley, 1993). Growth rate is estimated at 1m/year . Fairly drought tolerant (Johnson and Johnson, 1993).

Table 15. Cumulative ranking scores for plant species based on both lowest previous recorded LD_{50} toxicity levels for aqueous suspensions, and their cultivation potential.

PLANT SPECIES	LD_{50} FOR CRUDE AQUEOUS SUSPENSIONS (g/ℓ)		LOWEST PREVIOUS LD_{100} (g/ℓ)		AVAILABILITY OF LEAF MATERIAL		GERMINATION		GROWTH RATE		DROUGHT RESISTANCE		TOTAL (sum of ranks)
		RANK		RANK		RANK		RANK		RANK		RANK	
Warburgia salutaris	2.483	4	0.005 bark	1	evergreen	1	Good. Seeds susceptible to parasitic infestation.	2	50	3	prefers moderate to good rainfall	2	13
Gardenia thunbergia	0.057	1	0.1 fruit pulp	3	evergreen	1	good	1	30	5	yes	1	12
Apodytes dimidiata	1.251	2	0.1 leaves	3	evergreen	1	slow	3	70	2	yes	1	12
Rauvolfia caffra	1.943	3	0.1 root and stem	4*	deciduous (evergreen under favourable conditions)	2	good	1	120	1	no	3	14
Acacia nilotica	3.612	5	0.075 fruit pulp	2	deciduous	3	slow	3	60	3	yes	4	17

* Leaves inactive

Gardenia thunbergia was the only plant to approach WHO toxicity standards. WHO (1983) suggested a target activity of 0.1g/ℓ for crude aqueous suspensions. This suggestion must be based, in part, on the practical limitation of applying large volumes of plant material to waterbodies to achieve the desired effect. Belot *et al.* (1993) found that for *Ambrosia maritima,* an inordinate quantity of material would be needed to kill snails at test sites. Total consumption of dry material for experiments at two sites were 50kg and 180kg to achieve <80% reduction of the snail population.

At the levels of activity recorded in this study, a small pond or pool of standing water (5m x 3m x 0.5m = 7.5m^3 = 7500ℓ) to be treated would require the following amounts of dry leaf material to achieve a 50% reduction in snails over 48 hours:-

W. salutaris: 2.483g x 7500ℓ = 18.62kg
G. thunbergia: 0.057g x 7500ℓ = 0.42kg
A. dimidiata: 1.251g x 7500ℓ = 9.38kg

This example illustrates the relative degree of usefulness of these plants at standing water sites involved in the transmission of schistosomiasis. Invariably, transmission occurs where there is slow flowing water. Time-concentration relationships are therefore an important consideration for field evaluations. Plant material is unlikely to remain in a flowing water system for 24 hours. Nonetheless, under the conditions described above, *G. thunbergia* is the most practical species for use. The usefulness of *A. dimidiata* and *W. salutaris* could be limited to very small water contact points. According to minimum toxicity standards (WHO, 1965) rejection of the latter two species would be recommended. However, rejection based on predefined activity levels may be viewed as short-sighted since the availability of plant material, the nature of the waterbody to be treated, toxicity to non-target organisms, and acceptance of the plant in the community are other equally important considerations. The use of *W. salutaris* in such large quantities is controversial given its national status as an endangered plant (Hall *et al.*, 1980).

3.5 CONCLUSION

The toxicity of aqueous suspensions of leaf material of the six plants selected in Chapter 2 was assessed. *Gardenia thunbergia* was found to be the most active. Since three species could be subjected to further comprehensive evaluation, *A. dimidiata* and *W. salutaris* were also selected. The final choice of these three species was based on the toxicity of aqueous suspensions, the lowest previous record of toxicity, and on their cultivation potential.

CHAPTER 4

LABORATORY EVALUATION OF THE STABILITY OF AQUEOUS SUSPENSIONS OF *WARBURGIA SALUTARIS, GARDENIA THUNBERGIA* AND *APODYTES DIMIDIATA*

HYPOTHESIS

i. Molluscicidal activity is not altered by changes in temperature, exposure to sunlight, biodegradation, long-term storage and drying.

ABSTRACT

The activity of synthetic and naturally-derived molluscicides is known to be affected by physical and chemical factors. Before molluscicides can be promoted for use in the field these factors need to be assessed. In this investigation the effects of high temperature, sunlight, biodegradation, long-term storage and drying on aqueous suspensions of *W. salutaris, G. thunbergia* and *A. dimidiata* were evaluated. *Bulinus africanus* were used in all bioassays. Experimental and control treatments were compared using Student's t-tests. Probit analyses were used in the calculation of LD_{50}s and LD_{90}s for fresh leaf material.

Results indicated that the only variable which did not alter the activity of extracts of the three plants was exposure to sunlight. Both heating and long-term storage resulted in a loss in activity in *G. thunbergia* and *A. dimidiata*. Biodegradation was responsible for reduced activity in *W. salutaris* and *A. dimidiata*. The LD_{50}s and LD_{90}s of dry mass equivalents for fresh plant material indicated that dried material was more effective than fresh material. Dried material is also more practical for use in the field. When these three plants were ranked on the basis of their stability, *W. salutaris* ranked highest followed by *G. thunbergia* and *A. dimidiata*, each with an equal rank.

4.1 INTRODUCTION

The activity of molluscicides, whether synthetic or naturally-derived, is known to be adversely affected by environmental factors. As these factors may limit the usefulness of molluscicides, it is important that they be assessed prior to conducting pilot field trials (Sturrock and Duncan, 1987). The following environmental variables have been shown to reduce the molluscicidal potency of various compounds, and have therefore been recommended for evaluation (WHO, 1965):

Temperature	-	Endod (Lemma, 1970)
pH	-	Frescon® (Lemma and Yau, 1974b)
Ultraviolet irradiation	-	NaPCP, Bayluscide® (WHO, 1965), *Solanum aculeatum* (Mkoji *et al.*, 1989)
Biodegradation	-	Copper sulphate (Lemma and Yau, 1974b)

The WHO (1965) also recommended an assessment of the effects of minerals which influence water hardness such as calcium carbonate, the long-term stability of stored material and the application of fresh versus dry plant material (Duncan and Sturrock, 1987).

In this investigation the stabilities of crude aqueous extracts of three South African molluscicidal plants were assessed: *W. salutaris, G. thunbergia* and *A. dimidiata*. The investigation evaluated only crude aqueous suspensions since this is the form in which they are intended for use in rural communities. The selection of environmental factors and range of test exposures were constrained by the limited availability of *B. africanus* for bioassays. The physical and chemical factors which received priority included an elevated temperature, exposure to sunlight, and biodegradation. Long-term storage and the use of fresh as opposed to dry material were also included, given the important practical considerations of their field use.

Bulinus africanus was used in all bioassays. Unless otherwise indicated, all trials followed the procedures detailed in Appendix 1, and the preparation of plant extracts according to Appendix 2. Data for this chapter were collected from January to April, 1994. Fresh plant material and temperature data were gathered between March and September 1992.

4.2 TEMPERATURE

Changes in temperature have usually been assessed in relation to diurnal changes in water temperature in the field. Test parameters therefore range from 5 to 35 °C (Lemma, 1970). In this study, however, the primary concern was the effect of temperature changes occurring during the extraction process. Hot and cold water extracts of any plant molluscicide are the only preparation forms which are practical when organic solvents are

expensive and/or unavailable. As cooking facilities are available in any South African community, heating provides one of the few alternatives to the preparation of cold crude aqueous suspensions (Appendix 2).

A change in activity attributable to a large temperature increase provides some indication of the degree of stability of the active ingredient in the field. This information is also useful during the laboratory-based isolation and purification of the active compound(s), when exposure to higher temperatures may occur.

4.2.1 MATERIALS AND METHODS

Control extracts of the leaves of all three plants were prepared according to Appendix 2 (i.e extraction at 23°C for 3 hours). Experimental extracts of leaf material were extracted at 96°C for the same duration. The following concentrations (g/ℓ) were tested against *B. africanus*. Control and experimental concentrations were the same for each plant species; ranges occurred at least within the LD_{50}s for *B. africanus* (Chapter 3). The number of replicates is given in parentheses:

	CONTROL	EXPERIMENTAL
	EXTRACTION AT 23°C	EXTRACTION AT 96°C
Warburgia salutaris	10 (8), 5 (8), 1 (6), 0.5 (4), 0.1 (4)	10 (2), 5 (4), 1 (4), 0.5 (2), 0.1 (4)
Gardenia thunbergia	5 (6), 1 (3), 0.5 (6), 0.1 (2), 0.05 (2)	5 (2), 1 (2), 0.5 (2), 0.1 (2), 0.05 (2)
Apodytes dimidiata	5 (8), 1 (4), 0.5 (4), 0.1 (4), 0.05 (4), 0.01 (4)	5 (2), 1 (2), 0.5 (2), 0.1 (2), 0.05 (3), 0.01 (3)

The results of experimental and control trials were then compared using Student's t-tests.

4.2.2 RESULTS

4.2.2.1 *Warburgia salutaris*

The mortality of *B. africanus* following exposure to *W. salutaris* extracted at 23°C and 96°C is shown in Figure 10.1. Extraction at the higher temperature did not significantly reduce molluscicidal activity at any concentration tested.

4.2.2.2 *Gardenia thunbergia*

The mortality of *B. africanus* following exposure to *G. thunbergia* extracted at 23°C and at 96°C is shown in Figure 10.2. Extraction at the higher temperature did not reduce molluscicidal activity at high concentrations. However at concentrations required to kill 90% of *B. africanus* under control conditions (i.e. LD_{90} = 0.196g/ℓ, 95% confidence limits (C.L.): 0.12 - 0.45), there was a significant reduction in activity.

4.2.2.3 *Apodytes dimidiata*

The mortality of *B. africanus* following exposure to *A. dimidiata* extracted at 23°C and at 96°C is shown in Figure 10.3. Extraction at the higher temperature did not reduce molluscicidal activity at very high or at very low concentrations. There was however, a significant reduction in activity at concentrations within the range causing 50 to 90% mortality of *B. africanus* under control conditions (LD_{50} = 1.251g/ℓ, 95% C.L.: 0.79 - 1.71, LD_{90} = 3.404g/ℓ, 95% C.L.: 2.52 - 4.98).

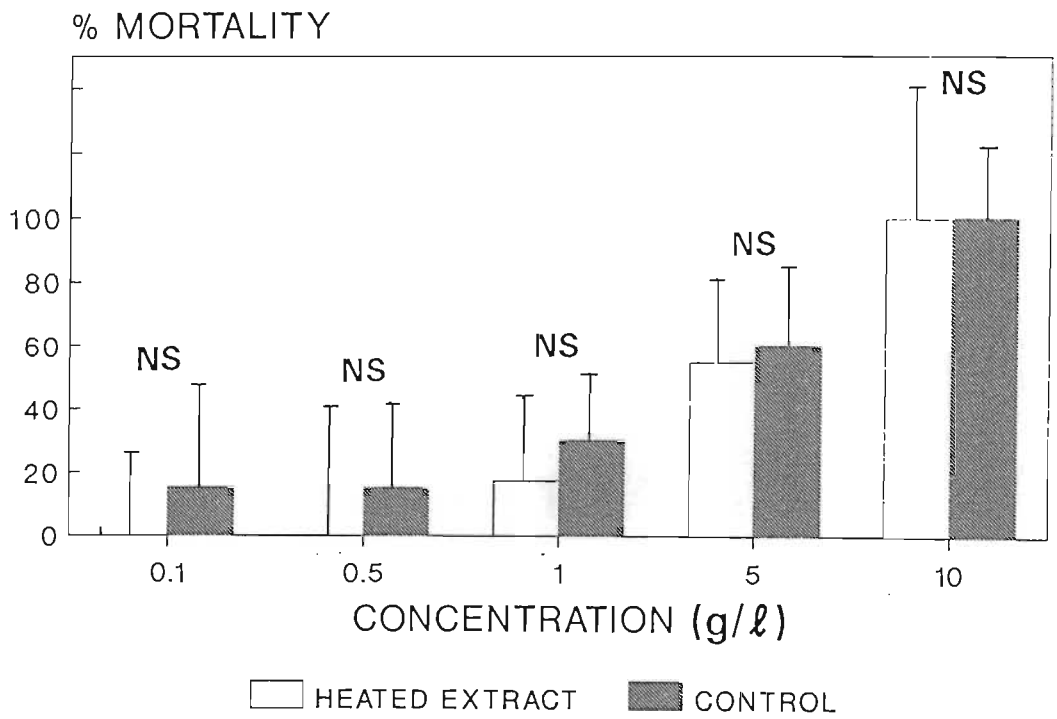


Figure 10.1 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Extractions at 96°C were compared with controls at 23°C (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

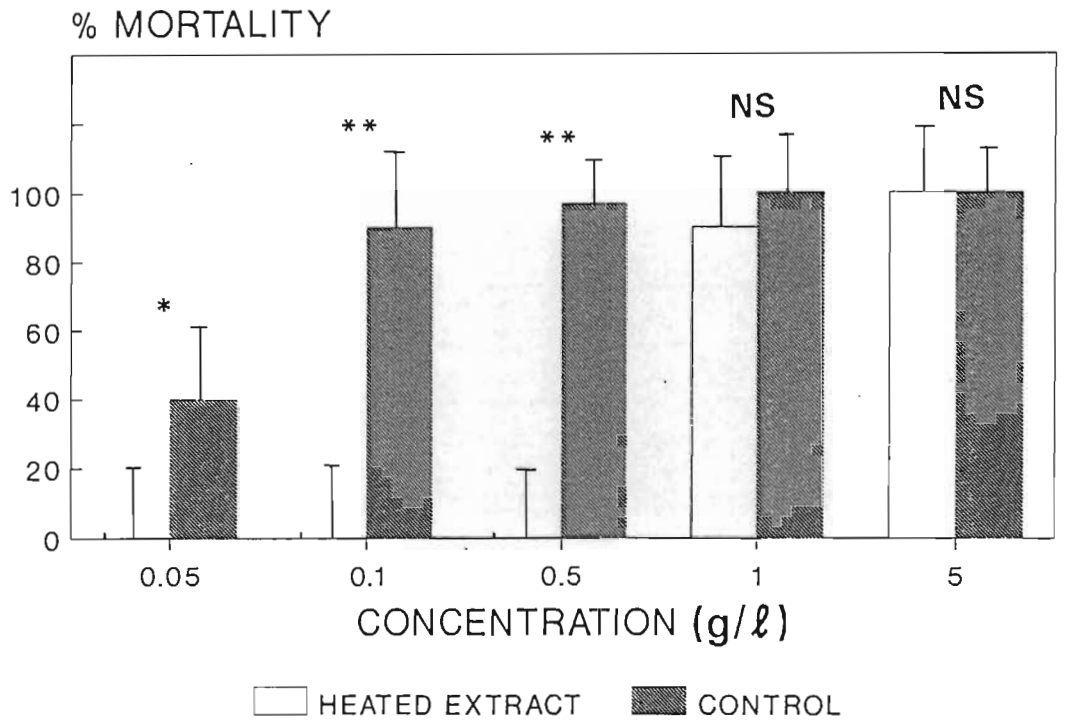


Figure 10.2 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Extractions at 96°C were compared with controls at 23°C (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

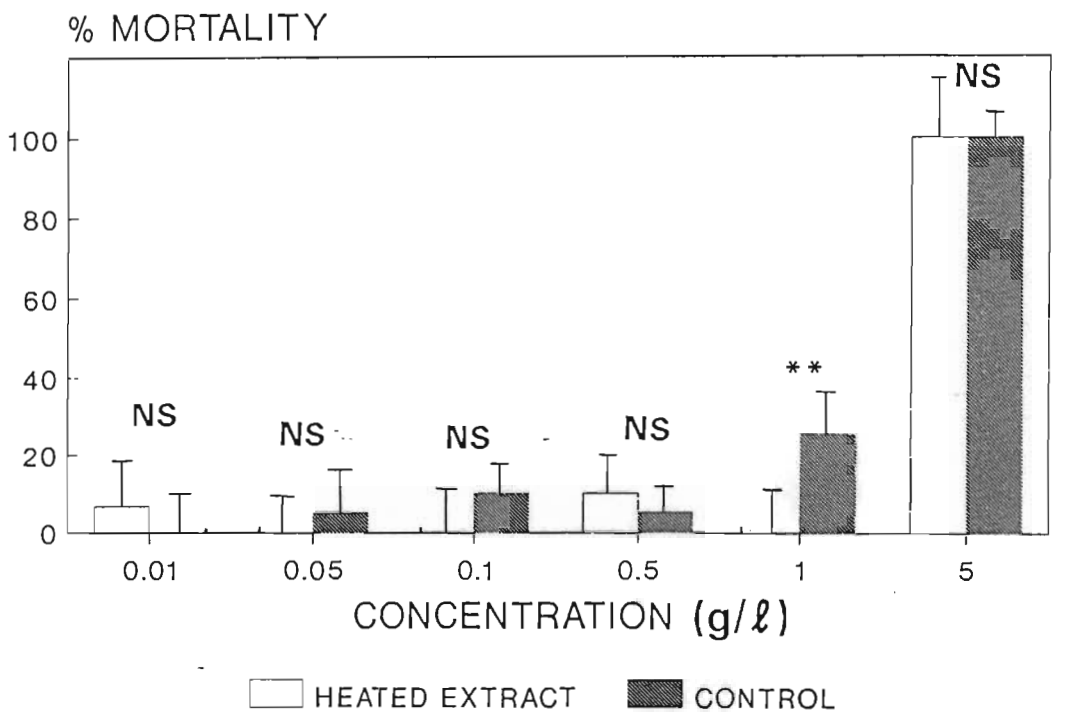


Figure 10.3 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Extractions at 96°C were compared with controls at 23°C (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

4.2.3 DISCUSSION

Boiling (or exposure to high temperature) did not improve the activity of any of the selected plants. There is therefore no practical reason to promote a hot extraction method. Heating either resulted in no change in activity, as in *W. salutaris*, or reduced activity at working concentrations (i.e. at the LD_{90}s calculated for *B. africanus* under control conditions), as for *G. thunbergia* and *A. dimidiata*. In *G. thunbergia* the effects of heating were more marked and also occurred at the LD_{50}s for *B. africanus* under control conditions. The sterilization of aqueous suspensions by boiling for the purpose of storage is therefore not advised for both *G. thunbergia* and *A. dimidiata*. Although the test temperature exceeded those of likely field conditions, the reduced activity is indicative of potential sensitivity to fluctuating field temperatures. Evaluation of the effects of temperature changes expected under field conditions warrants further investigation, at least with respect to *G. thunbergia* and *A. dimidiata*.

4.3 SUNLIGHT

4.3.1 MATERIALS AND METHODS

The procedures for testing the effect of direct sunlight have followed those of Shiff (1961), WHO (1965), Lemma (1970) and Yasuraoka (1971). Experimental and control extracts of the leaves of *W. salutaris, G. thunbergia* and *A. dimidiata* were prepared as in Appendix 2. Experimental extracts were then exposed to 4 hours of direct sunlight in large glass petri dishes (diameter - 15cm, depth - 2cm). Control extracts were exposed to fluorescent laboratory light (4 x 40W) for the same period. The following concentrations (g/ℓ) were tested against *B. africanus*. Control and experimental concentrations were the same for all plant species. The number of replicates is given in parentheses:

	CONTROL	EXPERIMENTAL
	EXTRACT EXPOSED TO LABORATORY LIGHT	EXTRACT EXPOSED TO DIRECT SUNLIGHT
Warburgia salutaris	10 (8), 5 (8), 1 (6)	10 (2), 5 (2), 1 (2)
Gardenia thunbergia	5 (6), 1 (3), 0.5 (6)	5 (2), 1 (2), 0.5 (2)
Apodytes dimidiata	5 (8), 1 (4), 0.5 (4)	5 (2), 1 (2), 0.5 (2)

The results of experimental and control trials were then compared using Student's t-tests.

4.3.2 RESULTS

There was no significant difference in mortality of *B. africanus* when treated with any of the three plant molluscicides exposed to either sunlight or laboratory light (Figures 11.1, 11.2, and 11.3).

4.3.3 DISCUSSION

Direct sunlight did not significantly alter molluscicidal activity of any of the three molluscicidal plants over a 4-hour period. Although this is indicative of potential stability under field conditions, trials assessing changes over a longer period need to be conducted. A problem encountered when assessing the activity of crude aqueous suspensions is the confounding effect of fermentation, which may be prevalent after long experimental periods (Cairns, 1982). One may then reasonably question whether molluscicidal activity can be attributed to the products of fermentation (the sources of which may be numerous), or to the presence of molluscicidal compounds. The most obvious solution here would be to re-evaluate the effects of ultraviolet irradiation following the successful extraction and identification of the active ingredient(s). Solutions of synthetic molluscicides and purified natural compounds, other than sugars, are likely to withstand fermentation over a longer period.

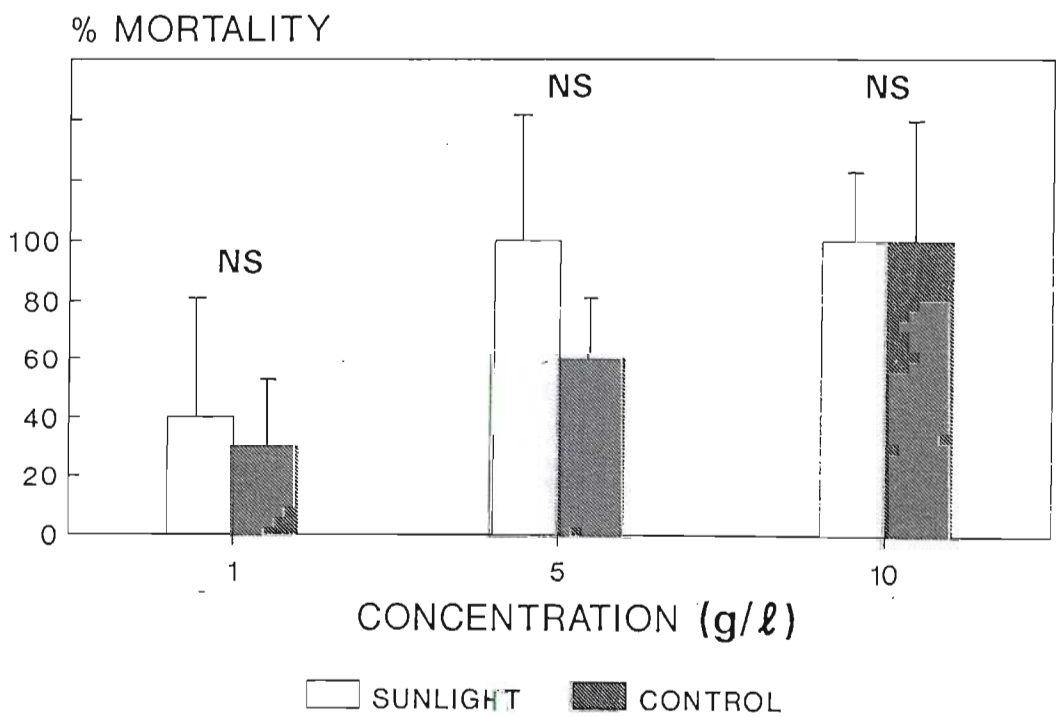


Figure 11.1 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Suspensions exposed to four hours of direct sunlight were compared with controls under laboratory lighting (NS = non significant.

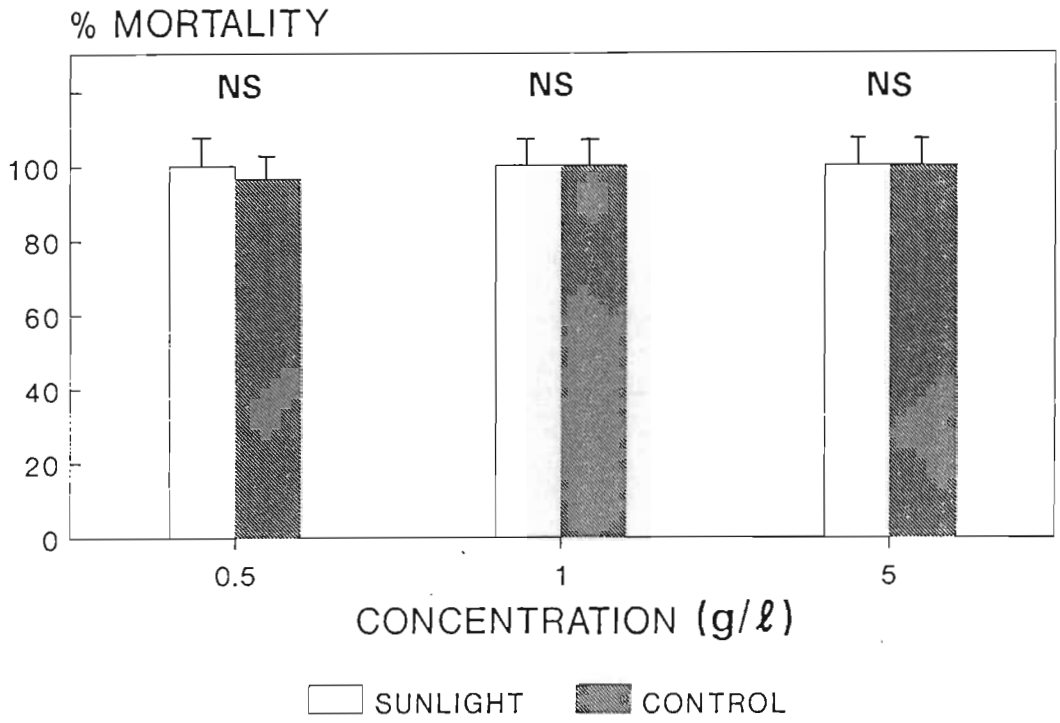


Figure 11.2 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Suspensions exposed to four hours of direct sunlight were compared with controls under laboratory lighting (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

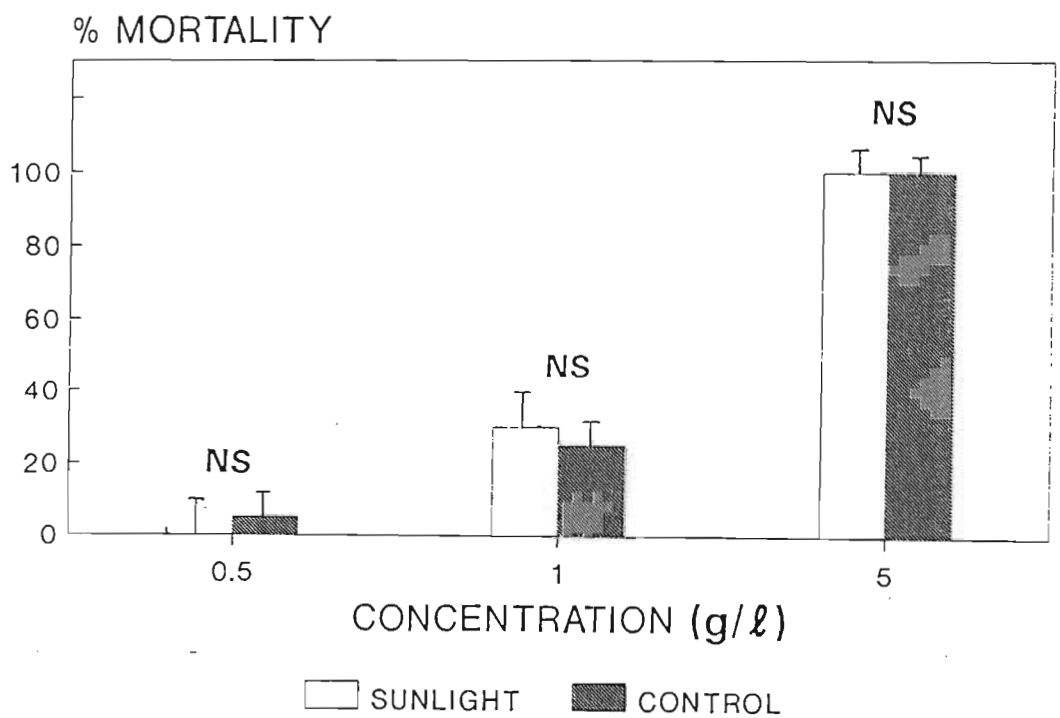


Figure 11.3 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Suspensions exposed to four hours of direct sunlight were compared with controls under laboratory lighting (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

4.4 BIODEGRADATION

Mud substrata, macrophytes and algae may all be responsible for absorption of molluscicides from solution in the natural environment. Such materials are not easy to define or standardize. The WHO (1965) suggested that faeces of rats, fed on a standard diet, represent the various contaminants in natural water. Fifty mg/ℓ was proposed as a standard concentration. Other suggested materials have included rabbit faeces (Lemma and Yau, 1974b), bentonite, powdered charcoal, kaolin and refined protein (WHO, 1983).

Rabbit faeces were selected for use in this study because of their availability and the indication (WHO, 1980) that faeces may be involved in physical adsorption, chemical binding or breakdown by bacteria. Recommended concentrations range between 500 and 4000ppm (Lemma and Yau, 1974b). An intermediate concentration of 2000ppm was employed in this study.

4.4.1 MATERIALS AND METHODS

Faeces from laboratory-reared rabbits which had been fed on a standard diet of vegetables were oven-dried at 60°C for 24 hours. Macerated plant material and faeces were then added to water to make up known concentrations of molluscicide and rabbit faeces at 2000ppm. Two sets of controls were prepared, one containing plant molluscicides alone and the other containing a solution of rabbit faeces. Test solutions were left to stand for four hours before exposure to snails. Longer standing times were avoided because of possible fermentation. Mortality due to the byproducts of fermentation could easily be confused with molluscicidal activity. The following concentrations (g/ℓ) were tested against *B. africanus*. Control and experimental concentrations were the same for each plant species. The number of replicates is given in parentheses:

	CONTROL	EXPERIMENTAL
	NO RABBIT FAECES	WITH RABBIT FAECES
Warburgia salutaris	10 (8), 5 (8), 1 (6)	10 (2), 5 (2), 1 (2)
Gardenia thunbergia	5 (6), 1 (3), 0.5 (6)	5 (2), 1 (2), 0.5 (2)
Apodytes dimidiata	5 (8), 1 (4), 0.5 (4)	5 (2), 1 (2), 0.5 (2)

Two replicates were conducted for controls containing rabbit faeces alone. The results of experimental and control trials were compared using Student's t-tests.

4.4.2 RESULTS

The mortality of *B. africanus* following exposure to treated and control solutions of the three plant molluscicides are given in Figures 12.1, 12.2 and 12.3. The presence of rabbit faeces reduced the activity of both *W. salutaris* (Figure 12.1) and *A. dimidiata* (Figure 12.3) at concentrations required to kill 50% of *B. africanus* (*W. salutaris*: $LD_{50} = 2.48 g/\ell$, 95% C.L.: 1.6 - 3.9; *A. dimidiata*: $LD_{90} = 1.25 g/\ell$, 95% C.L.: 0.7 - 1.7). At concentrations above and below this level there was no significant change in activity. The activity of *G. thunbergia* remained unaffected at all concentrations tested (Figure 12.2).

4.4.3 DISCUSSION

An organic substratum simulated by the use of rabbit faeces did not alter molluscicidal activity of *G. thunbergia*. This stability bodes well for its use under field conditions. In contrast, a significant reduction in molluscicidal activity of both *W. salutaris* and *A. dimidiata* was recorded. Concentrated solutions causing 100% mortality and those below the lower 95% confidence limits for LD_{50}s remained unaffected. Where absorption, chemical alteration or bacterial breakdown is likely taking place, there appears to be a threshold above and below which these processes do not occur. Since rabbit faeces simply simulate processes occurring in the field, the substratum at pilot study sites would require specific evaluation. The current findings do, however, warn of a potential loss of field activity.

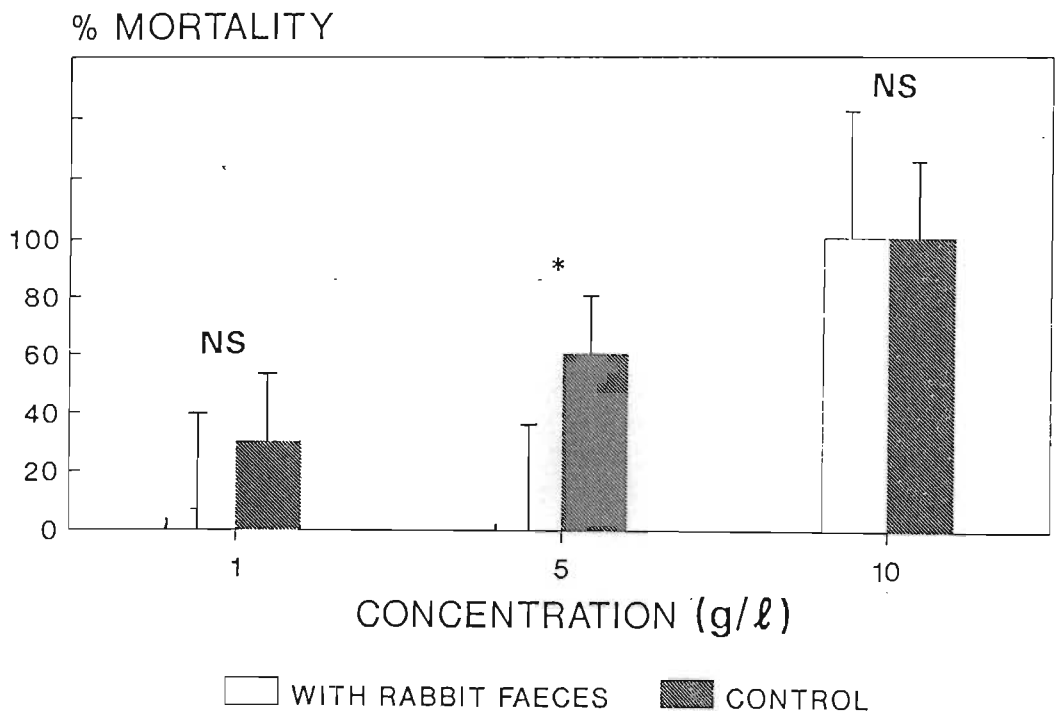


Figure 12.1 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Suspensions exposed to solutions of rabbit faeces at 2000ppm were compared with controls without

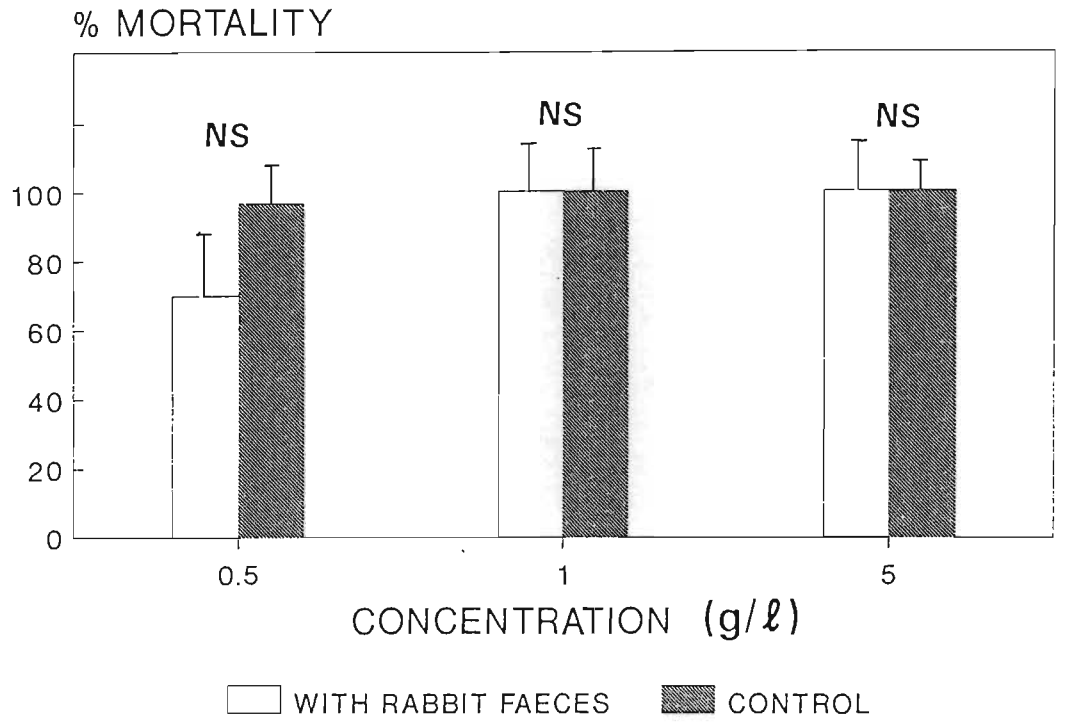


Figure 12.2 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Suspensions exposed to solutions of rabbit faeces at 2000ppm were compared with controls without faeces (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

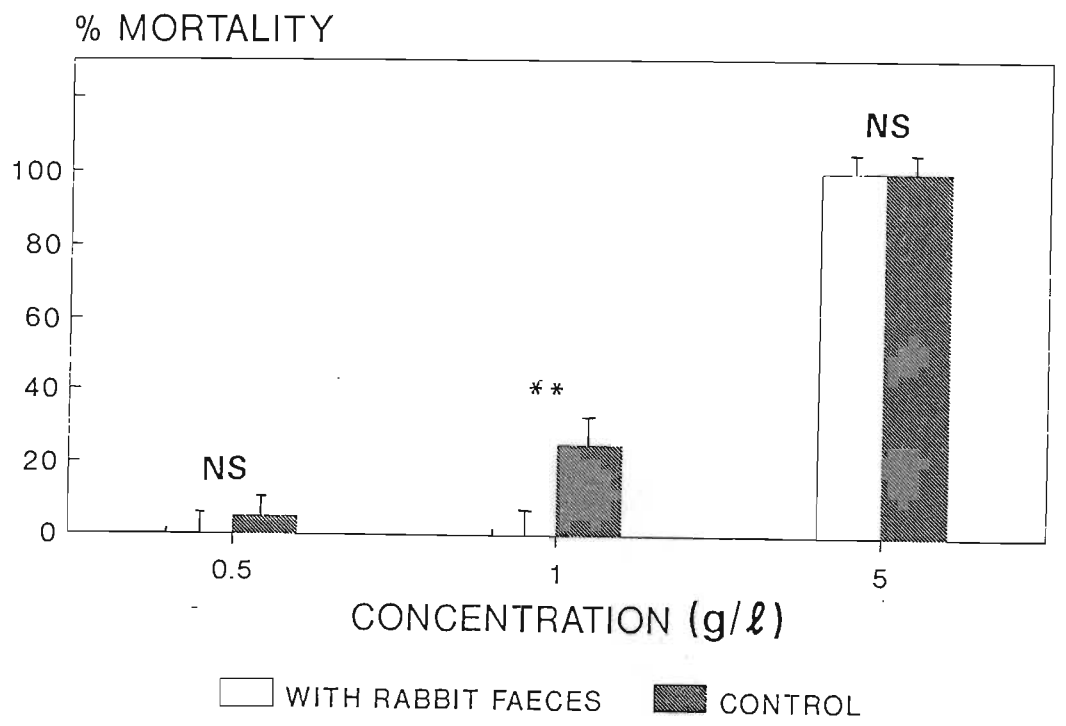


Figure 12.3 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Suspensions exposed to solutions of rabbit faeces at 2000ppm were compared with controls without faeces (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

4.5 LONG-TERM STORAGE

The WHO (1983) recommended that working dilutions of molluscicides be set aside for intervals of up to three years before testing. However, as aqueous extracts are subject to rapid fermentation, storage in this form under field and laboratory conditions is unsuitable. In a rural situation, dried material is the most convenient and practical form for storage.

4.5.1 MATERIALS AND METHODS

Macerated dried leaf material was stored at 23°C for 18 - 20 months prior to testing. Freshly dried material was used in control trials. The following concentrations (g/ℓ) were tested against *B. africanus*. Control and experimental concentrations were the same for each plant species and occurred within the LD_{50}s calculated earlier (Chapter 3). The number of replicates is given in parentheses:

	CONTROL FRESHLY DRIED PLANT MATERIAL	EXPERIMENTAL DRIED PLANT MATERIAL STORED FOR 17 - 19 MONTHS
Warburgia salutaris	10 (8), 5 (8), 1 (6), 0.5 (4), 0.1 (4)	19.6 months 10 (2), 5 (2), 1 (2), 0.5 (2), 0.1 (2)
Gardenia thunbergia	5 (6), 1 (3), 0.5 (6), 0.1 (2), 0.05 (2)	19 months 5 (2), 1 (2), 0.5 (2), 0.1 (2), 0.05 (2)
Apodytes dimidiata	5 (8), 1 (4), 0.5 (4), 0.1 (4), 0.05 (4)	18 months 5 (2), 1 (2), 0.5 (2), 0.1 (2), 0.05 (3)

The results of experimental and control trials were then compared using Student's t-tests.

4.5.2 RESULTS

4.5.2.1 *Warburgia salutaris*

Warburgia salutaris stored as dried material for 19.6 months did not lose activity (Figure 13.1).

4.5.2.2 *Gardenia thunbergia*

Lowered activity following 19 months storage of *G. thunbergia* resulted in a significant reduction in snail mortality (Figure 13.2). Concentrations affected were those required to produce 90% mortality (LD_{90} = 0.19g/ℓ, 95% C.L.: 0.12 - 0.45). At concentrations tested above this level, mortality remained unchanged.

4.5.2.3 *Apodytes dimidiata*

Apodytes dimidiata also lost activity after 18 months storage (Figure 13.3). Loss of activity occurred at concentrations required to produce 50% mortality (LD_{50} = 1.25g/ℓ, 95% C.L.: 0.79 - 1.71). At concentrations well above and below this level, mortality remained unchanged.

4.5.3 DISCUSSION

The storage of dried leaf material of *W. salutaris* for a period of 19.6 months did not result in a loss of molluscicidal activity. By comparison, *G. thunbergia* and *A. dimidiata* showed some loss in activity at working concentrations; storage of these taxa for similar time periods is not recommended. In reality, plant material would probably be stored over a period of only a few months. Accordingly, shorter storage intervals are recommended for further evaluation.

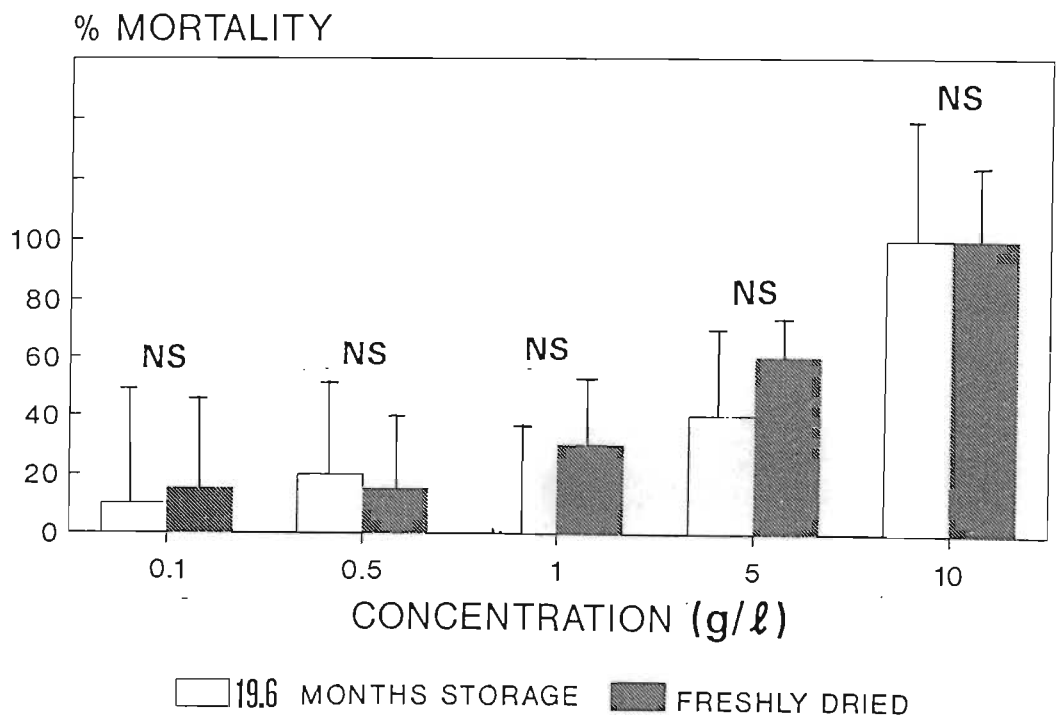


Figure 13.1 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Freshly dried material was compared with material stored for 19.6 months (NS = non significant, * = $P < 0.05$, ** = $P < 0.01$)

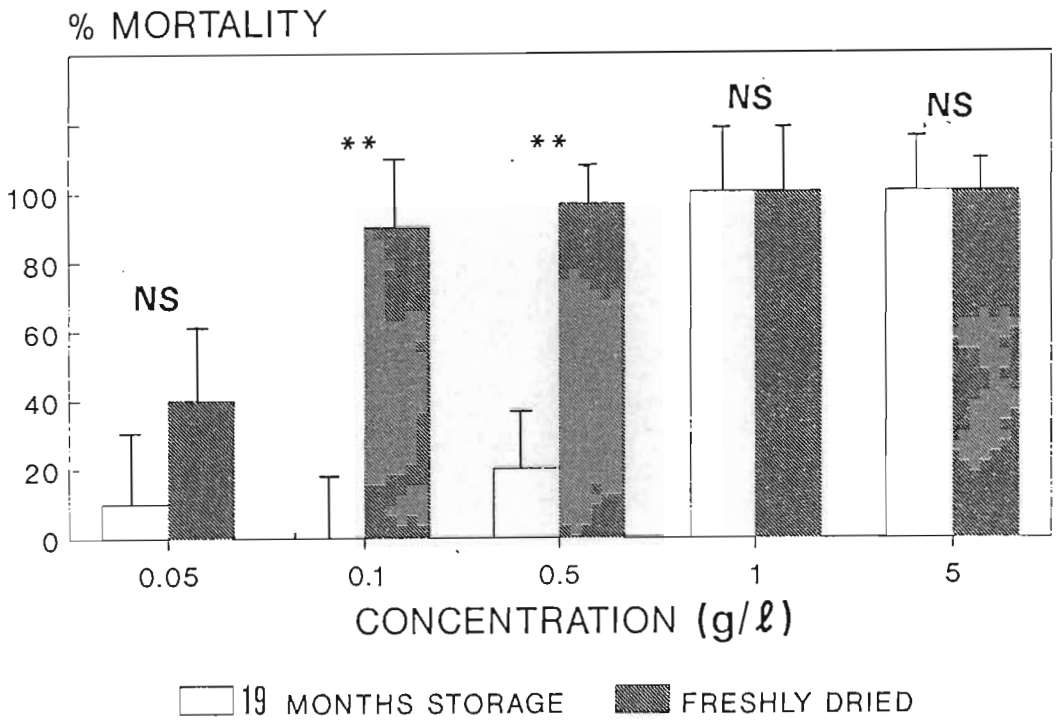


Figure 13.2 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Freshly dried material was compared with material stored for 19 months (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

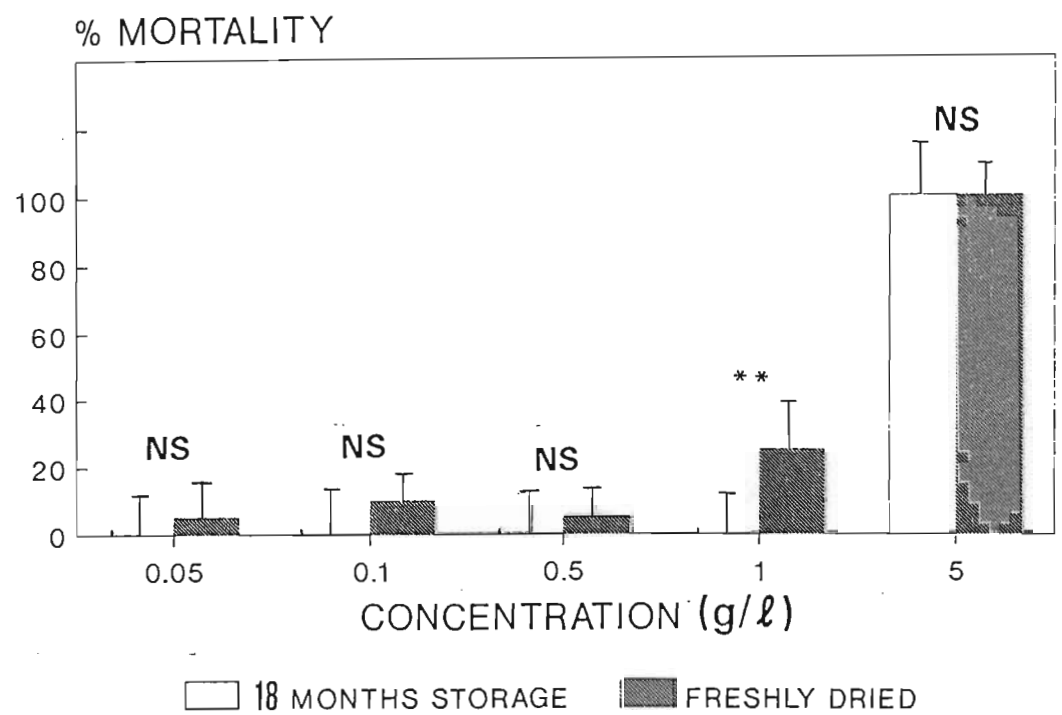


Figure 13.3 Mean % mortality and 95% confidence limits of *Bulinus africanus* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Freshly dried material was compared with material stored for 18 months (NS = non significant, * = $P<0.05$, ** = $P<0.01$).

4.6 DRYING

4.6.1 MATERIALS AND METHODS

Control dried leaf material was prepared according to the procedures in Appendix 2. In experimental trials, dried material was simply replaced by macerated fresh leaf material. LD_{50}s and LD_{90}s with 95% confidence limits were estimated using Fieller's method as implemented in the GENSTAT program (Payne, 1987). Further details of this analysis have been described (Section 3.2.1.2, Chapter 3). In order to compare the LD_{50}s and LD_{90}s obtained for dried material (as given in Chapter 3) and those obtained for fresh material, a correction for moisture content was necessary. Moisture content was calculated following weighing of fresh material, drying for 3 days at 40°C and reweighing. The mean moisture contents were based on sample sizes of 16 for *W. salutaris*, 23 for *G. thunbergia* and 18 for *A. dimidiata*.

The following concentrations (g/ℓ) were tested against *B. africanus*. The number of replicates is given in parentheses:

	FRESH LEAF MATERIAL
Warburgia salutaris	200 (2), 100 (2), 50 (2), 10 (2), 5 (2)
Gardenia thunbergia	100 (2), 40 (2), 10 (3), 2 (4), 1 (4)
Apodytes dimidiata	100 (2), 50 (2), 10 (4), 5 (4), 1 (3), 0.5 (3), 0.05 (3)

4.6.2 RESULTS

The mean percentage moisture content (± standard error) of fresh material was as follows:

W. salutaris	72.57 ± 0.97
G. thunbergia	62.83 ± 1.66
A. dimidiata	62.27 ± 1.61

The LD_{50} and LD_{90} values for crude aqueous suspensions of fresh material are presented in Table 16. Correction for moisture content permitted the calculation of dry mass equivalents (DMEs). Values for DMEs, together with those for dried material (Chapter 3) have been included for comparison in Table 16. Activities for DMEs of fresh material should theoretically be similar to values calculated directly from dry material.

It was found that the amount of DME of fresh material required was considerably higher than that of oven dried material, to produce the same mortality (Table 16). This result was unforseen since drying of material was expected to reduce activity.

ble 16. LD_{50} and LD_{90} values for crude aqueous suspensions of fresh leaf material of the three priority species.

LANT SPECIES	FRESH MATERIAL g/ℓ		FRESH MATERIAL CORRECTED FOR MEAN MOISTURE CONTENT (DRY MASS EQUIVALENT) g/ℓ		DRY MATERIAL (Results obtained in Chapter 3) g/ℓ	
	LD_{50} (95% confidence intervals)	LD_{90} (95% confidence intervals)	LD_{50} (95% confidence intervals)	LD_{90} (95% confidence intervals)	LD_{50} (95% confidence intervals)	LD_{90} (95% confidence intervals)
larburgia salutaris	44.07 (23.24 - 83.32)	255.1 (122.3 - 1384)	12.08 (6.374 - 22.85)	69.97 (33.54 - 373.63)	2.48 (1.63 - 3.96)	17.35 (8.80 - 71.10)
ardenia thunbergia	Too few partial mortalities to calculate LD_{50}s and LD_{90}s accurately. The following mortality was observed: 100% at 10g/ℓ and 60% at 2g/ℓ		100% mortality at 3.71g/ℓ 60% at 0.74g/ℓ		0.057 (0.031 - 0.096)	0.196 (0.120 - 0.451)
podytes dimidiata	4.74 (3.176 - 6.98)	16.53 (10.35 - 42.20)	1.78 (1.19 - 2.63)	6.23 (3.90 - 15.92)	1.25 (0.79 - 1.71)	3.40 (2.52 - 4.98)

It was therefore found more efficient to use dry material in molluscicidal as this form appears to contain more active ingredient than the dry mass equivalent of fresh material. The only exception recorded was the LD_{50}s for *A. dimidiata* which did not appear to show a large difference in activity when used as either fresh or dry material.

It is also apparent that the volumes of fresh material required to produce 50% mortality may be impractical for the treatment of either large or fast-flowing water bodies. For example, if a small pond or pool (5m x 3m x 0.5m = $7.5m^2$ = 7500ℓ) of standing water, were to be treated, the following amount of fresh leaf material required to achieve a 50% reduction in snail numbers over 48 hours would be:

W. salutaris: 44.07g x 7500 = 90.6kg
G. thunbergia: 2g x 7500 = 15kg (for a 60% reduction in snails)
A. dimidiata: 4.74g x 7500 = 35.55kg

4.6.3 DISCUSSION

Dry material of all three plant species contained higher concentrations of molluscicidal ingredient than the dry mass equivalent of fresh material. For *A. dimidiata* these differences were less pronounced. From this information, and the LD_{50}s and LD_{90}s for fresh material of these plants, the collection, storage, processing and application of fresh material is not likely to be practical in field situations. This is particularly so for *W. salutaris* which is currently classed as endangered in South Africa, in consequence of over-utilization as a source of herbal remedies (Cunningham, 1991).

4.7 CONCLUSIONS

The influence of physical and chemical factors on the activity of molluscicides is an important consideration when assessing field applications. Prior to their use under field conditions, the effect on molluscicides of a range of environmental factors (WHO, 1965) should be evaluated. In this investigation the effects of high temperature, exposure to sunlight, biodegradation, long-term storage, and drying were assessed.

The results indicated that the only variable which did not alter the molluscicidal activity of all three test subjects was exposure to sunlight. Both heat treatment and long-term storage resulted in loss of activity in *G. thunbergia* and *A. dimidiata*, but not *W. salutaris*. Resultant implications are that boiling or heating of aqueous suspensions will not improve the activity of extracts of the plants tested. Accordingly, the preparation of extracts should follow the procedure described in Appendix 2.

The storage of material in aqueous form is not feasible due to problems linked to fermentation, and sterilization by boiling at high temperatures is not advised given the observed loss of activity. Long-term storage of dried *G. thunbergia* and *A. dimidiata* (≥ 2 years) is also not advocated. Shorter storage period effects require evaluation.

The stability of molluscicides has also been shown to be affected by biodegradation. When simulated by solutions of rabbit faeces, biodegradation reduced the activity of *A. dimidiata* and *W. salutaris*. The activity of *G. thunbergia* remained unchanged.

An evaluation of suitable source materials for extracts was undertaken. For *G. thunbergia* and *W. salutaris*, fresh material, expressed as a dry mass equivalent, was less active than dried material. As dried material is therefore more efficient than fresh material, it can be applied in volumes more practical for use under field conditions.

Although limited in its assessment of environmental stability of these molluscicides, this study has provided some insight into the usefulness of aqueous suspensions in the field. The effect of water hardness, pH, a wider range of temperatures, longer exposures to sunlight, substrata from field sites and shorter periods of storage require thorough evaluation. A further research consideration is that of time-concentration relationships. This would provide information on concentrations required to kill snails over shorter periods of time. An understanding of this relationship is necessary when treating flowing water where molluscicides are unlikely to remain in the environment for time periods exceeding 24 hours (Shiff, 1961).

From the results of this study it is possible to prioritize the plants on the basis of their stability, and hence their suitability for use under field conditions. *W. salutaris* ranks more highly than both *G. thunbergia* and *A. dimidiata* which receive equal status.

CHAPTER 5

THE EFFECTS OF AQUEOUS SUSPENSIONS OF *WARBURGIA SALUTARIS, GARDENIA THUNBERGIA* AND *APODYTES DIMIDIATA* ON NON-TARGET FAUNA AND FLORA

HYPOTHESIS

i. Aqueous suspensions are non-toxic to non-target organisms.

ABSTRACT

The freshwater environments in which molluscicides are applied comprise numerous faunal and floral elements. As the function of molluscicides is to induce mortality of target snails, some imbalance to the biological equilibrium can be expected. It is impossible to assess the impact of these effects on all elements. Only representative species can be investigated.

In this study the toxicities of aqueous suspensions of *Warburgia salutaris*, *Gardenia thunbergia* and *Apodytes dimidiata* were assessed using the following standard test organisms and plants: *Daphnia pulex, Anopheles arabiensis, Helisoma duryi, Oreochromis mossambicus, Poecilia reticulata, Spirodela punctata* and *Lactuca sativa*. The effects of test concentrations of molluscicides were compared to controls using Student's t-tests.

It was found that concentrations equivalent to the LD_{50}s and LD_{90}s for *Bulinus africanus* had a detrimental effect on *D. pulex* and *A. arabiensis*. *Helisoma duryi,* although more molluscicide-tolerant than *B. africanus* itself (at LD_{50} levels), was also negatively affected. *Spirodela punctata* showed a significant reduction in growth rate when exposed to *W. salutaris* and *A. dimidiata* extracts. Although these results were indicative of a broader phytotoxicity affecting higher plants, germination and root elongation assays using *L. sativa* revealed no toxic effects. All three plant species were non-toxic to fish at molluscicidal concentrations. The overall order of increasing toxicity to non-target organisms, and hence decreasing suitability for field use was perceived to be: *G. thunbergia, A. dimidiata* and *W. salutaris*. The problems of extrapolating the results of limited laboratory trials to field situations are discussed, and recommendations made for further evaluations.

5.1 INTRODUCTION

Molluscicides of both natural and synthetic origin, undoubtedly disrupt biological equilibria since their function is to selectively kill snails (Hostettmann, 1984). The scientific basis

for the prediction of these effects has been the subject of considerable debate over the last decade. Numerous criteria for the selection and evaluation of test organisms have been set by a wide range of control bodies (CEC, OECD, FAO/SIDA, National Academy of Sciences) (Baudo, 1987). The final choice of organisms to undergo evaluation in toxicity trials is governed by a number of factors: availability and abundance of the correct age and size classes, economic and/or ecological importance, abiotic requirements (i.e. do they approach conditions found at the study site), availability of culture materials and methods for mass rearing, and knowledge of their physiological and nutritional requirements (Buikema *et al.*, 1982; Greenberg *et al.*, 1992). In addition, bioassays must be simple, rapid, inexpensive, and ultimately reproducible, if toxicity testing is to have any legal value (Hamburger and Hostettmann, 1991). Non-target organisms selected for this investigation included:

Invertebrates

Daphnia pulex (Leydig) Richard (CRUSTACEA: DAPHNIIDAE)

Anopheles arabiensis Patton (INSECTA: CULICIDAE)

Helisoma duryi (Wetherby) (MOLLUSCA: PLANORBIDAE)

Fish

Oreochromis mossambicus (= *Tilapia mossambica* Peters) (VERTEBRATA: CICHLIDAE)

Poecilia reticulata Peters (VERTEBRATA: POECILIIDAE)

Aquatic macrophytes

Spirodela punctata (G.F.W. Mey.) Thompson (ARALES: LEMNACEAE)

Terrestrial macrophytes

Lactuca sativa L. (ASTERACEAE: LACTUCEAE)

All crude aqueous suspensions used in bioassays in this study were prepared following procedures detailed in Appendix 1.

5.2 CRUSTACEA - *DAPHNIA PULEX*

Daphnia are reputedly one of the oldest tests organisms in the field of aquatic toxicology (Baudo, 1987). To date, most work has been conducted with *Daphnia magna* (Straus) and *D. pulex* (Baudo, 1987). De Bernardi and Peters (1987) recommended the use of

Daphnia because they survive well in culture, are easily sampled, and are important elements in the ecology of standing waters. In contrast with the other non-target organisms concurrently investigated in this study, standardized trial protocols for *Daphnia* are well established. DIN (German Standardization Organization), ISO (International Standardization Organization) and ASTM (American Society of Testing and Materials) have all proposed test procedures for *Daphnia*. There have defined culture, lighting, water quality, and test temperature conditions. Baudo (1987) clearly summarised these procedures. Other useful advice on *Daphnia* culture has been provided by Peters (1987).

5.2.1 MATERIALS AND METHODS

The methods used for static testing with *Daphnia* have been summarized (Table 17). This information was derived from Dave *et al.* (1981) and DIN, ISO and ASTM standards (Baudo, 1987). *D. pulex* was selected for evaluation because of its local availability.

5.2.2 RESULTS

The mortality of *D. pulex* over a 24-hour period of exposure to aqueous suspensions of *W. salutaris, G. thunbergia* and *A. dimidiata* are given in Figures 14.1, 14.2, and 14.3 respectively.

A significant increase in mortality for *Warburgia* at 5 and 10g/ℓ was noted when compared to controls (Figure 14.1). These concentrations were within the LD_{90} and its 95% confidence limits for *B. africanus*. Mortalities of *D. pulex* in excess of 80% were recorded at concentrations required to kill 90% of *B. africanus*. It is difficult to predict mortality of *D. pulex* at concentrations approximating the LD_{50}s for *B. africanus*, since there was a considerable increase in mortality between 1 and 5g/ℓ (Figure 14.1). If mortality is assumed to proportionally increase between these concentrations, then mortalities in excess of 50% would be expected.

Despite significant increases in mortality at 1 and 5g/ℓ of *G. thunbergia* (Figure 14.2), these concentrations were well in excess of those required to kill 50 to 90% of *B. africanus* snails. *D. pulex* is therefore likely to remain unaffected at molluscicidal concentrations.

A significant increase in mortality of *D. pulex* at 1 and 5g/ℓ of *A. dimidiata* was recorded (Figure 14.3). These concentrations were within the LD_{50}s and LD_{90}s required to kill snails. Accordingly, the mortality of *D. pulex* is expected to exceed 20% at molluscicidal concentrations.

Table 17. Summary of culture and trial conditions used for *Daphnia pulex* (adapted from Baudo, 1987).

CULTURE TECHNIQUES	
Culture vessel and medium	10ℓ glass aquaria filled with 7ℓ of dechlorinated tapwater
Photoperiod, light quality	12D ± 12L, natural
Food	*Selenastrum capricornutum* Printz and dry yeast
pH	7.4
Temperature	22.16 ± 0.136°C (n=17)
Aeration	yes
TRIAL CONDITIONS	
Test vessels	glass pill vials
Test volume	10mℓ
Individual age	<24 hours Twenty-four hours prior to experimentation, mature females were separated in glass pill vials. Neonates produced by these females during the following 12 hours (i.e. <24 hours old) were separated and used in trials.
Numbers of individuals	10-15 per vessel
Number of replicates	molluscicide treatments: 6 control: 16
Molluscicide concentrations tested	*W. salutaris*: 10, 5, 1, 0.5, 0.1, 0.05 *G. thunbergia*: 5, 1, 0.5, 0.1, 0.05, 0.01 *A. dimidiata*: 5, 1, 0.5, 0.1, 0.05, 0.01
Duration of trial	24 hours. Adema (1978) showed that mortality of unfed *Daphnia* after 24 hours was highly variable (i.e toxicity was confounded by mortality due to starvation). Preliminary trials for this study over a 48 hour period showed unpredictable mortality in controls. As this was possibly due to starvation, a reduced trial exposure period was employed.
Feeding	none
Temperature	22.16 ± 0.14°C (mean ± SD)
Photoperiod, light quality	12D ± 12L, natural
Aeration	none
Dilution water	aged, dechlorinated tapwater
Criterion of death	complete immobility

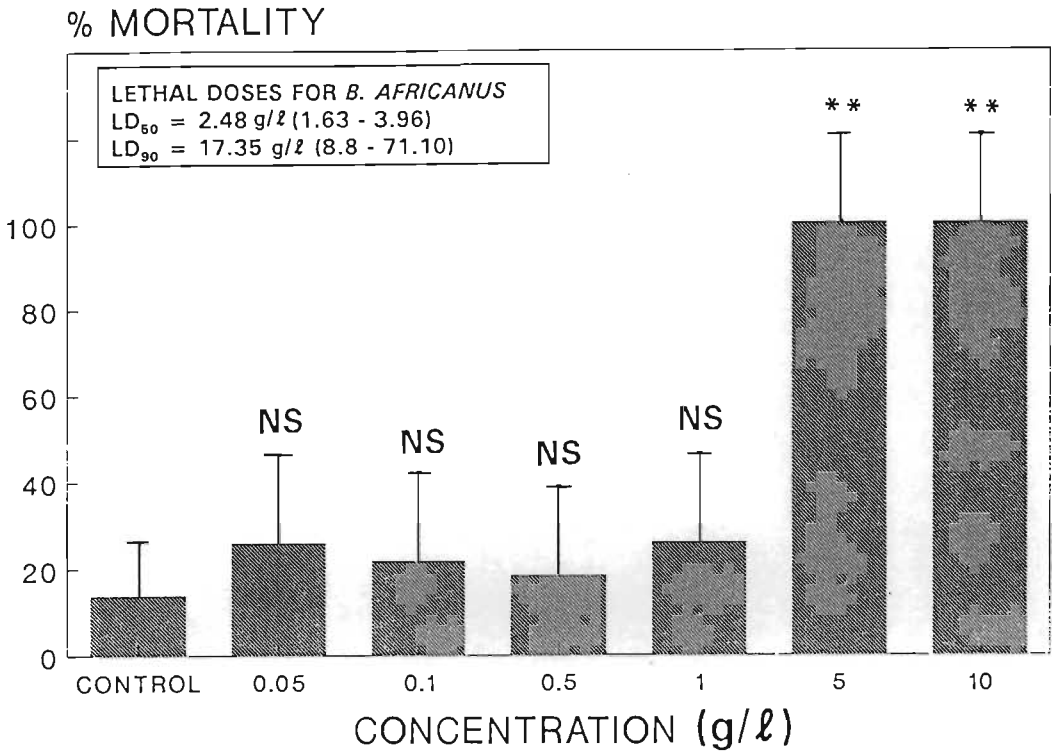


Figure 14.1 Mean % mortality and 95% confidence limits of *Daphnia pulex* in response to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

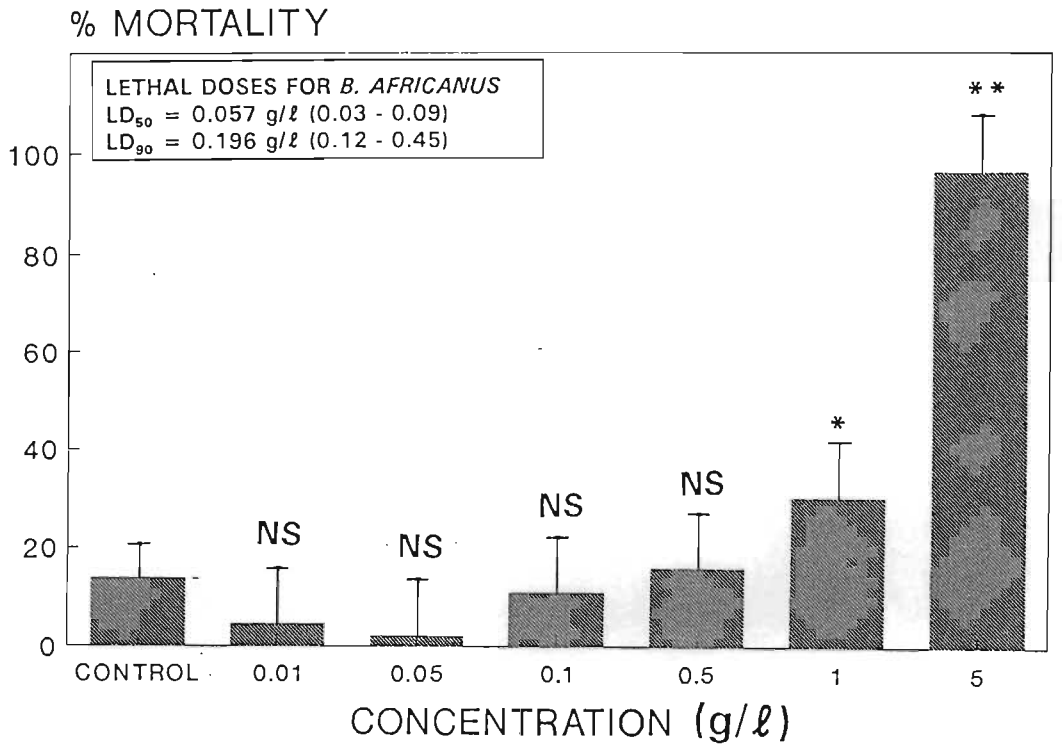


Figure 14.2 Mean % mortality and 95% confidence limits of *Daphnia pulex* in response to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$,

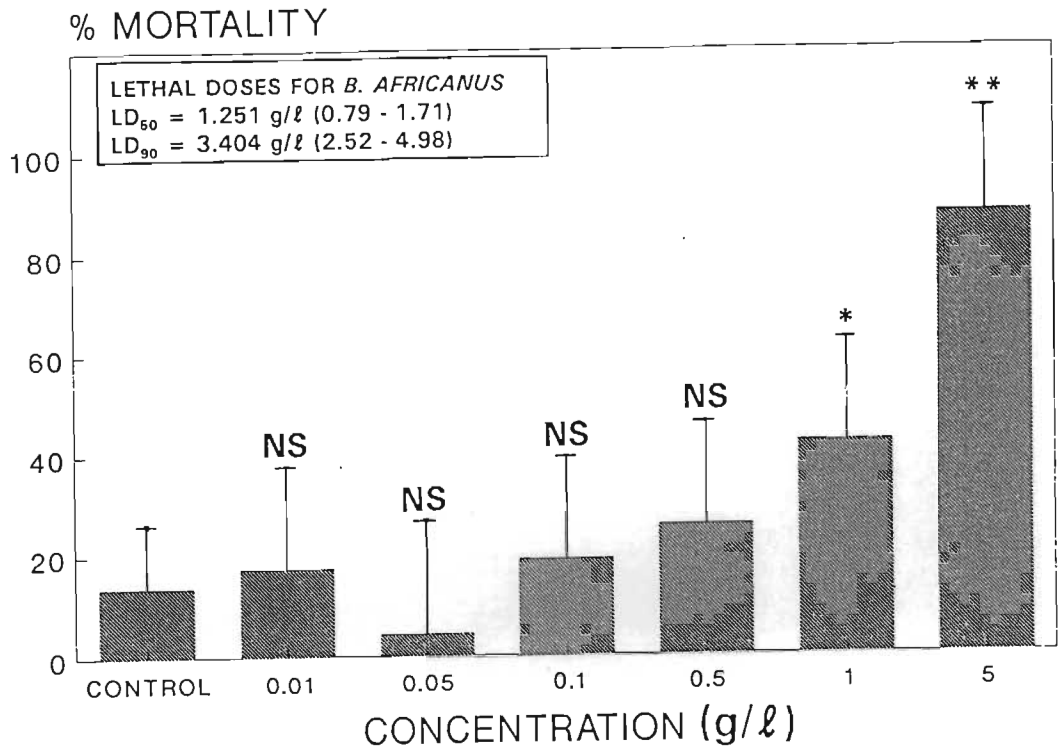


Figure 14.3 Mean % mortality and 95% confidence limits of *Daphnia pulex* in response to aqueous suspensions of *Apodytes dimidiata* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

5.2.3 DISCUSSION

The above findings based on *D. pulex* bioassays indicate that over a 24-hour exposure to molluscicidal concentrations of *W. salutaris* and *A. dimidiata*, *Daphnia*, and possibly other small crustaceans, would be adversely affected. Of these two plant species, *W. salutaris* is likely to have a greater detrimental effect at the LD_{90}s required to kill snails. In contrast, application of molluscicidal concentrations of *G. thunbergia* is unlikely to result in significant increases in mortality of *D. pulex*. On the basis of these findings, it is not possible to accurately predict the effect of molluscicides on the population dynamics of *D. pulex* in a natural environment. The results indicate the sensitivity of *Daphnia* only under standardized test conditions. Field investigations are required.

Shiff and Garnett (1961) reported that in field trials, NaPCP, niclosamide and copper sulphate all diminished planktonic life in ponds immediately following their application. However, population trends recovered to pretreatment levels within 32 days of applying NaPCP and niclosamide. Copper sulphate application resulted in a total elimination of cladocera and insects within this period. Research of this nature has highlighted the paradoxical resilience and sensitivity of invertebrates.

5.3 INSECTA - *ANOPHELES ARABIENSIS*

Mosquito larvae have conventionally been used in insecticidal assays because of their

sensitivity to small doses (Busvine, 1957). Although first and fourth instar larvae are usually employed, molluscicidal research has involved the full range of instars and pupae. Numerous species of mosquito have been used, including *Anopheles gambiae*, *A. quadrimaculatus* and *Aedes aegypti* (Spielman and Lemma, 1973; Lemma *et al.*, 1975; Tesfaye-Yohannes, 1976). In this study laboratory-reared *Anopheles arabiensis* were used.

5.3.1 MATERIALS AND METHODS

Trial procedures are detailed in Table 18 and have followed those of Spielman and Lemma (1973) and Lemma *et al.* (1975).

5.3.2 RESULTS

The mortality of the larvae and pupae of *A. arabiensis* in response to aqueous suspensions of *W. salutaris, G. thunbergia* and *A. dimidiata* are given in Figures 15.1, 15.2 and 15.3 respectively.

There was a significant increase in mortality at 10g/ℓ of *W. salutaris*, for all larval instars (Figure 15.1). As this concentration lies within the LD_{90} for *B. africanus* over the same time period, mortalities ≥ 15%, 80%, 50% and 20% are expected for first, second, third and fourth larval instars respectively. There was a significant increase in mortality for second and third larval instars at concentrations required to kill 50 to 80% of *B. africanus* over the same time period. Mortality was again higher for second instars. Pupae appeared to be most resistant at all molluscicidal concentrations (Figure 15.1). Although significant increases in mortality of all instars was recorded for *G. thunbergia* at 1 and 5g/ℓ (Figure 15.2), these concentrations well exceeded those required to kill either 50 or 90% of *B. africanus*. Only in second instar larvae was mortality significantly greater than controls, at concentrations required to kill 90% of the snails. At these concentrations, second instar mortalities can be expected to exceed 30%. As with *W. salutaris* extracts, pupae were again the most resistant at all molluscicidal concentrations. First instars and pupae were unaffected at the molluscicidal concentrations tested for *A. dimidiata* (Figure 15.3). Second instars were most severely affected; significant increases in mortality occurred at and below those concentrations required to kill 50 to 90% of *B. africanus*. Mortalities of second instar larvae in excess of 35% are expected at these concentrations. Some mortality is likely for third and fourth instar larvae at the LD_{90}s required to kill snails, since there is a significant increase in mortality at 5g/ℓ. Fourth instar larvae are likely to be equally affected, and mortalities in excess of 30% can be expected.

Table 18. Summary of trial conditions and procedures used for *Anopheles arabiensis*.

CULTURE TECHNIQUES	
Source of stock	laboratory cultures from the Malaria Programme of the Medical Research Council, Congella, Durban, South Africa
Culture medium and vessels	2ℓ plastic containers (200 x 150 x 80mm) filled with 1.5ℓ of distilled water
Photoperiod, light quality	12D ± 12L, cool fluorescent light (4 x 40W)
Food	macerated dried dog pellets
Temperature	26°C
Aeration	none
TRIAL CONDITIONS	
Test vessel	glass vials
Test volume	20mℓ
Molluscicide concentrations tested (g/ℓ)	*W. salutaris*: 10, 5, 1, 0.5, 0.1, 0.05 *G. thunbergia*: 5, 1, 0.5, 0.1, 0.05, 0.01 *A. dimidiata*: 5, 1, 0.5, 0.1, 0.05, 0.01
Individual age	First, second, third and fourth larval instars and pupae (Le Sueur, 1991)
Numbers of individuals	10 larvae per vessel 5 pupae per vessel
Number of replicates	molluscicide treatment: 4 for larvae, 3 for pupae controls: 6 for larvae, 4 for pupae
Duration of trial	24 hour exposure 24 hour recovery
Feeding	only during recovery
Temperature	26°C
Photoperiod, light quality	12D ± 12L, cool fluorescent light (4 x 40W)
Aeration	none
Dilution water and controls	distilled water
Criterion of death	inability to rise to the surface of the solution and lack of movement

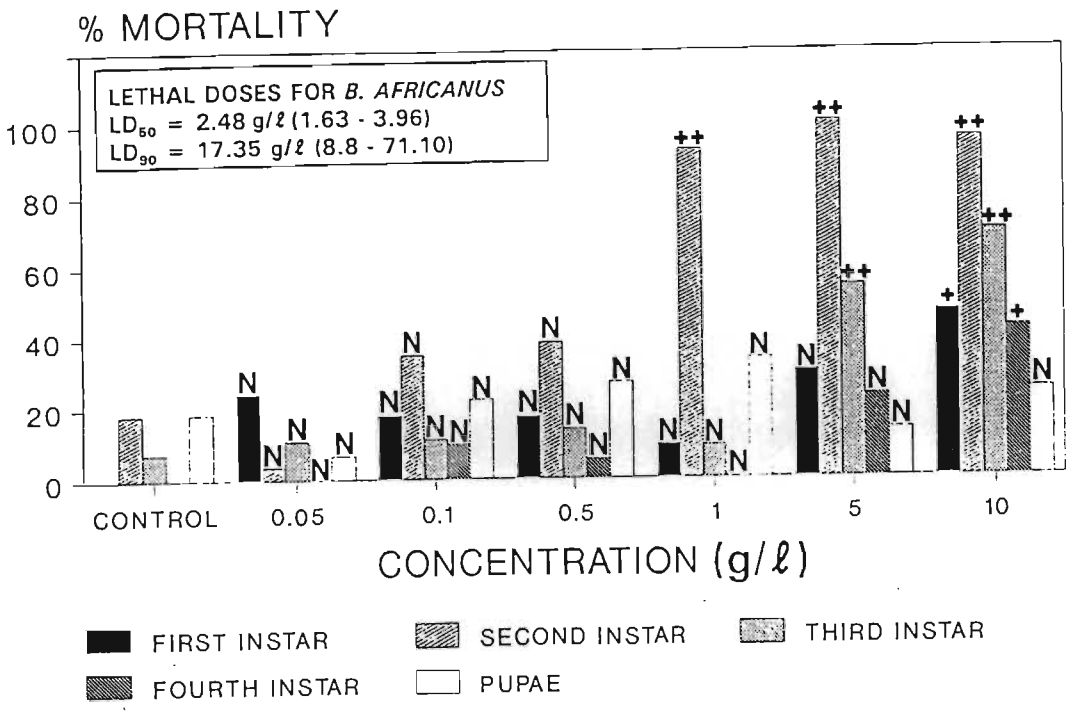


Figure 15.1 Mean % mortality of all larval instars and pupae of *Anopheles arabiensis* in response to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (N = non significant, + = $P<0.05$, + + = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

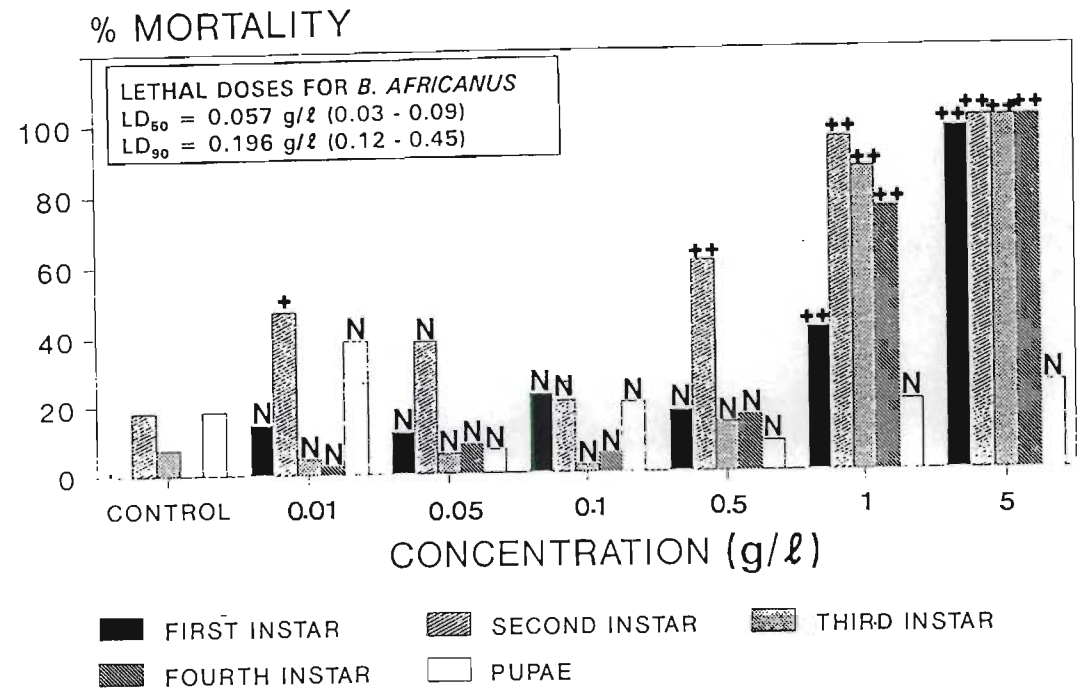


Figure 15.2 Mean % mortality of all larval instars and pupae of *Anopheles arabiensis* in response to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (N = non significant, + = $P<0.05$, + + = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

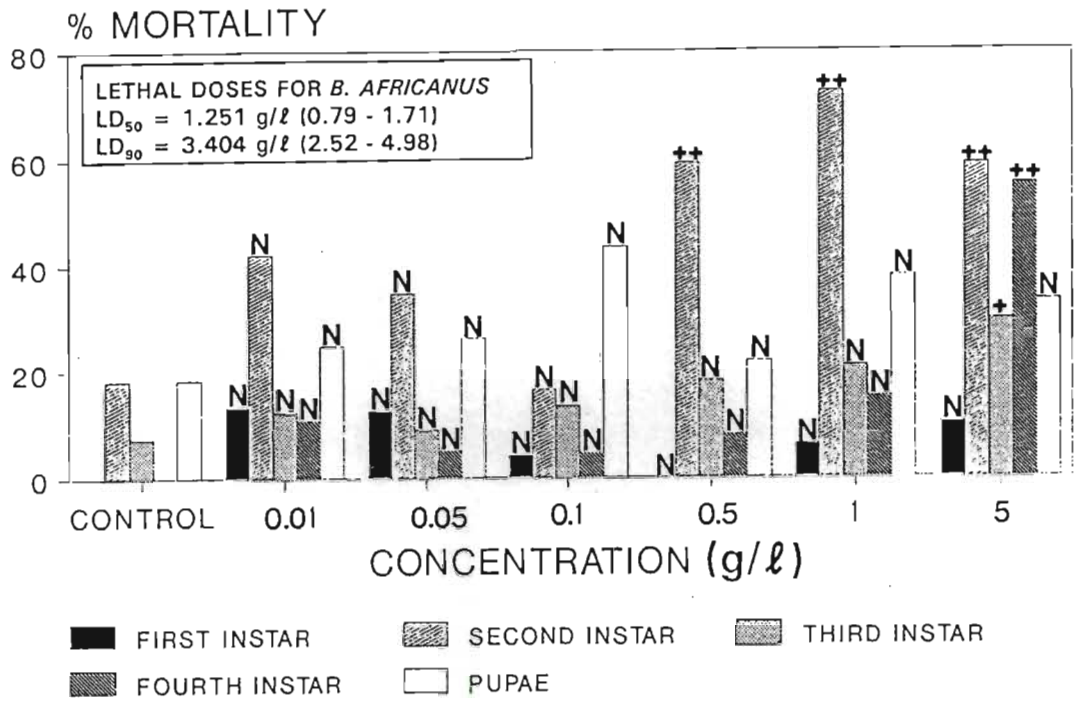


Figure 15.3 Mean % mortality of all larval instars and pupae of *Anopheles arabiensis* in response to aqueous suspensions of *Apodytes dimidiata* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (N = non significant, + = $P<0.05$, + + = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

5.3.3 DISCUSSION

Aqueous suspensions of the leaves of all plant species significantly increased second larval instar mortality at concentrations required to kill 90% of *B. africanus*. Of the three plant extracts, only *Gardenia thunbergia* did not significantly increase mortality at concentrations required to kill 50% of the snails. Second larval instars appeared, therefore, to be most sensitive to these molluscicides. Notably, all pupae remained unaffected. The current findings are in accord with those of Spielman and Lemma (1973) who recorded the greatest mortality for second instar larvae of *A. aegypti* exposed to endod. Pupae and eggs were similarly unaffected.

Gardenia thunbergia caused the least overall mortality of mosquitoes. The next least lethal was *A. dimidiata* which affected all but first larval instars. *Warburgia salutaris* affected all larval instars. Since at least one life stage was adversely affected by all three plant molluscicides, significant reductions in population numbers of mosquitoes could be expected. Although this effect may be welcomed when the eradication of mosquitoes is desired, it does however more importantly indicate the sensitivity of aquatic larval forms to these compounds. Field evaluations would again be important in clarifying the extent of this action.

5.4 MOLLUSCA - *HELISOMA DURYI*

Indigenous molluscs which are likely to be affected by field applications of molluscicides were not available in sufficient numbers for use in bioassays. However, *Helisoma duryi* had been well established in laboratory culture for over 2 years. This species was also of particular interest because it had been identified as a suitable model for investigations on the mode of molluscicide action (Chapter 6). Knowledge of mortality in response to these plant molluscicides was essential before use could be made of *H. duryi* in mode of action studies.

5.4.1 MATERIALS AND METHODS

Trial procedures followed those for *B. africanus* given in Appendix 1. The following concentrations (g/ℓ) were tested against both *H. duryi* and *B. africanus.* Concentrations fell within the LD_{90}s calculated for *B. africanus* (Chapter 3). The number of replicates is given in parenthesis:

	HELISOMA DURYI mean shell diameter ± SE: 12.58mm ± 0.35	*BULINUS AFRICANUS* mean shell height: as given in Appendix 1
Warburgia salutaris	10 (3), 5 (4), 1 (4)	10 (8), 5(8), 1 (6)
Gardenia thunbergia	5 (2), 1 (2), 0.5 (4), 0.1 (2)	5 (6), 1 (3), 0.5 (4), 0.1 (4)
Apodytes dimidiata	5 (3), 4 (2), 1 (4), 0.5 (4)	5 (8), 4 (2), 1 (4), 0.5 (4)

5.4.2 RESULTS

The mortalities of *H. duryi* relative to *B. africanus* are given in Figures 16.1, 16.2 and 16.3. Both *W. salutaris* and *A. dimidiata* extracts produced significantly lower mortalities of *H. duryi* at intermediate concentrations. These concentrations either closely approximated, or were within the range of LD_{50}s calculated for *B. africanus*. Higher concentrations of these two plant species would therefore be expected to kill 50% of *H. duryi*. However, at concentrations marginally in excess of the LD_{90}s for *B. africanus*, there was no significant difference in mortality between these two snail species.

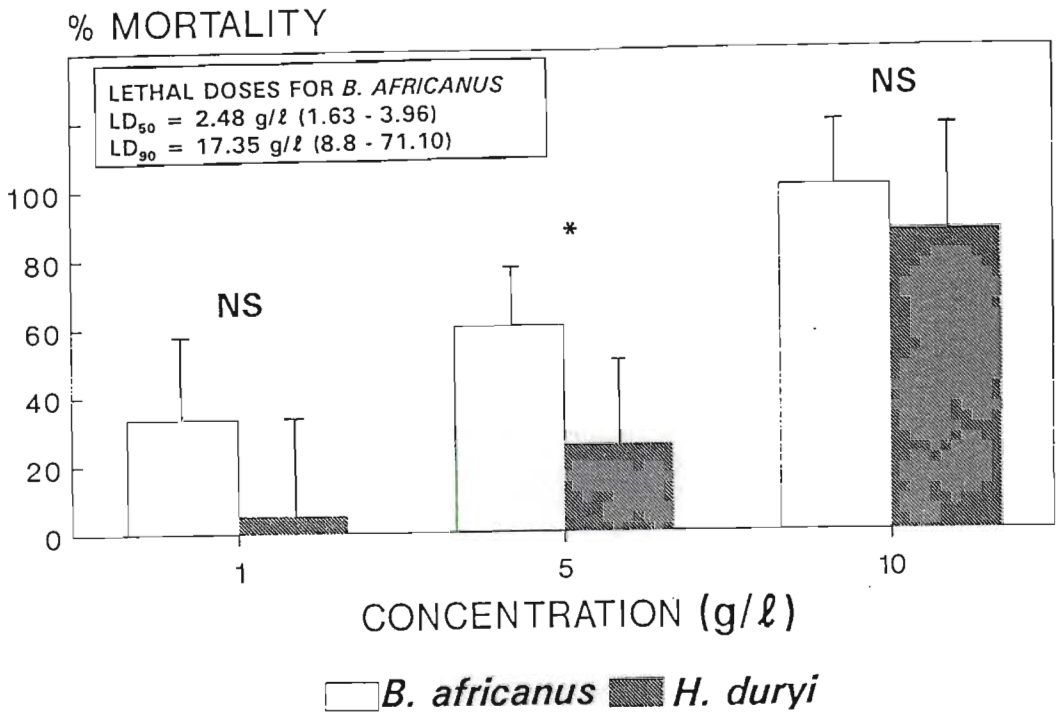


Figure 16.1 Mean % mortality and 95% confidence limits of *Bulinus africanus* and *Helisoma duryi* exposed to aqueous suspensions of *Warburgia salutaris* leaves (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

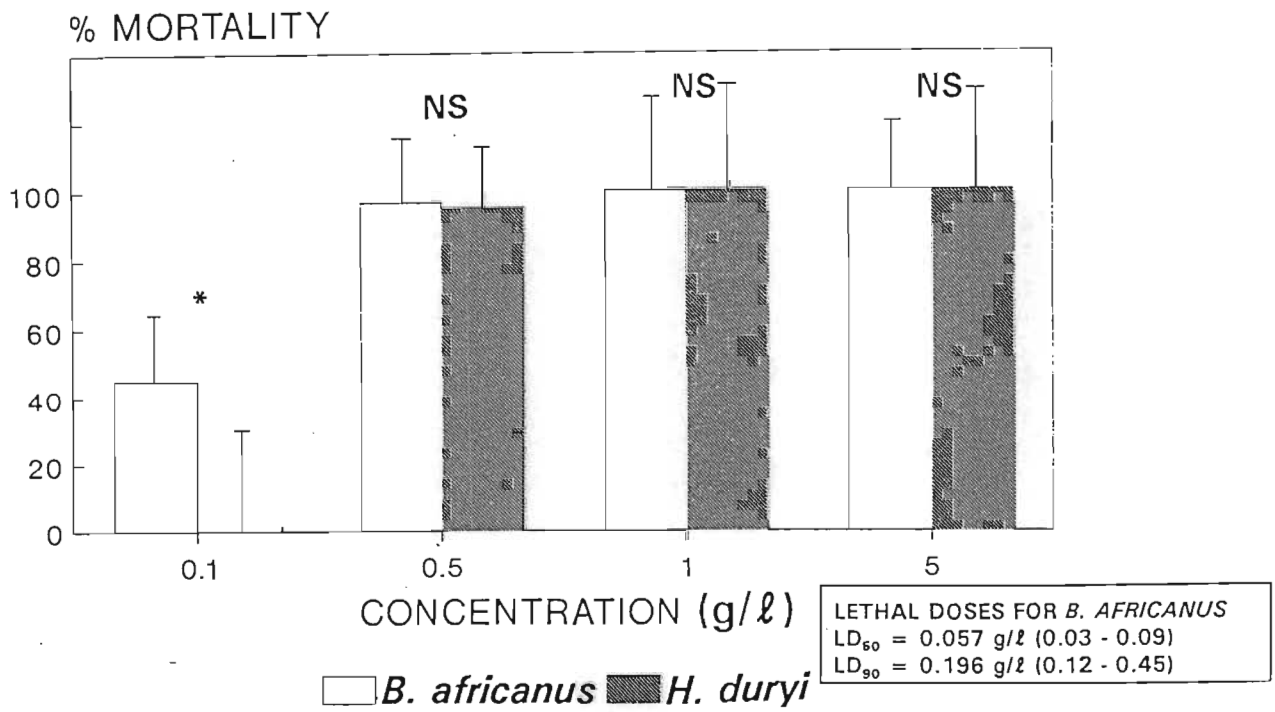


Figure 16.2 Mean % mortality and 95% confidence limits of *Bulinus africanus* and *Helisoma duryi* exposed to aqueous suspensions of *Gardenia thunbergia* leaves (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

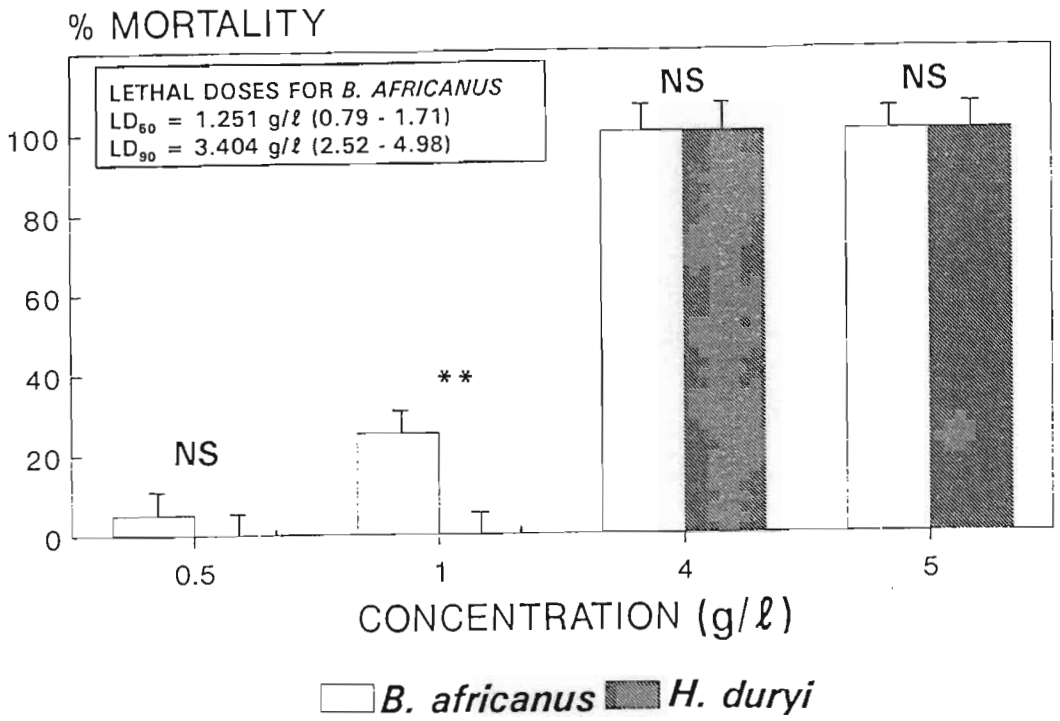


Figure 16.3 Mean % mortality and 95% confidence limits of *Bulinus africanus* and *Helisoma duryi* exposed to aqueous suspensions of *Apodytes dimidiata* leaves (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

Gardenia thunbergia also appeared to have less impact on *H. duryi* (Figure 16.2) at concentrations approximating the LD_{90}s calculated for *B. africanus*. As with *Warburgia*, concentrations in excess of the LD_{90}s resulted in no significant difference in mortality between the two snail species. Concentrations within the range of the LD_{50}s were not assessed; accordingly it is not known how activity would be affected.

5.4.3 DISCUSSION

Helisoma duryi exhibited a high tolerance to extracts of *W. salutaris* and *A. dimidiata* at concentrations required to kill 50% of *B. africanus*. Concentrations in excess of the LD_{50} are likely to result in similar mortalities for both species. *Gardenia thunbergia,* however, showed the least impact on *H. duryi* at concentrations approximating the LD_{90}s for *B. africanus*. The findings indicate that non-target snail populations are likely to be affected by these molluscicides, especially since laboratory rearing of *Helisoma* has revealed this species to be particularly vigorous and hardy. If the concentrations of molluscicides administered are maintained below the LD_{90}s for *B. africanus,* then effects on non-target molluscs may be less detrimental.

5.5 FISH - *OREOCHROMIS MOSSAMBICUS* AND *POECILIA RETICULATA*

5.5.1 MATERIALS AND METHODS

The methods have followed those of Shiff *et al.* (1967), and Joubert and Pretorius (1991). *Oreochromis mossambicus* has previously been used (Lemma and Yau, 1974a) and is common in schistosomiasis endemic areas in South Africa (Pretorius *et al.*, 1991). Extensive fungal infections in stock material essentially terminated the trials before evaluations were complete. However, useable results were still obtained for *W. salutaris* and *G. thunbergia*. This necessitated the selection of a second species for evaluation. *Poecilia reticulata* has also been similarly used in studies on the toxicology of molluscicides (Andrews *et al.*, 1987). This fish species is readily available from suppliers and is of a convenient handling size. The bioassay procedures followed have been given in Table 19.

5.5.2 RESULTS

The mortalities of *O. mossambicus* and *P. reticulata* following exposure to each plant extract are given in Figures 17.1 to 17.5.

Both fish species were unaffected by solutions of *W. salutaris* at all concentrations tested, including the LD_{50} for *B. africanus* (Figures 17.1 and 17.2). Both bioassay subjects showed a significant increase in mortality when exposed to high concentrations of *G. thunbergia* (Figures 17.3 and 17.4). However, concentrations tested were well in excess of that required to kill 90% of *B. africanus*. *Poecilia reticulata* was also unaffected by *A. dimidiata* extracts at concentrations exceeding the LD_{50} and LD_{90} required to kill *B. africanus* (Figure 17.5).

5.5.3 DISCUSSION

Both *O. mossambicus* and *P. reticulata* were unaffected by all three plant molluscicides at concentrations required to kill at least 50% of *B. africanus*. *Gardenia thunbergia* and *A. dimidiata* did not cause a significant increase in mortality at concentrations well in excess of the LD_{90}s for *B. africanus*. The negative findings for *A. dimidiata* are of particular interest since this plant species was identified by respondents of a rural community as a piscicide used in the KwaZulu-Natal south coast region (Chapter 1). During interviews no indications were given of how material was prepared, what plant parts were used, and at what concentrations the piscicide was applied. Further, Pretorius *et al.* (1991) reported that *O. mossambicus* died within one hour of exposure to aqueous suspensions of *A. dimidiata* at concentrations between 0.1 and 0.5g/ℓ.

Table 19. Summary of trial conditions and procedures used for ***Oreochromis mossambicus*** and ***Poecilia reticulata*** (guppy).

	OREOCHROMIS MOSSAMBICUS	*POECILIA RETICULATA*
Source of stock	commercial hatchery	commercial tropical fish supplier
Test vessel and volume	10ℓ glass aquaria containing 5ℓ of test solution	10ℓ glass aquaria containing 7ℓ of test solution
Molluscicide concentrations tested (g/ℓ)	*W. salutaris*: 7, 3.5, 0.7, 0.35, 0.07, 0.035 *G. thunbergia*: 5, 1, 0.5, 0.1, 0.05, 0.01 severe fungal infection of stock resulted in the cessation of further evaluation	*W. salutaris*: 10, 5, 1, 0.5 *G. thunbergia*: 5, 1, 0.5, 0.1 *A. dimidiata*: 5, 1, 0.5, 0.1
Individual size	*W. salutaris*: 54.82 ± 1.45mm (mean ± SE) (n = 39) *G. thunbergia*: 75.35 ± 1.31mm (mean ± SE) (n = 42) males and females used	28.17 ± 1.32mm (mean ± SE) (n = 11) females only (these are easily distinguished from the males)
Numbers of individuals	3 per vessel	5 per vessel
Number of replicates	2 for each molluscicide treatment and control	3 for each molluscicide treatment and control
Duration of trial	24 hour exposure (readings taken at 3, 6 and 24 hours), 24 hour recovery	
Feeding	fish flakes during recovery only	
Temperature	25°C	
Photoperiod, light quality	12D ± 12L, cool fluorescent light (4 x 40W)	
Aeration	yes	
Control, dilution and recovery water	aged dechlorinated tap water	
Criterion of death	no breathing and immobility	

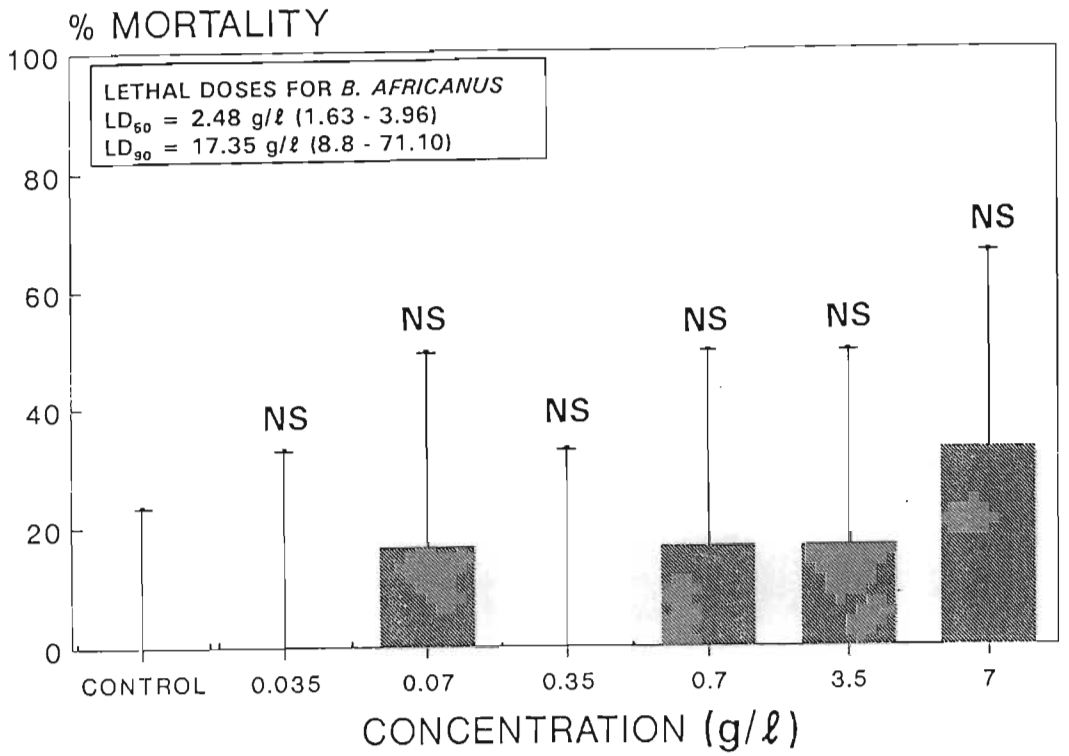


Figure 17.1 Mean % mortality and 95% confidence limits of *Oreochromis mossambicus* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

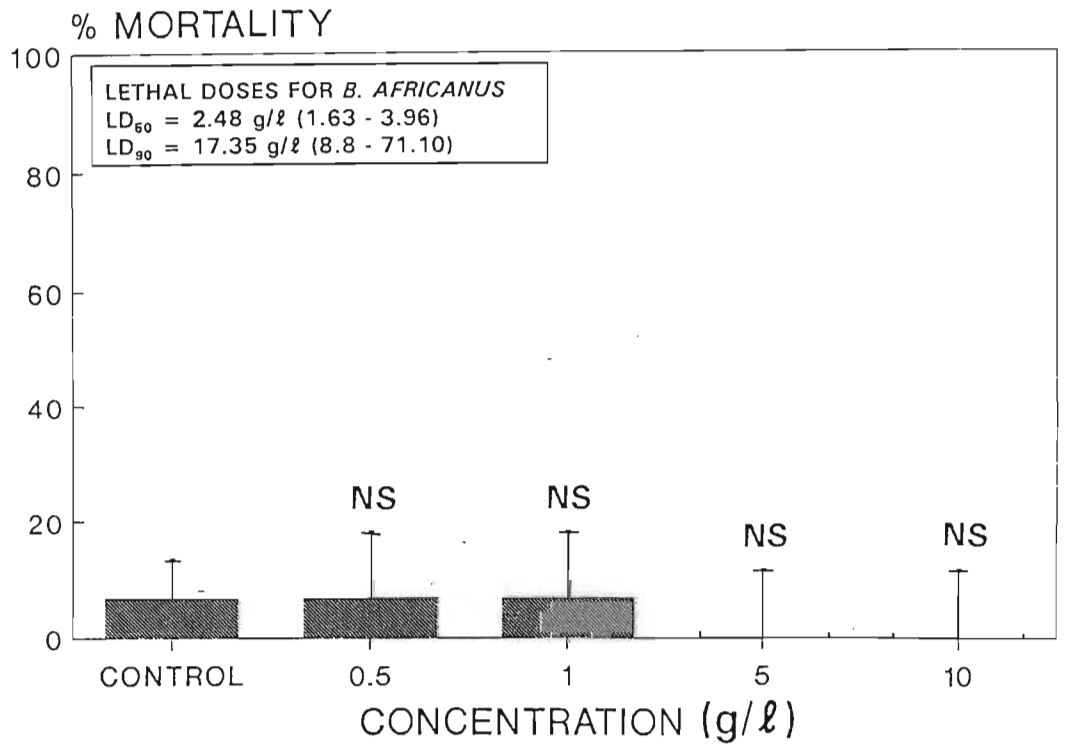


Figure 17.2 Mean % mortality and 95% confidence limits of *Poecilia reticulata* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$,

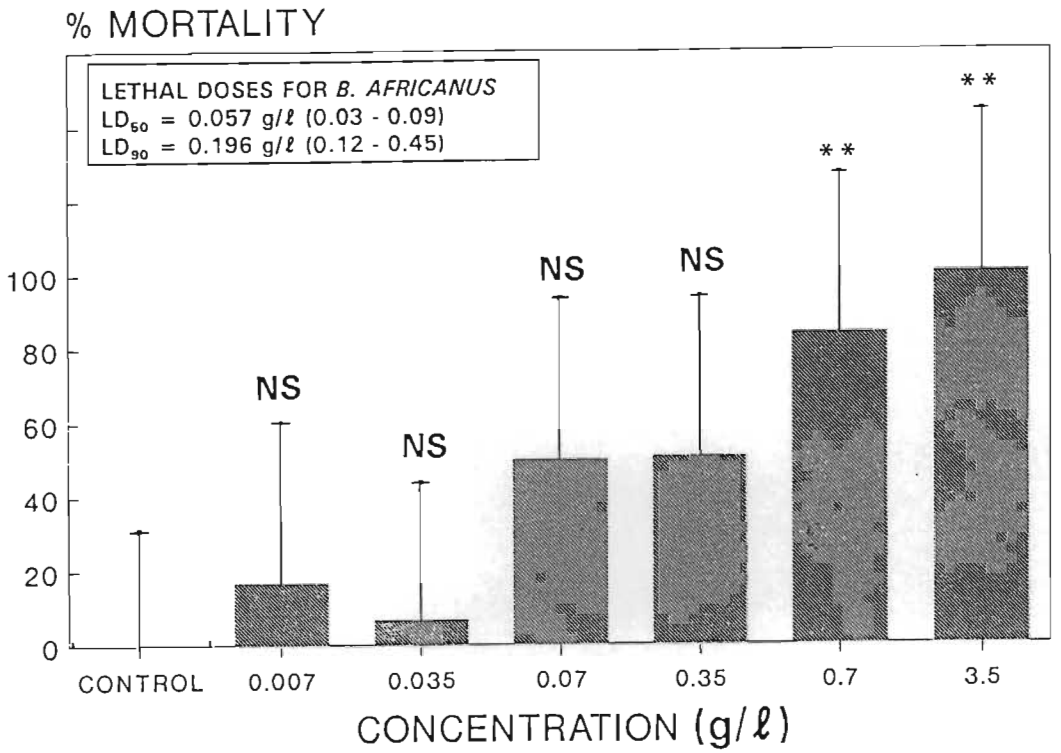


Figure 17.3 Mean % mortality and 95% confidence limits of *Oreochromis mossambicus* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

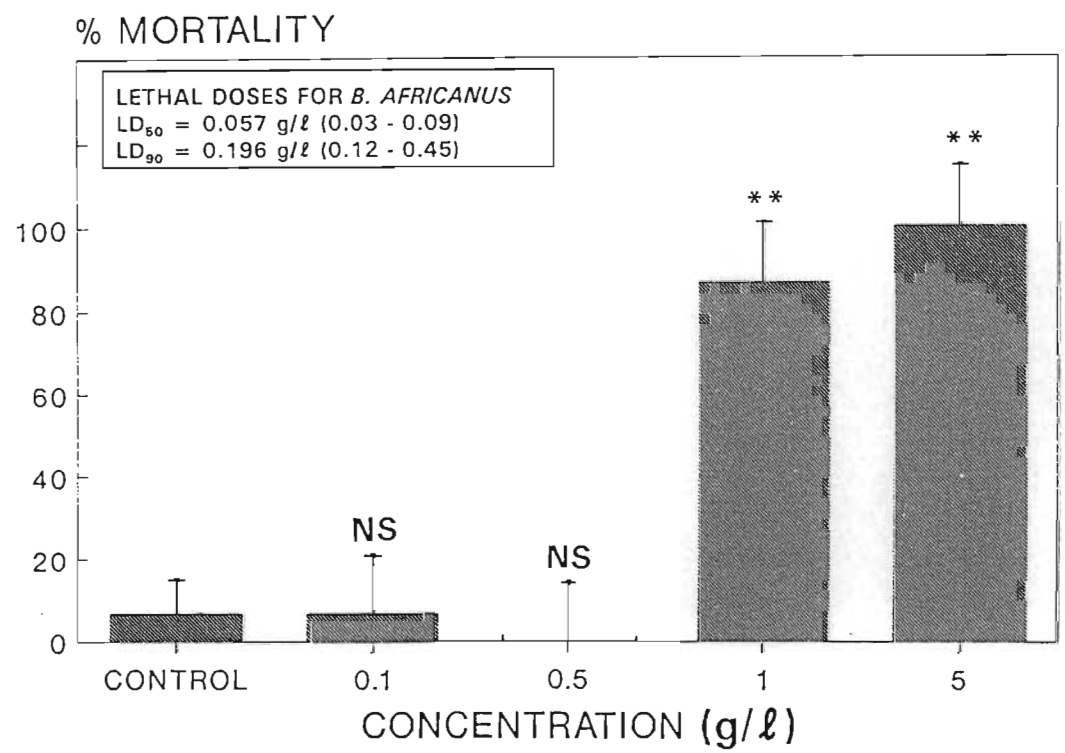


Figure 17.4 Mean % mortality and 95% confidence limits of *Poecilia reticulata* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for

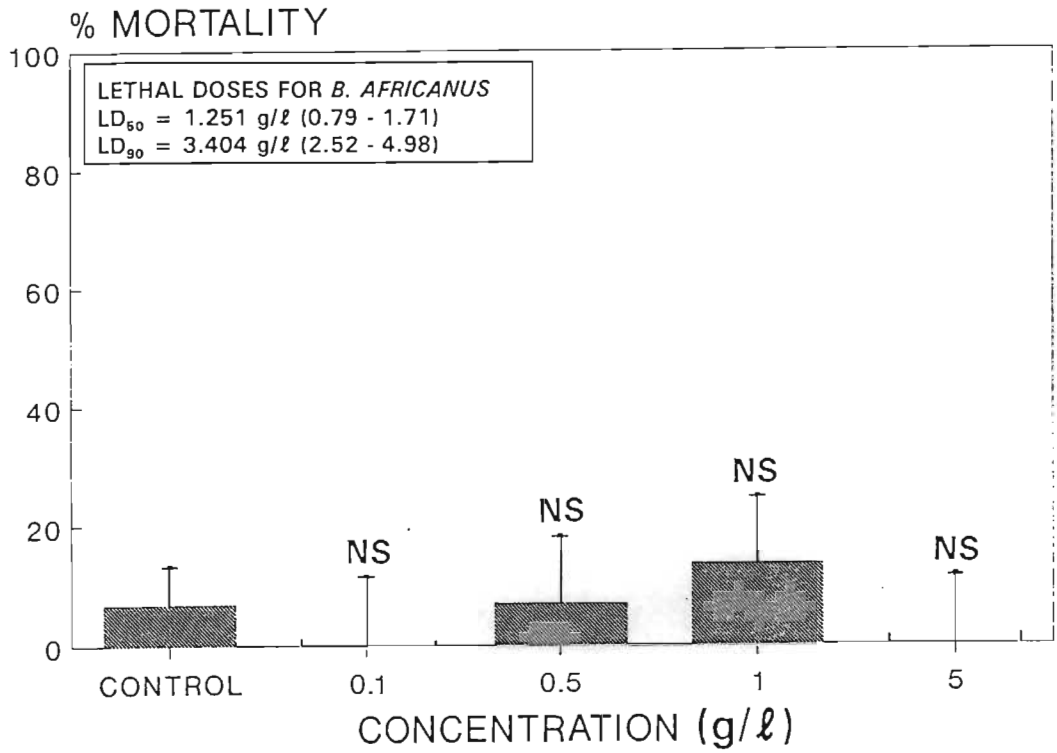


Figure 17.5 Mean % mortality and 95% confidence limits of *Poecilia reticulata* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Levels of significance indicate differences in mortality between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

Although a different test species (*P. reticulata*) has been used in the current investigation, mortality never occurred at levels below 5g/ℓ. Other discrepancies between the findings of Pretorius *et al.* (1991) and those from this investigation have been discussed in Chapter 2. The absence of toxicity to non-target vertebrates, such as fish, is encouraging.

5.6 AQUATIC MACROPHYTES - *SPIRODELA PUNCTATA*

The Lemnaceae are cosmopolitan, aquatic, free-floating, fast growing angiosperms (Hillman, 1961; Wang, 1991). They are important constituents of aquatic ecosystems because they harbour a variety of invertebrates and are eaten by waterbirds and fish (Hillman, 1961). Of the four genera recognized in this family, extensive experimental work (since as early as the 1920s) has been largely restricted to *Lemna* and *Spirodela*. Descriptive and experimental literature relating to the Lemnaceae has been reviewed by Hillman (1961) and Wang (1990; 1991). It has been claimed that toxicity bioassays utilizing the Lemnaceae can be compared favourably with those which employ fish and *Daphnia* (Wang, 1991). The selection of *Spirodela punctata* for this investigation was based on availability. Fronds of *Spirodela* are characteristically 3 - 5mm in length, and oval in shape. Each bears 2 or more roots. Frond-count increase is used as a test endpoint. The description of frond production is as follows: each "mother" frond produces

new "daughter" fronds alternately from 2 pockets on either side of the node (that end of the frond from which the roots arise) (Hillman, 1961). Each mother frond is capable of producing numerous daughter fronds during its lifetime. When all fronds which visibly project from mother fronds are counted, a typical doubling time obtained for *Lemna* species is 1 to 3 days under favourable conditions (Hillman, 1961).

5.6.1 MATERIALS AND METHODS

Studies of inhibition and toxicity require that control conditions permit growth at a rate which is high enough for the various levels of inhibition to be observed (Hillman, 1961). Accordingly, it was necessary to establish the concentration of nutrient algal medium required for optimal growth of *S. punctata*, prior to investigating the effects of plant molluscicides. The same medium was used for controls, and as the dilution solvent for molluscicides. Several media and concentrations have been recommended (Greenberg *et al.*, 1982; Wang, 1990; 1991). Bold's Basic Medium was selected because of convenience (used in the culture of *Nostoc* in our laboratory), and because of its similarity to Bristol's Medium which was recommended by Wang (1990). As two-fold and ten-fold concentrations have been recommended in the literature (Greenberg *et al.*, 1982; Wang, 1990; 1991), both concentrations were evaluated prior to toxicity trials (Section 5.6.2). Dechlorinated tap water alone was also investigated. From this initial investigation, a two-fold strength medium was subsequently selected for use in trials. Trial procedures are detailed in Table 20.

5.6.2 RESULTS

Results of the initial medium concentration trial (Figure 18.1) verified the use of a two-fold concentration of Bold's Basic Medium. Mean daily frond increase was 2.43 for a sample size of 21 fronds; the doubling time for individual fronds of *Spirodela punctata* was 9 days under the described experimental conditions. Both dechlorinated water and ten-fold concentrations of growth medium reduced frond growth rates. The mean daily frond increases of *S. punctata* exposed to aqueous suspensions of the three plants under investigation are given in Figures 18.2, 18.3 and 18.4. Both *W. salutaris* (18.2) and *A. dimidiata* (18.3) caused a significant reduction in frond production, and hence growth rate, at concentrations required to kill 90% of *B. africanus* snails. *Apodytes dimidiata* also significantly reduced growth rate at the LD_{50} required for *B. africanus*. *Gardenia thunbergia* did not affect the growth rate, even at concentrations well in excess of those required to kill 90% of snails.

Table 20. Summary of trial conditions and procedures used for *Spirodela punctata* (duckweed) (adapted from Wang, 1991).

CULTURE TECHNIQUES	
Source of stock	crude cultures established in 0.75m (diameter) x 1m high asbestos tubs which were kept outside. Innocula from these stocks were then transferred to culture medium for one week prior to use in trials.
TRIAL CONDITIONS	
Test vessels	glass petri dishes 90mm (diameter) x 10mm (height)
Test volume	50mℓ
Molluscicide concentrations tested (g/ℓ)	*W. salutaris*: 10, 5, 1, 0.5, 0.1, 0.05 *G. thunbergia*: 5, 1, 0.5, 0.1, 0.05, 0.01 *A. dimidiata*: 5, 1, 0.5, 0.1, 0.05, 0.01
Controls, culture medium and dilution water	A two-fold concentration of Bold's Basic Medium (Bold, 1942; Anonymous, 1978)
Numbers of individuals	22 fronds per vessel
Number of replicates	molluscicide treatment and controls: 4 replicates each
Duration of trial	120 hours (5 days), readings taken every 24 hours
Temperature	26°C
Photoperiod, light quality	continuous, cool fluorescent light (4x40W)
Endpoint	frond number increase per vessel

5.6.3 DISCUSSION

Investigations of optimal growth conditions for aquatic plants are prerequisites for macrophyte toxicity studies. Numerous media and concentrations are recommended for members of the Lemnaceae used in toxicity trials. In this study, a two-fold concentration of algal medium was required to achieve suitable growth rate of *S. punctata*. Exposure of *Spirodela* to the plant molluscicides under investigation revealed that both *W. salutaris* and *A. dimidiata* significantly reduced the growth rate. For all three molluscicides, growth was inhibited at least at the LD_{90}s for *B. africanus*, and also at the LD_{50}s following exposure to *A. dimidiata*. The above findings suggest that *G. thunbergia* is unlikely to result in any inhibitory effects on growth rate. As observed growth rate effects could be temporary it may be argued that such inhibition of macrophytes does not result in serious negative ecological consequences (Wang, 1991). The alternative view holds that in multi-species communities, weakened plants may be displaced by more tolerant species or may become prone to pathogenic attack. The ecological impacts are potentially severe. Field evaluation is an obvious course for future investigation. The current findings have revealed the potential hazard of applied molluscicides to aquatic macrophytes.

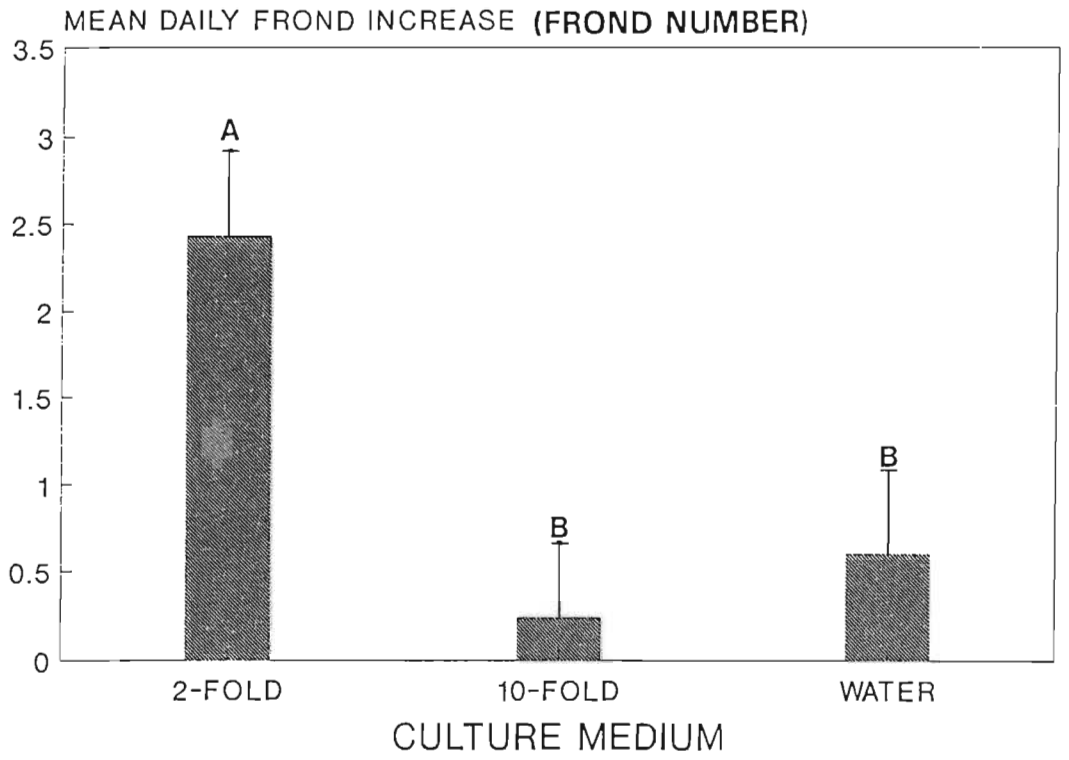


Figure 18.1 Mean daily frond increase and 95% confidence limits of *Spirodela punctata* cultured in two- and ten-fold concentrations of Bold's Basic Medium. A control containing no nutrient medium was included for reference. Means assigned the same letters are not significantly different at 99% probability levels.

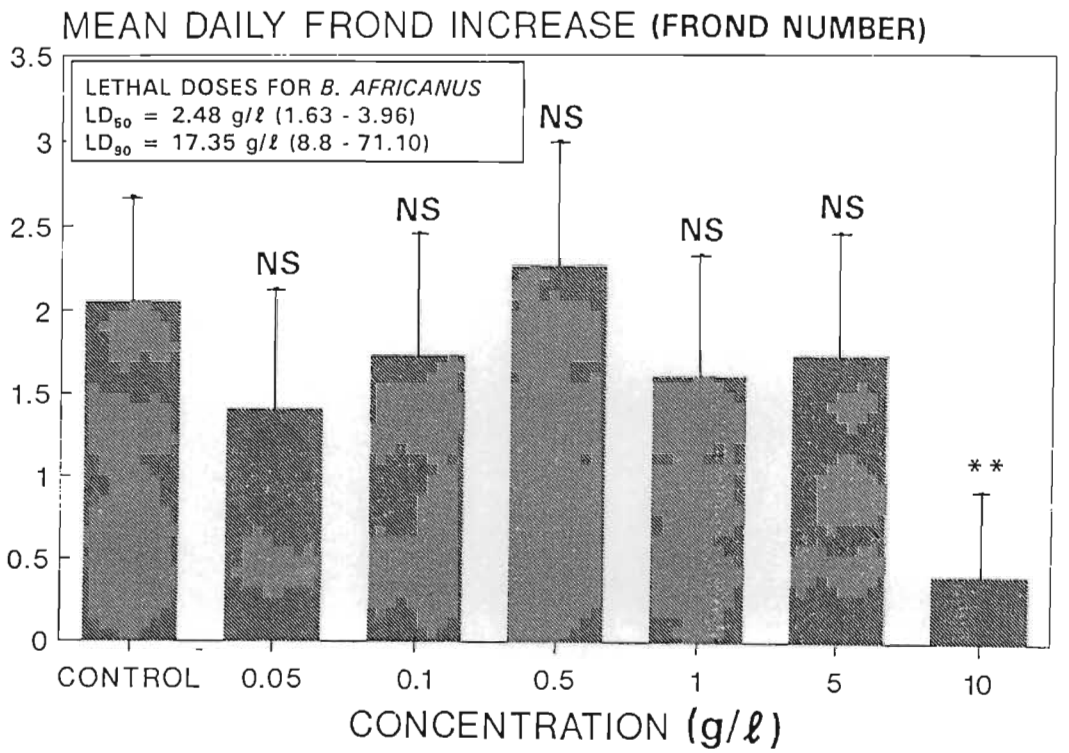


Figure 18.2 Mean daily frond increase and 95% confidence limits of *Spirodela punctata* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in frond growth between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence

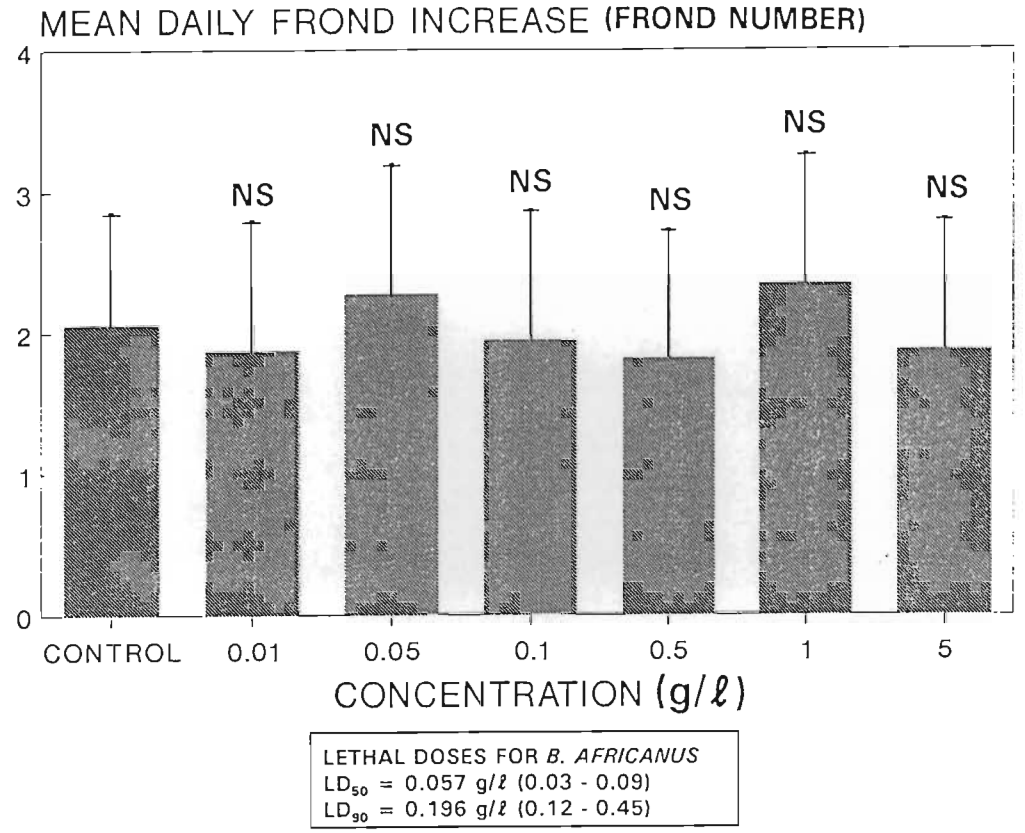


Figure 18.3 Mean daily frond increase and 95% confidence limits of *Spirodela punctata* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in frond growth between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

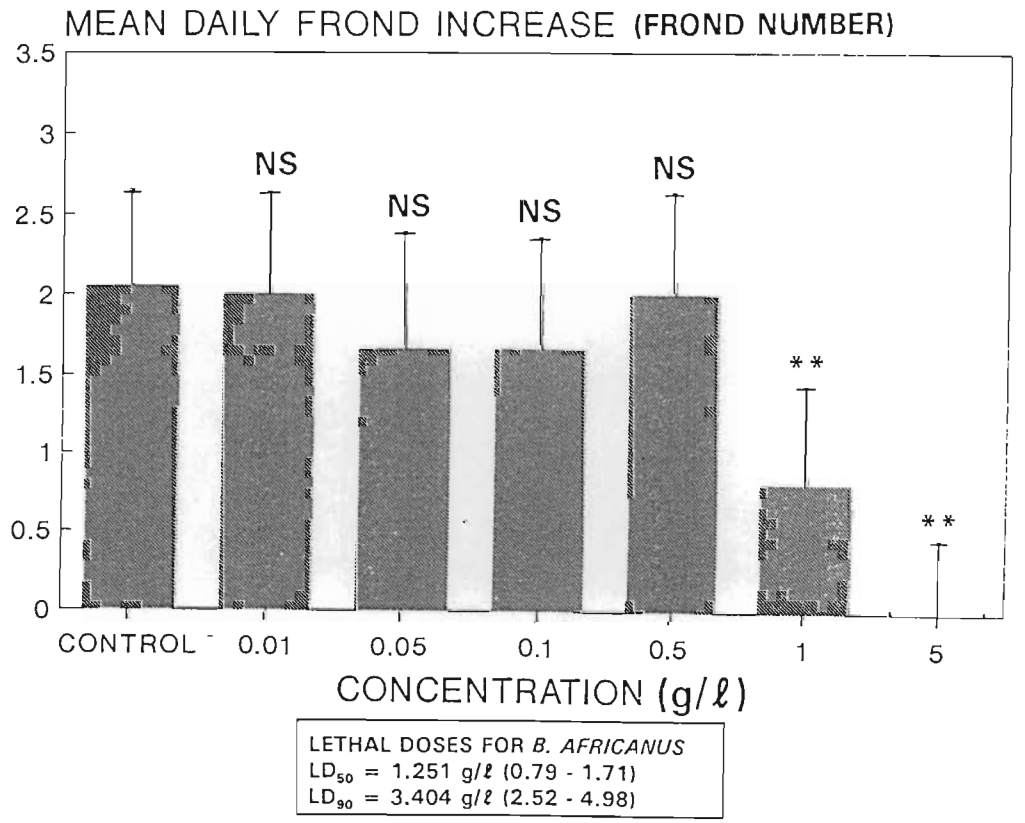


Figure 18.4 Mean daily frond increase and 95% confidence limits of *Spirodela punctata* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Levels of significance indicate differences in frond growth between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confid...

5.7 TERRESTRIAL PLANTS - *LACTUCA SATIVA*

Dormant dry plant seeds can often withstand harsh environmental changes without losing viability. Once hydrated under favourable conditions, processes of metabolism, nutrient transport and cell division proceed (Wang, 1991). At this stage seeds become highly sensitive to environmental stresses (Wang, 1991). Their value in toxicity trials is enhanced by their ready availability and low cost. Several plants have been recommended by the U.S. Environmental Protection Agency, the Food and Drug Administration and the Organization for Economic Cooperation and Development, for seed germination and root elongation trials. These taxa include cucumber, radish, lettuce, red clover and wheat (Wang, 1991). Lettuce was selected since it has been recommended for toxicity tests of unknown hazardous wastes (Wang, 1991).

5.7.1 MATERIALS AND METHODS

Trials procedures are detailed in Table 21.

Table 21. Summary of trial conditions and procedures used for static seed germination and root elongation trials of *Lactuca sativa* (lettuce) (adapted from Wang, 1991).

Source of stock	commercial seed suppliers
Test vessels	90mm (diameter) x 10mm (height) petri dishes lined with Whatman No. 1 filter paper
Test volume	7mℓ/vessel
Molluscicide concentrations tested (g/ℓ)	*W. salutaris*: 10, 5, 1, 0.5, 0.1, 0.05 *G. thunbergia*: 5, 1, 0.5, 0.1, 0.05, 0.01 *A. dimidiata*: 5, 1, 0.5, 0.1, 0.05, 0.01
Control and dilution water	dechlorinated tap water
Numbers of individuals	15 seeds per vessel
Number of replicates	seed germination molluscicide treatments: 6 seed germination controls: 8 root elongation molluscicide treatments: 3 root elongation controls: 4
Duration of trial	96 hours (3 days), readings taken every 24 hours
Temperature	23°C
Photoperiod, light quality	continuous darkness
Endpoint	seed germination: primary root 1mm or more root growth: primary root length (mm)

5.7.2 RESULTS

5.7.2.1 Germination

Germination rates for *L. sativa* exposed to *W. salutaris, G. thunbergia* and *A. dimidiata* are shown in Figures 19.1, 19.2 and 19.3 respectively. For each molluscicide tested there was no significant reduction in germination rate at concentrations in excess of those required to kill 50 to 90% of *B. africanus*.

5.7.2.2 Root elongation

Changes in root length were measured over three days of exposure to aqueous suspensions of *W. salutaris* (Figure 20.1), *G. thunbergia* (Figure 20.2) and *A. dimidiata* (Figure 20.3). Within 24 hours, *W. salutaris* significantly promoted root growth at intermediate concentrations (Figure 20.1). This promotive effect continued over the succeeding 48 hours.

Root growth was neither significantly promoted or inhibited following exposure to *G. thunbergia* (Figure 20.2) and *A. dimidiata* (Figure 20.3). Although a slight promotion of root growth occurred after 24 hours of exposure to on intermediate concentration of *A. dimidiata*, this was not sustained.

5.7.3 DISCUSSION

Germination appeared to be unaffected by exposure to the three plant molluscicides. Unexpectedly, root elongation was slightly promoted in response to *W. salutaris* extracts. *Apodytes* also showed an initial promotion which was, however, not sustained. The absence of inhibitory effects is of greater immediate interest than the presence of root growth promoters.

5.8 OVERALL DISCUSSION

Freshwater environments are composed of hundreds, if not thousands of coexistent species. If this biota is to be protected against the hazards of applied chemicals then the sensitivity of all species needs to be assessed. This task is impossible, and the choice of sufficiently sensitive indicator species is no less easy, given that diverse species are differentially sensitive to a range of chemicals. Baudo (1987) claimed that the relative susceptibility of 22 freshwater organisms (bacteria, algae, protozoans, crustaceans, insects, coelenterates, molluscs, fish and amphibians) in sub-acute toxicity tests with 15 chemicals, varied up to a factor of 9000. No single species can therefore be expected to represent entire ecosystems in terms of sensitivity to toxic compounds.

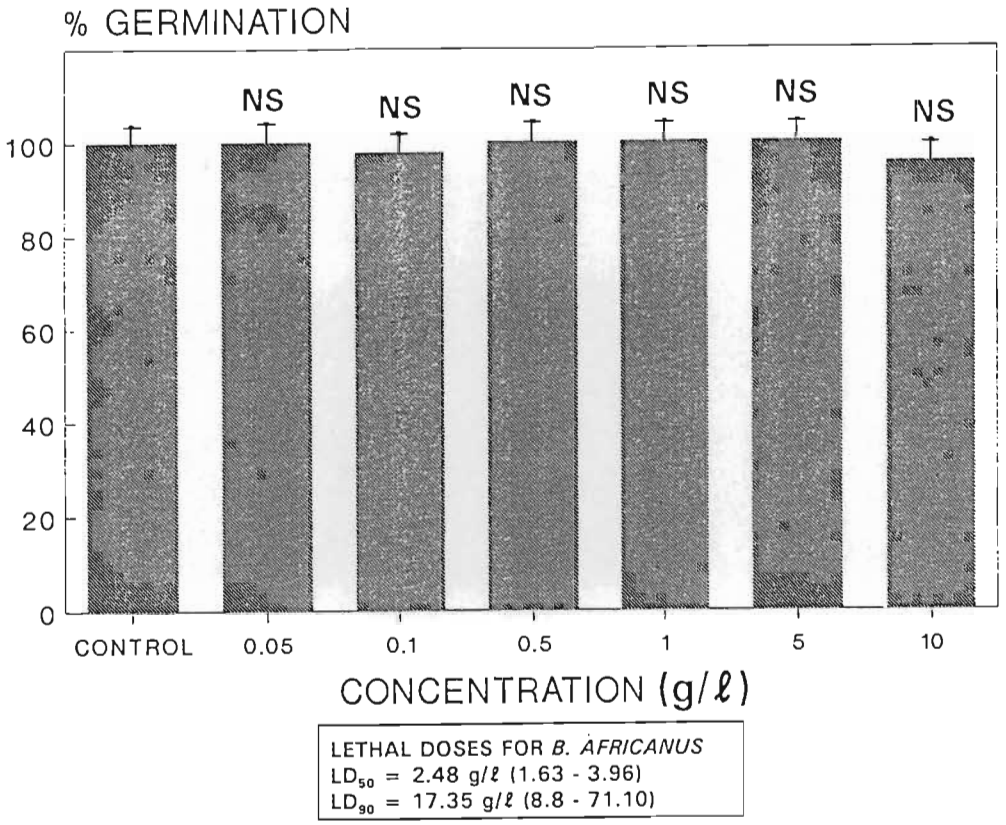


Figure 19.1 Mean % germination and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Warburgia salutaris* leaves. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

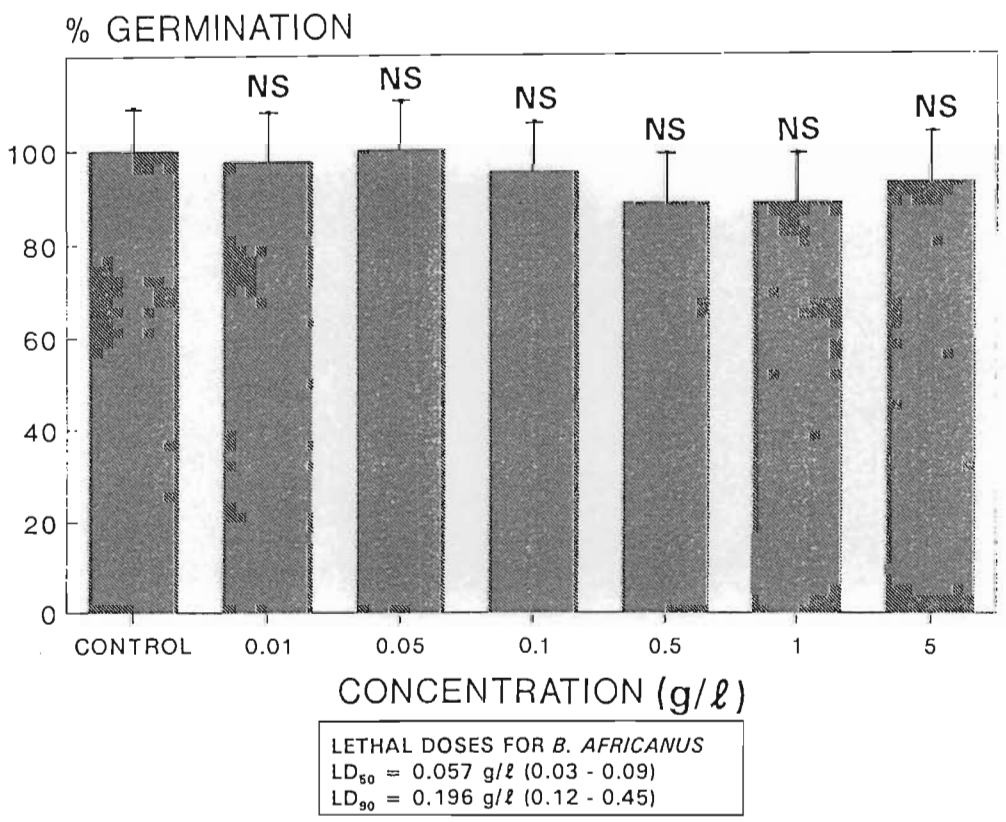


Figure 19.2 Mean % germination and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Gardenia thunbergia* leaves. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for

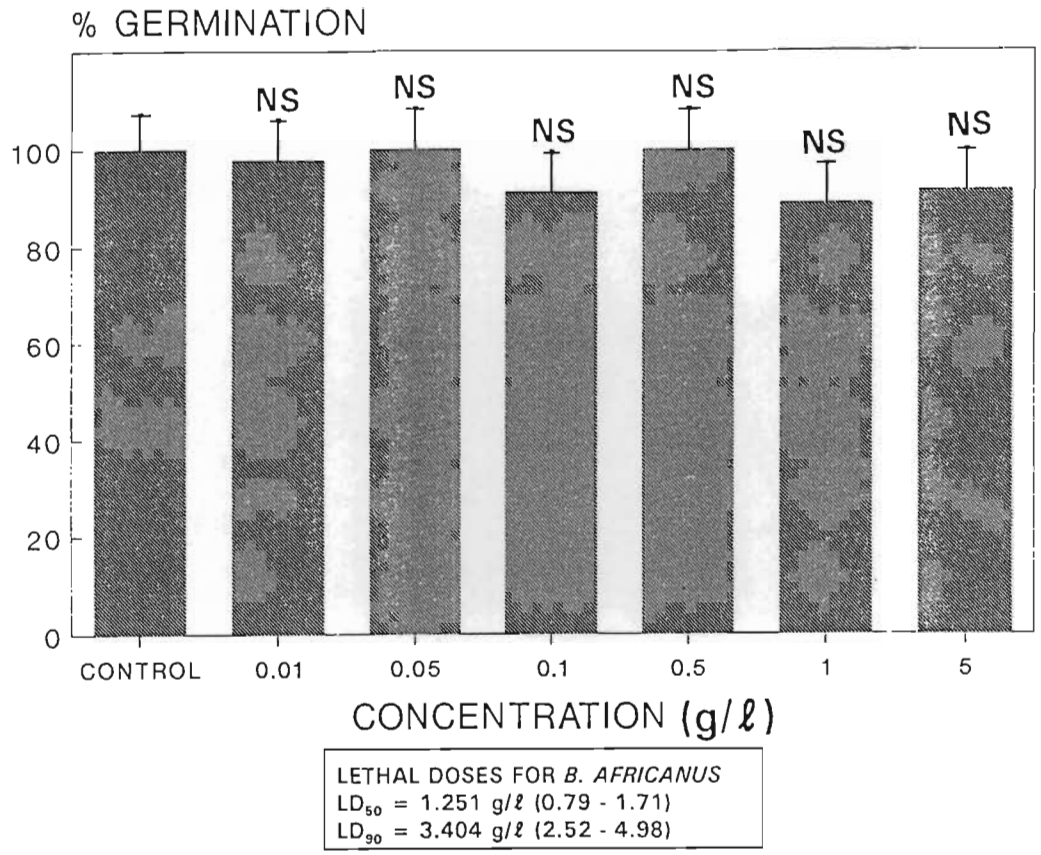


Figure 19.3 Mean % germination and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Apodytes dimidiata* leaves. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

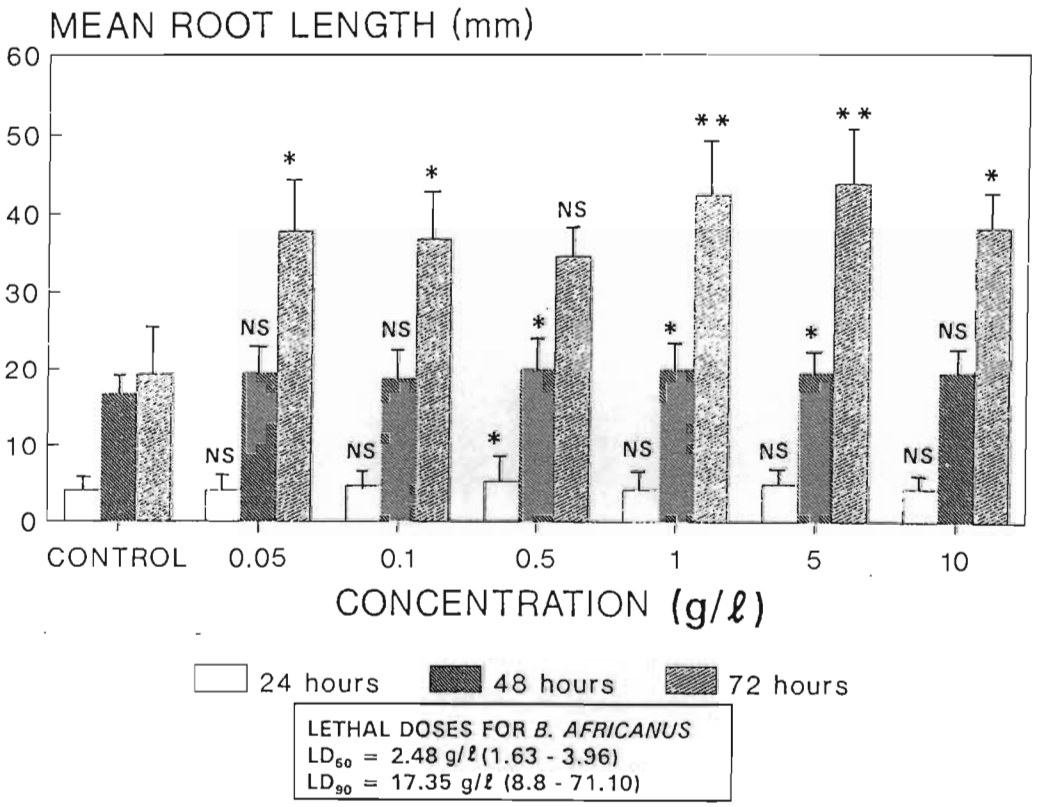


Figure 20.1 Mean root length and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Warburgia salutaris* leaves and measured over three days. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The

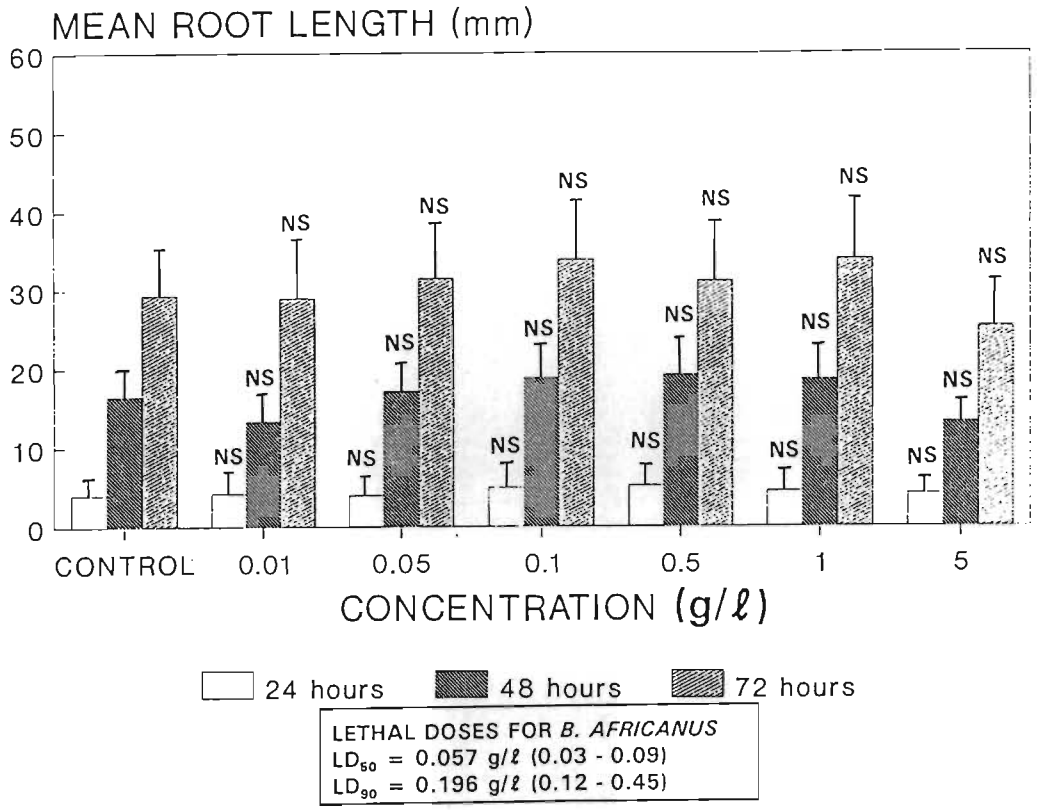


Figure 20.2 Mean root length and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Gardenia thunbergia* leaves and measured over three days. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95% confidence limits for *Bulinus africanus* are given for reference.

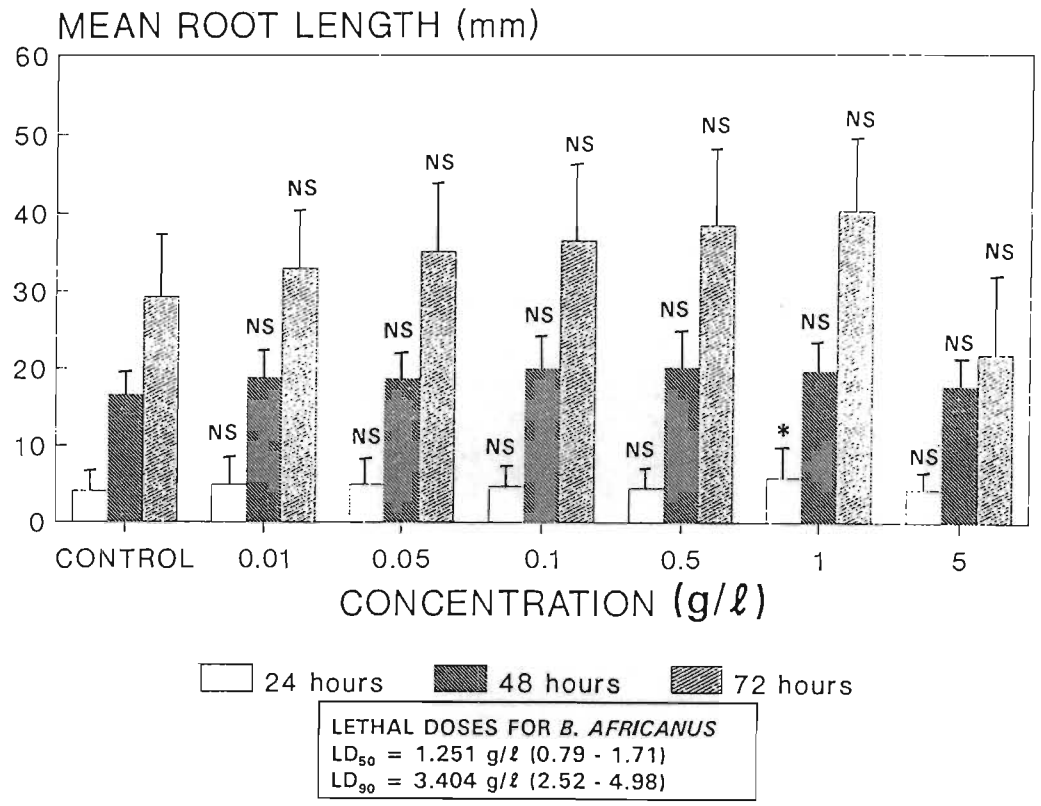


Figure 20.3 Mean root length and 95% confidence limits of *Lactuca sativa* exposed to aqueous suspensions of *Apodytes dimidiata* leaves and measured over three days. Levels of significance indicate differences in germination between test concentrations and controls (NS = non significant, * = $P<0.05$, ** = $P<0.01$). The LD_{50}s, LD_{90}s and 95%

Furthermore, single-species testing ignores species/population interactions. Multispecies testing as an alternative is expensive and extrapolation to field conditions difficult (Baudo, 1987). Baudo (1987) recommended a step-by-step approach where single species are tested first and subsequent tests are undertaken only if a given lethality is achieved. The test species selected for this investigation cover a wide range of taxonomic and trophic groups. All tests followed previously established methods, and where practical, followed the guidelines given by international standardization organizations. The results of these investigations are summarized in Table 22.

Gardenia thunbergia had the least impact on non-target organisms when tested under laboratory conditions (Table 22). *Warburgia salutaris* was the most impacting, followed by *A. dimidiata* (Table 22).

The above tests are considered first tier investigations. The results of this work together with those of Chapters 2 and 3 should provide sufficient information on which to base further evaluations of these plants in the field. Further issues warranting investigation prior to field evaluation are discussed below.

5.8.1 ALGAL ASSAYS

Three basic tests are used routinely by effluent toxicity regulating agencies: a fish, a macroinvertebrate and an alga (Wang, 1991). The use of *Selenastrum capricornutum* Printz has been recommended (Greenberg *et al.*, 1982). Chlorophyll *a* is used as an indicator of algal biomass and may be determined spectrophotometrically. An algal inoculum is added to concentrations of test substances and changes in algal biomass are then monitored. In the current investigation the use of crude aqueous suspensions in such an evaluation was impractical because of sample turbidity and pigmentation. Although filtering could reduce turbidity, this procedure decreases the integrity of the sample (Greenberg *et al.*, 1982). Algal bioassays are therefore best performed once the compound(s) responsible for molluscicidal activity have been isolated and identified.

5.8.2 MAMMALIAN TOXICITY

Mammalian toxicity is an area of considerable importance given that humans, stock animals and wildlife may contact molluscicides. This discipline has had to attend to significant ethical dilemmas. Acute oral LD_{50} toxicity in Sprague Dawley rats alone is not considered indicative of the effects of molluscicidal compounds on mammalian systems.

Table 22. Summary of results of investigation of toxicity to non-target fauna and flora. Results are simply identified as "yes", if negative impacts were recorded, and "no", if non-target organisms were unaffected.

TEST	*W. SALUTARIS*		*G. THUNBERGIA*		*A. DIMIDIATA*	
	LD_{50}	LD_{90}	LD_{50}	LD_{90}	LD_{50}	LD_{90}
INVERTEBRATES *DAPHNIA PULEX*	yes	yes	no	no	yes	yes
ANOPHELES ARABIENSIS						
1st INSTAR	no	yes	no	no	no	no
2st INSTAR	yes	yes	no	yes	yes	yes
3st INSTAR	yes	yes	no	no	no	yes
4st INSTAR	no	yes	no	no	no	yes
PUPAE	no	no	no	no	no	no
OTHER MOLLUSCS *HELISOMA DURYI*	yes	yes	-	yes	yes	yes
FISH *OREOCHROMIS MOSSAMBICUS*	no	-	no	no	no	no
POECILIA RETICULATA	no	-	no	no	no	no
AQUATIC MACROPHYTES *SPIRODELA PUNCTATA*	no	yes	no	no	yes	yes
CROP PLANTS *LACTUCA SATIVA* GERMINATION	no	no	no	no	no	no
LACTUCA SATIVA ROOT ELONGATION	no	no	no	no	no	no

Studies must include observations on the clinical signs of toxicity (Gralla, 1980); gross necropsy, dermal sensitization, eye and inhalation tests, and mutagenicity (such as Ames test or sister chromatid exchange tests) all require evaluation (Lambert *et al.*, 1991). Public pressure and the high cost of *in vivo* experiments have encouraged the use of *in vitro* systems (Anton *et al.*, 1986). Such cytotoxicity trials may not provide the definitive answer; these tests represent purely cellular events since it is difficult to recreate the complex pharmacokinetics which may occur *in vivo* (Freshney, 1986). The enormity of the field of toxicology and the debates which surround their application, the necessity for appropriate skills, animals and cell lines (Lambert *et al.*, 1991), have resulted in exclusion of studies on mammalian toxicity from this investigation. However, such research will have to be performed prior to progressing to field studies.

5.8.3 OVICIDAL ACTIVITY

Ovicidal activity with respect to the eggs of target snails has been included in this discussion since it should be a component of either first or second tier investigations.

Should plant extracts be shown to be toxic to the eggs of target molluscs then this would provide additional motivation for their use in the field. The same would apply to cercariacidal and miracidal activity.

The viability of eggs of *B. africanus* were assessed during the course of first tier investigations. Procedures followed those of Hopf *et al.* (1967), Lemma and Yau (1974a), and Lugt (1986). Egg packs of *B. africanus* (mean number of eggs per pack ± standard deviation: 14.65 ± 3.114) were exposed to a range of concentrations of plant molluscicides for 24 hours. Development and hatching were observed during the subsequent two weeks. Six replicates of data were gathered for all three plants. The results of this work have not been presented due to inexplicably high control mortalities (mean % mortality ± standard deviation: 64 ± 32.9). The patterns of response failed to make sound biologically sense and it is suspected that mortality effects were being obscured by pathogenic bacterial and/or fungal infections. Poor hatchling survival rates are discussed in Appendix 1. Attempts to rectify this situation have proven unsuccessful. As the source of mortality has not been identified and controlled, ovicidal trials are currently impractical in our laboratories.

Further studies on the toxicity of molluscicides to non-target organisms could include many other test systems. However, the most important classes of test subjects have been reviewed in this investigation.

CHAPTER 6

SOME PHYSIOLOGICAL EFFECTS OF AQUEOUS SUSPENSIONS OF *WARBURGIA SALUTARIS, GARDENIA THUNBERGIA* AND *APODYTES DIMIDIATA* ON A FRESHWATER SNAIL (*HELISOMA DURYI*)

HYPOTHESES

i. Clinical signs of toxicity follow a previously described "distress syndrome" which is characteristic for molluscicidal activity.

ii. Molluscicidal activity manifests itself in changes in oxygen consumption, heart rate and water uptake.

ABSTRACT

This study investigated the behavioural and physiological responses of a freshwater snail, *Helisoma duryi* (Wetherby), to solutions of three South African medicinal, molluscicidal plants (*Warburgia salutaris*, *Gardenia thunbergia* and *Apodytes dimidiata* subsp. *dimidiata*). Documented responses to synthetic and naturally-derived molluscicides are reviewed and discussed in relation to potential mode of action. Although mortality is the desired effect, death appeared to result via different physiological routes. Clinical signs of toxicity, oxygen consumption, heart rate and water uptake were used to assess commonality of response to three plant molluscicides.

All plants produced symptoms in *H. duryi* characteristic of the distress syndrome described by Harry and Aldrich (1963). Haemolysis, however, occurred only in response to *G. thunbergia* extracts. Oxygen consumption in snails exposed to *W. salutaris* and *G. thunbergia* did not differ significantly from the controls, while sublethal concentrations of *A. dimidiata* caused a rapid increase in oxygen consumption. Respiratory failure in snails exposed to the former two plants did not appear to be the primary cause of mortality. The disparity in response to different concentrations of *A. dimidiata* may be due to two independent effects whose occurrence is dose dependant. All three plants produced a significant lowering of heart rate at lethal concentrations. Water imbalance, which is a commonly suggested effect, did not occur. The difficulty of inferring mode of action from observed effects is emphasized. Despite common patterns of response for these three molluscicides, the differences clearly indicate the potential for alternative modes of action.

6.1 INTRODUCTION

Mode-of-action studies aim to discover which molluscan systems are affected by molluscicides. These systems include activity at the cellular level, uptake into the snail, distribution, metabolism and excretion (Duncan, 1987a). Not only is this knowledge basic to the search for new synthetic and naturally-derived molluscicidal compounds, but is also the result of a fundamental curiosity as to why, and how, some compounds are able to induce mortality in freshwater snails.

Copper compounds were employed as molluscicides as early as the 1920s, and nothing was understood of their mode of action at this time. Fifty years later the situation was little improved. Ignorance, however, proved to be no deterrent in its wide scale application (Cheng and Sullivan, 1974). Some 20 years hence, and despite some notable progress (Österberg, 1987), the specific molecular activity of these compounds on snail tissues remains ill-defined. The use of copper compounds as molluscicides has since been succeeded other safer and more effective synthetics (e.g. Frescon®, Bayluscide®) and by plant molluscicides (saponins being the most notable (Marston and Hostettmann, 1991)). With this development has come the question of the mechanism(s) of death.

The first attempts to address modes of action (Harry *et al.*, 1957; Harry and Aldrich, 1963) were accounts of behavioural changes in *Biomphalaria glabrata* (Say.), reported a "distress syndrome". This condition was evoked at sublethal concentrations of metallic ions (notably copper) (discussed further in Section 6.1.1). De Villiers and Mackenzie (1963) classified molluscicides according to two further characters: poisons of enzymes which rely on sulfhydryl groups (e.g. copper sulphate), and those which interfere with osmoregulation (e.g. Bayluscide®, Pentachlorophenol and most organic molluscicides). These two responses are, however, not mutually exclusive. Copper, for example, has been implicated with respect to both (Österberg, 1987). Numerous other responses have since been recorded (Webbe, 1987a), mainly from the study of synthetic compounds, as little work has been done on naturally-derived compounds. Adewunmi and Adesogan (1986) provided one of the few examples of work on plant molluscicides. These effects have been summarized (Figure 21) and are discussed in the following subsections. Besides aiding in the interpretation of results, these recorded effects serve to highlight the complexity of snail physiology, and the difficulty of providing conclusive evidence for mode of actions without further histological and neurological research.

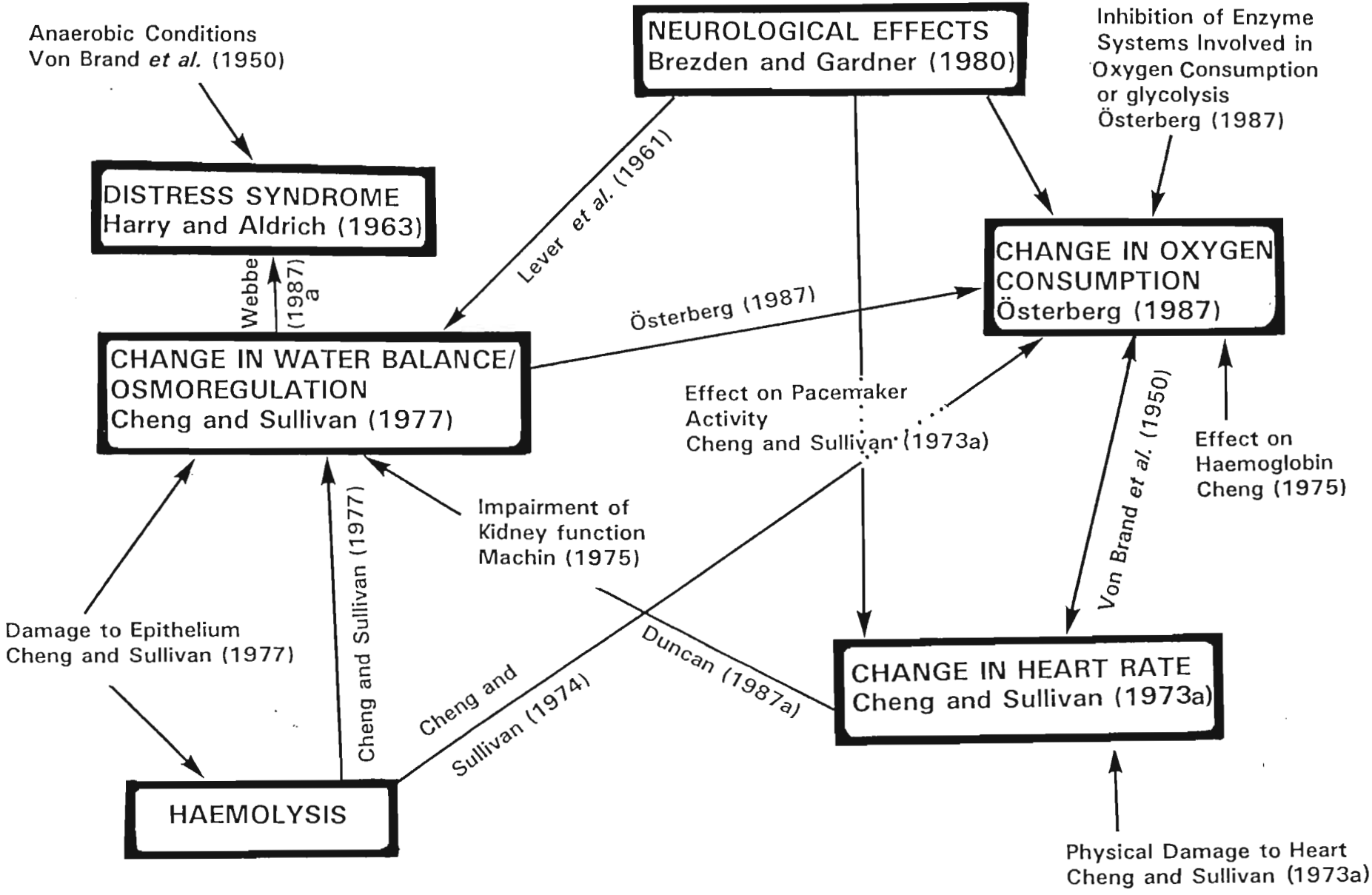


Figure 21 Summary of possible causes of mortality in snails due to molluscicides. The most common physiological effects are enclosed in boxes. The relationships between effects and suggested modes of action are indicated by arrows.

6.1.1. THE DISTRESS SYNDROME

Harry *et al.* (1957) first reported a "distress syndrome" for snails exposed to sublethal concentrations of candidate molluscicides. Harry and Aldrich (1963) described this more fully for numerous inorganic compounds, copper being among the most active. Researchers have since described similar clinical signs of toxicity for a wide variety of other compounds (Michelson, 1957; Van der Schalie, 1958; Cheng and Sullivan, 1973a; Malek and Cheng, 1974; Duncan, 1987b; Webbe, 1987a). These signs are summarized as follows:

i. extension of the cephalopedal mass, either fully or partially, from the shell aperture
ii. inability to attach to the substrate
iii. release of haemolymph into the water
iv swelling of tentacles and sloughing of cells at the tentacular bases
v. sand grains normally retained in the stomach are defecated
vi. heart rate slows down
vii. excessive production of mucus

Webbe (1987a) recorded the above symptoms as being characteristic for all molluscicides and further suggested that they indicated a loss of water balance control. Such claims have not been unambiguously substantiated. Rather, these symptoms have been recorded under completely different circumstances. The behaviour of a wide range of snail species (including *H. duryi*) under anaerobic conditions has shown that all snails become immobile and extruded from the shell (Von Brand *et al.*, 1950). Although able to recover following a return to aerobic conditions, if an anaerobic state persists, snails begin to haemorrhage and finally retract into their shells. The latter snails do not recover (Von Brand *et al.*, 1950). Extreme care must therefore be taken in the interpretation of molluscicidal effects. Here the distress syndrome is shown to occur under completely different circumstances and hence the interpretation of changes in water balance becomes questionable in the absence of direct evidence. The complexity of snail physiology is such that very often effects can only be inferred. The molluscicidal effects presented here simply highlight the limitations of attempts to accurately define the mode of action.

6.1.2 WATER BALANCE

Given that the haemolymph of freshwater pulmonates is hyper-osmotic to the external

medium and that most molluscan tissues are highly permeable, maintenance of water balance must be continuous (Machin, 1975). Any compound interfering with the mechanisms maintaining water balance will therefore lead to a rapid increase or decrease in body weight. The swelling of body tissues following the application of molluscicides has been suggested as a failure of water balance control (McMullen, 1952; Harry *et al.*, 1957; De Villiers and Mackenzie, 1963; Hopf *et al.*; 1967; Cheng and Sullivan, 1977; Webbe, 1987a). The osmotically induced inflow of water in *B. glabrata* due to copper has been proposed as the cause of mortality (Cheng and Sullivan, 1974). How then is osmoregulation being impaired?

Molluscicides may act directly on the membranes. Cheng and Sullivan (1977) found experimental snails exposed to copper to have a higher ratio of wet to dry weight. They also observed a distention of the rectal ridge, apparently due to osmotic influx. In this case, damage to epithelial surfaces and consequent increases in permeability of epithelial membranes could have led to the accumulation of water in tissues (Cheng and Sullivan, 1977). Damage may also lead to loss of haemolymph, and hence water. However, this latter effect would result in loss rather than uptake of water. Van Aardt and Coertze (1981) concluded that the effects of copper were not limited to particular organs, tissues or cells in *Bulinus tropicus* (Krauss) but occurred throughout the snail body. They postulated that the basic cause of gross disturbances of water balance are due to changes in the permeability of cell membranes, particularly epithelial cells. Copper was indeed shown to inhibit the ATP-ase activity necessary for active membrane transport.

Analysis of treated snails for copper deposits showed that copper accumulated in the epithelial covering of the head-foot and rectal ridge (Cheng and Sullivan, 1974). One suggestion was that this area may be involved in oxygen uptake (Österberg, 1987). Copper would therefore affect snails, as it does with fish, by interfering with respiration (Österberg, 1987). The effect on oxygen uptake may be secondary. It has also been suggested that copper disrupts osmoregulation, resulting in swelling which then leads to impaired respiration and death by asphyxiation (Österberg, 1987).

Since the kidney is the principal water volume regulator in freshwater pulmonates (Machin, 1975), water imbalance may relate to the impairment of kidney function. This has been shown for nembutal and MS222 anaesthesia which rapidly bring about a decrease in urination, and hence an increase in body weight (5% within 8 minutes) (Machin, 1975). The effects may again be indirect. For example, copper poisoning, which is known to decrease the heart rate would, impair the efficiency with which the heart-kidney is able to remove excess body water (Duncan, 1987a).

Molluscan water balance, as for other invertebrates, is ultimately under neurosecretory control (Lever *et al.*, 1961; Lever and Joosse, 1961). The pleural ganglia in *Lymnaea stagnalis* (L.) are thought to be responsible. Accordingly, damage to the ganglia or neurosecretion could also result in water imbalance. Removal of the pleural ganglia has been shown to reduce the ability to control water balance (Lever *et al.*, 1961). Frescon® has been shown to reduce neurosecretory activity in *Bulinus truncatus* (Audouin) (Duncan, 1987b), and copper sulphate in *Indoplanorbis exustus* (Deshayes) (Webbe, 1987a). This stress alone is considered sufficiently great to cause mortality. The reduction of normal water flow may also precipitate other physiological disturbances.

Further, water imbalance could arise from the excessive production of mucus when the snail encounters the toxin. This suggestion is unsubstantiated because of a lack of quantitative data relating mucus production to the maintenance of water balance (Machin, 1975).

6.1.3. RESPIRATION AND CARBOHYDRATE METABOLISM

Oxygen consumption has been proposed as one of best indicators of overall metabolic activities (Von Brand *et al.*, 1949). Gaseous exchange in pulmonates can take place both cutaneously and through the "lung", a modified vascularized area in the mantle cavity (Cheng and Sullivan, 1974; Ghiretti and Ghiretti-Migaldi, 1975). The relative importance of these two sites in oxygen consumption has been debated by Alberts (1966). He concluded that cutaneous respiration was most important in meeting the oxygen requirements of pulmonates. Any damage to cutaneous respiration would therefore result in changes in oxygen consumption.

As with osmoregulation, the site of entry for oxygen may be affected by molluscicides. As previously mentioned (Section 6.1.2) the accumulation of copper in the epithelium could result in asphyxiation (Österberg, 1987). The epithelial lining of the head-foot and rectal ridge is, however, poorly vascularized and possibly not the primary area for oxygen uptake (Österberg, 1987).

Molluscicide effects on haemoglobin, the oxygen carrier in the Planorbidae, could alter oxygen consumption. Cheng (1975), however, found that although copper sulphate ions destroyed pigment cells in the connective tissue of the rectal ridge, there was no observed lowering of haemoglobin concentration.

The correlation between heart rate and oxygen consumption, at least in *B. glabrata* (Von Brand *et al.*, 1950), also presents problems in making assumptions about possible effects. Is observed mortality ultimately due to effects on the heart, or on osmoregulation

(Von Brand *et al.*, 1950; Lee and Cheng, 1971)? And is the inhibition of oxygen uptake indeed equivalent to killing snails (Andrews *et al.*, 1987), since sublethal concentrations of niclosamide showed no effect on the survival of *B. alexandrina* (Ehrenberg) over 96 hours (Ishak and Mohamed, 1975)?

Studies have shown that particular compounds employed, such as napthoquinones, inhibit enzyme systems involved in oxygen consumption or glycolysis in various invertebrates (Von Brand *et al.*, 1949). The activity of copper has been attributed to inhibition of oxidative phosphorylation due to its effects on enzymic sulfhydryl groups (McCullough and Mott, 1983). Since snails have a low sulfhydryl content in their tissues, they are sensitive to enzymes utilizing -SH groups. Inhibition and stimulation of oxidation has been shown for other synthetic molluscicides; pentachlorophenol and niclosamide (Duncan, 1987a).

6.1.4 HEART RATE

The slowing of heart rate is a feature of the distress syndrome described by Harry and Aldrich (1963), and has been demonstrated both *in vitro* and *in vivo* (Banna and Plummer, 1978). Cheng and Sullivan (1973a) graded the advance of the distress syndrome using heart rate and suggested that it was a good quantitative method for determining the effects of candidate molluscicides. However, this characteristic does not indicate the mode of action.

Suggestions have included direct effects on the heart, or pacemaker activity itself (Cheng and Sullivan, 1973a). These authors further proposed that retraction into the shell leads to a reduction in heart rate. Caution is required in accepting this suggestion. Notably, the experimental conditions of Cheng and Sullivan (1973a) bore little resemblance to those operating when snails are in a toxic solution; the heart rate was measured in snails which were forced to remain retracted within their shells through the use of paraplast film.

The relationship between heart rate and oxygen consumption has already been considered (Section 6.1.3). The relative contributions of oxygen consumption and heart rate to mortality are difficult to assess independently.

6.1.5 HAEMOLYSIS

The discharge of haemolymph during exposure to some molluscicides, e.g. copper (Harry *et al.*, 1957), indicates rupture of external membranes. The site of discharge, however, has not been identified and requires further histological investigation. If the foot of *L.*

stagnalis is severely stimulated, haemolymph is exuded in the process of rapid withdrawal into the shell (Jones, 1975). Lever and Bekius (1965) suggested that in emergency situations, haemolymph is released through the haemal pore (located in the pneumostome close to the nephridiopore (Hymen, 1967)). Van Aardt and Frey (1981) proposed that haemolymph is exuded from a haemal pore in *Bulinus globosus* (Morelet), based on the findings of Lever and Bekius (1965), although no such structure has yet been described for this species.

6.1.6 NEUROLOGICAL EFFECTS

The nature of the molluscan nervous system is such that damage to it could result in a wide range of effects, including those systems already discussed, e.g. changes in heart rate, oxygen consumption and water uptake (Hassall, 1982). Mention has been made of water imbalance through damage to neurosecretory cells and pleural ganglia (Section 6.1.2). Damage to pacemaker activities of the heart also implicates neurological effects (Section 6.1.4).

According to Hanumante *et al.* (1979) copper sulphate increases the nuclear diameter of certain neurosecretory cells in *I. exustus*. This probably results in an increase in the rate of transport and release of neurosecretory material. Brezden and Gardner (1980) have shown that Frescon® is capable of disrupting the *in vitro* electrophysiological activity of the *Lymnaea* central nervous system (CNS). Severe disruption of the CNS could cause the observed snail deaths.

Neurological investigations into the effects of molluscicides is a neglected field which may be able to clarify the original symptoms described for the distress syndrome, and for mode(s) of action in general.

The above account of potential and established modes of action clearly indicates the interpretation difficulties experienced by researchers in their attempts to solve the fundamental question of how snails die. The complexity of snail physiology makes definitive conclusions difficult to draw. However the identification of common patterns of response is important in both confirming and suggesting potential modes of action. From the diversity of possible molluscicidal effects (Figure 21), the clinical signs of toxicity, oxygen consumption, heart rate and water uptake were selected as criteria for evaluating the molluscicidal effects of aqueous suspensions of *Warburgia salutaris, Gardenia thunbergia* and *Apodytes dimidiata*.

6.2 MATERIALS AND METHODS

Although *Bulinus africanus* (Krauss) has been used as the target snail for all other toxicological work in this study, *Helisoma duryi* was used here as an experimental subject for the following reasons:

i. Sufficient numbers of laboratory reared snails of equal size (shell diameter: 6-9mm, mean ± SD = 7.38 ± 0.101) were available.

ii. The shell is sufficiently transparent to observe ventricular contraction for the assessment of heart rate.

iii. The toxicity of the aqueous suspensions under study have been confirmed for this snail species (Chapter 5, Section 5.4).

iv. Like *B. africanus*, *H. duryi* is a member of the family Planorbidae.

v. *H. duryi* is a cosmopolitan species which has been employed in other physiological investigations (Von Brand *et al.*, 1948).

Unless otherwise indicated, trials followed the protocol given in Appendix 1. The procedure for the preparation of aqueous extracts of molluscicidal plants is given in Appendix 2. Concentrations of test molluscicide were chosen to include a lethal or high sublethal concentration and a low sublethal concentration. Lethal concentrations produced 100% mortality of snails, high sublethal concentrations produced 50 - 90% mortality and low sublethal concentrations produced <50% mortality.

The data for this investigation were collected during February to May, 1994.

6.2.1 CLINICAL SIGNS OF TOXICITY

The data were collected during toxicity trials described in Chapter 3 (*B. africanus*) and Chapter 5 (*H. duryi*). Four replicates of five snails each were subjected to selected concentrations of test molluscicides. Lethal and sublethal concentrations were tested. Concentrations in g/ℓ were 10, 5, 1 for *W. salutaris* and 5, 1, 0.5 for both *G. thunbergia* and *A. dimidiata*. Lethal concentrations were 10g/ℓ for *W. salutaris*, 1g/ℓ for *G. thunbergia* and 5g/ℓ for *A. dimidiata*. Behavioural signs were noted following a 24-hour exposure and 24-hour recovery period.

6.2.2 OXYGEN CONSUMPTION

A Warburg differential respirometer was used for the measurement of oxygen consumption. The methods for measuring oxygen consumption in relation to molluscicidal activity have been widely reported on (Von Brand *et al.*, 1948; Von Brand *et al.*, 1949; Edwards, 1958; Lee and Cheng, 1971; Cheng and Sullivan, 1973b; Malek and Cheng,

1974). Seven to 12 specimens of *H. duryi* were placed in individual reaction flasks containing 6mℓ of the test molluscicide. Concentrations in g/ℓ were 10 (lethal) and 5 (high sublethal) for *W. salutaris*, 0.5 (high sublethal) and 0.2 (low sublethal) for *G. thunbergia* and 4 (high sublethal) and 1 (low sublethal) for *A. dimidiata*.

Two-tenths of 1mℓ of 20% Potassium Hydroxide was placed in the centre well of each flask, together with a filter paper fan (Whatman No. 1). The KOH, functioned to absorb the CO_2 evolved. A thermobarometer, consisting of an empty flask containing only KOH was included in each experiment to compensate for short-term fluctuations in atmospheric pressure. Any changes recorded were added or subtracted from experimental values, depending on whether they represented positive or negative pressure changes. Flasks were submerged in a waterbath maintained at 26°C, and shaken at 80 oscillations per minute. They were then allowed to equilibrate for two hours. After this period the respirometric readings were recorded at 20-minute intervals for a two-hour period. Although an equilibration time of 15 minutes has frequently been cited (Lee and Cheng, 1971; Cheng and Sullivan, 1973b), preliminary trials for this experiment revealed that stabilization of manometer readings took between 1 and 1.5 hours. Handling of snails is known to result in stress with resultant changes in oxygen uptake (Hayes *et al.*, 1992). Recordings were effectively taken two to four hours from the start of the exposure period. A standard two-hour recording period was employed. Longer recording periods were not feasible given the low liquid volume of the reaction flask. The WHO (1965) recommended that for toxicity trials the volume per snail should not be less than 40mℓ. Confinement to the Warburg flasks employed in the current study may have caused stress to snails (Section 6.4.3). At the conclusion of the observation period the snails were dried for 24 hours at 70°C, and weighed. The raw respirometric data were converted to $\mu\ell$/g dry weight/hour. Respirometric data were similarly obtained for control snails immersed in dechlorinated tap water.

Differences in oxygen consumption were compared using Student's t-tests, F-tests and analysis of variance (ANOVA) in STATGRAPHICS (STSC Inc., 1985-1991).

6.2.3 HEART RATE

Methods for measuring heart rate have followed Malek and Cheng (1974); five to seven specimens of *H. duryi* were placed in 250mℓ of test solution at 26°C. As heart rate is inversely proportional to size (Lee and Cheng, 1971), care was taken to choose snails of the same size for a trial. The following lethal and sublethal concentrations were tested (in g/ℓ): 10 (lethal), 5 (high sublethal), 1 (low sublethal) for *W. salutaris*, 0.5 (high sublethal),

0.2 (low sublethal), 0.1 (low sublethal) for *G. thunbergia* and 4 (high sublethal), 1 (low sublethal), 0.5 (low sublethal) for *A. dimidiata*.

The time required to complete 5 ventricular contractions was recorded. The mean of three records was calculated for each snail following 2, 5, 8, 11 and 24 hours of exposure to molluscicide, and again after a 24-hour recovery period. The times recorded were then converted to beats/minute.

Differences in heart rate were compared between dosage and exposure times using Student's t-tests, F-tests and analysis of variance (ANOVA) in STATGRAPHICS (STSC Inc., 1985-1991).

6.2.4 WATER UPTAKE

Methods for the measurement of water uptake have followed Cheng and Sullivan (1977). Five to seven specimens of *H. duryi* were placed in 250mℓ of test solution for a 24-hour exposure, followed by a 24-hour recovery period. The following sublethal concentrations were tested (gℓ): 5, 1 for *W. salutaris*, 0.2, 0.1 for *G. thunbergia* and 1, 0.5 for *A. dimidiata*. At the end of this period snails were dried with filter paper (Whatman No. 1) for two minutes to standardize the amount of moisture present, and weighed. Snails were dried at 70°C for 24 hours, and then weighed again. The ratio of wet to dry weight was then calculated and compared using non-parametric Mann-Whitney U-tests because of the small sample sizes (Student's t-tests did however produced the same results).

6.3 RESULTS

6.3.1 CLINICAL SIGNS OF TOXICITY

Clinical signs of toxicity to the test molluscicides have been summarized (Table 23). At lethal concentrations (for 24-hour exposure and 24-hour recovery period) snails were completely inactive and remained retracted within their shells from the experiment outset. Haemolysis was an obvious response in *G. thunbergia* and snails were unable to recover.

At sublethal concentrations there was little activity. Some retracted, others were partially extruded but slow to respond to mechanical stimulation. Attempts by snails to attach to the substrate were unsuccessful. Mucus production was obvious in all cases, except in response to *W. salutaris* at 10gℓ. Haemolysis occurred predominantly in snails exposed to *G. thunbergia*.

ble 23. Patterns of response shown by *Bulinus africanus* and *Helisoma duryi* during a 24-hour exposure and 24-hour recovery period. Unless otherwise dicated comments refer to both species (Haem = haemolysis, + = normal, + + = obvious, + + + = excessive)

Plant species	Conc. g/ℓ	24-hour EXPOSURE PERIOD			24 hour RECOVERY PERIOD		
		Movement	Mucus	Haem.	Movement	Mucus	Haem.
Warburgia alutaris	10	no activity, position unchanged from experiment start, fully retracted into shell, not attached to substrate	none	none	*Helisoma:* no activity (80% mortality), partially extruded from the shell but not attached to substrate *Bulinus:* 100% mortality	+ +	none
	5	*Helisoma:* no activity, position changed from experiment start, fully retracted into the shell but attached to substrate *Bulinus:* as for 10g/ℓ	+ + +	none	*Helisoma:* 40% mortality, remainder active or inactive but all attached to substrate *Bulinus:* 100% mortality	+	none
	1	*Helisoma:* all attached to substrate and active *Bulinus:* 60% slow to respond to mechanical stimulation but attached to substrate, 40% partially retracted into shell, inactive	+ +	none	active or inactive but attached to substrate, *Bulinus*: 40% mortality	+	none
ardenia unbergia	5	100% mortality, position unchanged from experiment start, fully retracted into shell, not attached to substrate	+ + +	+ +	-	-	-
	1	20% attached to substrate and showing some movement during exposure period, all others fully retracted into shell and not attached to substrate	+ +	+ +	100% mortality	+ +	+ +
	0.5	*Helisoma:* 75% active, 20% inactive but attached to substrate, 5% with foot extruded from shell but unable to attach to substrate *Bulinus:* 90% retracted into shell, inactive and unattached to substrate 10% with foot extruded but unable to attach to substrate	+ +	+ +	*Helisoma:* 5% mortality, remainder active and attached to substrate *Bulinus:* 100% mortality	+	none

Plant species	Conc. g/ℓ	24-hour EXPOSURE PERIOD			24 hour RECOVERY PERIOD		
		Movement	Mucus	Haem.	Movement	Mucus	Haem.
Apodytes dimidiata	5	100% mortality *Helisoma:* 20% with foot extruded from shell but unattached to substrate, remainder are fully retracted, all inactive *Bulinus:* 100% with foot loosely retracted, all inactive	+ +	none	-	-	-
	1	active and attached to substrate *Bulinus:* 20% with foot retracted (5% showing haemolysis)	+ +	none	active and attached to substrate *Bulinus:* 20% mortality	+	none
	0.5	active and attached to substrate	+	none	active and attached to substrate	+	none
Control		active and attached to substrate	+	none	active and attached to substrate	+	none

6.3.2 OXYGEN CONSUMPTION

6.3.2.1 Oxygen consumption over the two-hour exposure period

Oxygen consumption is illustrated in Figure 22 and differences in consumption at tested concentrations given in Table 24. *Warburgia salutaris* and *G. thunbergia* did not cause significant changes in oxygen consumption at lethal and high sublethal concentrations while low sublethal concentrations of *A. dimidiata* did result in a significant mean increase in oxygen consumption relative to the controls (Table 25). The results of the F-tests showed that variation in oxygen consumption by snails exposed to molluscicides was significantly greater than the natural variation shown by control snails. Again *A. dimidiata* was the exception, with the variation not significantly different for snails exposed to 4g/ℓ.

6.3.2.2 Oxygen consumption for 20-minute intervals over the two-hour exposure period

Mean oxygen consumption recorded at intervals are given in Figures 23.1, 23.2 and 23.3. ANOVAs for significant differences in oxygen consumption at 20-minute intervals are given in Table 26. Mean oxygen consumption by snails exposed to both *W. salutaris* and *G. thunbergia* did not differ significantly with time at lethal and high sublethal concentrations, respectively. At high sublethal concentrations for *W. salutaris* and low sublethal concentrations for *G. thunbergia* both produced a significant fluctuation in oxygen consumption. In contrast, *A. dimidiata* (Figure 23.3), produced significant changes in oxygen consumption at both high and low sublethal concentrations.

6.3.3 HEART RATE

6.3.3.1 Mean heart rate over the 48-hour observation period

Mean heart rates are given in Figure 24. ANOVAs for heart rates at different concentrations are given in Table 27. Mean heart rate differed significantly for different concentrations for all plant extracts. At lethal concentrations all plant extracts reduced heart rate significantly below that of the controls (Table 28). Lower concentrations, however, resulted in a slight increase in heart rate, or remained non-significantly different from the controls. As was found for oxygen consumption, the variation as measured by the F-test was significantly greater for snails exposed to molluscicides than the natural variation shown by controls. Again *A. dimidiata* was the exception.

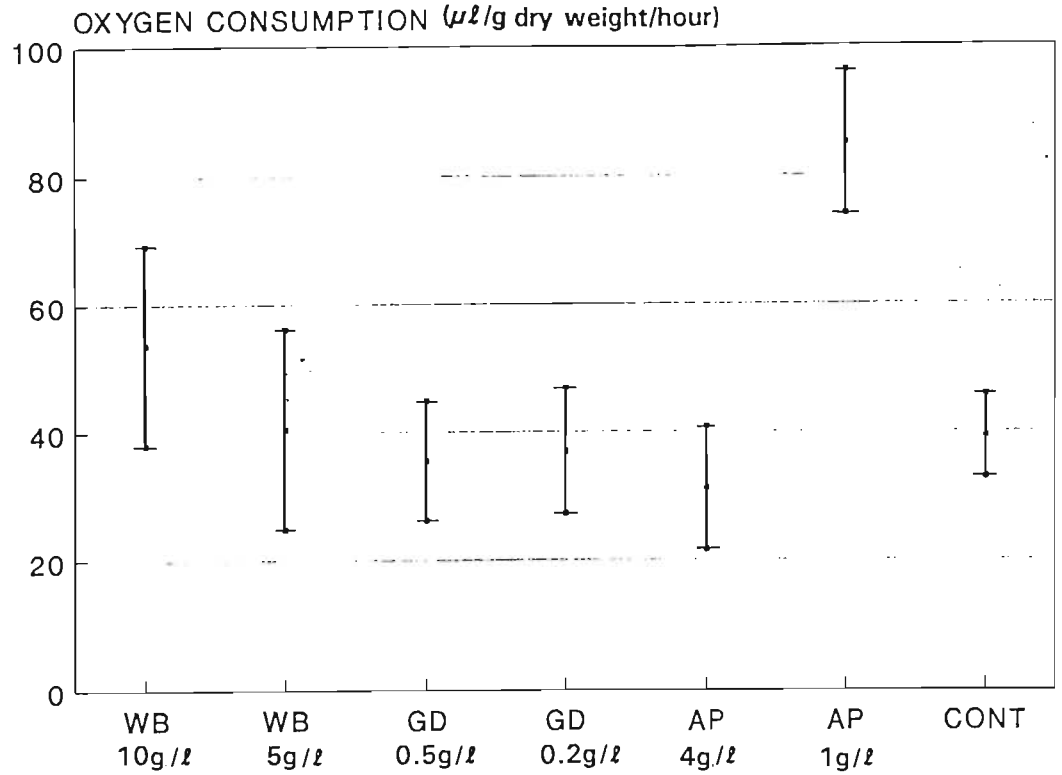


Figure 22 Mean oxygen consumption and 95% confidence limits for *Helisoma duryi* exposed to aqueous suspensions of leaves over a two-hour observation period (WB = *Warburgia salutaris*, GD = *Gardenia thunbergia*, AP = *Apodytes dimidiata*, CONT = control).

Table 24. Results of t-tests for oxygen consumption (µℓ/g dry weight/hour) at test concentrations after a two-hour exposure period (NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Plant species	Concentrations compared	Difference between means	t-value	Significance level
Warburgia salutaris	10 g/ℓ and 5 g/ℓ	13.12	1.18	NS
	control and 10 g/ℓ	-14.32	-1.78	NS
	control and 5 g/ℓ	-1.19	-0.17	NS
Gardenia thunbergia	0.5 g/ℓ and 0.2 g/ℓ	-1.49	-0.22	NS
	control and 0.5 g/ℓ	3.76	0.61	NS
	control and 0.2 g/ℓ	2.26	0.41	NS
Apodytes dimidiata	4 g/ℓ and 1 g/ℓ	-53.85	-7.28	***
	control and 4 g/ℓ	8.03	1.60	NS
	control and 1 g/ℓ	-45.82	-6.40	***

Table 25. Results of t-tests and F-tests for differences between experimental and control oxygen consumption ($\mu\ell$/g dry weight/hour) taken after a two-hour exposure period (df = Degrees of Freedom, NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

		MEANS			VARIANCES		
Plant species versus control	Conc. (g/ℓ)	Difference between means	t-value	Signif. level	F-ratio	df	Signif. level
Warburgia salutaris	10	14.32	1.78	NS	4.76	42, 66	**
	5	1.19	0.17	NS	2.95	42, 66	**
Gardenia thunbergia	0.5	-3.76	-0.61	NS	2.71	72, 66	**
	0.2	-2.26	-0.41	NS	1.95	66, 66	**
Apodytes dimidiata	4	-8.03	-1.606	NS	1.53	72, 66	NS
	1	45.82	6.406	***	3.80	54, 66	**

Table 26. Results of ANOVA for oxygen consumption ($\mu\ell$/g dry weight/hour) at 20-minute intervals over a two-hour exposure period (df = Degrees of Freedom, NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Plant species	Concentration (g/ℓ)	F-ratio	df	Significance level
Warburgia salutaris	10	0.696	5, 36	NS
	5	4.215	5, 36	**
Gardenia thunbergia	0.5	1.305	5, 48	NS
	0.2	4.601	5, 60	**
Apodytes dimidiata	4	5.960	5, 66	***
	1	4.844	5, 48	**
Control	0	2.344	5, 60	NS

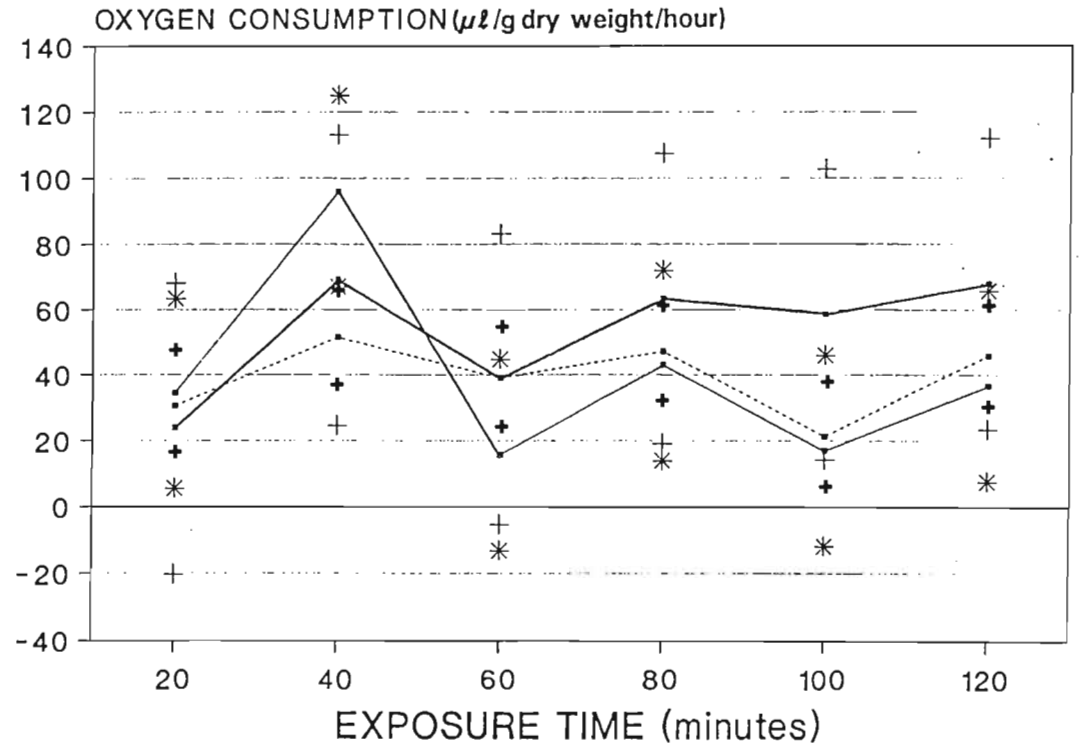


Figure 23.1 Mean oxygen consumption and 95% confidence limits for *Helisoma duryi* exposed to *Warburgia salutaris* leaves at intervals during two hours of observation (MEAN: ---= control, —= 10g/ℓ, —= 5g/ℓ. CONFIDENCE LIMITS: ✚ = control, ┼ = 10g/ℓ, ✳ = 5g/ℓ).

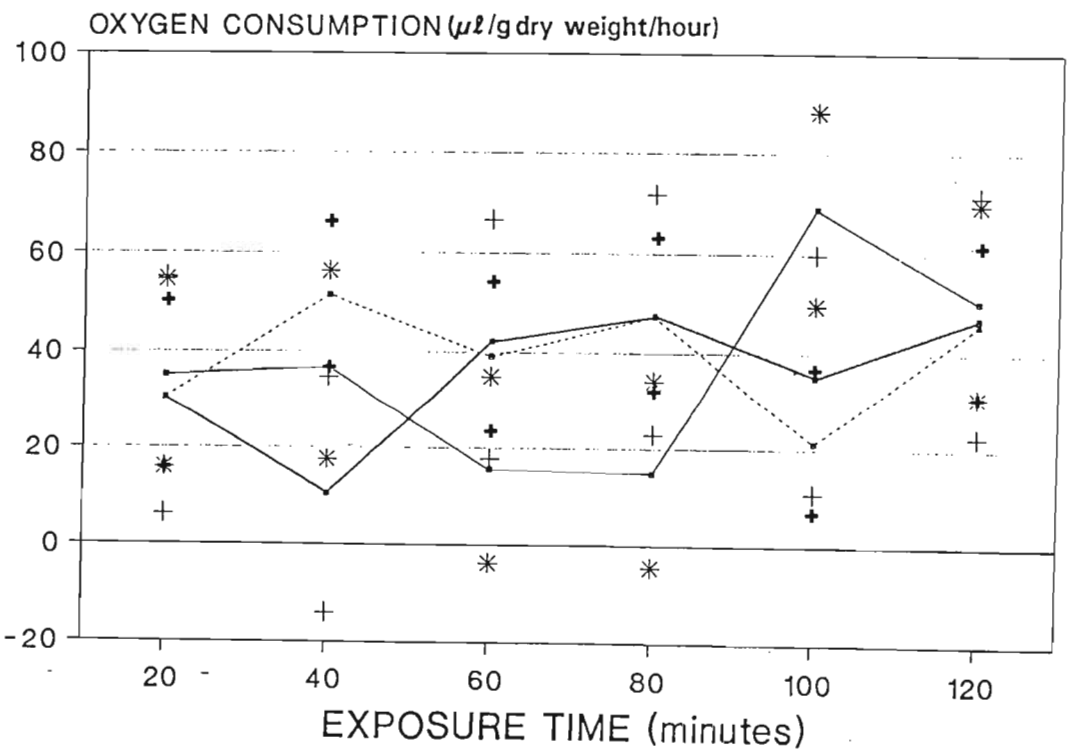


Figure 23.2 Mean oxygen consumption and 95% confidence limits for *Helisoma duryi* exposed to *Gardenia thunbergia* leaves at intervals during two hours of observation (MEAN: ---- = control, —= 0.5g/ℓ, —= 0.2g/ℓ. CONFIDENCE LIMITS: ✚ = control, ┼ = 0.5g/ℓ, ✳ = 0.2g/ℓ).

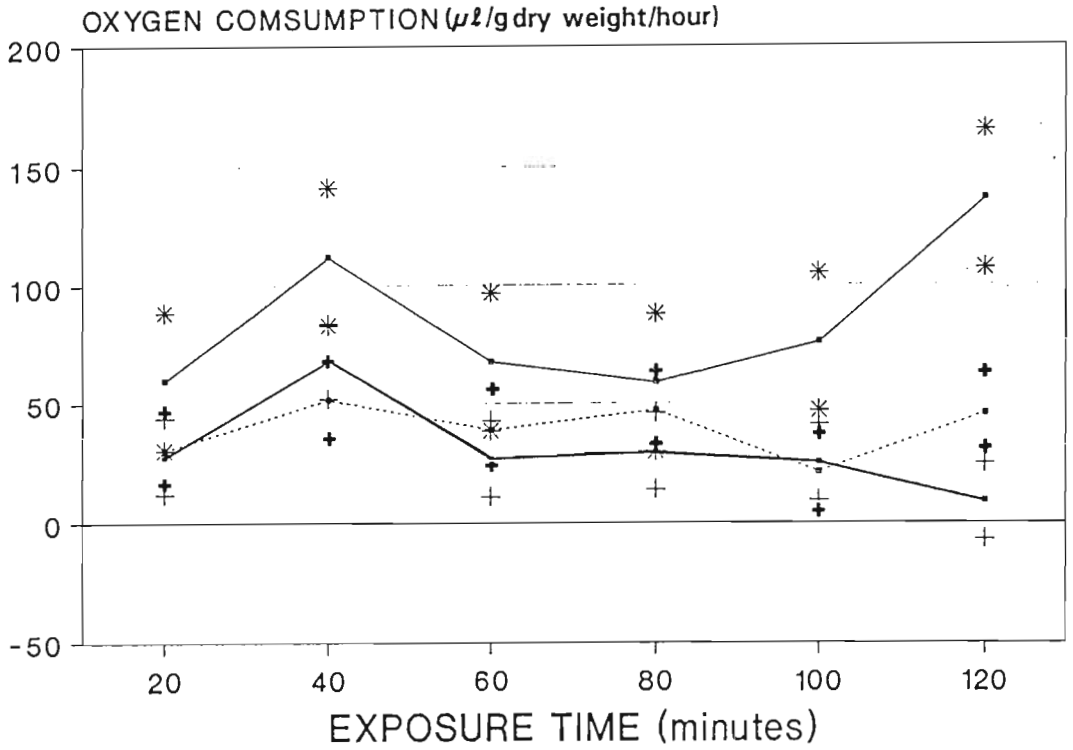


Figure 23.3 Mean oxygen consumption and 95% confidence limits for *Helisoma duryi* exposed to *Apodytes dimidiata* leaves at intervals during two hours of observation (MEAN: ----= control, —— = 4g/ℓ, —— = 1g/ℓ. CONFIDENCE LIMITS: ✚= control, ┼ = 4g/ℓ, ✳= 1g/ℓ).

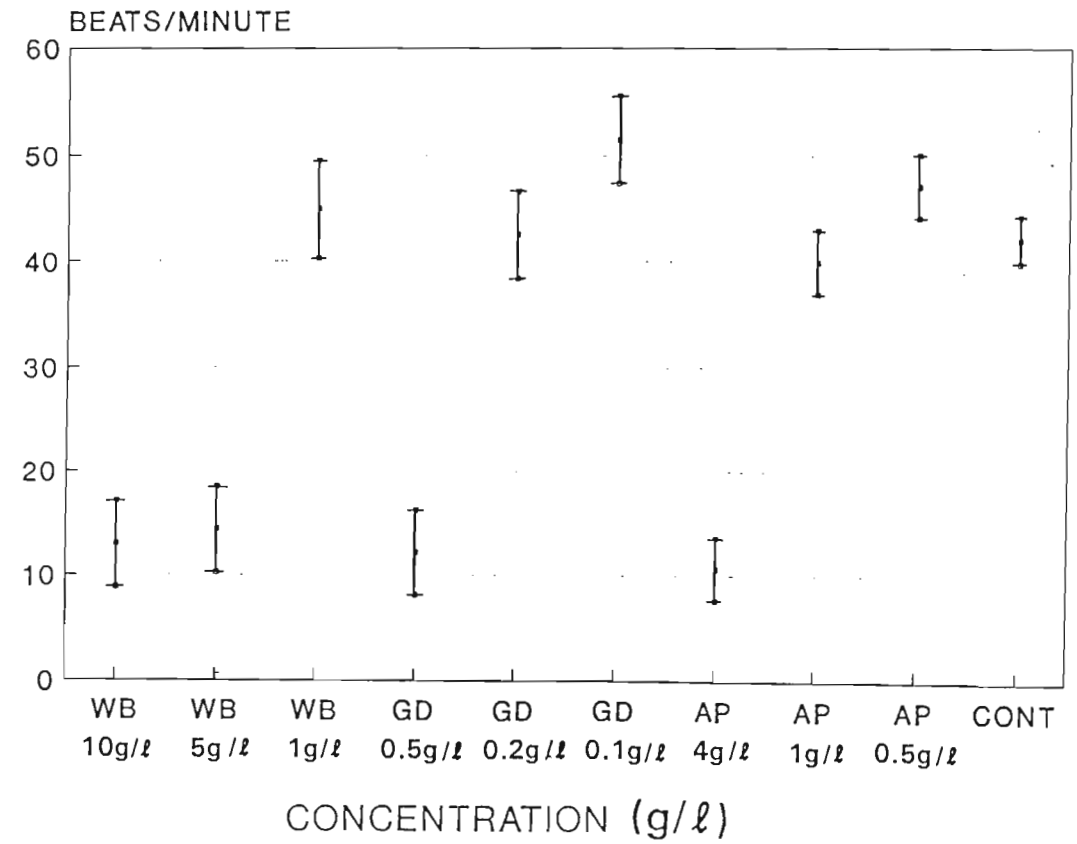


Figure 24 Mean heart rate and 95% confidence limits for *Helisoma duryi* exposed to aqueous suspensions over a 48-hour observation period (24-hour exposure and 24-hour recovery) (WB = *Warburgia salutaris*, GD = *Gardenia thunbergia*, AP = *Apodytes dimidiata*, CONT = control).

Table 27. Results of ANOVA for heart rate (beats/minute) for test concentrations over the total 48-hour observation period (df = Degrees of Freedom, NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Plant species	Concentrations tested (g/ℓ)	F-ratio	df	Significance level
Warburgia salutaris	10, 5, 1	64.64	2, 87	***
Gardenia thunbergia	0.5, 0.2, 0.1	101.01	2, 87	***
Apodytes dimidiata	4, 1, 0.5	165.96	2, 87	***

Table 28. Results of t-tests and F-tests for differences between experimental and control heart rate (beats/minute) taken over a 48-hour period (df = Degrees of Freedom, NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

		MEANS			VARIANCES		
Plant species versus Control	Conc. (g/ℓ)	Difference between means	t-value	Signif. level	F-ratio	df	Signif. level
Warburgia salutaris	10	-29.07	-13.61	***	2.70	30, 42	**
	5	-27.67	-12.66	***	2.52	30, 42	**
	1	2.82	1.24	NS	2.71	24, 42	**
Gardenia thunbergia	0.5	-29.87	-14.66	***	2.16	30, 42	**
	0.2	0.43	0.2	NS	2.48	30, 42	**
	0.1	9.37	4.12	***	3.05	30, 42	**
Apodytes dimidiata	4	-31.42	-19.06	***	0.93	30, 42	NS
	1	-2.1	-1.06	NS	1.96	30, 42	**
	0.5	5.02	2.86	***	1.24	30, 42	NS

6.3.3.2 Mean heart rate taken at selected intervals during the 48-hour observation period

Mean heart rates for selected intervals are shown in Figures 25.1, 25.2, and 25.3. Confidence limits are given in Appendix 4. These graphs should be viewed in relation to the results for ANOVAs given in Table 29. In *W. salutaris*, heart rate was significantly lower than the controls at lethal and high sublethal concentrations, and fluctuated little from one interval to the next (Figure 25.1). At low sublethal concentrations mean heart rate was similar to the controls but fluctuated significantly over the observation period. Heart rate gradually increased with time but returned to normal during the recovery period.

High sublethal concentrations of *G. thunbergia* (Figure 25.2) and *A. dimidiata* (Figure 25.3) produced a significant reduction in heart rate over 48 hours. Heart rate dropped and was unable to recover during the recovery period. Low sublethal concentrations resulted in a significant increase in the heart rate. Heart rate increased initially but returned to normal levels during the recovery period.

The variation in mean heart rate for different plants and concentrations was generally significantly greater than that of the controls. This indicates that even if mean heart rate does not differ significantly from controls, snails were battling to maintain their heart rates at normal levels.

A significant reduction in heart rate for control snails was recorded over the 48-hour exposure period (Table 29). This has very important consequences in the interpretation of snail mortalities during laboratory trials.

6.3.4 WATER UPTAKE

Over a 48-hour observation period there was no observable water uptake or loss in snails exposed to any plant extract, at any concentration investigated (Figure 26). Results of statistical analyses are given in Table 30.

6.4 DISCUSSION

6.4.1 CLINICAL SIGNS OF TOXICITY

Snail response to lethal and sublethal concentrations of these plant molluscicides was shown to follow previously described patterns of distress. A clear distinction among the three plants was the occurrence of haemolysis in snails exposed to *G. thunbergia*. This was the first indication that, despite the similarity of clinical signs of toxicity, the mode of action for at least one of the plants tested may be occurring via an distinct route.

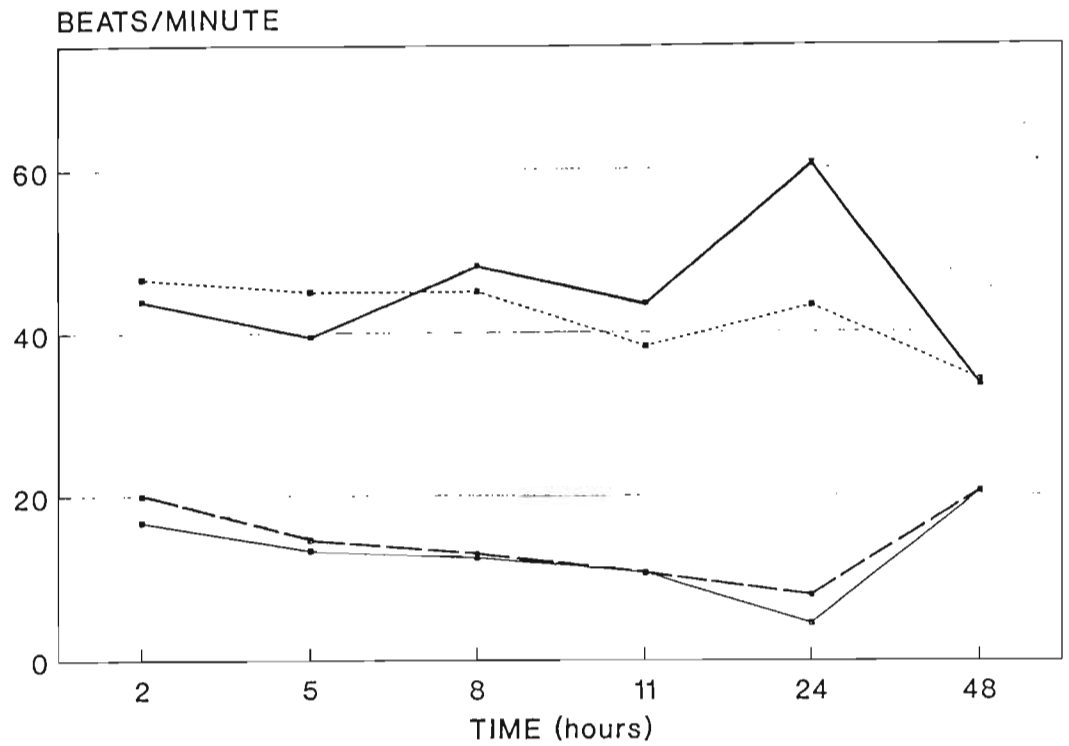


Figure 25.1 Mean heart rate for *Helisoma duryi* exposed to *Warburgia salutaris* leaves at intervals during 48 hours observation (MEAN:---- = control, — = 10g/ℓ, — — = 5g/ℓ, ——— = 1g/ℓ). Confidence limits are given in Appendix 4.

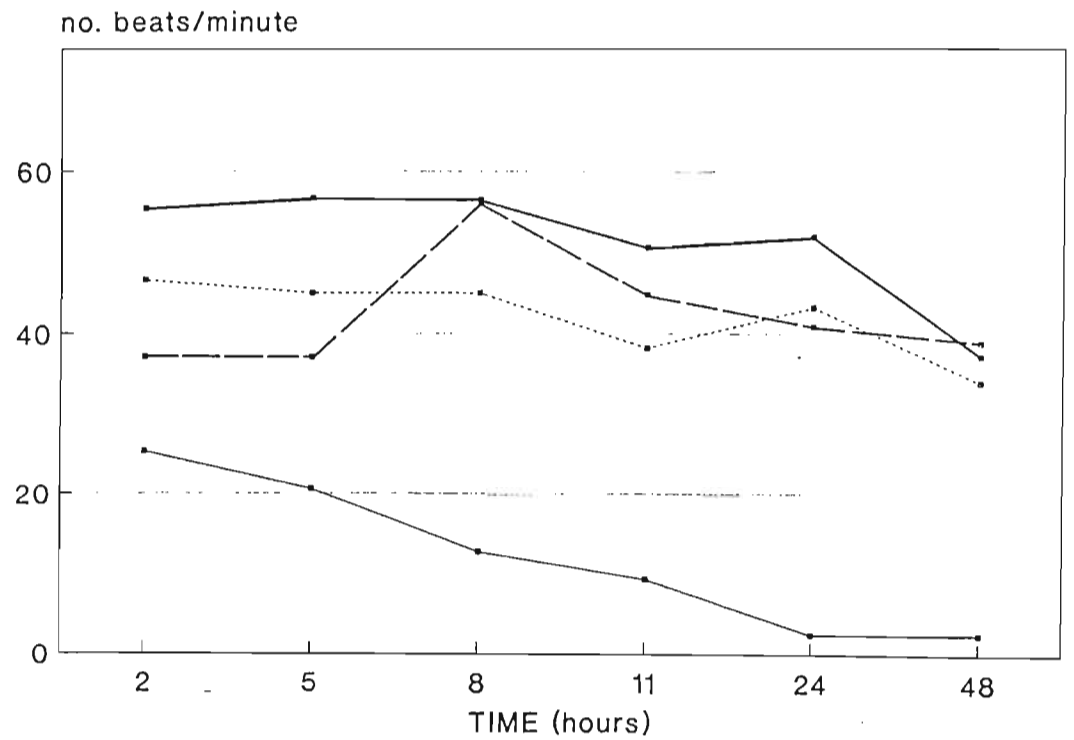


Figure 25.2 Mean heart rate for *Helisoma duryi* exposed to *Gardenia thunbergia* leaves at intervals during 48 hours observation (MEAN:---- = control, — = 0.5g/ℓ, — — = 0.2g/ℓ, ——— = 0.1g/ℓ). Confidence limits are given in Appendix 4.

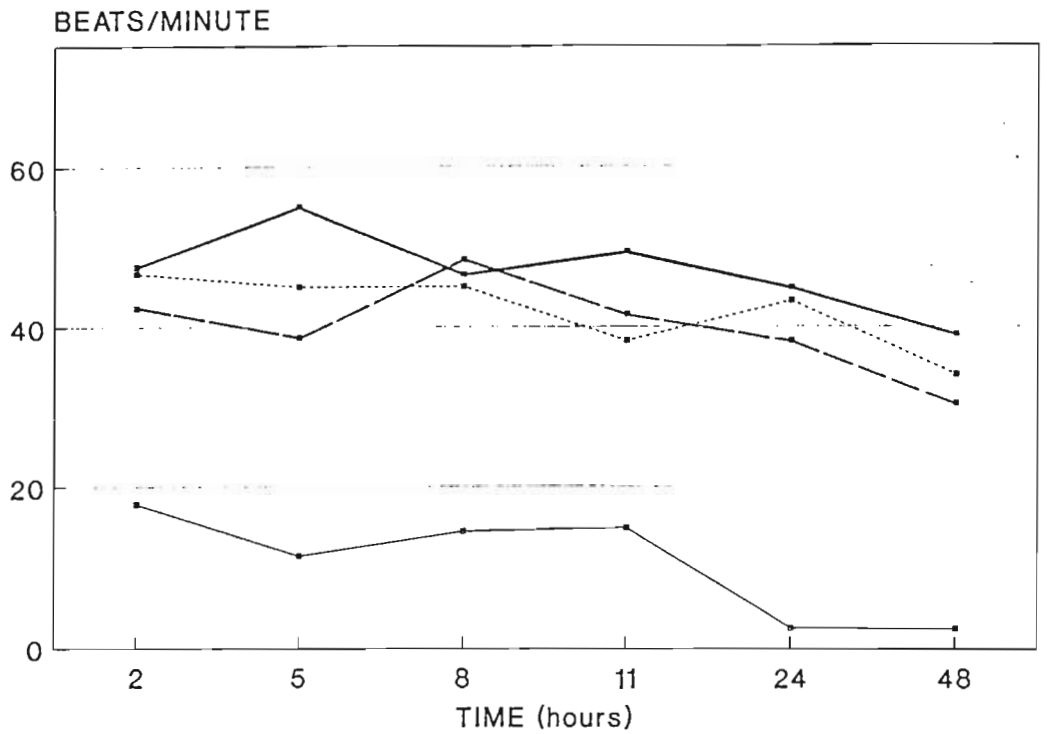


Figure 25.3 Mean heart rate for *Helisoma duryi* exposed to *Apodytes dimidiata* leaves at intervals during 48 hours observation (MEAN:----= control, —— =4g/ℓ, — —= 1g/ℓ, ▬ =0.5g/ℓ). Confidence limits are given in Appendix 4.

Table 29. Results of ANOVA for heart rate (beats/minute) taken at 2, 5, 8, 11 and 24 hours after exposure to molluscicides, and following a 24-hour recovery period (df = Degrees of Freedom, NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Plant species	Concentration (g/ℓ)	F-ratio	df	Significance level
Warburgia salutaris	10	1.266	5, 25	NS
	5	0.971	5, 25	NS
	1	4.378	5, 18	**
Gardenia thunbergia	0.5	11.989	5, 25	***
	0.2	2.864	5, 25	*
	0.1	2.189	5, 25	NS
Apodytes dimidiata	4	22.099	5, 25	***
	1	2.201	5, 25	NS
	0.5	3.131	5, 25	*
Control	0	5.116	5, 37	**

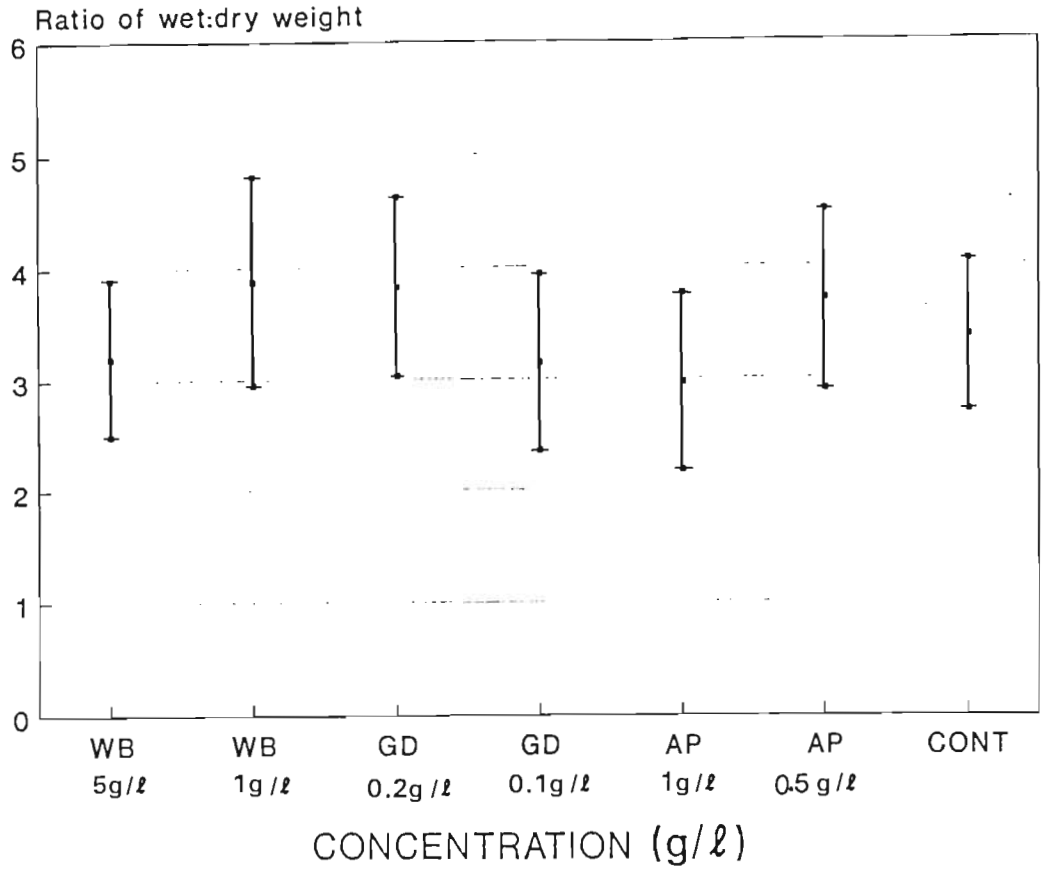


Figure 26 Mean ratio of wet to dry weight and 95% confidence limits for *Helisoma duryi* exposed to aqueous suspensions over a 48-hour observation period (24-hour exposure and 24-hour recovery) (WB = *Warburgia salutaris*, GD = *Gardenia thunbergia*, AP = *Apodytes dimidiata*, CONT = control).

Table 30. Results of Mann-Whitney U-tests for water uptake measured as the ratio of wet to dry snail weight over a 48-hour observation period (NS = Non Significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Plant species	Concentrations compared	z	Significance level
Warburgia salutaris	5 g/ℓ and 1 g/ℓ	1.606	NS
	control and 5 g/ℓ	-0.094	NS
	control and 1 g/ℓ	0.433	NS
Gardenia thunbergia	0.2 g/ℓ and 0.1 g/ℓ	-1.253	NS
	control and 0.2 g/ℓ	0.721	NS
	control and 0.1 g/ℓ	-0.162	NS
Apodytes dimidiata	1 g/ℓ and 0.5 g/ℓ	0.835	NS
	control and 1 g/ℓ	-0.974	NS
	control and 0.5 g/ℓ	0.721	NS

The absence of mucus production in snails exposed to 10g/ℓ of *W. salutaris* suggests that the debilitating effects of the toxin may occur before snails are able to respond by producing mucus.

6.4.2 OXYGEN CONSUMPTION

With extracts of *W. salutaris* and *G. thunbergia,* snail response to the presence of lethal and sublethal concentrations of molluscicides did not manifest itself in a change in oxygen consumption during the first four hours of exposure (i.e. two hours equilibration and two hours exposure). The ability to maintain oxygen consumption despite changes in heart rate (Section 6.4.3) is noteworthy. The observed variation in oxygen consumption indicated that the snails experienced difficulty sustaining oxygen consumption at regular levels. This was verified by a significant fluctuation in oxygen consumption measured at intervals during exposure to sublethal concentrations. Despite the fluctuations, snails were still able to maintain a mean oxygen consumption at levels not significantly different from the controls. These results suggest that for these two plant species, oxygen consumption in snails is not an immediate physiological response, and hence that respiration may not be directly responsible for mortality.

In contrast, oxygen consumption for the first four hours of exposure to low sublethal concentrations of *A. dimidiata* was considerably higher than that for high sublethal concentrations. This is quite the reverse of what one would intuitively expect. One explanation for the disparity of response is that molluscicidal activity is operating via two paths. These paths may be dose-dependant and affect different systems at different concentrations. Duke (1992) suggested that most biologically active compounds have several dose-dependant bioactivities: some medicinal and some pesticidal.

6.4.3 HEART RATE

The significant reduction in heart rate occurring in control snails, particularly during the recovery period, warns of potential confounding of mortality with a stress factor when mortality is measured under laboratory conditions. Trial protocols prescribe that over a 48-hour period, feeding and aeration are not required. The volume of test fluid has been set at a minimum of 40mℓ per snail (WHO, 1965), a much larger volume than employed in this study. Accordingly, the reduction in heart rate for control snails in this study has been interpreted as stress-related. The exact source of this stress is not known. Without preliminary trials under local laboratory conditions, using local snails, one cannot reasonably disregard the effects of starvation, oxygenation and container size. The effect

of this reduction in heart rate on the susceptibility of snails to test molluscicides is not known.

Lethal and high sublethal concentrations of all test molluscicides significantly reduced heart rate within two hours of exposure while increasing or maintaining it relative to the controls at low sublethal concentrations. The obvious difference in heart rates at high and low concentrations can be accounted for by the fact that extreme concentrations were selected for the very purpose of highlighting changes. Intermediate doses may not have produced such obvious effects.

Variation in heart rate at set intervals was also significantly greater than controls, suggesting difficulty in regulating heart rate at normal levels.

Another interesting observation was the apparent independence of heart rate and oxygen consumption (except for sublethal concentrations of *A. dimidiata*). This is remarkable since they should be physiologically correlated (Section 6.1.3). It is possible that although heart rate is affected, their physical inactivity results in lower demand such that oxygen consumption can be maintained as long as they remain immobile. The reduction in heart rate for *G. thunbergia* and *W. salutaris* suggests possible neurological effects (particularly in relation to pacemaker activity). Without further neurological research, however, inferences are all that can be made.

6.4.4 WATER UPTAKE

In contrast with earlier findings (Section 6.2.4), no significant changes in water uptake were observed over the 48-hour experimental period. It may be that perturbations in water uptake occur during the exposure period but return to normality during the recovery period. Interpretation of such findings is difficult, given that the concentrations tested, although sublethal, would produce mortality of some snails and should therefore result in some measurable effects on osmoregulation. Measurement of water uptake during the exposure and recovery period may improve our understanding of observed changes in water balance.

6.5 CONCLUSIONS AND SUGGESTED MODES OF ACTION

Snails exposed to these three plant molluscicides do follow a previously described distress syndrome, but the occurrence of haemolysis in snails exposed to *G. thunbergia* is the first indication that the mode of action may not be the same for all three plants.

Respiratory failure does not appear to be a primary cause of mortality in response to *W. salutaris* and *G. thunbergia* extracts. However, there is evidence that snails have

difficulty maintaining oxygen consumption at regular levels. The independence of oxygen consumption and heart rate parameters for snails exposed to these two plants, suggests that mortality may result either from direct effects on the heart, or through indirect effects on pacemaker activity. Indirect neurological effects should also not be ruled out.

A. dimidiata, at low sublethal concentrations alone, causes a substantial increase in oxygen consumption. This disparity in oxygen consumption at high and low sublethal concentrations may be due to the occurrence of more than one mode of action for the plant extract. The particular mode(s) of action would then be dose-dependant. Reduced heart rates at lethal concentrations suggests that mortality may occur through effects on the heart (either directly or indirectly through oxygen consumption), neurosecretion or the inhibition of enzyme systems involved in oxygen uptake.

Water balance does not appear to be an important factor in snail mortality although frequent mention is made of its potential involvement with respect to other molluscicides.

Despite the common fate of snails exposed to these three molluscicides, the process of death appears to occur via three distinct routes. Changes in heart rate, oxygen consumption and water uptake in snails are difficult to interpret in terms of mode of action because of the complex physiological inter-relationships which exist (Figure 21). Without further neurological investigations or more direct attempts to identify the site(s) of activity it is not credible to propose exact modes of molluscicidal action.

CHAPTER 7

IDENTIFICATION OF THE COMPOUNDS RESPONSIBLE FOR MOLLUSCICIDAL ACTIVITY IN *APODYTES DIMIDIATA*

ABSTRACT

A knowledge of the source of molluscicidal activity in plants is basic to the understanding of its toxicology and potential development. Material from all plant parts of *Apodytes dimidiata* was subjected to differential solvent extraction using hexane, dichloromethane, ethyl acetate and ethanol. Column chromatography was used in the separation of conspicuous compounds. Bioassays of *Helisoma duryi* were used to monitor molluscicidal activity. Two sources of activity were identified: genipin, first isolated from *Genipa americana* L. (RUBIACEAE), and a complex of four compounds. The most prominent constituent was found to be (S)(-)-2-hydroxypropanoic acid ethyl ester or (S)(-) ethyllactate. The latter source of activity appeared to result from synergistic effects, as in isolation, each of the four compounds were inactive. Genipin has not previously been recorded as molluscicidal, and although extracted from *Gardenia* spp. from the East, has not yet been identified as responsible for activity in *Gardenia thunbergia* in South Africa. The identification of (S)(-) ethyllactate as a natural product represents a novel finding. Prior to this report this compounds was known only from synthesis and had no known biological activity. Prospects for future development of genipin, and its toxicity relative to other plant and synthetic molluscicides, are discussed.

7.1 INTRODUCTION

Knowledge of the compounds responsible for molluscicidal activity are fundamental to studies on toxicity, stability, structure-activity relationships, and to an understanding of their effect on snail physiology (Marston and Hostettmann, 1991). It is has only been in the last 5 to 10 years that concerted efforts to isolate and characterize the active ingredients of plant molluscicides other than *Phytolacca dodecandra*, have been made. Progress in this field has been documented by Marston and Hostettmann (1985; 1991) and Hostettmann and Marston (1987). Some 80 molluscicidal compounds have been isolated from plants (Farnsworth, *et al.*, 1987), from many chemical classes, including saponins, spirostanol saponins, sequiterpene lactones, alkaloids, steroid glycoalkaloids, diterpenes, monoterpenes, iridoids, naphthoquinones, alkenyls phenols, chalcones, flavenoids, furanocoumarins, isobutylamides and tannins (Kloos and McCullough, 1987). The saponins appear to offer the greatest potential (Webbe and Lambert, 1983). Three

of seven plants which have been used in field trials, have triterpene saponins as molluscicidal constituents: *Phytolacca dodecandra, Tetrapleura tetraptera* and *Swartzia madagascariensis*. The chemical constituents of the other four plants mentioned by Marston and Hostettmann (1991) include the sequiterpene lactones from *Ambrosia maritima*, tannins from *Acacia nilotica* (north African subspecies *tomentosa* and *astringens*) and phenols from *Anacardium occidentale*. The activity of these compounds is quite comparable with those of synthetic molluscicides. Examples follow:

Bayluscide® WP 70 - 1ppm

Bayluscide® EC 250 - 0.6ppm

Phytolacca dodecandra - Oleanoglycotoxin-A, lemmatoxin and lemmatoxin-C are active between 1.5 and 3ppm (Marston and Hostettmann, 1991)

Tetrapleura tetraptera - *N*-acetylglycosides are active between 2.5 and 20ppm (Marston and Hostettmann, 1991)

Swartzia madagascariensis - Saponins are active between 3 and 50ppm (Marston and Hostettmann, 1991)

Warburgia salutaris - 5ppm (Nakanishi, 1984)

Whether such compounds are primary or secondary metabolites, and whether they play a beneficial "role" in plants, has been the subject of considerable debate (Haslam, 1986; Harborne, 1990). Most secondary compounds identified in plants show some bioactivity, particularly in plant-plant or plant-animal interactions (Duke, 1990). Of the molluscicidal compounds mentioned above, Bisset (1991) suggested that saponins function as growth regulators and allelochemicals. The terpenes have been implicated in insect antifeedant activity (Duncan, 1987a), as phytoalexins, defence agents, allelochemicals, and in pheromone production in insects (Harborne, 1991).

In this investigation attention has focused on the compounds responsible for molluscicidal activity in *A. dimidiata*, since no information is available on these active constituents. Questions which arise include whether these compounds bear any relationship to those extracted from other molluscicidal plants. Further, what is their

relative toxicity, and what other biological activity has previously been described, particularly with respect to environmental toxicity?.

Warburgia was excluded from evaluation since the sequiterpenes, warburganal and muzigadial, have already been implicated with respect to molluscicidal activity (Table 9, Chapter 3). These compounds are also powerful antifeedants for herbivorous insects (Brattsten, 1986).

Little information is available for *G. thunbergia*, a species widespread in Africa. Ahmed *et al.* (1984) listed active compounds as alkaloids, saponins, sterols and/or triterpenes, but gave no further details. Research on compounds from South African *G. thunbergia*, although excluded from discussion here, is proceeding in the Department of Chemistry and Chemical Technology (University of Natal).

The results of this investigation are the product of a collaborative effort between the abovementioned department and the Department of Zoology and Entomology. All procedures relating to the identification and isolation of the active ingredients (Section 7.2.1) were conducted by Mr L. Kayonga, under the supervision of Professor S.E. Drewes.

7.2 METHODS

7.2.1 ISOLATION AND PURIFICATION OF ACTIVE INGREDIENTS

The procedure followed for the extraction, isolation and identification of active compounds is summarized in Figure 27. Leaves, bark, flowers and small branches of *A. dimidiata* were air-dried at room temperature (23°C) and macerated. Material was then subjected to differential solvent extraction using hexane, dichloromethane, ethyl acetate and finally, ethanol. Fractions, for all plant parts, were continually assessed to ensure that activity had not been lost during extraction. The relative toxicity of plant parts was also assessed.

Thin layer chromatography (TLC) of the most active fractions was used to identify prominent compound(s) present. Column chromatography was used in the separation of these compounds, and the purity of active fractions confirmed by TLC. The purest fractions, as indicated by TLC, were then crystallized from a saturated solution. Nuclear magnetic resonance (NMR), infrared (IR) and mass spectroscopy (MS) were used in the identification of compounds of interest.

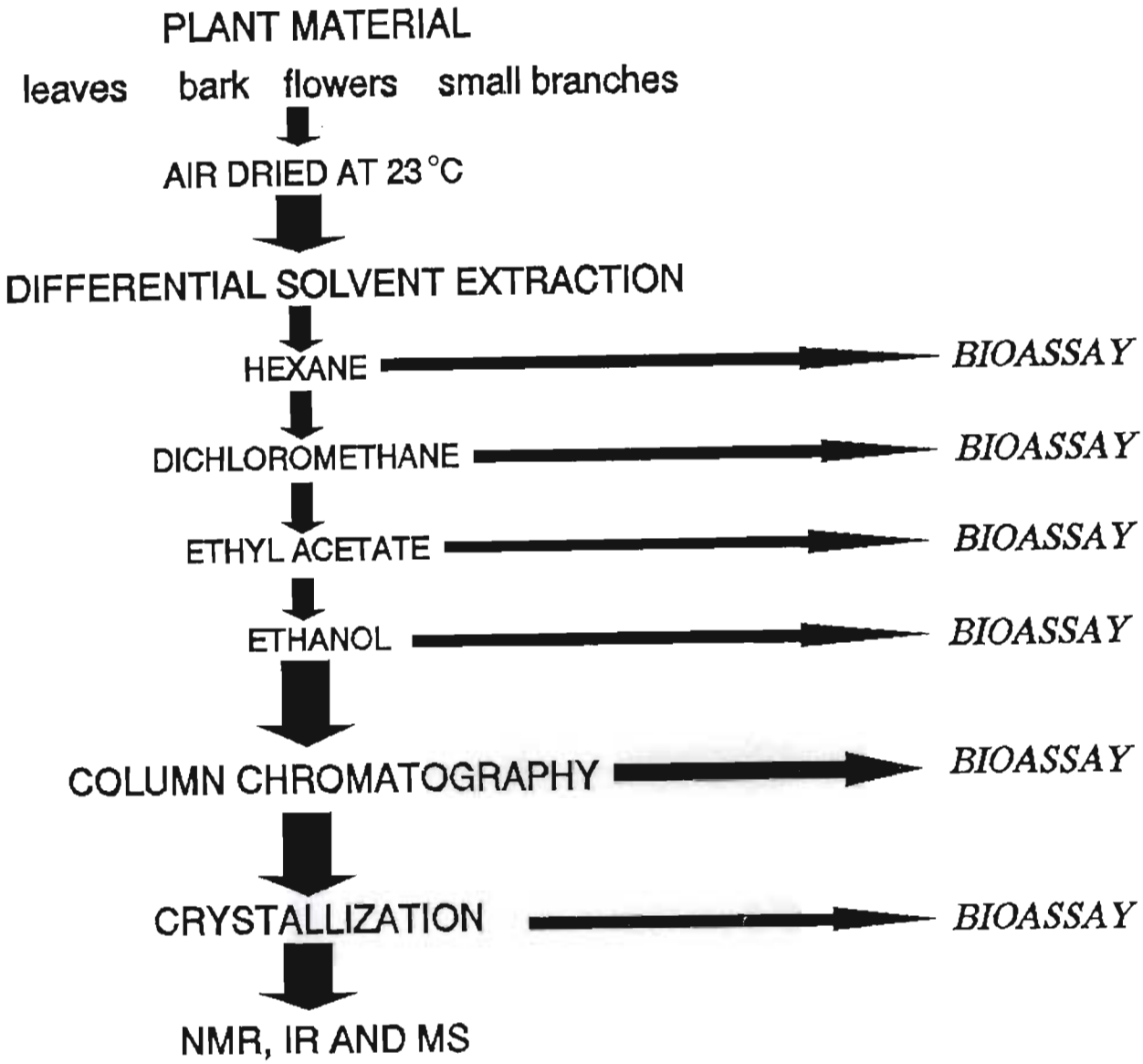


Figure 27 Summary of procedures followed in the isolation and identification of molluscicidal constituents of *Apodytes dimidiata*.

7.2.2 BIOASSAYS FOR MOLLUSCICIDAL ACTIVITY

Protocol for bioassays of molluscicidal activity are given in Appendix 1. *Helisoma duryi* was used as a target snail. Although not responsible for the transmission of schistosomiasis, this species breeds more readily under laboratory conditions, belongs to the same family as *Bulinus africanus* (the intermediate snail host of *Schistosoma haematobium*), and has been shown to be similarly affected by the aqueous suspensions of these plant molluscicides (Chapter 5, Section 5.4).

Solutions of extracts were made up in aged dechlorinated tap water, on a weight to volume basis (mg/ℓ or ppm). Where extracts could not be taken down to dryness, due to the presence of oils, solutions were made up volume for volume (mℓ/ℓ). The density

of liquid extracts has been assumed to be approximately equal to that of water. Accordingly, these concentrations were converted to mg/ℓ or ppm for comparative purposes.

Snails were exposed in groups of 5 in glass Petri dishes containing 50mℓ of test solution. The exposure period of 24-hours was followed by a 24-hour recovery period. The number of replicates and range of test concentrations used was dependant on the quantity of extract available. A minimum of 2 replicates were completed for all test concentrations. These exploratory investigations were used to identify active concentrations and the presence/absence of activity following extraction. Following identification of active fractions, a greater number of intermediate concentrations were assessed.

LD_{50}s and LD_{90}s were calculated for active compounds using Fieller's method as implemented in the GENSTAT 5.0 program (Payne, 1987). The analysis of dose-response data has been discussed (Chapter 3, Section 3.2.1.2).

7.3 RESULTS

The results of exploratory bioassays for molluscicidal activity following solvent extraction are given in Table 31. All fractions, except for the hexane fraction of bark, showed some activity between 500 and 1000ppm. The most active fraction was a dichloromethane bark extract. The broad spectrum of activity detected for all plant parts indicated that the active compound(s) are widely distributed throughout the whole plant. Activity in numerous fractions suggested that either solvents were not adequately extracting all traces of the compound(s), or that more than one compound was responsible for activity.

On the basis of these results a fresh ethanolic extract of the bark was further purified, and activity subsequently assayed for. Four compounds, coded for convenience as DBX_1, DBX_2, DBY and CBZ, were isolated, crystallized, and bioassayed. The results of these bioassays are presented in Table 32. DBX_1 was identified as a monoterpene, **genipin** (Figure 28) and DBX_2 as **(S)(-) ethyllactate** (Figure 29). The molluscicidal activity of (S)(-) ethyllactate was not verified following bioassays with the commercially available S-isomer. The commercial product showed no effect on snails at 50ppm, 10ppm and 5ppm. This inactivity prompted reassessment of the purity of DBX_2, and an additional three compounds were found: DBX_3, DBX_4 and DBX_5. Each compound was bioassayed independently following purification (Table 33). Each isolated fraction was inactive which suggested that molluscicidal activity is a consequence of two or more of these compounds acting synergistically.

Table 31. Exploratory bioassays for molluscicidal activity. Mean % mortality (± SD) of *Helisoma duryi* exposed to *Apodytes dimidiata* following differential solvent extraction (- = insufficient volumes of extract for a minimum of 2 replicates).

SOLVENT FRACTION	CONCENTRATION ppm (mg/ℓ)	LEAVES	SMALL BRANCHES	BARK	FLOWERS
Hexane	1000	100 ± 0	70 ± 0	0 ± 0	55 ± 7.07
	100	0 ± 0	-	0 ± 0	-
	10	0 ± 0	-	0 ± 0	-
Dichloro-methane	1000	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	100	0 ± 0	-	70 ± 14	-
	10	0 ± 0	-	40 ± 28	-
Ethyl acetate	1000	100 ± 0	100 ± 0	100 ± 0	-
	500	-	-	-	100 ± 0
	100	0 ± 0	-	0 ± 0	-
	10	0 ± 0	-	0 ± 0	-
Ethanol	1000	-	-	90 ± 14	-
	500	100 ± 0	48 ± 38	22.5 ± 3.5	-
	400	60 ± 0	-	-	-
	200	30 ± 14	-	-	-
	100	0 ± 0	10 ± 14	10 ± 14	-

Table 32. Mean percentage mortality (± SD) of *Helisoma duryi* when exposed to four compounds isolated from *Apodytes dimidiata*.

DBX_1 GENIPIN		DBX_2 (S)(-) ETHYL LACTATE (in combination with DBX_3, DBX_4, DBX_5)		DBY UNIDENTIFIED		CBZ UNINDENTIFIED	
CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY
40	100 ± 0	2000	100 ± 0	200	10 ± 14*	100	0 ± 0
20	30 ± 28	50	100 ± 0	100	10 ± 14*	50	0 ± 0
15	20 ± 34*	20	100 ± 0	50	10 ± 14*	20	0 ± 0
10	10 ± 14*	10	100 ± 0	10	0 ± 0	10	0 ± 0
8	0 ± 0	5	22 ± 38*	5	0 ± 0	5	0 ± 0
5	0 ± 0	1	15 ± 30*	0.1	0 ± 0	1	0 ± 0
1	0 ± 0	0.5	0 ± 0			0.1	0 ± 0
0.1	0 ± 0						

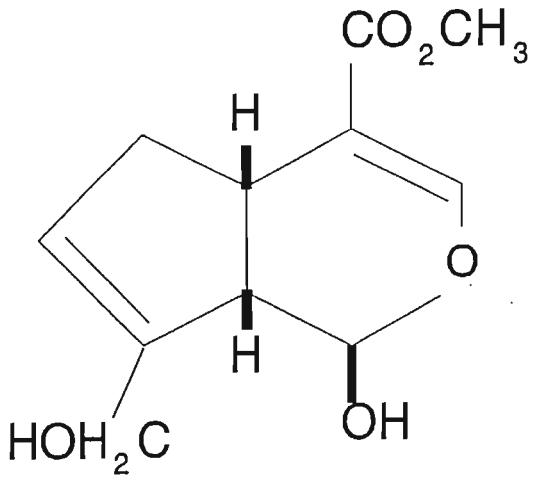
* Large standard deviations are the product of a low number of replicates. The number of replicates was limited by the volume of the extract available.

able 33. Mean percentage mortality (± SD) of *Helisoma duryi* when exposed to the four ompounds which constitute DBX_2.

(S)(-) ETHYLLACTATE		DBX_3 UNIDENTIFIED		DBX_4 UNIDENTIFIED		DBX_5 UNIDENTIFIED	
CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY	CONC. ppm	% MORTALITY
1000	0 ± 0	1000	0 ± 0	1000	0 ± 0	1000	0 ± 0
100	0 ± 0	100	0 ± 0	100	0 ± 0	100	0 ± 0
30	0 ± 0	30	0 ± 0	30	0 ± 0	30	0 ± 0

(+)-Genipin

$C_{11}H_{14}O_5$



LETHAL DOSE 95% CONFIDENCE LIMITS

LD = 21.04 ppm (17.50 - 28.04)

LD = 35.88 ppm (27.24 - 74.04)

Figure 28 Chemical structure and lethal dosages for genipin extracted from *Apodytes dimidiata*.

(S)(-)-ETHYL LACTATE

LETHAL DOSE 95% CONFIDENCE LIMITS

(when in combination with DBX_3 , DBX_4 , DBX_5)

LD_{50} = 10.88ppm (7.73 - 15.16)

LD_{90} = 33.86ppm (22.54 - 72.66)

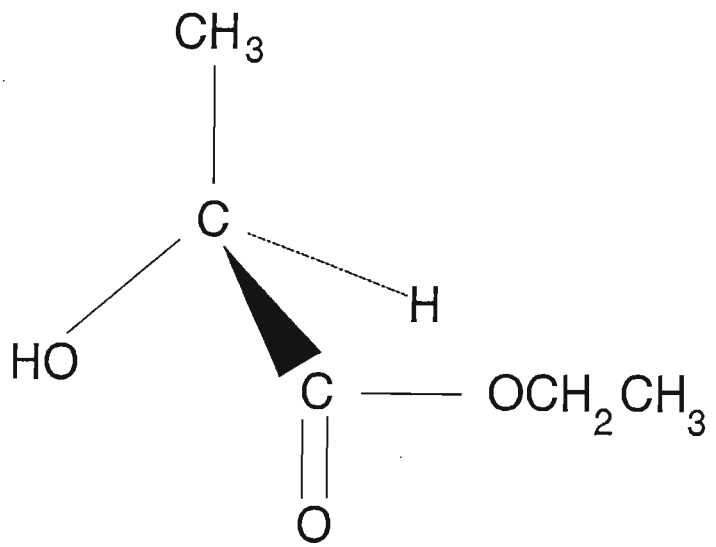


Figure 29 Chemical structure of (S)(-)-ethyllactate extracted from *Apodytes dimidiata* and lethal dosages for this compound in combination with DBX, DBX, and DBX

7.4 DISCUSSION

7.4.1 GENIPIN

Genipin has been isolated from the ripe fruits of *Genipa americana* and the structure elucidated by Djerassi *et al.* (1960). *Genipa americana* is native to tropical central and South America. The fruit is elliptical, edible and popular as a beverage (Ueda *et al.*, 1991). Genipin has since been extracted from the fruit of *Gardenia jasminoides* Ellis (RUBIACEAE), and is used in the production of blue pigments (Fujikawa *et al.*, 1987). Other monoterpenes identified from these two plants include: geniposidic acid (*G. americana*), geniposide (*G. jasminoides*), 1-gentiobioside (*G. jasminoides*), genipic acid and genipinic acid (*G. americana*) (Dev *et al.*, 1982). Notably, Yamano *et al.* (1990) reported that the conversion of geniposide to genipin was causally related to the hepatotoxicity of geniposide in rats. This discovery highlights the necessity for completing mammalian toxicity trials on aqueous suspensions before advocating field use of *A. dimidiata*. In contrast to the findings of Yamano *et al.* (1990), Ueda *et al.* (1991) found this compound to be antitumourous. No other biological activity has been recorded for genipin. Of the other cyclopentanoid monoterpenes mentioned, genipic acid has been identified as a growth inhibitor of a variety of gram-positive and gram-negative bacteria, a fungus, an alga, and a protozoan (Dev *et al.*, 1982).

7.4.2 (S)(-) ETHYLLACTATE, DBX_3, DBX_4, DBX_5

(S- or R-)-2-hydroxypropanoic acid ethyl ester has been synthesized for use as a solvent for cellulose acetate and nitrocellulose (Windholz, 1983). Apparently, no medical or pharmacological properties have been demonstrated. Although
molluscicidal activity is apparent following extraction from *A. dimidiata*, its commercial derivative is not active. The presence of 3 additional compounds, initially unobserved and as yet unidentified, are required for activity. As it is not known which combinations are required for activity, a molluscicidal role for (S)(-) ethyllactate is questionable. The identification of this compound as a biological product represents a novel finding.

7.4.3 RELATIVE MOLLUSCICIDAL ACTIVITY

LD_{50} and LD_{90} values showed that both genipin and DBX_2 (i.e. the combination of (S)(-) ethyllactate, DBX_3, DBX_4, DBX_5) were active at concentrations comparable to plant molluscicides currently under field investigation elsewhere. However, they are not as active as commercially produced synthetics, such as Bayluscide®, but do approximate the WHO (1965) standards for toxicity to snails, i.e. activity $\leq$20ppm for solvent extracts.

The moderate toxicity of crude aqueous suspensions of *A. dimidiata* (Chapter 5) indicate that these compounds, although less active than Bayluscide®, are likely to have less environmental impact on non-target fauna and flora. An obvious problem with respect to rural community use, is that the process of extraction of the active ingredient is wholly impractical under typical rural conditions. The use of *A. dimidiata* would be of considerably greater value if applied in its crude form. Application in this form should be profitable for small, still or very slow-flowing waterbodies (Chapter 3, Section 3.4).

7.5 CONCLUSIONS

Two sources of molluscicidal activity have been identified from *A. dimidiata*: genipin, which was first isolated from *G. americana*, and a complex of (S)(-) ethyllactate together with 3 additional unknown compounds. In the latter case the relative contribution of each compound towards activity is unknown.

Genipin has been implicated with respect to hepatotoxicity and tumour inhibition. The former warns of potentially hazardous mammalian toxicity and the need for further investigation before considering its field application, in either a pure or crude form. (S)(-) ethyllactate has previously been known only from synthesis, and had no known bioactivity. Although its role in molluscicidal activity remains questionable, its identification as a natural product is significant.

The activity of these extracts was comparable to that of other plant molluscicides currently under field investigation. Both sources of activity were, however, less active than Bayluscide®, the only synthetic molluscicide currently endorsed by the WHO. The commercial production of either compound is therefore unfeasible. The complexity of extraction procedures also precludes their application as pure compounds. Their use in aqueous suspensions would likely be the most important means of application in rural communities.

GENERAL DISCUSSION AND CONCLUSIONS

The results of this study show that a rural South African coastal community in a schistosomiasis endemic area does not share the indifference of the former South African health-care system towards the disease. These perceptions were assessed in relation to actual levels of prevalence and intensity of infection. In the study area, concern for schistosomiasis was matched by a mean prevalence of 75.14% in children and young adults (2 - 25 years old). It is unlikely, however, that all communities have the same regard for the disease since its impact appears to be focal (Appleton, 1985). Infection is also related to dependence on rivers and streams to meet water requirements. Increased access to piped water has shifted health-care priorities towards concern for diarrhoea and other helminth infections.

Although traditional medication is an integral part of health-care in the Mtwalume community, little confidence was shown in its ability to cure schistosomiasis. Transport and treatment costs were significant limiting factors for those seeking western medication. The few who had succeeded in obtaining western medicine expressed disillusionment with its efficacy. This highlights the problem of reinfection and the need for health education. The frustration of communities in the lack of alternative solutions to the problem of schistosomiasis, is reflected in their willingness to become actively involved in reducing infection themselves, through the use of plant molluscicides.

Plant molluscicide use is not a new concept. Despite decades of research on some plant species, such as, *Phytolacca dodecandra*, none have been officially endorsed for wide-scale application by the WHO. The reasons for this are complex and discussed in greater detail below. Despite their irresolute status in extensive National Control Programmes, plant molluscicides may yet have a convincing contribution to make in small-scale community control programmes. Motivation for their use in South Africa is centred on the status of both the disease, and the South African health-care system.

SOUTH AFRICAN HEALTH-CARE

Despite the fact that most African countries allocate substantial portions of their Gross National Product (GNP) to health, sustained improvements in community health-care and access to health services have not been obvious (Good, 1987). South Africa spent 5.4% of the GNP in 1985-1986 on health, compared to 2-3% spent by other developing countries (Department of National Health and Population Development, 1987). Since the mid-1980s expenditure has increased, and in 1990 sub-Saharan Africa spent 4.5% of the

GNP on health (World Development Report, 1993). In South Africa the imbalance in health services provided to Black and Coloured communities during the apartheid era (Department of National Health and Population Development, 1987) meant that these communities did not experience the full benefits of this health budget. A recent estimate of population density for rural communities on the KwaZulu-Natal coast (e.g. at Mtwalume) was 334 people per square kilometre (Kvalsvig and Connolly, 1993) and yet in 1987 KwaZulu-Natal had only 323 Primary Health Care clinics for the entire province (Department of National Health and Population Development, 1987). For developing countries with limited financial and manpower resources, PHC clinics are the most effective way of improving the general health of the total population. Improved access to PHC clinics could be achieved through the establishment of a greater number of clinics, while better use needs to made of the existing infrastructure, particularly with respect to health education.

South Africa's new Reconstruction and Development Programme (RDP) has pledged improved access to PHC facilities. This has already been initiated through the introduction of free health-care for pregnant women and children under the age of six years. However, this is unlikely to seriously impact on the prevalence of schistosomiasis since peak prevalence and intensity is known to occur in children between the ages of 10 and 15 years (Bergquist, 1992). Even if an effective chemotherapy programme could be implemented through PHC structures, the cost of treatment (cost of the drugs and examination), may still be beyond the financial resources of patients and health ministries (Gryseels, 1989; Straub and Walzer, 1992). Nonetheless, a positive impact on schistosomiasis would be the improved network through which health education could be disseminated.

A further consideration is the status of schistosomiasis relative to other infections in this country. The current system of National Disease Research Programmes in South Africa channels financial resources for research into diseases which result in high nationwide morbidity and mortality. In this way funding is prioritized, and focused attempts are made to provide solutions whilst simultaneously attempting to ensure that South Africa produces internationally competitive research. Past priorities set in the health sector in South Africa and in other developing countries often reflected national and international goals, but neglected local community problems (Tanner, 1989b). This approach, while being practical given the constraints of a limited health budget, did not address the problems of health-care at a local and regional level. Evaluation of local health problems could result in regional health prioritization, the results of which would be felt

by a greater number of people. Tanner (1989b) reiterated this by suggesting that little success would be achieved without more positive moves to decentralise health management. This point highlights the need for further knowledge, attitude and perception investigations such as that described in Chapter 1. Such studies can assist in setting priorities for health and development problems at the district level. Acceptable health decisions cannot take place in a social vacuum. Communities have limited resources and priorities which may compete with health (Cohen, 1989). These must be established before attempting to implement control programmes.

Given the low priority of schistosomiasis in the South African health-care system, there is an obvious need for community-orientated programmes. These programmes need to be simple, cost-effective and culturally acceptable. Synthetic molluscicididing and chemotherapy require long-term financial and logistical commitment, neither of which can be guaranteed from an already overburdened health budget. The philosophy of independence and self determination which underlies the use of plant molluscicides lends support to South Africa's quest for social, political and economic reconstruction and development.

PLANT MOLLUSCICIDE USE IN SOUTH AFRICA

Having identified the need for alternative control options, and the potential for plant molluscicide use in South Africa, it was necessary to include investigations of the following:

- The establishment of a selection procedure for the identification of South African molluscicidal candidates.

Such a system should incorporate indigenous knowledge and use of local plants, should make use of the wealth of data already available in the literature, and should be based on WHO (1965; 1983) criteria and guidelines for good plant molluscicides. The scoring system described in Chapter 2, although not without its limitations, should be accepted as an improvement on the random mass screening efforts of the past. The result of this selection procedure was not only the selection of *Warburgia salutaris, Gardenia thunbergia* and *Apodytes dimidiata* for further investigation, but also the prioritization of other South African plants for future evaluation.

- **The application of plant molluscicides as aqueous suspensions for use in rural community situations.**

Crude aqueous suspensions of plant material are the most practical form of application in rural situations. However, extraction with water as a solvent does not always result in maximal extraction of the active ingredients. Efficient extraction may only occur with the use of expensive and inaccessible organic solvents. Where water is an inefficient solvent, impractically large quantities of plant material may be needed to kill target snails. In Chapter 3 the three selected plants were evaluated on the basis of their toxicity as aqueous suspensions, their occurrence within the schistosomiasis endemic area and their cultivation potential. *Gardenia thunbergia*, followed by *A. dimidiata* and *W. salutaris*, ranked most highly on these three criteria.

Warburgia salutaris is unlikely to be of any value in the field as a crude aqueous suspension, since large volumes of leaf material would be required to significantly reduce snail numbers. This plant species is currently classed as "endangered" in South Africa (Hall *et al.*, 1980; Cunningham, 1991) and a major reason for its disappearance from the wild is that it is one of the most popular (and expensive) herbal remedies in south-eastern Africa (A.B. Cunningham, University of Namibia, Windhoek, *pers. comm.* with C.C. Appleton, 1991). Extensive cultivation of this species would be required if it were to be promoted as a molluscicide. In addition, communities are unlikely to see the value of treating water with plant material from which they could make financial gain, if sold locally.

Gardenia thunbergia was the only plant to closely approximate the activity levels recommended by the WHO (1965; 1983). These are recognized by researchers as guidelines alone, and as such, care should be taken not to dismiss plants exclusively on this basis. Abundance of plant material, familiarity and usefulness of the plant to the communities where it is to be applied, toxicity to non-target fauna and flora, and stability under wide-ranging environmental conditions are other factors worthy of consideration before excluding plants from possible control efforts.

-**The stability of crude aqueous suspensions under changing environmental conditions.**

The physical and chemical factors likely to alter molluscicidal activity in the field are numerous. The aspects investigated in Chapter 4 were temperature, sunlight, biodegradation, long-term storage, and drying. *Warburgia salutaris*, followed by *A. dimidiata* and *G. thunbergia*, was found to be the most stable under laboratory

simulations of these conditions.

- **The likely imbalance to the ecological equilibrium through effects on non-target fauna and flora.**

Some imbalance to the ecological equilibrium should be expected, since the function of a molluscicide is to induce mortality in snails. Extrapolation of the results of laboratory toxicity evaluations to effects in the field is extremely difficult. The effects of plant extracts on representative fauna and flora gives only an indication of likely disturbances, since it is impossible to assess the effects on all community elements. In Chapter 5 the susceptibility of a diverse range of non-target organisms were assessed, including invertebrates, fish and macrophytes. *Gardenia thunbergia*, followed by *A. dimidiata* and *W. salutaris* showed the least overall impact on the non-target test subjects.

- **To gain understanding of the physiological effects of plant molluscicides on snails.**

An understanding of how these plant extracts affect snails not only satisfies an academic curiosity, but may also provide useful clues for the future development of new molluscicides. Despite the use of molluscicides for more than half a century (e.g. copper sulphate since the early 1920s), elucidations of precise modes of action remain obscure. Some trends have been revealed: increased water uptake and evidence of a "distress syndrome". However, the complexity of snail physiology is such that actual physiological effects can only be inferred. An investigation of the action of the three plant molluscicides indicated that despite following a previously described distress syndrome, the effects on heart rate, oxygen consumption and water uptake were quite distinct for each plant species. Indications are that more complex neurological and histological investigations are required to clarify the mode(s) of action.

- **To establish what compound(s) were responsible for molluscicidal activity.**

A chemical and structural knowledge of the compounds responsible for activity is particularly useful when assessing toxicity to non-target organisms, especially mammals. Of the three plants under investigation, *A. dimidiata* was given priority because of the absence of any information relating to the chemistry of its activity. Limited information was available on *G. thunbergia*, whilst the chemistry of *W. salutaris* is well known. A monoterpene, genipin, was found to be the major component responsible for activity in *A. dimidiata*. Evidence of further activity, possibly involving (S)(-) ethyllactate in a synergistic effect, was also detected.

PLANT MOLLUSCICIDES: WHERE TO NOW?

This study has contributed to our understanding of not only South African molluscicidal plants, but to selection procedures, toxicology, mode of action and the chemistry of molluscicidal activity. It is now almost the 30th anniversary of Lemma's first report on *Phytolacca dodecandra* and despite the wealth of information generated during this period, no single plant molluscicide has yet been endorsed for wide-scale use! The proponents of plant molluscicides have continued to promote their potential (cost-effectiveness, availability, cultural acceptability, the development of community independence, self-determination, etc), and yet the communities desperately needing this "technology" are still not receiving the benefits of this research. It is not unlikely that this "potential value" has been a convenient guise for substantiating the selfish endeavours of those who simply wish to enjoy natural products research. Reasons for changes in research emphasis in plant molluscicides have been discussed in Chapter 2 (Section 2.4.5), but do not explain why the available information has not being incorporated in small-scale community control programmes. One possible reason may be that researchers are not always able to carry the products of their research efforts to completion. Incomplete data sets and the need for further research are obvious limitations. Additionally, without contractual agreement between themselves and funding bodies, researchers are under no obligation to provide recommendations on technology transfer to the affected communities. In the absence of moral or social conscience towards the communities to which their research is "directed", researchers will likely not see the products of their work through. Further, even if researchers show willingness to implement their research findings, they are often poorly equipped with the necessary communication skills, or the necessary contacts with policy-makers and community representatives who can facilitate the transfer of information. It understandably takes time to translate a good idea into a workable product or an efficient social service (Jequier, 1981). Solutions to these problems are not easily found, but need to be addressed if the mass of information generated from plant molluscicide research is to ultimately benefit rural communities. Given the results of this investigation and the above discussion, the following recommendations are made for plant molluscicide research and implementation in South Africa.

1. Finding a solution, i.e. a suitable plant which is effective throughout the schistosomiasis endemic area in South Africa is unlikely because of the nature of plant habitat requirements, local availability, geographical differences in activity and local acceptability of the plant. Instead of searching for nation-wide solutions more

effort should be concentrated on utilizing the wealth of data and information already available in the literature to provide local solutions. Priority should be given to the most severely infected communities and the implementation of community-based control programmes in these areas. The usefulness of plant molluscicides will be best assessed following small-scale pilot programmes. The results of such investigations are likely to naturally permeate surrounding communities.

2. Plant molluscicide use should ideally be part of an integrated approach to control which would include health education, chemotherapy, and improved sanitation and water supplies. With respect to chemotherapy, transport and treatment costs are currently prohibitive. Although the widespread provision of piped water in rural areas is ideal it is an unrealistic expectation in the near future. Health education would however be a prerequisite for the successful implementation of plant molluscicide control, since community involvement is obviously dependant on the understanding of the role of freshwater snail control in reducing infection. In Chapter 1 none of the respondents could make the association between poor hygienic habits and infection. This is the case for many other infections involving a parasite in a complex life-cycle of one or more hosts (Kvalsvig, 1991b). Policy-makers face a considerable challenge if they are to raise educational standards to a level capable of having a positive impact on health. It is not only the task of the Department of National Health. A realistic approach would be to incorporate health education through already established facilities: (a) through PHC clinics and (b), through the school curriculum (Department of Education). Effective health education requires training, manpower, and finances, and these in turn require commitment from health ministries at central government and provincial levels.

3. An analysis of cost-effectiveness is necessary for field-tested plant molluscicides. Specialized control programmes of any description are only justified if an evaluation demonstrates cost-effectiveness (Korte *et al.,* 1986). The following costs should be considered: plant material (if any), transport, personnel, health education, the number of individuals likely to benefit from molluscicidal application and the efficiency of the molluscicide (Korte *et al.*, 1986; Prescott, 1987). Herrin (1986) and Bundy and Guyatt (1992) discussed some of the problems with analyses of cost effectiveness. The simplest estimate of cost is usually given as the purchase price of anthelminthics. However, drug costs should include the costs of central

and peripheral storage, transportation and the administration required to achieve this, personnel involved in diagnosis, and the identification of target communities. Cost analysis would be most appropriate once a field trial site had been identified. Despite claims of the potential cost-effectiveness of plant molluscicides, chemotherapy may still prove to be a cheaper form of control in some areas, albeit in the short-term (Webbe and Lambert, 1983).

4. Priorities for future evaluations of plant molluscicides from the current study would be the assessment of mammalian toxicity, and efficacy in the field. An exhaustive mammalian toxicity investigation should be a prerequisite to pilot field applications, as it is necessary to ensure that molluscicides do not have a detrimental effect on communities using this water.

5. Mammalian toxicity is also a prerequisite for the registration of extracts for use as plant molluscicides. One of the major shortcomings of plant molluscicide applications appears to be a legal problem in endorsing natural products for local use. Registration of active ingredients requires serious consideration if the WHO is to endorse its use, and if local authorities are to be satisfied with the safety of its application. Bureaucracy, and the absence of well defined registration guidelines for natural products in developing countries, pose formidable obstacles. It seems logical to identify the tests required by national authorities for the registration of chemical compounds in those countries. Not only should these authorities offer regulations on standard tests, but if these are properly completed and the plant can be found in all other respects to be suitable for community use, then the active compounds should be registered for use nationally; ultimately to be endorsed by the World Health Organization.

6. Every effort should be made to appropriately transfer the findings of this study, and other studies of this kind, to the communities for whom it was intended. A wealth of data exists which can and should be incorporated into small community-based pilot control programmes. Local solutions should be sought to ensure that plant material can be harvested, processed and applied to infected waterbodies. Emphasis should be given to focal control, not only because it is less labour intensive but also because it restricts the possible impacts of toxicity on non-target fauna and flora (Klumpp and Chu, 1987; Tanner, 1989a).

Appendix 1. Definitive laboratory screening procedures for the evaluation of molluscicidal activity (adapted from WHO, 1965).

1.	Snail species	*Bulinus africanus* (Krauss). Dr D.S. Brown (Natural History Museum, London) confirmed the identity of the snails used.
2.	Snail source and stock maintenance	Uninfected locally available field source. Snails were reared in the laboratory according to De Kock and Van Eeden (1980) and Van der Schalie and Berry (1973). Laboratory rearing efforts did not prove to be sufficiently productive. While adults and juveniles survived well under laboratory conditions, hatchlings died before reaching 2mm in shell height. Ten to 15 adult snails were maintained in glass aquaria containing 10ℓ of dechlorinated tapwater. Water was aerated continuously and tanks were cleaned every week. Black plastic strips for egg laying were placed in each tank. Snails were fed alternately on fishflakes and dried lettuce. In an attempt to improve survival rate, an inoculum of *Nostoc* sp. was obtained from the USAID/SRP Biological Supply Programme (Theodor Bilharz Institute, Cairo). *Nostoc* cultures were established using half-strength Bold's Basic Medium (Bold, 1942; Anonymous, 1978) and stock cultures maintained on agar under sterile conditions. Although adults and juveniles thrived off this alga, the hatchling survival rate did not improve. Possible fungal and bacterial infections were discounted through the sterilization of aquaria and other equipment. Malacologists at the Instituto de Medicina Tropical "Pedro Kouri", Havana, Cuba, experienced similar problems rearing *Biomphalaria* spp. on *Nostoc* (G. Perera *pers. comm.* with C.C. Appleton, 1993). Poor hatchling survival under our laboratory conditions is an issue seriously in need of further investigation.
3.	Trial containers	Glass Petri dishes. Beakers used initially were unsuitable because of the need for a gauze cover. Because toxicity, and not protective behaviour was being tested, a cover was required to ensure that snails were continuously exposed to the test solution. Concern for the possible influence of cover material on molluscicidal action led to the use of glass Petri dishes.
4.	Container size	Large (250mℓ) diameter - 15cm, height - 2cm. Small (50mℓ) diameter - 9cm, height - 1cm (used only in chemistry assays - Chapter 7).
5.	Volume of solution/snail	A minimum of 40mℓ per snail has been recommended (WHO, 1965). Large Petri dishes allowed 50mℓ per snail. The use of small Petri dishes did not comply with recommendations but was unavoidable when only limited quantities of purified extracts were available (Chapter 7). Controls were run under the same conditions for each trial.
6.	No. of snails/ container and no. of replicates	Five snails per container. A minimum of 2 replicates for each test concentration, and a control (Duncan and Sturrock, 1987). Wherever possible 3 to 10 replicates were conducted. The dependence on field populations of snails and the need for 3000 to 4000 snails during the study meant that often insufficient snails were available to increase the numbers of replicates.

7.	**Snail size (measured as shell height)**	Adults 9.6mm ± 2.14 (mean ± SE).
8.	**Water used for controls, dilutions and washing snails following exposure**	Dechlorinated tapwater since the amount of molluscicide taken up by snails is significantly reduced in distilled water (Duncan and Sturrock, 1987).
9.	**Aeration**	None. Not considered necessary during either exposure or recovery periods.
10.	**Light**	Fluorescent lighting, 12 hour light : 12 hour dark cycle.
11.	**Temperature**	26°C
12.	**Exposure period**	24 hours
13.	**Recovery period**	24 hours
14.	**Criterion of death**	Lack of response to mechanical stimulus (Duncan and Sturrock, 1987).
16.	**Reference molluscicides**	**Endod-S**, a standard spray-dried water extract of *Phytolacca dodecandra* from Dr. C.B. Lugt (The Hague, Netherlands). LD_{100} should be approximately 6 - 7ppm (Balaawy, 1972). **Bayluscide® EC 250** (emulsifiable concentrate containing 250 g a.i./ℓ. Active Ingredient: Niclosamide. Bayer, AG, Germany). LD_{100} should be approximately 6ppm.

Appendix 2. Procedure for the preparation of aqueous suspensions for all toxicity tests.

1. Freshly collected plant material dried in an oven at 40°C.
2. Dried material macerated and used within 9 weeks (else discarded).
3. Known quantities of dried material added to dechlorinated tap water to make up the desired concentrations.
4. Material allowed to soak for three hours at 23°C whilst on a magnetic stirrer. A one-hour extraction period was used by Dossaji *et al.* (1977). Although duration of extraction is an important source of variability it has not been formally standardized (Kloos and McCullough, 1987).
5. The solution filtered through muslin cloth to remove coarse particulate matter, and then applied to snails.

Appendix 3. Analysis of deviance tables showing the fit of dose-response data to the probit model. Where the data showed a significant lack of fit (i.e. $P < 0.05$ for the residual deviance), a complementary log-log model was used. The improved residual deviances are also given (NS = Non Significant, df = Degrees of Freedom).

MODEL	SOURCE	df	DEVIANCE	SIGNIFICANCE LEVEL
WARBURGIA SALUTARIS				
Probit	Log Dose	1	35.887	$P < 0.01$
	Residual	3	6.245	NS
	Total	4	42.132	
GARDENIA THUNBERGIA				
Probit	Log Dose	1	70.6763	$P < 0.01$
	Residual	6	3.6328	NS
	Total	7	74.3091	
APODYTES DIMIDIATA				
Probit	Log Dose	1	132.333	$P < 0.01$
	Residual	5	18.697	$P < 0.01$
	Total	6	151.020	
Complementary log-log	Log Dose	1	143.107	$P < 0.01$
	Residual	5	7.912	NS
	Total	6	151.020	
RAUVOLFIA CAFFRA				
Probit	Log Dose	1	111.676	$P < 0.01$
	Residual	4	7.717	NS
	Total	5	119.393	
ACACIA NILOTICA				
Probit	Log Dose	1	25.9694	$P < 0.01$
	Residual	4	3.3814	NS
	Total	5	29.3509	
ENDOD				
Probit	Log Dose	1	18.030	$P < 0.01$
	Residual	3	8.192	$P < 0.05$
	Total	4	26.222	
Complementary log-log	Log Dose	1	20.472	$P < 0.01$
	Residual	3	5.75	NS
	Total	4	26.221	

Appendix 4. Mean oxygen consumption and 95% confidence limits for *Helisoma duryi* exposed to test concentrations of plant molluscicides. Results are given for intervals during a 48-hour observation period.

Warburgia salutaris

TIME (hours)	CONCENTRATION (g/l)								
	10			5			1		
	Mean	95% Confidence Limits		Mean	95% Confidence Limits		Mean	95% Confidence Limits	
2	16.77	6.73	26.8	19.99	5.56	34.42	43.94	34.74	53.13
5	13.28	3.25	23.31	14.60	0.17	29.02	39.48	30.28	48.67
8	12.42	2.39	22.45	12.80	-1.62	27.22	48.21	39.02	57.41
11	10.53	0.50	20.56	10.50	-3.91	24.93	43.53	34.34	52.73
24	4.44	-5.58	14.47	7.90	-6.52	22.32	60.62	51.42	69.81
48	20.46	10.42	30.49	20.55	6.12	34.97	33.54	24.35	42.74

Gardenia thunbergia

TIME (hours)	CONCENTRATION (g/l)								
	0.5			0.2			0.1		
	Mean	95% Confidence Limits		Mean	95% Confidence Limits		Mean	95% Confidence Limits	
2	25.33	19.74	30.92	37.15	28.29	46.00	55.44	45.16	65.72
5	20.61	15.02	26.21	37.12	28.27	45.98	56.71	46.43	66.99
8	12.76	7.17	18.35	56.09	47.23	64.94	56.53	46.25	66.80
11	9.41	3.82	15.00	44.84	35.99	53.69	50.68	40.40	60.96
24	2.5	-3.09	8.09	40.87	32.02	49.72	51.97	41.69	62.25
48	2.5	-3.09	8.09	38.88	30.03	47.73	37.27	26.99	47.55

Apodytes dimidiata

TIME (hours)	CONCENTRATION (g/l)								
	4			1			0.5		
	Mean	95% Confidence Limits		Mean	95% Confidence Limits		Mean	95% Confidence Limits	
2	17.79	14.89	20.68	42.41	34.18	50.65	47.58	47.42	53.73
5	11.47	8.58	14.37	38.66	30.43	46.90	55.06	48.91	61.22
8	14.51	11.61	17.40	48.41	40.18	56.65	46.56	40.40	52.71
11	14.99	12.10	17.88	41.57	33.33	49.80	49.4	43.24	55.55
24	2.57	-0.31	5.47	38.18	29.94	46.41	44.87	38.72	51.03
48	2.5	-0.39	5.39	30.47	22.23	38.70	39.04	32.89	45.20

Control

TIME (hours)	Mean	95% Confidence Limits	
2	46.67	40.80	52.54
5	45.08	39.21	50.95
8	45.05	39.18	50.92
11	38.25	32.38	44.12
24	43.23	37.36	49.10
48	34.07	28.20	39.94

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