Triterpene glycosides chemically defend the South African kelp bed sea cucumber, *Pseudocnella insolens* (Echinodermata: Holothuroidea), against predation.

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A thesis submitted in fulfilment of the requirements for the degree M.Sc. in the

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Date: 15.05.97.

I declare that

"Triterpene glycosides chemically defend the kelp sea cucumber,

***Pseudocnella insolens* (Echinodermata: Holothuroidea), against predation in**

South Africa."

is my own work and that all the sources I have used or quoted

have been indicated and acknowledged by means of complete references.

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LITERATURE REVIEW

1. History of chemical ecology

The last three decades have witnessed the growth of a new inter-disciplinary field of study which has developed as an offshoot from natural products chemistry (Manes *et al.*, 1985). This field of study is called ecological biochemistry or chemical ecology and it is concerned with the biochemistry of plant-plant, plant-animal and animal-animal interactions in the natural environment (Harborne, 1988).

Harborne (1988) ascribes the growth of this new discipline to the development and improvement of biochemical techniques. The union between dissimilar disciplines such as ecology and biochemistry may seem a puzzling association at first glance. Ecology is concerned with the interactions between living organisms in their natural habitats (Starr & Targett, 1989). By contrast, biochemistry is essentially experimental and is primarily concerned with interactions at the molecular level (Conn *et al.*, 1987). Despite differences, these two disciplines have in amalgamated with great success (Harborne, 1988).

For over a century the elucidation of the chemistry of natural products has been a dominant theme in organic chemistry (Haslam, 1985) but the emergence of chemical ecology has shifted the focus to investigating the rôle that natural products play in the ecology of living organisms. In natural products chemistry the emphasis of research has been on the structure, chemical properties, and the synthesis of natural products.

The term 'natural product' is easily understood and appreciated by the organic chemists, for the roots of their science lie in the chemistry of natural products. This science has evolved through the study of substances isolated from living organisms (Haslam, 1985). Scientists in the late eighteenth century began to develop theories which suggested possible sources of these natural products. To suggest probable origins from simple precursors, they laid the basis of the biogenetic theory and they brought coherence to groups of apparently unrelated natural products by suggesting biochemical relationships (Haslam, 1985).

From the early twentieth century organic scientists began to separate, purify, and finally analyse those compounds produced in living cells (Torsell, 1983). Separation methods such as various analytical and chromatographic methods were developed, and without a doubt natural products chemistry has led to the development of the refined techniques that are available to chemical ecologists today (Miyamoto *et al.*, 1992). These methods have made it possible to isolate natural products and study their chemical and biological properties.

The success of chemical ecology depends on the co-operation of scientists from different fields of study. It is also apparent that interdisciplinary studies between ecologists and chemists are academically rewarding because they seek the important mechanisms behind ecological principles (Bakus *et al.*, 1986). An example of the co-operation between these scientists from different disciplines is that of Mark Hay, a marine ecologist, and William Fenical, an organic chemist. The success of this association is revealed in numerous publications from the field of marine chemical ecology (eg. Hay *et al.*, 1987; Hay & Fenical, 1988; Hay *et al.*, 1990a,b; Hay & Fenical, 1996). Marine chemical ecology, which is an emerging sub-discipline, seeks to achieve an integrated understanding of chemical biological interactions occurring in the sea.

In recent years, ecologists have demonstrated that naturally occurring products have a significant rôle in the complex interactions occurring between organisms in the natural environment. In animals, naturally occurring products are important in influencing food selection and are also involved in social and reproductive behaviour. Higher plants compete with each other for moisture, light and nutrients in the ecosystem. In the course of this struggle, they have developed various means of defence against their neighbours. Within work done in chemical ecology there are a number of examples of chemically guided behaviours in animals and plants in their environment (Torsell, 1983; Harborne, 1988; Fenical, 1988; 1996).

2. Comparison between primary and secondary metabolites

Naturally occurring compounds found within living organisms have been categorised as either primary or secondary metabolites. Compounds that are essential to life processes are referred to as primary metabolites (Salisbury & Ross, 1985). These include natural products such as fatty acids, sugars, and nucleic acids. Fatty acids are components of lipid structures. Amino acids are building blocks of proteins, and purine and pyrimidine bases are units of nucleic acid structure which constitute the genetic code. All these products are essential for living organisms and life cannot exist without these basic building blocks (Yudkin & Offord, 1973).

Organisms also contain compounds that are not required for normal growth and therefore have no obvious rôle in the welfare of the organism which produces them (Bulock, 1980). The substances in this group are commonly referred to as secondary metabolites because of their apparent secondary rôle (Manitto, 1981; Salisbury & Ross, 1985). These include compounds such as alkaloids, terpenes, pigments, phenols and myotoxins that occur naturally.

This distinction amongst the chemical constituents of living organisms has long been recognized (Yukin & Offord, 1973), and is useful in explaining the different biological rôles of these compounds (Haslam, 1985). These categories of primary and secondary metabolites are however, merely opposite ends of a continuum of metabolic

function. Because they exist along a continuum, opinions often differ as to the specific functioning and status of a particular metabolite (Yukin & Offord, 1973).

A large portion of research on secondary metabolites has focused on plant-animal interactions, especially herbivory (Rosenthal & Janzen, 1979; Bernays, 1981; Fox, 1981; Denno & McClure, 1983). Rosenthal and Jenzen (1979) and Haslam (1985) noted that Stahl, in the late eighteenth century appears to have been the first person to suggest that some of the chemical substances found in plants may have evolved for protection against attack by herbivorous animals. This research served as a starting point in understanding the rôle of secondary metabolites in the life of plants and animals in their natural environment. The secondary metabolites of plants and animals have been recognised to possess biologically important properties and it is now known that organic compounds are responsible for many of these biological properties (Fenical, 1982).

Herbivory in terrestrial communities can be very intense (Rosenthal & Janzen, 1979; Denno & McClure, 1983). Herbivory may reduce the growth and survival of individual plants and can influence interspecific competition and community structure (Rockwood, 1973; Morrow & Fox, 1980; Rausher & Feeny, 1980; Lubchenco & Gaines, 1981; Coley, 1983; Coley *et al.*, 1985). The importance of secondary metabolites in defending terrestrial plants against herbivores is well studied and generally accepted as one of the most effective form of defence mechanism (Sondheimer & Simeone, 1970; Levin, 1976; Harborne, 1978, 1988; Rosenthal & Janzen, 1979; Denno & McClure; 1983).

Studies of chemical ecology in the marine environment were not started until the invention of SCUBA, which allowed scientists to witness directly the biologically diverse and ecologically complex marine environment (Fenical, 1982). Although research on marine secondary metabolites and their ecological rôles has a short history relative to similar investigations in terrestrial systems, more than 1 000 secondary metabolites have been isolated and structurally identified (Elyakov *et al.*, 1973; Fenical & Norris, 1975; Hellou *et al.*, 1982; Faulkner & Ghiselin, 1983; Faulkner, 1984, 1986).

Benthic marine macro-algae contain wide variety of secondary metabolites (Faulkner, 1984, 1986; Hay & Fenical, 1988). Although many of these compounds in algae have been proposed to function as defences against herbivores (Steinberg, 1984; Paul & Van Alstyne,1988), until recently there was little known about their ecological effects. However, at least some algal secondary metabolites have harmful and deterrent effects against marine herbivores, and thus can function as defences (Targett & McConnell, 1982; Steinberg, 1984; Paul & Fenical, 1986; Hay *et al.*, 1987). Clearly anything which reduces herbivory on a plant can increase the plant's overall fitness and should be strongly selected for. However, it is still not known whether secondary metabolites that convey defensive ability evolved in response to the selection pressure exerted by herbivory, or if secondary metabolites function as antifouling agents of pathogen inhibitors (Hay &Fenical, 1988; Davis & Wright, 1990; Hay, 1991).

Plants are richer than animals in the diversity of secondary metabolites which they

produce (Luckner, 1984). Although, secondary metabolism occurs in animals, over four-fifths of all presently known natural products are of plant origin (Swain, 1974; Robinson, 1980). Over 12 000 different natural products of varied biogenetic origins are produced by terrestrial plants (Harborne, 1988). Harborne (1988) ascribes this richness in secondary metabolites to the simple fact that plants are rooted in the soil and cannot move; they cannot respond to the environment in the ways open to mobile animals.

3. Defence against consumers

Consumers are widespread in the natural environment, and play a significant rôle in determining the distribution and abundance of the organisms which are their food (Paine, 1971; Vince *et al.*, 1976; Menge & Lubchenco, 1981; Bingham & Braithwaite, 1986; Hay *et al.*, 1988). In response to the selection pressure of consumption, protective and defensive features evolved that increased the fitness of these organisms. These features include, structural defence, habitat selection and chemical defence.

3.1. Structural Defence

Plants and animals produce an array of structural defences that reduce their susceptibility to damage from predators (Duffy & Paul, 1992). For example in the marine environment, many sessile organisms produce structural material such as mineral skeletons or organic fibres that are commonly believed to serve a protective function

(Hay, 1984; Paul & Hay, 1986). Some seaweeds may concentrate calcium carbonate which make them very difficult for many marine herbivores to consume (Duffy & Paul, 1992; Schupp & Paul, 1994). All classes in the phylum Echinodermata have spicules or spines in their skin, although this characteristic is developed to different degrees in the various classes (Barnes, 1987). In the Echinoidea their bodies are encased in a hard, calcium carbonate shell or test, covered with spines. In the Holothuroidea the spines are reduced to spicules. In the terrestrial environment the supportive and strengthening function of lignin in plant tissues is well known, but there is also evidence that lignin also gives protection against attack by pathogens and consumption by herbivores, both insect and mammalian (Swain, 1974). Thus there may be a dual structural and defensive function for lignin in plants. Structural defences may not deter all consumers, but they provide some protection against certain consumers.

3.2 Habitat and other kinds of defence mechanisms

Prey species use a variety of mechanisms to escape from their predators. To persist in marine communities, prey must escape, deter or tolerate consumption. The ecological and evolutionary importance of spatial and temporal escapes has been extensively studied for seaweeds (Gaines & Lubchenco, 1982; Hay, 1984, 1985; Lewis, 1986; Hay & Fenical, 1988). Seaweeds may reduce the impact of herbivorous fish by occupying habitats where fish rarely feed or by occurring in habitats so favourable for growth that production exceeds herbivory even if the rates of grazing are high (Hay,

1991). Numerous studies have documented vastly different rates of grazing on different areas of a coral reef system and have noted the importance of these between habitat differences in generating spatial refuges for seaweeds that are highly susceptible to grazing fish (Hay *et al.*, 1983; Lewis, 1986;Morrison, 1988). Spatial patterns of fish grazing can occur on smaller spatial scales within habitats that are otherwise relatively uniform (Hay, 1991).

Interactions between fish and the physical environment are less thoroughly studied but also appear to be important in generating within-habitat refuges for seaweeds. Studies have shown that grazing rates of herbivorous reef fish are correlated with water temperature (Carpenter, 1986; Klumpp & Polunin, 1989). If lowered temperature depresses feeding rates more than rates of algal production, then palatable seaweeds will have a greater probability of escaping herbivorous fish during cooler periods of the year. Other kinds of defence, particularly among invertebrates include: nocturnal activity and parental protection (Fishlyn & Phillips, 1980). It seems likely that an organism may employ a number of these defences to reduce consumption from diverse consumers (Duffy & Paul, 1992).

3.3 Chemical defence

Organisms possess a number of mechanisms which reduce the impact of consumers (Hay, 1984; Paul & Hay, 1986; Paul, 1987), many of which may have evolved in response to the intense selection pressure exerted by consumers (Lewis, 1986). One of the most important of these mechanisms is the production of secondary metabolites, which can function as chemical defences against consumers (Hay & Fenical, 1988; Hay *et al.*, 1988; Hay *et al.*, 1990a,b; Steinberg & Paul, 1990; Duffy & Paul, 1992). Secondary metabolites have been shown to have a negative effect on consumer fitness or to deter consumption (Rosenthal & Janzen, 1979). This reduction in consumption increases the chances of survival of the producing organisms thereby increasing their overall fitness.

Metabolites responsible for the chemical defence of an organism are frequently not uniformly distributed within the organism (Van Alstyne *et al.*, 1994). Variation in chemical defence composition and concentration is often seen in marine plants and invertebrates (Mckey, 1979; Paul & Hay, 1986; Hay *et al.*, 1987). Differences in the distributions of chemical defence within marine organisms provide an opportunity for assessing some of the constraints and selective forces that have influenced the evolution of these defences (Harvell *et al.*, 1988). For example, Harvell *et al.* (1988) have proposed a model for the evolution of chemical defences within Caribbean gorgonians. This model is based on the distributions of secondary metabolites within octocoral species. They

suggest that the use of secondary metabolites as defences evolved in response to selective pressures imposed by predators. According to their model, the high concentrations of secondary metabolites in the polyps of some octocorals resulted from selective pressures from fishes. The concentration of secondary metabolites are lower in the bases because predation by fish at the base is less intense. The distribution and concentration of metabolites are therefore related to the extent of consumer pressure.

Organisms may use one of two chemical defence strategies to allocate resources for antipredator defences (Paul & Van Alstyne, 1992). The two strategies are continuous and acquired chemical defence. Continuous chemical defence refers to the use of secondary metabolites as a chemical defence to reduce predation when predation rates are consistent and predictable. This type of strategy describes most kinds of chemical defence. Acquired chemical defence refers to the use of secondary metabolites as a chemical defence to reduce predation when predation rates are temporarily unpredictable. One type of acquired chemical defence that has been described in several marine organisms is the production of predator-induced defences (Schultz, 1989; Adler & Harvell, 1990). Usually this type of defence operates when an attack by a predator acts as a cue for stimulating the synthesis of defensive compounds (Paul & Van Alstyne, 1992). In marine organisms, predator-induced chemical defences have been reported only in the brown seaweed *Fucus* (Van Alstyne, 1988). The different chemical defence strategies allow organisms to deal more effectively with predators.

3.3.1. Alternative and additional functions of secondary metabolites

Seaweed secondary metabolites have been demonstrated to function as herbivore deterrents, but some of these compounds may have additional functions (Habermehl & Krebs, 1990). Secondary metabolites have also shown to play a rôle in microbial and epiphytic deterrence (Targett *et al.*, 1983; Hay & Fenical, 1988; Davis & Wright, 1990; Hay, 1991). Despite the various effects of secondary metabolites it is not known whether the primary function of secondary metabolites is chemical defence. It is likely that many secondary compounds have multiple functions and thus form the basis of a complex chemical defence system (Standing *et al.*, 1982; Rittscof *et al.*, 1985).

3.3.2 Cost of chemical defence

The production of chemical defences against consumers could incur metabolic costs for the defended organism if significant energy or materials have to be diverted from other functions to the production of chemical defence. Thus, defences are hypothesised to be costly and in the absence of consumers less defended individuals will have higher fitness than do heavily defended individuals (Lubchenco & Gaines,1981; Coley *et al.*, 1985). If this reasoning applies to seaweeds, then habitats that serve as predictable escapes from herbivory should be populated primarily by species that are highly susceptible to herbivore damage. Low susceptibility to herbivory should be characteristic of those species or individuals that occur in habitats where herbivory is predictably high.

This pattern occurs in a number of marine communities (Lubchenco & Gaines, 1981; Hay *et al.*, 1983; Hay, 1984; Paul & Fenical, 1986), and experimental decreases in herbivory result in the more herbivore-susceptible seaweeds dominating the more herbivore-resistant forms (Lubchenco & Gaines, 1981; Lewis, 1986).

There are no direct experimental assessments of the costs of chemical defences in seaweeds. The currencies in which to measure the costs associated with chemical defences are difficult to separate and quantify, but probably involve more than the chemical energy stored in the molecular bonds of secondary metabolites (Hay & Fenical, 1988; Fagerstrom, 1989; Cronin & Hay, 1996). Since seaweeds are often nitrogen limited but seldom carbon limited (Hay & Fenical, 1988), it is unclear that allocation of energy (i.e. carbon bonds) will entail any direct costs. However, substantial costs could be involved in the synthesis and storage of compounds. It is interesting to note that seaweeds rarely produce the nitrogen containing compounds that are defences in some terrestrial plants. Nitrogen limitation may make nitrogen-based defences too costly, especially when carbon-based defences work well. With few exceptions, the only seaweed secondary metabolites that contain nitrogen are produced by the blue-green algae (Moore; 1981; Faulkner, 1984), many of which are nitrogen fixers. However, substantial costs could still be involved in synthesis, and storage. The allocation of chemical defences to different organism parts (Paul & Fenical, 1986; Hay *et al.*, 1988), the activation of chemical defence following attack (Van Alstyne, 1988), and the competitive interactions between chemically defended and undefended organisms in the absence of

consumers (Lewis, 1986) all suggest that chemical defences are costly.

4. Categories of compounds, their functional and evolutionary significance

The major classes of secondary metabolites have been assigned various rôles in the complex interactions occurring between organisms in the natural environment (Harborne, 1988). The major classes and their physiological activity include:

1. Nitrogenous compounds: many toxic and bitter tasting;
2. Terpenoids: many toxic, bitter and haemolyse blood;
3. Phenolic compounds: anti-microbial, anti-digestive agents and pigments;
4. Miscellaneous compounds: some toxic.

These compounds have served as a starting point for many studies of chemical-ecological interactions.

Marine metabolites in general structure do not differ from those produced in terrestrial organisms and there are many biosynthetic pathways which are common to organisms from both realms. The general classes of compounds are outlined below.

Nitrogenous compounds. Nitrogenous compounds have nitrogen as a basic element, and many are known for their toxicity. The simplest nitrogen-based toxins are the non-protein amino acids. These are widely present in plants and may be directly toxic to the organism eating them. In the simplest case, azetidine 2-carboxylic acid (Fig. 1),

may be mistakenly incorporated into proteins during synthesis so that the organism produces unnatural proteins which cannot function properly so the organism dies (Harborne, 1988). Although, the production of nitrogen containing compounds are hypothesized to be costly in the marine environment, a wide variety of nitrogenous compounds have been isolated from marine organisms, ranging from simple compounds such as tetramine to complex ones such as tetrodotoxin (Woodward, 1964; Shimizu, 1978). The latter are strong neurotoxins which inhibit sodium passage through membranes (Chevolet, 1981). Other simpler nitrogenous compounds play important rôles in marine biological interactions. For example, gamma-amino-butyric acid, produced by encrusting coralline algae, influences settlement and metamorphosis in abalone larvae (Morse & Morse, 1984). The most familiar class of nitrogen-based toxins are the alkaloids. Alkaloids isolated from marine acorn worms function as a chemical defence against micro- and macro-organisms (Higa *et al.*, 1980). Nitrogen-based compounds are significant feeding deterrents to grazing animals, particularly when their presence is often associated with a bitter taste.

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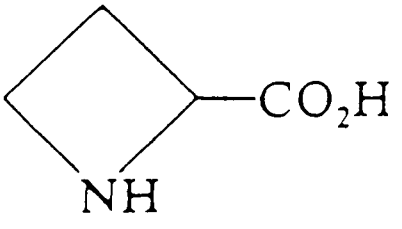

Fig. 1. azetidine 2-carboxylic acid (Harborne, 1988)

Terpenoids. Terpenoids represent the largest and biologically most important class of natural products (Rosenthal & Janzen, 1979). These compounds are multiples of C_5 units linked together 'head to tail' (Fig. 2). They have been found in all living organisms so far examined for them. Although most terpenoids occur free in nature, often being accumulated in specific tissues, many are conjugates of organic acids, sugars, and many other constituents (Rosenthal & Janzen, 1979). Some researchers call these conjugates terpenes, but this term should be reserved for the terpenoids that are true hydrocarbons (Robinson, 1980).

Terpenoid compounds have been isolated from algae, sponges, molluscs, coelenterates, and especially the holothurians (Bakus *et al.*, 1986). Triterpene glycosides are known to be vital for numerous plants with a broad range of biological activity. Nigrelli *et al.* (1955) and Yamanouchi (1955) were the first to discover that marine animals, sea cucumbers in particular were the source of triterpene glycosides. More than 60 sesquiterpenes have been identified from algae (Bakus *et al.*, 1986). Most typically, halogen containing terpenes are found in algae in the genus *Laurencia* where they are thought to function in part as herbivore feeding deterrents (Fenical, 1975; Erikson, 1983). The sea slugs *Aplysia* spp. are one of the few herbivores adapted to grazing on *Laurencia* spp. The slug sequesters this halogenated metabolite from the plant and is thought to use it as a means of defence against its own predators (Fenical, 1975). In general, terpenoid compounds are thought to function in antipredation, competition for space and possible antifouling (Rosenthal & Janzen, 1979 Bakus *et al.*, 1986).

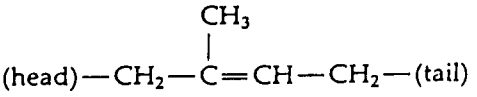

Fig. 2. Terpenoids, multiples of C_5 units linked together head to tail (Salisbury & Ross, 1985).

Phenolics. All phenolic compounds have an aromatic ring that contains various attached substituent groups, such as hydroxyl, carboxyl, and methoxyl groups (Fig. 3). They arise via a variety of biosynthetic pathways. Their most obvious characteristic is the presence and abundance of halogenated substituents. Bromine is the halogen most frequently incorporated. Halogenated and non-halogenated phenolics have been shown to have a multiplicity of potential ecological rôles. Phenolic compounds occur in algae, sponges, and annelids. The well known ones occur in brown and red algae, and sponges (Higa *et al.*, 1980). More than 200 phenolic compounds have been described from marine organisms (Steinberg,1984; Van Alstyne, 1988; Steinberg & Paul, 1990). Phenolic compounds are known to function as feeding deterrents in certain marine macrophytes (Phillips & Towers, 1982; Steinberg, 1984; 1985) and they also function as antifouling and antimicrobial agents in sessile organisms (Davis & Wright, 1990; Hay, 1991).

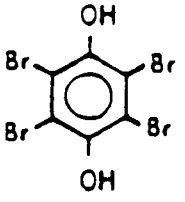

Fig. 3. Phenolic compound with an aromatic ring and characteristic substituents (Bakus *et al.*, 1986).

Miscellaneous compounds and those of mixed origins. Many compounds are derived from the condensation of several products each arising from a different biogenetic pathway. An example of a compound of mixed origin is asterosaponin A (Fig. 4), which is an oligosaccharide with a steroidal aglycone (Ikegami *et al.*, 1972). It is thought to be responsible for the obvious avoidance behaviour of a number of marine species when placed in close proximity with a starfish (Burnell & Apsimon, 1983). Miscellaneous compounds include acetylenes and lipids. A variety of rôles have been attributed to these compounds, including chemical defence and species recognition (Muller, 1979; Gerhart, 1984).

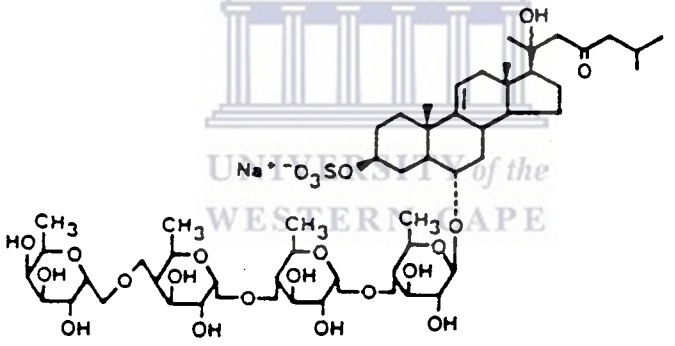

Fig. 4. Compound of mixed orgin: astersaponin A (Ikegami *et al.*, 1972)

5. Defence mechanisms in holothurians

Holothurians have developed a number of antipredator defence mechanisms, including thick integument, toxicity, evisceration, and unpalatability (de Vore & Brodie, 1982). The presence of a thick and/or tough integument may discourage most predation

on holothurians. Associated with a thick integument is the ability of some species to contract the body into a ball when harassed (Bakus, 1973). Certain sea cucumbers secrete mucus from their skin. These mucosal secretions may be toxic in some species and thereby play a rôle in the animal's defence (Russell, 1966). More than 100 toxic holothurian species have been reported (Nigrelli & Jakowska, 1960). A toxic substance, holothurin, in crystalline form was isolated from various sea cucumbers and was examined for its toxicity towards several animals (Hashimoto, 1979). These toxins are species specific and the concentration of these toxins may vary on a seasonal basis (de Vore & Brodie, 1982). The Japanese holothurian *Holothuria leucospilota* has a toxin in every part of the body; however, all the organs, especially the ovaries and Cuverian tubules, show higher concentrations in July and during the breeding season (Bakus, 1973). Another defence mechanism in some holothurians is evisceration which has been observed under stress conditions or predator attack (Nigrelli & Jakowska, 1960). For evisceration to be advantageous, holothurians must possess rapid regeneration powers.

Compounds isolated from the epidermis of holothurians may serve as a chemical defence system (Lucas *et al.*, 1979; Garneau *et al.*, 1983; Encarnacion *et al.*, 1989). These isolated compounds, called triterpene glycosides, belong to a group of compounds termed saponins. They acquired their name from their property for forming soapy emulsions in water (Torsell, 1983). Holothurians were the first source of saponins from an animal (Garneau *et al.*, 1983). Triterpene glycosides were found in the epidermis of holothurians and few biological assays have shown that these compounds could serve as

a chemical defence (Lucas *et al.*, 1979; Garneau *et al.*, 1983; Encarnacion *et al.*, 1989).

Feeding trials conducted with the subtidal sea cucumber *Psolus chitonoides* demonstrated that this holothurian contain chemical substances which make it unattractive as a prey item (Bingham & Braithwaite, 1986). Protection of *P. chitonoides* is based on distasteful chemicals which make them highly unpalatable to fish. In the feeding trials, gelatin cubes flavoured with *P. chitonoides* pieces were immediately rejected by test fish while control cubes were readily accepted. Yamaguchi (1975) found that test fish absolutely discriminated against echinoderm larvae. This discrimination is thought to be attributed to the saponins present in the larvae. De Vore & Brodie (1982) demonstrated that the deterrent compounds are mainly concentrated in the integument of holothurians. Distastefulness of the integument would increase the survival of holothurians more than distastefulness of other tissues because, the integument is the first barrier that the predator is likely to encounter.

For more than thirty years triterpene glycosides from holothurians have attracted the attention of chemists, biochemists, pharmacologists and taxonomists. The majority of the known triterpene glycosides in the class Holothuroidea have aglycones of a lanostane skeleton system (Avilov *et al.*, 1994). They are classified as the so-called holostane series (Hablemehl & Volkvein, 1971). Recently several new structural types of aglycones have been discovered (Avilov *et al.*, 1994).

The biological activity of holothurian glycosides and physical properties of these substances indicate an important external defensive function (Avilov *et al.*, 1994). A strong membranotropic action and high solubility of most triterpene glycosides from holothurians, associated with the presence of monosaccharide units, sulphate groups and a functional oxygen group in a aglycone moiety, suggest an important "external" function. Bakus (1970, 1981) developed the hypothesis of an ecological cause for the origin of deterrent compounds in marine invertebrates to their protective action against fish predators. Attack by a predator leads to some species of holothurians to expel Cuverian tubules that contain an extremely high concentration (up to 10-20% dry weight) of triterpene glycosides (Elyakov *et al.*, 1973) that may lead to the deterrence of predation or the death of the predator (Bakus, 1973).

An important outcome of this study is the direction it can provide for further research into the chemical ecology of kelp bed sea cucumbers. In the past, studies of marine natural products isolation have focused on the identification of novel classes of compounds, with little regard to the biological function of the metabolites. This study provides a base from which to isolate specific, ecologically relevant compounds in a biologically directed fashion.

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NAME OF JOURNAL

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Running Head: Chemical defence in a sea cucumber

TRITERPENE GLYCOSIDES CHEMICALLY DEFEND THE SOUTH AFRICAN KELP BED SEA CUCUMBER, *PSEUDOCNELLA INSOLENS*, AGAINST PREDATION.

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Abstract: The small, subtidal sea cucumber, *Pseudocnella insolens* appears to have no obvious protection from predation, yet it occurs in dense colonies covering rocky areas and appears to have few predators. Laboratory-based feeding trials were developed to assess the deterrent effects of various purified sea cucumber extracts. Extracts were examined by thin-layer chromatography to determine the presence or absence of secondary metabolites. Feeding trials were conducted with crude, lipid-soluble, and water-soluble extracts at natural concentrations by applying these extracts to palatable food. Assays demonstrated that the crude and water-soluble material deterred feeding by the test-fish, *Clinus superciliosus*. Additional trials were conducted with silica-gel purified, water-soluble fractions. Feeding trials demonstrated that *P. insolens* extract is composed of four potentially deterrent water-soluble fractions. The presence of foaming of the extract is consistent with the presence of triterpene glycosides. All four fractions were not equally deterrent. Fractions ii and iii were highly deterrent and appear to be the two main metabolites. Secondary metabolites may thus account at least for part of the apparent low predation on *P. insolens*.

Key words: antipredation, chemical defence, holothurian ecology, kelp bed, *Pseudocnella insolens*, triterpene glycosides, unpalatability.

INTRODUCTION

Predation is an important factor affecting the abundance of marine invertebrates (Connell, 1961; Menge & Lubchenco, 1981; Young & Bingham, 1987). To avoid predation many organisms have developed defensive features which protect them from potential predators (Bingham & Braithwaite, 1986). Holothurians have evolved a number of antipredator mechanisms, including unpalatability (Russell, 1966), toxicity (Bakus, 1968; Burnell & Apsimon, 1983), evisceration (Byrne, 1985) and a thick and/or tough integument (de Vore & Brodie, 1982). It is generally believed that most holothurians use one or more of these features to avoid predation (de Vore & Brodie, 1982; Bingham & Braithwaite, 1986), although very few studies have tested this assumption experimentally(Russell, 1966; Burnell & Apsimon, 1983; Avilov *et al.*, 1994).

Most sea cucumbers are elongate, relatively soft-bodied as the spines in their skin are reduced to spicules (Day, 1968). Although the skin is flexible it also has the ability to stiffen if the animal is harrassed. The skin is criss-crossed by ligament-like collagen fibres. These fibres may become chemically bonded to one another. This bonding allows the collagen fibres to contact causing the skin to stiffen (Branch & Branch, 1988).

Pseudocnella insolens (Théel) belongs to a group of suspension feeders that are common sublitorally on the South African south and west coasts (Barkai, 1991). *Pseudocnella insolens* inhabits rocky and sandy substrata from the intertidal zone down to 40 m, where it occurs in dense colonies covering rocky areas (Day, 1968; Barkai, 1991; Branch *et al.*, 1994). It occurs from Port Nolloth to Port Elizabeth (Branch *et al.*, 1994; Fig. 1).

Many echinoderms have developed secondary metabolites in various parts of their body, some of which have been shown to provide a defence against predators (Lucas *et al.*, 1979; de Vore & Brodie, 1982; Garneau *et al.*, 1983; Encarnacion *et al.*, 1989; Habermehl & Krebs, 1990). The persistence of sea cucumbers in areas with high levels of predation has often been attributed to their production of anti-predator secondary metabolites (Russell, 1966; Bakus, 1968), although this assumption has rarely been tested experimentally (Russell, 1966; Burnell & Apsimon, 1983; Avilov *et al.*, 1994). The study of secondary metabolites in holothurians began with the work of Cooper in the latter part of the eighteenth century (Halstead, 1978), although research on these metabolites was initiated by Yamanouchi in 1929. The characteristic chemical feature is that these metabolites are triterpene glycosides (Chanley *et al.*, 1960; Elyakov *et al.*, 1973; Garneau *et al.*, 1983; Encarnacion *et al.*, 1989; Stonik, 1986).

Saponins were first isolated in plants and are known to be vital to numerous plants with a broad range of biological activity (Elyakov *et al.*, 1973). Saponins consist of triterpene glycosides, steroid or steroidal glycoalkaloid molecules bearing one or more sugar chains (Hostettman *et al.*, 1991; Osbourn, 1996). Nigrelli (1955) and Yamanouchi (1955) were the first to discover that holothurians were a source of saponins (triterpene glycosides). Since Yamanouchi's pioneering studies on sea cucumber saponins, several species of various families of the phylum Echinodermata have been shown to possess these biologically active saponins but, the triterpene glycosides have only been identified for the sea cucumbers (Garneau *et al.*, 1983)

For more than thirty years triterpene glycosides from sea cucumbers have attracted the attention of chemists, biochemists, taxonomists, and pharmacologists. Research has mainly focussed on the isolation, structural elucidation, and the description these novel compounds. These compounds are specific for different taxonomic groups of sea cucumbers and make them a very convenient model for biochemical evolutionary studies (Stonik & Elyakov, 1988). In addition to the chemotaxonomic studies, pharmacologists have demonstrated a wide spectrum of biological effects for triterpene glycosides: antifungal, antitumour, and immunomodulatory activities (Avilov *et al.*, 1994). Despite the extensive research on triterpene glycosides, little is known about the rôle of these metabolites in chemical defence (Russell, 1966; Burnell & Apsimon, 1983; Avilov *et al.*, 1994). This could be due to the fact that triterpene glycosides are water-soluble and are difficult to work

with from an ecological perspective. Tests of their effects against consumers usually involve mixing water-soluble compounds in an agar matrix. The agar matrix contains plant extracts that could stimulate feeding (Hay & Fenical, 1988).

The purpose of this study is to test the hypothesis that *P. insolens* contains secondary metabolites that deter predation, and if so isolate them and carry out a preliminary investigation of whether they are triterpene glycosides and in what concentration they are effective.

MATERIALS AND METHODS

Collection of animals. *Pseudocnella insolens* (Théel) was collected by hand using SCUBA (16 to 18m) at Partridge Point along the False Bay coast (34° 15' 4" S, 18° 25' 46" E) in July 1995. The identification was confirmed by Prof. A. Thandar of the Zoology Department, University of the Durban-Westville where a voucher specimen is kept.

General extraction, isolation and purification procedures. Preliminary work showed that the use of previously published isolation procedures (Apsimon *et al.*, 1973) are less appropriate *P. insolens.* The glycosides are specific for different taxa and require a general isolation procedure. The lack of versatilility of these methods led V. Kalinin (*personal communication*) from the Pacific Institute of Bio-organic Chemistry, Far East Division of the Russian Academy of Sciences to suggest more general procedures (Fig. 2 and 3). Details on the isolation procedure are given in Appendix II.

Extraction. Towel dried fresh sea cucumbers (3 kg, 844.4 g dry weight) were cut in approximately one centimetre portions and kept in 3 l of absolute ethanol for one day at room temperature. The extract was decanted and stored in a coldroom at 4°C. The hard material was extracted with an additional 3 l of absolute ethanol under reflux. This was done in a waterbath at 100°C for 1 h. The procedure was repeated 3 times. The combined crude extract was evaporated using a rotary evaporator with a heating bath at 45°C. Small amounts of butanol were added to the extract to prevent foaming. Foaming

is an indication for the presence of triterpene glycosides (Basu & Rastogi, 1967; Rosenthal & Janzen, 1979; Torsell, 1983).

The dried crude residue was separated into lipid and water soluble material under hydrophobic chromatography. The extract was made into a paste with 100 ml of distilled water. The paste was applied to a 1 l column and chromatographed with 500 g of the nonionic polymeric resin absorbent, Amberlite XAD-2. Inorganic salts and polar impurities were eluted from the column with 2 l of distilled water.

Chemical analysis. To test whether secondary metabolites existed in the extracts, all extracts were analysed by thin-layer chromatography (TLC). The TLC plates were viewed under an ultraviolet (UV) lamp to observe any UV activity. The secondary metabolites were present as purple spots.

Isolation. A mixture of glycoside fractions were eluted from the column with 50 % ethanol. The glycoside fractions were evaporated to dryness and purified by column chromatography on silica gel in a chloroform-ethanol-water (100:100:17) system. The saponin mixture was dissolved in 30 ml of the above solvent mixture and applied to the column. The column was eluted with the same solvent mixture under low pressure. The first 400 ml of elute was discarded. Thin layer chromatography showed that the next 14 x 40 ml fractions showed UV activity at 254 nm. The following fractions were combined and concentrated under vacuum as they showed similar UV activity: 1-4

(0.2501 g), 5-6 (0.125 g), 7-8 (0.0173 g) and 9-14 (0.1185 g). Fractions 1-4 yielded oils which were rejected after TLC analysis as the compounds responsible for feeding deterrence were water-soluble. Details on the isolation procedures are given in Appendix III.

Purification. The glycosides were isolated with thin-layer (0.5 mm) chromatography plates (20 cm x 20 cm) with a fixed silica gel layer with luminescer (254 nm). The plates were developed in a chloroform-methanol-water (100:100:17) system. The crude glycoside fractions 5-6, 7-8, 9-14 were dissolved with 1 ml of 100 % methanol and then 1 ml of 90 % methanol. The glycoside mixtures were applied to three separate plates.

The glycoside mixtures (2 ml) were applied as a 1 cm wide streak on the plate. The solvent in which the glycosides were dissolved was evaporated with the aid of a hair dryer at a cool setting. The dried plates were developed in separate chromatography tanks with 50 ml of the solvent system. It took approximately 2 hrs for the plate to develop. The developed plates were removed and pencil lines were drawn at the solvent fronts. Table 2 shows the R_f values for the individual fractions.

Extraction of purified fractions. On the basis of TLC comparisons, fractions having similar R_f values were considered to be similar and were combined. The silica gel of fractions i, ii, iii and iv were scraped off the plates as a series of bands. The silica gel

of similar fractions were added together in a 50 ml centrifuge tube. The centrifuge tubes containing the individual fractions were filled to the 50 ml mark with 90 % methanol. The centrifuge tubes were put on a Griffin flask shaker at high speed. The first extraction was done overnight. The extracts were centrifuged for 15 min at 3000 rpm The supernatant was poured off and stored in a coldroom. This extraction procedure was repeated 3 times. After the extraction was completed the supernatants were collected, added to the previous extractions and were dried under vacuum. The purified fractions i, ii, iii and iv were assayed against the test fish.

Test animals. The fish chosen for laboratory assays was *Clinus superciliosus,* one of the most abundant predatory fish found present during the collection of the sea cucumbers and one which is easily obtained and kept under laboratory conditions. Attempts to keep other predators, *Pachymetopon blochii* (hottentot), *Chrysoblephus laticeps* (red roman), and *Spondyliosoma emarginatum* (steentjie), in the lab were unsuccessful. Fish were captured with hand nets by baiting tidal pools. After capture the fish were placed in buckets containing aerated sea water and transported to the aquarium. The fish were placed in an aquarium supplied with flowing aerated sea water at ambient temperature. Fish of equal size (60-90 mm long) were used as for as possible.

Aquarium conditions. The aquaria were (50 cm x 32 cm x 23 cm) overall and were divided into three sections with black perspex so that the visual influence of other fish would not affect the assays. Sea water at ambient temperature flowed sequentially

through these dividers from one compartment to the next. Aeration was continuous and salinities were checked weekly.

Maintenance feeding. The fish quickly acclimatised and began accepting food pellets after a few days. The fish were fed exclusively with Tetrapond floating food sticks, which are composed of plant and animal ingredients. Each day, pellets were dropped on the water, and were consumed within two minutes.

Biological assays. The experiment ran for 3 consecutive days with 30 test fish. One offering of the control and the treatment pellet was made to each fish every 24 h. Only one feeding trial could be tested per day, because preliminary analysis showed that if more trials were run, the fish even ignored the control, based presumably on their experience of the test compound. Russell (1966) reported similar findings in an investigation of the palatability of some marine invertebrates.

Known concentrations of each test compound (20 µl) were applied to 0.0073 g food pellets and were evaporated at room temperature. Control pellets were treated with only the solvent. The control and treatment pellets were presented to the fish by dropping it at the top of the individual compartments with a pair of forceps. Crude, lipid-soluble, water-soluble and purified water-soluble extracts were assayed against test fish. Natural concentrations of extracts were applied to the food pellets as far as possible.

Observations. The reaction of the fish to each pellet was observed for a period of 2 min. Normal consumption of a food offering was followed by normal respiratory movements, while rejection of a food offering included flaring of the gill covers and was sometimes followed by erratic swimming motion.

A modified method of Lucas *et al.* (1979) was used to record the responses of the fish during the biological assays. Four different results were recorded in the series of 60 food particles fed to the fish.

1) Retained: the fish took and immediately retained the food particle.

2) Recaptured: the fish spat out the food particle and recaptured it, often several times before retaining it.

3) Rejected: the fish rejected the food particle, sometimes after spitting and recapturing it several times.

4) Untouched: the food particle was ignored, or approached but not mouthed.

Food particles that were retained or recaptured were regarded as being accepted by the fish. Finally, the results were scored as rejected or accepted and the results were tested for statistical significance using χ^2 analysis for 2 x 2 contingency tables (Zar, 1974).

RESULTS

The compositions of the extracts and the presence (+) or absence (-) of secondary metabolites from *P. insolens* are listed (Table 1). The presence of secondary metabolites in the extracts is associated with low susceptibility to feeding. The concentration of the extract of *P. insolens* will be referred to as the natural concentration.

Palatability tests with crude, lipid and water soluble extracts. All the control pellets were eaten by the test fish in every assay (Figs 4, 8, 9). Food pellets that were treated with crude extract were highly unpalatable, with none of the treatment pellets consumed (Fig. 4). Therefore, crude extracts effectively deterred fish at natural concentrations (Fig. 4; χ^2=60, P<0.05).

The crude extract was separated into lipid and water soluble extracts and analysed for feeding deterrence. The lipid soluble extract showed no deterrent activity, as all the treatment pellets were consumed (Fig. 4). The water soluble extract was highly unpalatable, with none of the treatment pellets consumed (Fig. 4). Therefore, the water soluble extract effectively deterred feeding at natural concentrations (Fig. 4; χ^2=60, P<0.05). Subsequently, each fraction of the water soluble extract was analysed for feeding deterrence.

Purification analysis. Plate 1: The glycoside mixture 5-6 separated into two distinct fractions (Fig. 5), of which fraction i proved to be more mobile than fraction ii.

Plate 2: The glycoside mixture 7-8 separated into 4 distinct fractions (Fig. 6), of which fractions i and ii were present including an additional two fractions (iii and iv). Fractions i and ii on plate 2 were similar to fractions i and ii on plate 1 because they had similar R_f values (Table 2). Fractions iii and iv were considered to be different fractions because they formed two separate bands (independent of fractions i and ii), were less mobile (Fig. 6) and showed different R_f values (Table 2).

Plate 3: The glycoside mixture 9-14 separated into 3 distinct fractions (Fig. 7) These three fractions had similar R_f values to fractions i, ii, and iii and were therefore considered to be identical to that of the fractions found on plates 1 and 2 (Fig. 5 and 6).

Palatability tests with purified water soluble fractions. Thin-layer chromatography indicated that the water soluble extracts of *P. insolens* contained four secondary metabolites of varying polarity (Fig. 5 to 7). These metabolites were isolated and tested for feeding deterrence. At natural concentrations these fractions did not deter feeding, as all the treatment pellets were consumed (Fig. 8). The feeding trials of each of the fractions were repeated with pellets coated with a 2-fold greater concentration of extract. Each of the fractions showed feeding deterrence at these concentrations. This suggests that the test fish were sensitive to changes in dry weight concentrations.

Although, all four fractions showed feeding deterrent activity at these concentrations, fractions ii and iii were significantly more distasteful (greater χ^2 value) than fractions i and iv (Fig. 9). Therefore, fractions ii and iii proved to be the major metabolites isolated from *P. insolens* that deter predation. Samples of the deterrent compounds have been sent to the Pacific Institute of Bio-organic Chemistry, Far East Division of the Russian Academy of Sciences for description and structural elucidation.

DISCUSSION

The results of this study clearly indicate that *Pseudocnella insolens* contains secondary metabolites which deter predators. This sea cucumber has a small, soft body and is "bite size" for many predatory fish, and it should also be easy prey for crabs and lobsters. Despite this, *P. insolens* occurs at high density in kelp beds and on shallow reefs around the Cape Peninsula. Its high abundance and the apparent lack of significant predation is consistent with the rôle of these secondary metabolites as feeding deterrents in nature. The results presented here are consistent with the hypothesis that triterpene glycosides are the active compounds in *P. insolens*.

The water- soluble extract of *P. insolens* is composed of four potentially deterrent secondary compounds. The presence of two minor and two main UV active bands were detected by TLC which is indicative of the presence of secondary compounds (Table 1). The work of several authors have shown sea cucumbers to contain triterpene glycosides that are detected by TLC (Elyakov *et al.*, 1973). The triterpene glycosides isolated for various sea cucumbers were stichoposides A and C (Elyakov et al., 1969) and cucumarioside C (Elyakov *et al.*, 1973). Maltsev *et al.*, (1984) noted in their extensive studies on the glycosides of the Pacific, Indo-Pacific and Atlantic sea cucumbers and in analyses of literature data, the glycoside fractions from the animals collected in the different areas consisted of the same compounds. The lipid-soluble extract showed no

feeding deterrence, while the water-soluble extract did effectively deter feeding. The purified water-soluble components were only deterrent at concentrations double that of the calculated natural concentration. Coating the pellets with a 2 fold greater concentration of extract is reasonable as the concentration was determined by whole body extracts, whereas triterpene glycosides tend to be concentrated in the integument (Ikegami *et al.*, 1972; Mackie *et al.*, 1977; Lucas *et al.*, 1979). In addition it seems probable that some of the compound is not recovered from the silica-gel. Regretfully, there are no standardised percentage yields for secondary metabolites so there is no baseline for comparison.

One of the most significant ecological problems involved in these assays is determining the natural concentration of the compound. The actual concentration of secondary metabolites has not been well documented since most of the literature has been generated by chemists interested primarily in describing new compounds, rather than carefully documenting their natural concentrations (Hay & Fenical, 1988). These scientists rarely list the yield of the compound. When yields are given, they are conservative since (a) extraction is rarely complete, (b) most isolation and purification techniques entail a loss of significant quantities of the metabolites, and (c) handling of specimens before and during extraction may result in compound degradation if the compounds are relatively unstable (Paul & Fenical, 1987; Hay & Fenical, 1988).

All four purified fractions showed significant feeding deterrence towards the test fish in the feeding trials. Fractions i and iv were less effective as a feeding deterrent compound, although other defensive rôles for these compounds may be possible. The deterrent effects of *P. insolens* water-soluble extract appear to be the result from the moderately polar compound (fraction ii and iii). The water soluble fractions at the extremes of the TLC plate showed less feeding deterrence. The two major metabolites could account for the deterrence observed in the feeding trials. However, the deterrent effects of the minor metabolites in these extracts were not examined. It is also known that some compounds are more deterrent in combination than individually, and higher concentrations of the individual mixture may be needed to show individual deterrence (Geiselman & McConnell, 1981; Paul *et al.*, 1987; Van Alstyne *et al.*, 1994). Although, the secondary metabolites deterred feeding by the test fish, some compounds may be effective toward one species but may be ineffective toward other species (Paul and Fenical, 1987). Further studies would be necessary to determine if *P. insolens* is indeed avoided by a broad range of predators on the basis of these secondary compounds.

This is one of a few studies which investigates the effects of triterpene glycosides on fish in which the triterpene glycosides have been included in fish food. In previous studies the fish have been immersed for periods in saponin solutions, e.g. Hashimoto (1979) and Mackie *et al.*, (1975, 1977). Immersion in saponin solution does not occur in the field. This approach has been useful in screening for compounds with toxic properties, but provides inadequate information about deterrent compounds. Therefore,

to assess the deterrent potential of a compound it is important to conduct an ingestive assay. The reason for this paucity of literature regarding the ecological effects of triterpene glycosides (purified saponins) could be attributed to the fact that these compounds are water-soluble and are difficult to work with from an ecological perspective. Since these compounds are water-soluble, tests of their effects against consumers usually involve mixing water-soluble compounds in an agar matrix which contain plant extracts that may stimulate feeding (Hay & Fenical, 1988).

Bakus (1968) suggested that if an invertebrate species lacks behavioural (e.g. cryptic) or constructural (e.g. shell, tube) defence mechanisms, yet lives exposed to some potential predator, then it is likely that some alternative form of defence mechanism will have evolved. In many soft-bodied holothurians, biological assays suggest that triterpene glycosides make the tissues unpalatable (Mackie *et al.*, 1977; Lucas *et al.*, 1979; de Vore & Brodie, 1982). Chapman & Blaney (1979) discuss perception of secondary metabolites by predators and suggest that the response of the sense cells to secondary metabolites is inborn. A behavioural response, resulting in avoidance or acceptance to the compound, is also often inborn, although animals can learn to avoid ingesting secondary metabolites after an 'unpleasant' experience (Wylie & Paul, 1988).

In many holothurians the presence of a distasteful integuments discourages most predation. Studies conducted by de Vore & Brodie (1982) on the sea cucumber *Thyone briareus* showed that the integument was avoided by fish predators. Lane (1968)

suggested that most sea cucumbers contain compounds in their integument that may render the animal distasteful. Regretfully, the compounds were not identified. Some organisms ensure the survival of their progeny by concentrating distasteful compounds within their ova. Gonads of *T. briareus* were generally avoided by fish in experiments conducted by de Vore & Brodie (1982). Portions of *T. briareus* consisting of the stomach, longitudinal muscle bands, intestines, and retractor muscles were generally eaten by fish indicating that the tissues tested, only integument and gravid ovaries were unpalatable to the test fish. When the feeding trials were conducted with purified extracts from *P. insolens* at concentrations of whole body extracts, all the treatment pellets were consumed (Fig. 8). However, feeding deterrence occurred at 2x concentration. Triterpene glycosides tend to be concentrated in the integument (Ikegami *et al.*, 1972; Mackie *et al.*, 1977; Lucas *et al.*, 1979). Distastefulness of the integument would increase the survival of sea cucumbers more than distastefulness of other tissues.

This study indicates the potentially important rôle that secondary compounds may play in the defence of holothurians. The demonstration that the main secondary terpenoid compounds of *P. insolens* have strong ichthyodeterrent properties towards the test fish is only the first step in unravelling the complex chemical and defence adaptations of these holothurians. Several questions arise from this study. What are the synergistic effects of the triterpene glycosides? In what tissues are the triterpene glycosides concentrated? Do these compounds discourage other predators and what are the effective concentrations against other predators?

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Table 1. Composition of extracts from P. insolens , and an indication of whether UV active spots were visible on the TLC plates.

extract	yield in grams	% dry weight	TLC
crude	100	11.84	+
lipid soluble	0.3735	0.04	-
water soluble	7.4693	0.88	+
fraction i	0.0209	0.002	+
fraction ii	0.0031	0.0004	+
fraction iii	0.0368	0.004	+
fraction iv	0.0229	0.003	+

Table 2. Properties of TLC fractions

glycoside mixture no.	plate no.	fraction	R_f value
5-6	1	i	0.8
		ii	0.68
7-8	2	i	0.79
		ii	0.66
		iii	0.51
		iv	0.37
9-14	3	i	0.8
		ii	0.69
		iii	0.5

FIGURE CAPTIONS

Figure 1. Geographic range of *P. insolens* (after Branch *et al.*, 1994).

Figure 2. Outline of the extraction and isolation procedure used for the glycosides of *P. insolens.*

Figure 3. Outline of the purification procedure used for the glycosides of *P. insolens.*

Figure 4. Biological assay to show the percentage of pellets rejected and accepted by test fish for crude, lipid-soluble, and water-soluble extracts at natural concentrations.

Figure 5. TLC diagram of plate 1 (glycoside mixture 5-6).

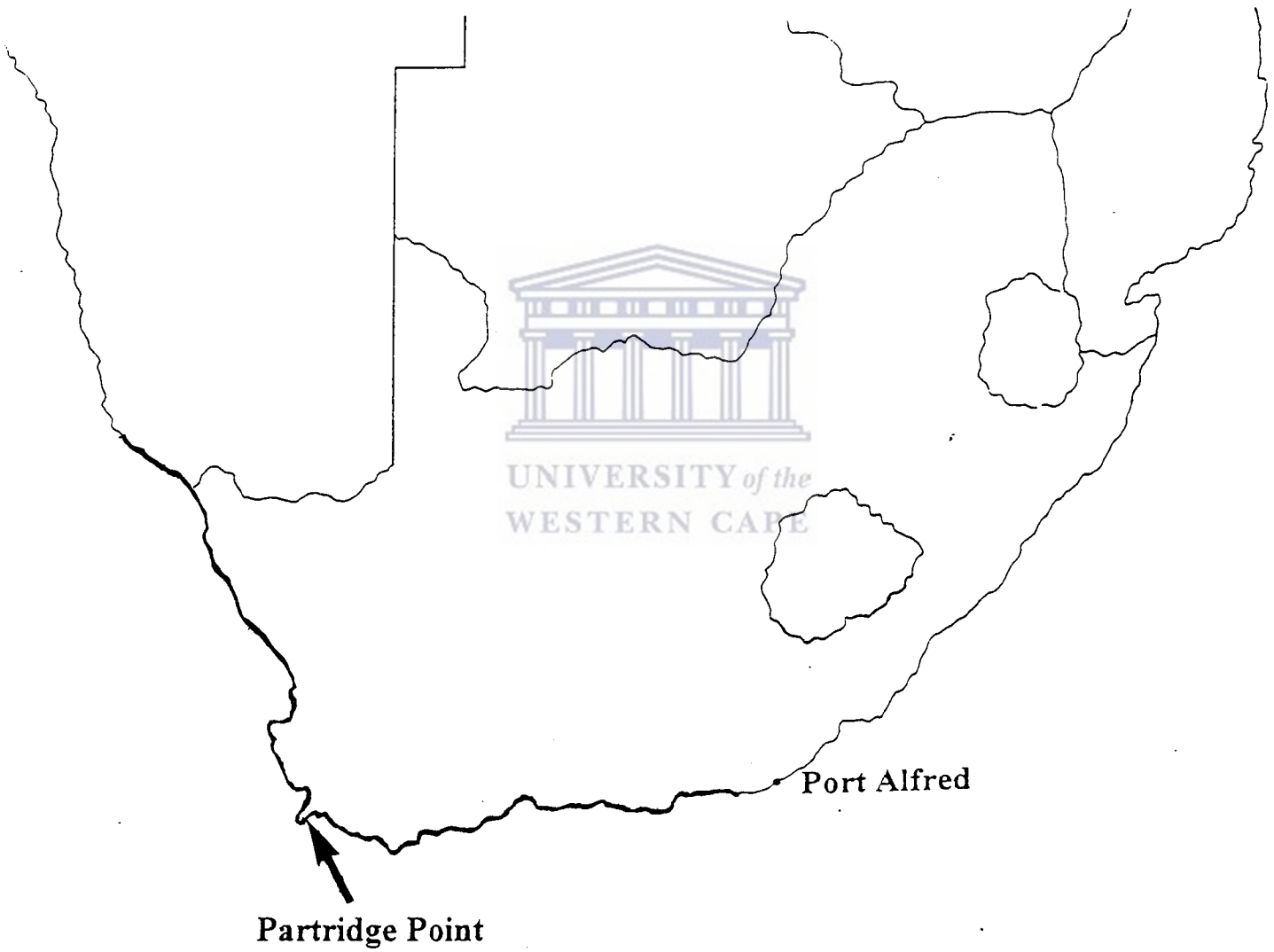
Figure 6. TLC diagram of plate 2 (glycoside mixture 7-8).

Figure 7. TLC diagram of plate 3 (glycoside mixture 9-14).

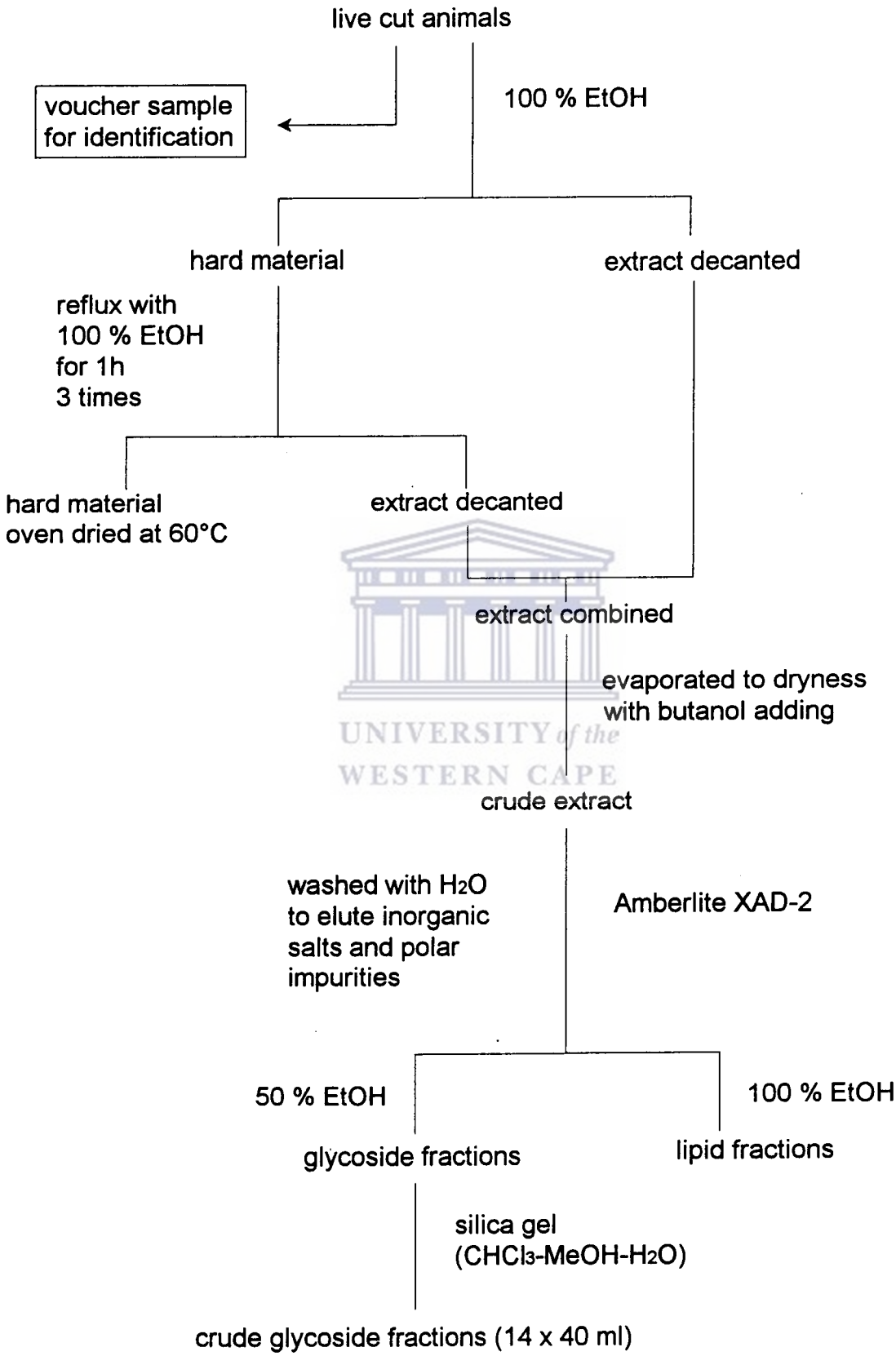
Figure 8. Results of a biological assay to show the percentage of pellets rejected and accepted by test fish for purified fractions i to iv at natural concentrations.

Figure 9. Results of a biological Biological assay to show the percentage of pellets rejected and accepted by test fish for purified fractions i to iv at a 2-fold greater concentration of the dry weight.

P. insolens



Brown, R.E. Fig. 1, 66



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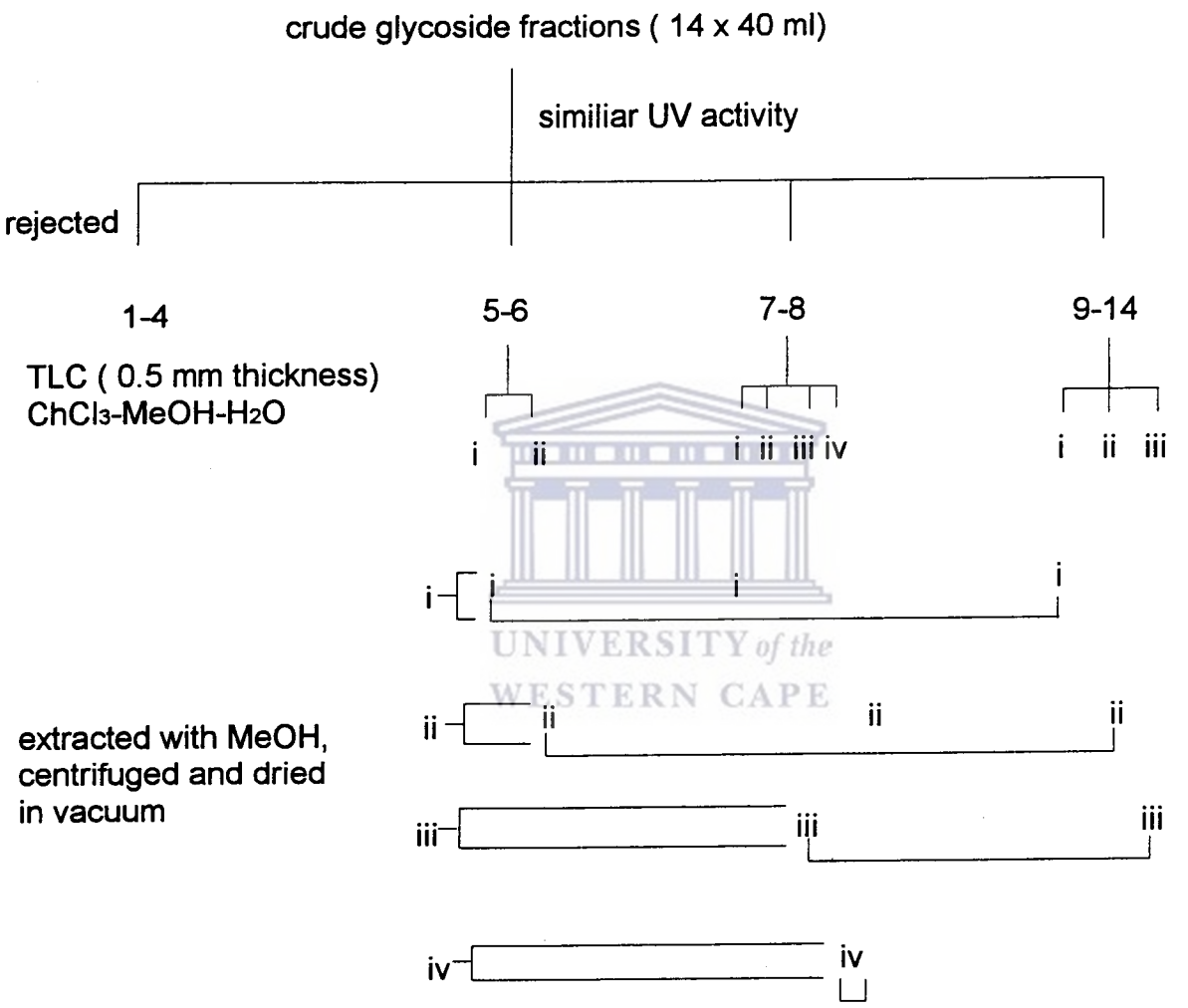

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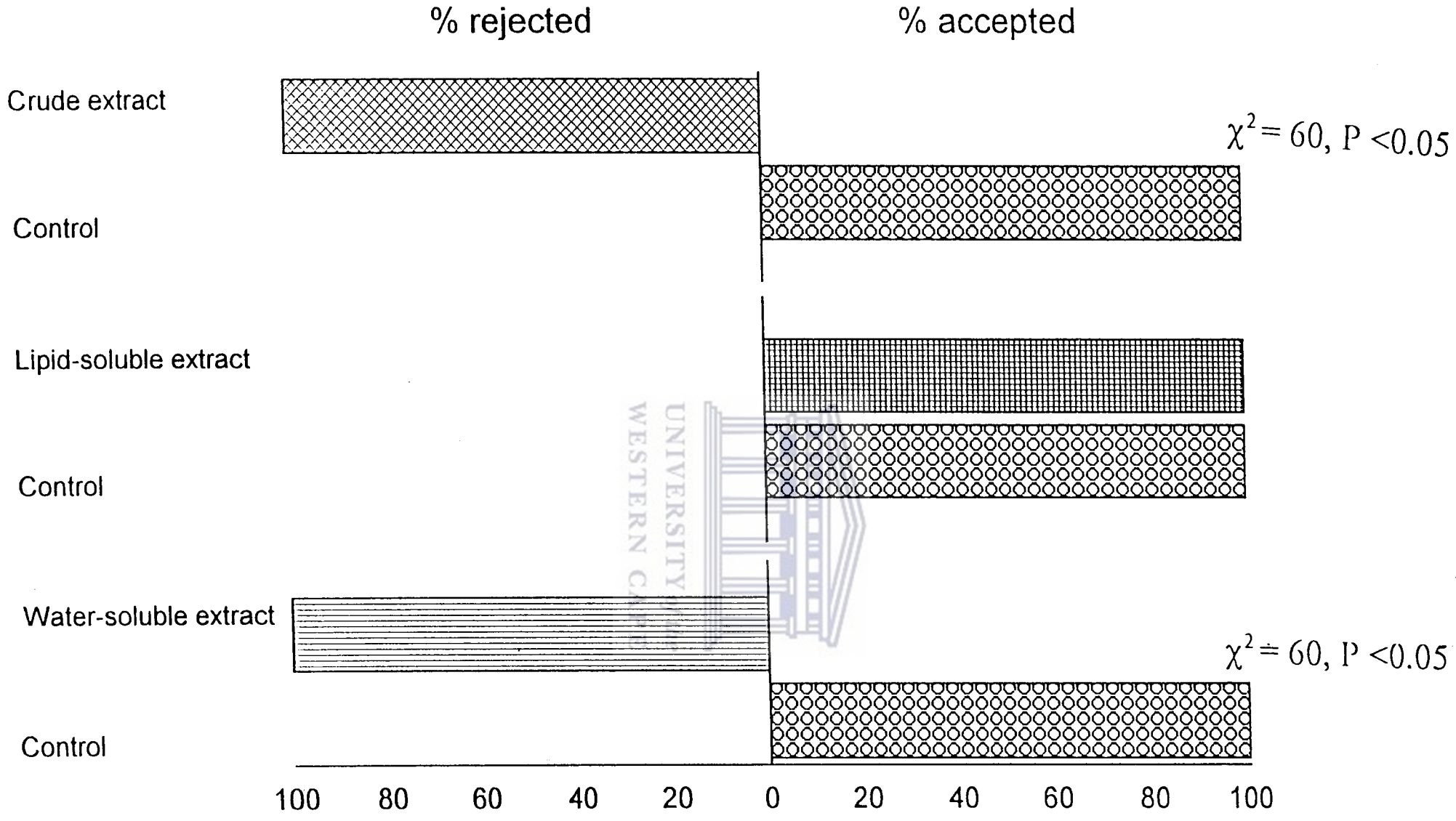

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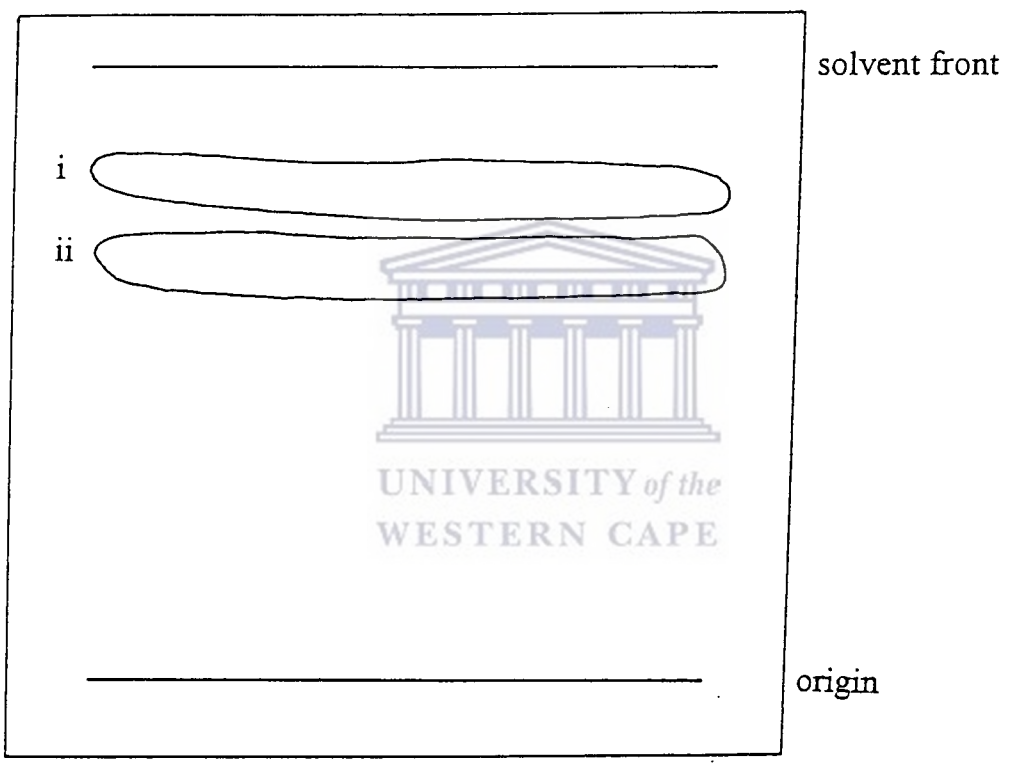

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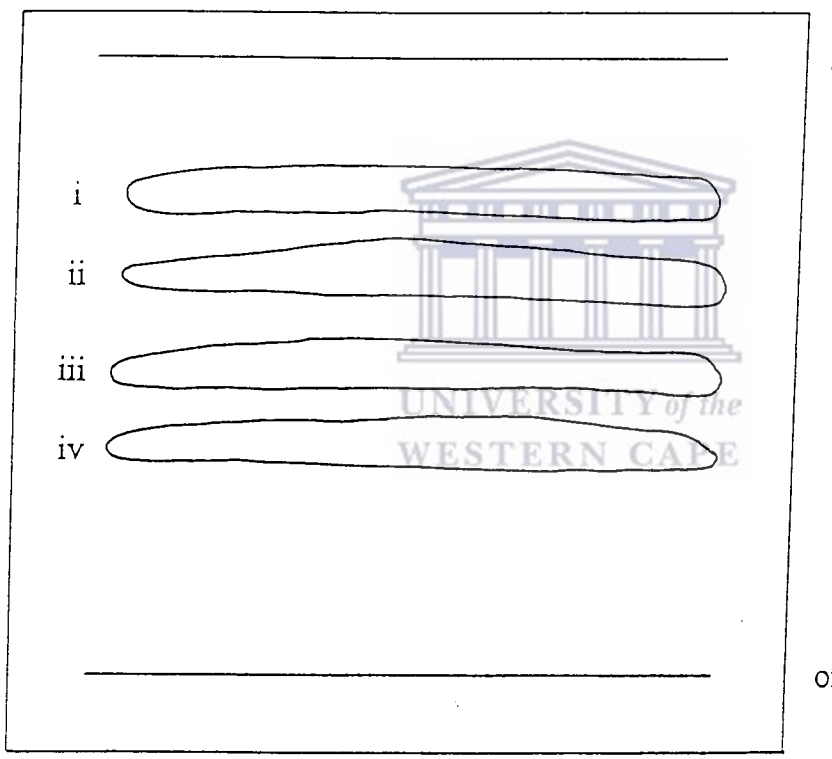

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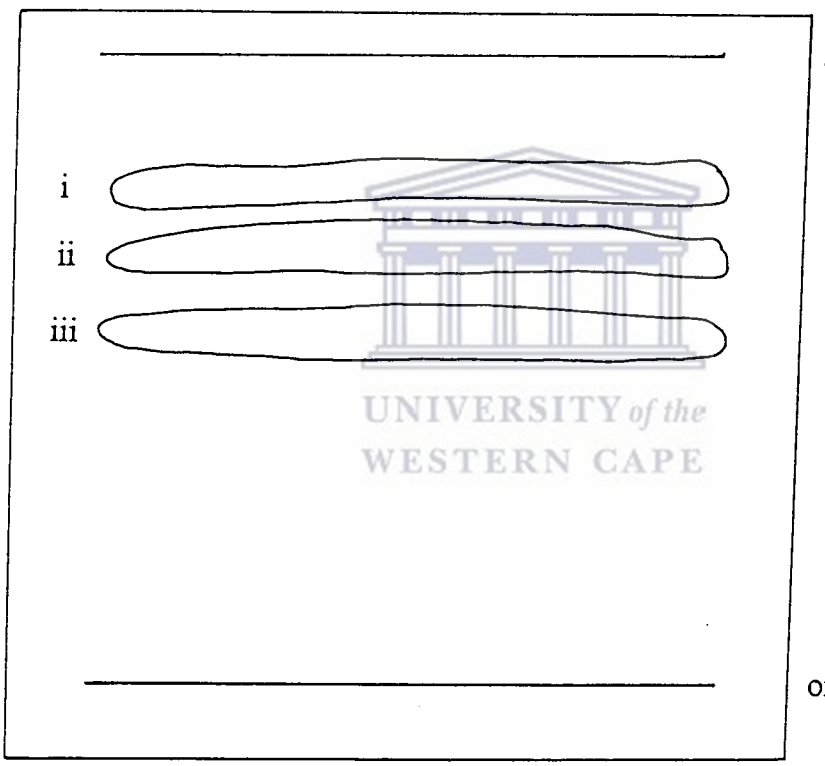

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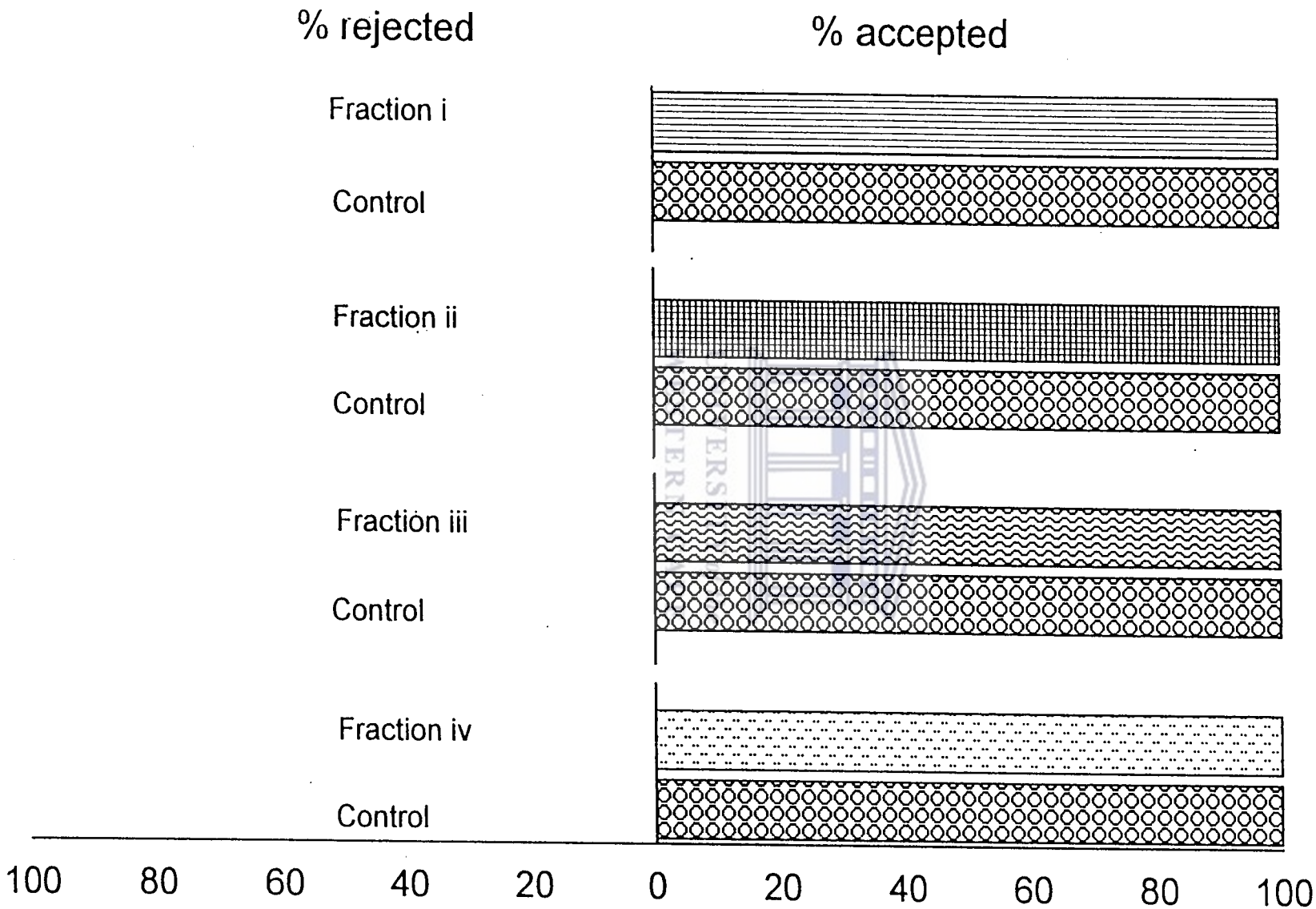

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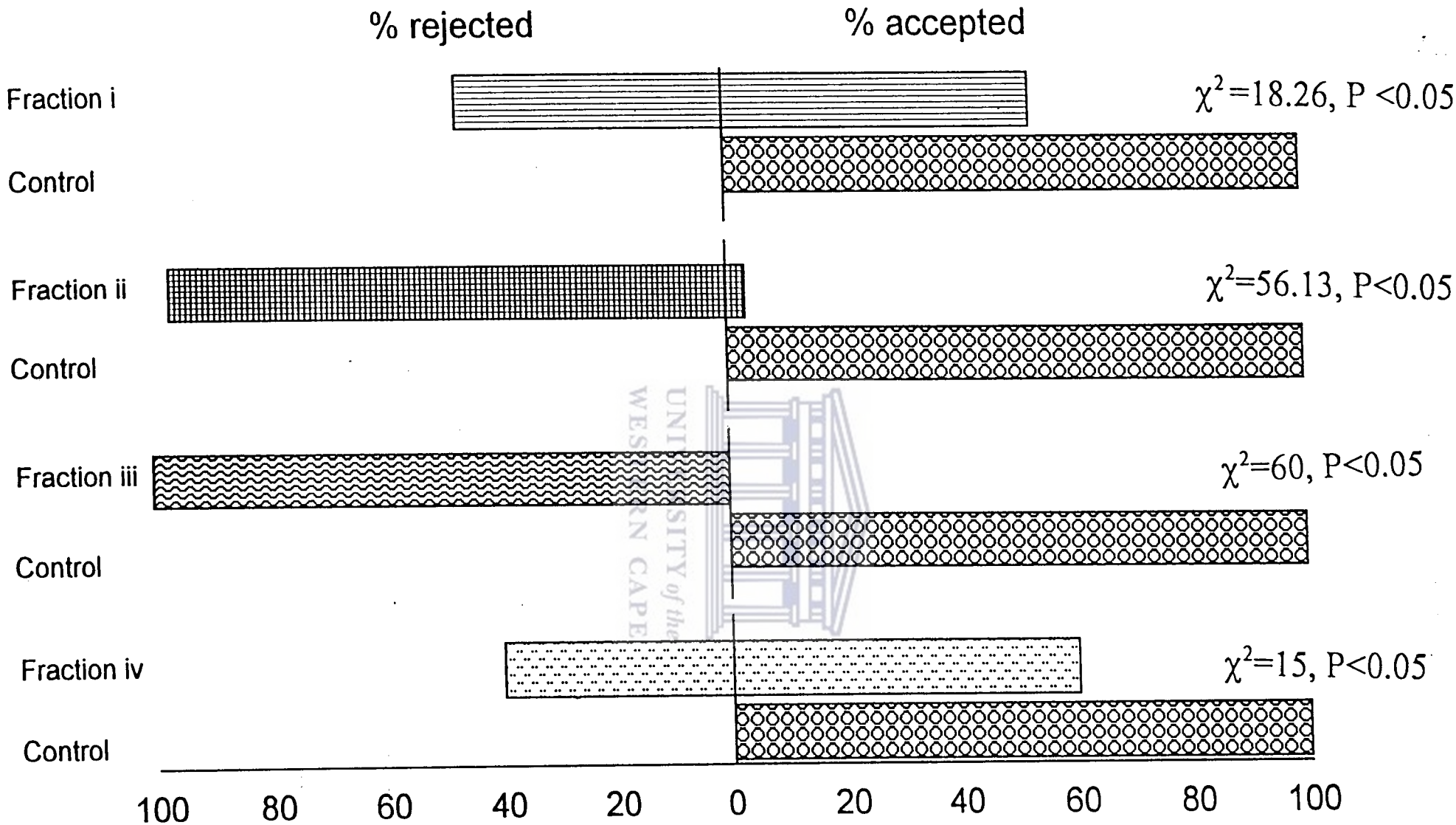

Brown, R.E. Fig. 9, 74

APPENDIX I

JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

Harold Barnes, Founder Editor

Aims and Scope

The journal provides a forum for work in the biochemistry, physiology, behaviour, and genetics of marine plants and animals in relation to their ecology; all levels of biological organization will be considered, including studies of ecosystems and ecological modelling. The main emphasis of the journal lies in experimental work, both from the laboratory and the field. Descriptive studies will, however, be acceptable if they elucidate general ecological principles. Papers describing important new techniques, methods, and apparatus will also be considered. All papers will be refereed by experts before acceptance for publication. In all cases proofs will be sent to authors. The editors, referees, and publisher will make every effort to expedite publication and the cooperation of authors in this task is welcomed.

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APPENDIX II

Isolation data (R. E. Brown, *unplublished*) of the previously published Classical and Rapid isolation method (Apsimon *et al.*, 1973):

Classical Method

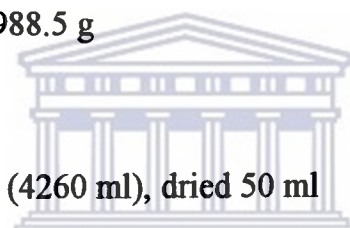
The frozen block of *P. insolens* (4222.06 g, 988.94 g dry weight) was divided into small pieces and thawed for 4-5 h under methanol:water (3:1) solution (6 l). The thawed mixture was homogenized using a Waring Commercial blender and allowed to stand for 3 h. The animal pulp was separated by suction filtration and extracted twice more with methanol: water (3 l). The filtrates were stored in a cold room overnight at 4 °C and filtered once more. The filtrate was concentrate (to 3400 ml) by rotary evaporation, using bath temperatures of 45°C or less to minimize thermal decomposition. The resulting mixture of oils and aqueous solution was diluted to 5 l with water and extracted with ether (3 l) and then benzene (3 l). In both extractions emulsions were obtained: the ether emulsions separated in 2-4 h but those of benzene required 12-48 h for acceptable separation. The defatted aqueous solution was made up to 5 % sodium chloride and then the pH adjusted to 4 with dilute hydrochloric acid. The resulting mixture was extracted with water-saturated 1-butanol (3 times 1 l) and the water layer discarded. The butanol extract was suction-filtered through Whatman 1 filter paper and then gravity filtered through a cotton plug to remove the sticky suspension. The butanol filtrate was concentrated (to 100-200 ml) by rotary evaporation and the glycoside precipitated by the

addition of benzene (1.5 l). The supernatant solution was decanted and the precipitate washed with acetone and collected by suction filtration. This precipitate contained large amounts of NaCl and was freed of inorganic salts and small organic molecules by dialysis is a cellophane bag against running tap water for 24 h. The glycoside mixture (0.1742 g) was freeze dried.

percentage glycoside yield:

wet weight of *P. insolens* = 4222.06 g

dried weight of *P. insolens* = 988.5 g



evaporation of butanol extract (4260 ml), dried 50 ml

evaporation of supernatant solution of benzene and acetone (5760 ml), dried 50 ml

evaporation of benzene extract (3240 ml), dried 50 ml

evaporation of ether extract (1680 ml), dried 50 ml

dried contents of butanol extract (50 ml) = 3.05 g

dried contents of supernatant solution of benzene and acetone (50 ml) = 0.09 g

dried contents of benzene extract (50 ml)= 0.36 g

dried contents of ether extract (50 ml)= 0.72 g

freeze dried glycoside mixture = 0.1742 g

total dried contents of butanol extract

in 1 ml = 3.05g / 50 ml

= 0.061 g/ml x 4260 ml

= 259.86 g

total dried contents of supernatant solution of benzene and acetone

in 1 ml = 0.09 g / 50 ml

= 0.0018 g/ml x 5760 ml

= 10.368 g

total dried contents of benzene extract

in 1 ml = 0.36 g / 50 ml

= 0.0072 g/ml x 3240 ml

= 23.328 g

total dried contents of ether extract

in 1 ml = 0.72 g / 50 ml

= 0.0144 g/ml x 1680 ml

= 24.192 g

freeze dried glycoside mixture = 0.1742 g

dried weight of *P. insolens* = 988.5 g

total weight of dried contents = 1306.4222 g

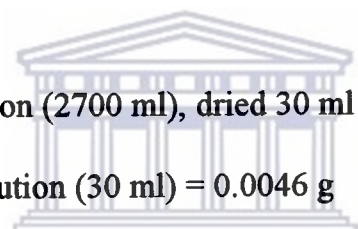
percentage glycoside yield = 0.1742 g / 1306.4222 g x 100

= 0.013 % dry weight

Rapid Isolation Method

wet weight of *P. insolens* = 507.3 g

dried weight of *P. insolens* = 91.74 g



evaporation of resulting solution (2700 ml), dried 30 ml

dried contents of resulting solution (30 ml) = 0.0046 g

total dried contents of resulting solution

in 1 ml =0.0046 g / 30 ml

= 0.00015 g/ml x 2700 ml

=0.414 g

evaporation of supernatant solution = 1900 ml

dried contents of supernatant solution = 0.0205 g

total dried contents of supernant solution

in 1 ml = 0.0205 g / 30 ml

= 0.00068 g/ml x 1900 ml

= 1.2982 g

dried contents of glycoside mixture = 0.1785 g

dried weight of *P. insolens* = 91.74 g

percentage glycoside yield = 0.1785 g/ 93.6307 g x 100

= 0.19 % dry weight

APPENDIX III

Isolation data of the general procedure as proposed by V. Kalinin of the Pacific Institute of Bio-organic Chemistry, Far East Division of the Russian Academy of Sciences.

percentage glycoside yield:

wet weight of *P. insolens* = 394.7 g

dried weight of *P. insolens* = 71.38 g

evaporation of ethanol/butanol extract (3080 ml), dried 30 ml

dried contents of ethanol/butanol extract (30 ml) = 0.0053 g

total dried contents of ethanol/butanol extract

in 1 ml = 0.0053 g/30 ml

= 0.000176 g/ml x 3080 ml

= 0.544 g

dried contents of glycoside mixture = 24.4598 g

dried weight of *P. insolens* = 71.38 g

glycoside yield = 24.4598 g/ 96.3838 g

$V_1 = V_2 \times M_1/M_2$

$= 1 \text{ ml} \times 24460/125$

$= 195.68 \text{ ml}$

carried out feeding trial with calculated concentration

Took left over crude and applied to Amberlite column.

weight of water soluble extract = 20.5764 g

weight of lipid soluble extract = 1.5683 g

total mass of crude extract after feeding trial = 22.1447 g

% water soluble yield = 20.5764 g/22.1447g x 25.38 %

= 23.5826 %

% lipid soluble yield = 1.5683 g/22.1447g x 25.38 %

= 1.7974 %

weight of each pellet = 0.0073 g

therefore, weight of compound to add to each pellet = 0.2538 x 0.0073/ 1-0.2538

= 0.0025 g per pellet

= 2.5 mg per pellet

water solubles:

21.1447 g crude extract contains 20.5764 g water solubles

therefore, 22144.7 mg crude extract contains 20576.4 mg water solubles

therefore, in 2.5 mg

= 2.5 mg/22144.7 mg x 20576.4 mg

= 2.3229 mg

pellet should contain 2.3229 mg water

concentration: 2.3229 mg water solubles/20 μl

= 20576.4/2.3229 x 20

= 177.208 ml water

to get 2.3229 mg water soluble extract/ 20 μl, dissolve 20576.4 mg water soluble extract/ 177.208 ml water

lipid solubles:

22.1447 crude extract contains 1.5683 g lipid solubles

therefore, 221447 mg crude extract contains 1568.3 mg lipid solubles

therefore, in 2.5 mg

= 2.5 mg/221447 mg x 1568.3 mg

= 0.1771 mg

pellet should contain 0.1771 mg solvent (diethyl ether)

concentration: 0.1771 mg lipid solubles/20 μl

= 1568.3 mg/0.1771 mg x 20

= 177.1089 ml diethyl ether

to get 0.1771 mg lipid soluble extract/ 20 μl, dissolve 1.5683 mg lipid soluble extract/ 177.1089 ml diethyl ether

The general procedure produced the highest percent dry weight of glycosides, therefore this procedure was repeated with 3 kg of *P. insolens*.