544341

**Department of Environmental Biology**

# A Search for Biologically Active Compounds in *Acacia* (Mimosaceae) species

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# ABSTRACT

Indigenous Australians were also known to use plants for medicinal purposes. For thousands of years, Indigenous Australians have used native plants as a source of medicinal agents. Some tribes living in Central Australia still, to this day, prefer to use traditional medicines in favour of the more common and readily available western medicines.

A number of plant species endemic to Australia are listed in various Aboriginal pharmacopoeias, with approximately one-third of those species belonging to two genera, *Acacia* and *Eremophila*. Of the 1100 recognised species of *Acacia*, approximately 900 occur in Australia. At least thirty of these species were utilised by the Indigenous Australians as a source of medicine.

Extracts of 8 *Acacia* species were screened using four frontline bioassays. These were the brine shrimp lethality test, the crown gall tumour assays, the disc diffusion antibiotic assay and the seed germination test to determine if any of the species were biologically active. Of all the species screened, *Acacia pruinocarpa* showed the most promise. The species demonstrated significant activity at concentrations at low as 3.7ppm, which is well below the standard 400ppm exhibited by potassium dichromate (Sam, 1993). *Acacia adsurgens* and *A. dictophleba* were the next two promising species exhibiting activity at concentrations of 16.12ppm and 37ppm respectively. This was a trend that was also observed in the Lettuce seed germination test for allelopathy with these three species showing the most promise. Interestingly the potency of *A. pruinocarpa* extract decreased significantly when it was re- screened after being put through a polyamide column. It can therefore be suggested that as tannins are removed by the polyamide column, the biological activity exhibited by *A. pruinocarpa* is a result of the tannin content in the species (2%), although more testing is required.

Both *A. pruinocarpa* and *A. adsurgens* showed promise as anti-tumour activity when used in the Crown Gall Tumour Assay (CGTA). *Acacia pruinocarpa* and *A. adsurgens* both exhibited significant activity when compared to the control producing inhibition percentages of 31% and 37% respectively.

Surprisingly, only one of the *Acacia* species tested inhibited pathogenic growth when tested on the common pathogens *Staphylococcus aureus*, *Streptococcus pyogens* and *Candida albicans*. *Acacia bivenosa* was the only species to exhibit any activity when tested on the

pathogens. This activity, however is not considered to be significant, as the species was only active against one of pathogens tested, *Staphylococcus aureus*. In order to be considered to be significant, a species must be active against two or more pathogens. It is however, worthy of further evaluation.

*Acacia* species are among the large number of plants that have long been regarded sources of biological activity. This study was guided by the indigenous use of *Acacia* species as sources of medicine, which led to the use of front-line bioassays. All of the species tested exhibited some form of biological activity.

*Acacia pruinocarpa* demonstrated the most promise as a source of novel biologically active compounds exhibiting activity at very low concentrations. Such compounds have not been determined as it was outside the scope of this study to identify the active constituents of this species. However, it has been suggested that tannins are responsible for eliciting some of the activity observed in *A. pruinocarpa*.

All of the species screened in this study are worthy of further evaluation. The bioassays used in this study are good examples of front-line bioassays. All of the tests used in the study fulfil the criterion, which defines a good test.

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Traditional Medicine

The use of plant and other organisms as sources of medicinal agents has occurred throughout most of the human species history (Latz, 1995; Moran, 2002). Traditional health care practices have universally recognized that the mind and body are intrinsically linked by the core belief that to prevent ill health there is a need to maintain a balance with Nature's life force. The use of plants to maintain this balance is well documented. For example, the first known use of plants as medicinal agents dates back to Neanderthal times (Akerele, 1993; Plotkin, 1992). The remains of eight different species of plant were found in close proximity to the grave of a 50,000 year old Neanderthal male in north eastern Iraq. Seven of these species are still in use for medicinal purposes.

The first systematic register of medicines, however, dates back to ancient Greek and Egyptian times. In such societies it is apparent that the individuals that made and distributed the agents possessed power and influence over their civilisations (Plotkin, 1992). The most extensive of the early records is thought to be the Code of Hammurabi by the King of Babylon of 1728 – 1686 BC. Hammurabi had many of his records carved into stone, parts of which can now be found in the Louvre in France (Plotkin, 1992). This code features not only the laws pertaining to medical practice, but numerous references to curative plants such as henbane, liquorice and mint. These still feature prominently in modern pharmacopoeias (Plotkin, 1992).

In Ancient Greece, in contrast, medicine was practised in large temples built in the honour of Asclepias, the God of healing. This was usually by physicians known as the sons of Asclepias. Such treatments were religious events often involving incantations, fasting and bathing (Low *et al.*, 1994). This type of medicine continued until Hippocrates, considered by most to be the father of modern medicine, came into prominence and shifted Greek medicine from being based on mystery and religion to one based on science (Plotkin, 1992). Hippocrates believed that the four elements fire, water, earth and air were represented in the human body by yellow bile (choleric), black bile (melancholy), phlegm (phlegmatic) and blood (sanguine). Ancient Greek medicine aimed at obtaining a balance of these four elements in the human body. Illnesses were caused by an imbalance of the elements. It was believed that such imbalance could only be restored by bleeding, vomiting or sweating (Plotkin, 1992). Hippocrates also recognised, however, the potential of using plants to treat ill health. His treatise comprised between 300 and 400 plant species (Plotkin, 1992). It is estimated that over 75% of medicinal agents derived from plants were discovered through examining the use of such plants in traditional medicine.

### 1.1.1 Traditional Australian Ethnobotany: Medicinal and other uses of plants by Indigenous Australians

Indigenous Australians were also known to use plants for medicinal purposes (Barr, 1993; Latz, 1995; Low, 1990, Everist, 1974). For thousands of years, these astute botanists have used native plants in their medicinal practices (Pennacchio and Ghisalberti, 1997). Some tribes of Indigenous Australians living in Central Australia still, to this day, prefer to use traditional medicines in favour of the more common and readily available western medicines (Latz, 1995).

Several plant species endemic to Australia are listed in various Aboriginal pharmacopoeias, with approximately one-third of those species belonging to two genera, *Acacia* and *Eremophila* (Latz, 1995). The remaining two-thirds are represented by a wide range of species including *Hakea spp* and *Atriplex spp* (Low, 1990; Barr, 1988; Barr 1993; Bindon, 1996; Latz, 1995). Unlike western medicines, which are usually used for specific purposes, bush medicines were often used for a variety of purposes. This is due, in part, to the traditionally nomadic lifestyle of the Indigenous Australians, who rather than carry a number of plant species with them, preferred to carry species that had a broad spectrum of uses (Latz, 1995). For example species such as *Eucalyptus camaldulensis* were used to treat a variety of complaints ranging from skin disorders to chest complaints such as colds and coughs. It is important to note however, that the use of oils from the leaves of *Eucalyptus* spp has only recently become a practice of Indigenous Australians (Latz 1995). *Eucalyptus miniata* is another species favoured by Indigenous Australians. It to was used as a treatment for ailments ranging from skin complaints to respiratory infections (Barr, 1993).

Although work investigating the medicinal qualities of native Australian plants is limited, interesting biological activities have been reported (Lassak and McCarthy, Barr, 1992; Latz, 1995; Low, 1990). A number of different types of compounds that exhibit some type of biological activity have been isolated including monoterpenes, sesquiterpenes, flavones and iridoid glycosides (Syah and Ghisalberti, 1995; Pennacchio *et* al., 1996,1997). These researchers reported that some *Eremophila* species, in particular *E.alternifolia* and *E.longifolia*, exhibit biological activity when tested on the hearts of Langendorff rats (Pennacchio *et al.*, 1996; Kemp, 1999). Verbascoside, a phenylethanoid glycoside was isolated from the leaves of *E.alternifolia* has been shown to be one of the active constituents of *Eremophila alternifolia* (Pennacchio *et al.*, 1996). It was reported to induce significant increases in heart rate, contractile force and coronary perfusion rate (CPR) (Fig 1.1).

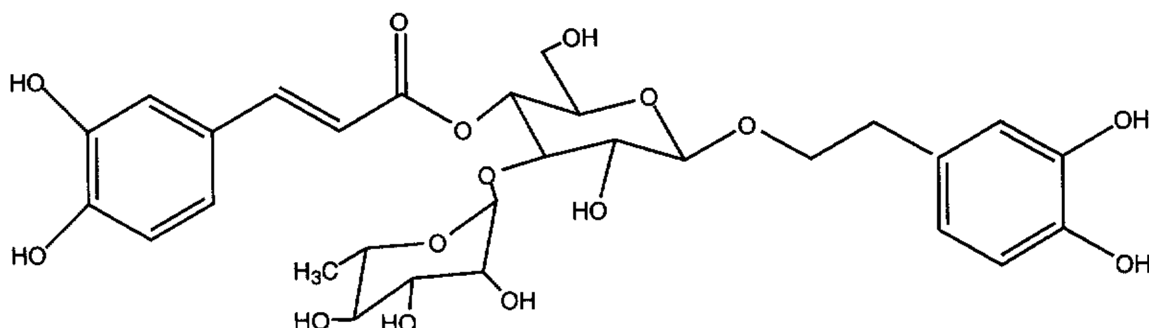


**FIGURE 1.1**: The chemical structure of Verbascoside (syn: acteoside; kusaginin)

Other *Eremophila* species also exhibit cardioactive properties. However, in the case of *Eremophila longifolia* these properties had an inhibitory effect on the heart (Pennacchio *et al.*, 1996). An iridoid compound, Geniposidic acid was responsible for the cardioactive properties exhibited by *E.longifolia* (Pennacchio *et al.*, 1996)(Fig 1.2).

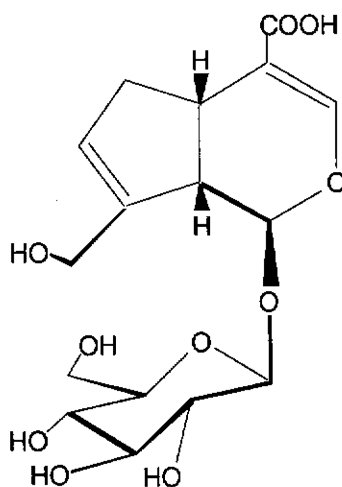


**FIGURE 1.2:** The chemical structure of Geniposidic Acid.

Unlike verbascoside, geniposidic acid exhibited negative effects on the hearts of Langendorff rats (Pennacchio *et al.*, 1996). Geniposidic acid induced a significant inhibitory effect on heart rate, contractile force and coronary perfusion rate.

Four other iridoid glycosides have been isolated from *Eremophila* species and have been shown to have an effect on the activity of the heart. Melampyroside and ferruloyajugol were isolated from *E. pantonii*. Verminoside was isolated from *E.ionantha* while catapol was isolated from *E.maculata* (Pennacchio *et al.*, 1997). All four of these exhibited stimulatory effects on heart rate.

The use of plants by the indigenous Australians was not only for medicinal purposes (Low,1990; Barr,1992; Latz,1995). Plants also played a major role in the diet, with numerous species being utilised for this purpose. The indigenous Australians utilised the whole plant from the seeds and roots to the leaves. Species such as *Solanum ellipticum*, *S.centrale* and *S.esuriale,* which is less common than the other species, were harvested for their fruit, which is a good source of vitamins and minerals (Latz, 1995). Over 70 species of plants were utilised for the edible seeds in Central Australia. These are an important source of proteins, oils, vitamins and fibre. *Acacia* species contribute to 50% of the total seed content of the diet of Indigenous Australians, with the remaining 50% being comprised of grasses, such as *Panicum decompositum* and other species like *Tecticornia verrucosa* (Latz, 1995).

Some species were also harvested for their gum, which was used as a food source. The tannin content was also important because it was used in the manufacturing process of clothes and leather. Seigler (2002) has reported that a number of *Acacia* species have a high level of both tannins and gums, both of which are high in proteins and minerals.

Being hunters and gatherers, the Indigenous Australians are highly dependent on wooden implements and weapons. Like the plant species they are made from, many of these had a number of uses. Over 33 species of plant were used by indigenous Australians in the manufacture of weapons and tools (Latz, 1995). Some species, such as *Acacia aneura* were used to manufacture a number of different products. For example, this species was used to construct shields, boomerangs, woomeras, digging sticks and sacred boards known as churingas. Others, such as *Pandorea spp,* were used in the manufacture of spears due to the unbranched stems that are characteristic of these species (Latz, 1995).

Plants also played a major role in the religion, ceremony and mythology of Indigenous Australians. These also were used in ornaments and decoration (Latz, 1995). Hundreds of species of plant were used in this way. For example, species, such as *E.longifolia*, considered to be the most sacred, mystical and magical of plants, were often used in ceremonies both for decoration and as part of the ritual (Latz, 1995). *E.freelingii*'s, flowers were used in head- wear during ceremonies. *Helipterum tietkensii* was also used

for ceremonial purposes. This species provides down, which was used as a substitute for feathers in body decoration (Latz, 1995). A number of other plants also feature in the mythology of Indigneous Australians.

## 1.2 *Acacia* species used by Indigenous Australians

Of the 1100 recognised species of *Acacia*, approximately 900 occur in Australia (Maslin, 1997). Thirty of these species were used medicinally by the Aboriginal people. Unlike many other medicinal plants, *Acacia* species can be used both internally and externally (Table 1.1). For example, *A. aneura* was used by Indigenous Australians as a smoke treatment for new born babies to increase the immune system of the child. This was to promote good health and strength (Table 1.1)(Barr, 1993), *A. holosericia* was a multi purpose species, which was used to treat a number of complaints including laryngitis, skin infections, sore throats and headaches (Table 1.1) (Barr, 1993), *A.ancistrocarpa*, was ideal in treating used to treat skin infections, headaches, joint pain and swelling. This species was prized as a smoke treatment for diarrhoea in babies (Barr, 1992; Latz, 1995). A number of *Acacia* species, including *A.dictopleba* and *A.pruinocarpa* were reported for their ability to induce lactation, to stop post-partum bleeding and to strengthen the mother and baby (Table 1.1) (Barr 1993).

The leaves of the plant species were the major source of medicines in the aboriginal pharmacopoeia. However, the bark and wood were also used. For example, the bark of *A.cuthbertsonii* was used as splints and bandages for bone injuries while the bark of *A.estrophiolata* was used as a treatment for burns (Table 1.1) (Barr, 1993).

**TABLE 1.1:** *Acacia* species which are known to be used for medicinal purposes by Indigenous Australians. Those species marked with an asterisk (*) were screened for biologically active compounds.

| SPECIES | PART USED | PREPARATION | TREATMENT | REFERENCES |
|---|---|---|---|---|
| *A.adurgens* (*) | Leaves and twigs | Smoke treatment | To treat babies for diarrhoea | Low (1990) |
| *A.ancistrocarpa* (*) | Leaves and twigs | Bashed and soaked | Skin sores and headaches | Reid and Betts (1979) |
| | Leaves and twigs | Heated | Swellings and pain | Bindon (1996) |
| | | Smoke treatment | To treat babies for diarrhoea | Low (1990) |
| *A.aneura* (*) | Leaves and twigs | Smoke treatment | Strengthens mothers and babies | Barr (1993) |
| *A.auriculoformis* (*) | Leaves | Decoction | Antiseptic cleanser | Barr (1993) |
| | Leaves and twigs | Lather | Pruitis and allergy rash | |
| | Leaves and pods | Lather | Pain in legs and body | Levitt (1981) |
| *A.bivenosa* (*) | Bark and roots | Infusion | Cough and cold medicine | Cribb and Cribb (1981) |
| *A.coriacea* (*) | Trunk and lateral roots | Ash | Chewed with pituri | Cleland and Tindale (1958) |
| *A. cuthbertsonii* | Bark | Unknown | Toothache & pain | Reid and Betts (1979) |
| | Bark | Stripped | Rhuematism | |
| | Bark | Moistened | Splint and bandages | |
| | | | Headache | Barr (1993) |
| *A.dictopleba* (*) | Leaves | Infusion | Coughs, colds and headaches | Bindon (1996) |
| | Leaves and branches | Smoke treatment | Strengthens mothers and babies | Meggitt (1962) |
| | | | | Low (1990) |
| *A.difficilis* | Bark | Stripped | Bandages and splints | Barr (1993) |
| *A.estrophiolata* | Bark from branches | Decoction | Antiseptic for boils scabies and sore eyes | Barr (1993) |
| | Bark | Moistened | Bandage for sores and burns | |
| | Root bark | Infusion | Headache, sore throat,cold and alimentary discomfort | |
| *A.holosericia* (*) | Roots | Infusion | Laryngitis | Reid and Betts (1979) |
| | Leaves | Decoction | Cleanser, deodorant, and mild antiseptic fo skin sores | Barr (1993) |
| | Roots | Infusion | Sore throats | |
| | Pods and Seeds | Lather | Relieves itchiness | |
| | Pliable Bark | Moistened | Headaches | |
| *A.inaequilatera* (*) | Bast under cork bark | Infusion | Skin sores, cuts, chicken pox | Reid and Betts (1979) |
| *A.kempeana* | Leaves | Decoction | Wash for severe colds | Barr (1993) |
| | Leaves and twigs | Smoke treatment | Strengthens mothers and babies, helps to stop post partum bleeding | |

Table 1.1 continued

| SPECIES | PART USED | PREPARATION | TREATMENT | REFERENCES |
|---|---|---|---|---|
| *A.leptocarpa* | Green Leaves | Infusion | Sore eyes | Reid and Betts (1979) |
| *A.liguata* | Leaves and branches | Smoke treatment | Diaphoretic for several complaints including those of the nervous system and after childbirth | Barr (1993) |
| | Bark | Decoction | Cough medicine | |
| *A.lysophloia* | Leaves and twigs | Infusion | Body wash for colds and flu | Barr (1993) |
| | Leaves and branches | Scorched on hot embers | Relieves pain and aches when applied externally | |
| | Young leaves and twigs | Decoction | Anodyne of post-natal therapy | |
| | Phyllodes | Smoke treatment | Strengthens mothers and babies | |
| *A.melanoxylon* | Bark | Infusion | Skin wash | Low (1990) |
| *A.monticola* | Branches | Decoction | Coughs and colds | Webb (1995) |
| | Roots | Infusion | Cough medicine | Reid and Betts (1979) |
| *A.multisiliqua* | Leaves | Boiled or crushes | Vapour inhaled for nasal congestion | Barr (1993) |
| *A.oncinocarpa* | Leaves | Decoction | Respiratory illness | Barr (1993) |
| *A.pellita* | Pods | Lather | Pruritic skin | Barr (1993) |
| *A.pruinocarpa* (*) | Leaves and branches | Smoke treatment | Strengthens mothers and babies | Low (1990) |
| *A.pyrifolia* | Bark | Decoction | Skin sores | Webb (1995) |
| *A.salicina* | Leaves | Unknown | Considered to have medicinal properties | Latz (1995) |
| *A.spondylophylla* | Unknown | Unknown | Skin Wash | O'Connell *et al.*, 1983 |
| *A.tetragonophylla* | Inner bark | Infusion | Cough medicine | Webb (1969) |
| | Wood | Burnt to ashes | Antiseptic | Reid and Betts (1979) |
| | Leaves | Chewed | Dysentary | |

## 1.3 Diseases of Indigenous and other Australians

Since the main aims of this study were to search for novel medicinal agents with antibiotic and anti cancer properties and how they affect Indigenous Australians, a brief description of diseases related to these have been included.

A disease is defined as any disorder with a specific cause and recognisable signs and symptoms, or as any bodily abnormality or failure to function properly except that resulting from physical injury (Woods and Guiterrez, 1993). Diseases can affect all parts of the body including the skin. Skin diseases can be caused by a number of pathogenic agents, the most common being bacteria and fungi (Woods and Guiterrez, 1993).

Individuals carry an extensive population of microflora on the skin, which, in healthy individuals, excludes many other bacteria and other would-be pathogens from becoming infectious (Mims, 1987). These organisms are able to exist on the skin without causing an inflammatory response in the individual (Mims, 1987). Small portions of these do, however, give rise to pathogenic changes or disease. The skin, therefore, can be considered as the body's primary barrier against infection caused by pathogenic agents. However, when the individuals immune system is compromised in some way, as is the case with cancer and AIDS, or have a predisposition to infection, this front-line barrier breaks down and does not function to the full potential (Mims, 1987).

Pathogens can also have an effect internally. For example, the common pathogen *Staphylococcus aureus*, is responsible for 90% of all bone infections in the indigenous Australians. Along with *Streptococcus pyogens*, it is responsible for the childhood skin complaints, such as impetigo (Webb, 1995). Indigenous Australians have one the highest rates of post-streptpcoccal glomerulonephritis in the world, a kidney disease and rheumatic fever, both of which, are a result of a severe streptococcal infection (Gardiner and Sripakash, 1996). Unlike other members of the community, where the infection rate of children is much lower, indigenous children are still highly susceptible to such pathogens with an infection rate of approximately 70% (Gardiner and Sripakash, 1996).

Members of the *Staphylococcus* genus are gram-positive bacteria that belong to the family Micrococcaceae (Woods and Guiterrez, 1993). They are generally immotile, non-spore forming bacteria approximately 0.5-1.5 micrometres in diameter (Woods and Guiterrez, 1993). Of the 27 recognised species of *Staphylococcus*, 14 are known to infect human skin and mucous membranes. *Staphylococcus aureus* is a component of the normal microflora of humans. They inhabit the anterior nasal cavity and the perineum of the axilla without causing any symptoms. Most infections caused by *S.aureus* are endogenous, i.e. the organism has transferred from a site of colonisation where dermal breaks provide a site of entry (Woods and Guiterrez, 1993).

Infections caused by *S.aureus* have the ability to affect multiple organ systems. The skin, however, is the most common organ that is affected by this organism. In severe

cases, it can infect the bone tissue of the individual. Impetigo is one of the more common skin conditions caused by *S.aureus* affecting mainly children and young adults. The disease is a superficial cutaneous disorder that is transmissible through contact with an infected party (Woods and Guiterrez, 1993).

Treatment of infection depends both on the location of the infection and the severity of it. Superficial infections and uncomplicated urinary tract infections are adequately controlled with anti microbial therapy using ointments such as Bacitracin, Neomycin and Polymyxin. More serious invasive infections require antimicrobial agents such as penicillin, cloxacillin, erythromycin, and methicillin.

*Streptococcus pyogens* is also a gram-positive bacterium. Like most species of this genus, they are facultative anaerobes and can, therefore, survive in both aerobic and anaerobic conditions. *S.pyogens* is a non-motile spherical bacterium that forms chains (Woods and Guiterrez, 1993). The components of the cell wall, the enzymes and toxins it exudates, as well as the host responses are involved in the pathogenesis of streptococcal infections (Woods and Guiterrez, 1993).

*Streptococcus pyogens* is responsible for a number of skin diseases in humans, but has the ability to infect other parts of the body as well. For example, pharyngitis, the inflammation of the throat behind the pharynx is a disorder that is caused by the bacterium. Indigenous Australians are particularly susceptible to Streptococcal infections with approximately 70% of children having the cutaneous skin infection known as Impetigo. Indigenous Australians also have the highest rates of acute rheumatic fever and rheumatic heart disease in the world with the annual rates at 2.5 per 1000 children and 10.4 per 1000 adults (Gardiner and Sriprakash, 1996). Acute post-streptococcal glomerulonephritis is also prevalent in indigenous communities with 10% of indigenous children affected by it (Gardiner and Sriprakash, 1996). Infections caused by *Streptococcus pyogens* are routinely treated with antibiotics such as penicillin and erythromycin.

Despite bacteria being common skin pathogens, some species of fungi can also be pathogenic to humans. In particular, the yeast *Candida albicans* and the keratinophilic mould *Trichophyton rubrum* are to such pathogens.
Although information on the statistics of infection in the Indigenous Australian communities is limited, reports of the use of plant material antiseptic skin washes (Barr, 1993), suggests that this group were susceptible to fungal and other infections of the skin, and yeast infections such as those caused by *Candida albicans* did occur.

The genus *Candida* was first identified in oral lesions in the late 1830's and early 1840's and was recognised as a potential cause of deep-seated infection (Woods and Guiterrez, 1993). Most species of *Candida* are part of the normal microflora of the human body, inhabiting the gastrointestinal tract, mouth, vagina and skin. Of the 150 recognised species of *Candida*, ten have been recognised as human pathogens with *Candida albicans* being the most common (Woods and Guiterrez, 1993). Recent reports suggest that approximately one third of the worlds population suffer from infections caused by *Candida albicans* (Woods and Guiterrez, 1993).

*Candida albicans* becomes infectious in humans only when the human immune system is compromised. People who suffer from disorders such as diabetes, cancer, HIV, or women that are pregnant are particularly susceptible (Brunner, 1987). Infections of *C.albicans*, or Candidiasis, can involve the mucocutaneous surface, i.e. the cutaneous surfaces or the deep organs (Woods and Guiterrez, 1993). Mucosal infections affect several sites of the human body. These sites include the mouth, gastrointestinal tract and vagina. Cutaneous infections include paronchchia, onychomycosis and nappy rash. Deep organ infections or mucotaneous infections are those that can cause the most harm to humans. *Candida albicans* can infect the lungs, heart, urinary tract, musculoskeletal system, abdomen and eyes (Martin, 1990; Woods and Guiterrez, 1993).

Treatment of candidiasis is via a number of anti-fungal agents. The use of the agents depends on the location and severity of the infection. For example, the antibiotic Nystatin is effective in the treatment of superficial *Candida* infections and Amphotericin B is effective against systemic, skin and nail infections (Brunner, 1987).

Where *Tinea pedis* or "athletes' foot" is concerned, two fungal species are responsible for infection. The main species responsible is *Trichophyton rubrum*, but ususally works in conjunction with *T.mentagrophytes* (Brunner, 1987). Both species are keratinophilic moulds that parasitise the cutaneous surfaces of the body especially those that have the ability to secrete keratinases eg, the toes. Ringworm, also caused by *Trichophyton* spp, was one of the first skin diseases to be recognised (Woods and Guiterrez, 1993).

Two forms of *Tinea pedis* are known to infect humans. A simple form, where the disease is caused primarily by fungi with some bacteria present, is characterised by mild to moderate skin peeling. The other, a complex form where the bacteria genera, *Proteus* and *Pseudomonas* are the principal pathogens, *Trichophyton* plays a minor role (Brunner, 1987).

The latter form is characterised by the maceration of tissue, inflammation and fissuring. Some individuals are prone to chronic forms of this complaint. Susceptibility to *Trichophyton rubrum* and other forms of Tinea is not, however, universal (Woods and Guiterrez, 1993). The sensitivity of a person to the disorder is enhanced by moisture, warmth, skin chemistry, composition of the sebum and perspiration, youth, heavy exposure, genetic predisposition and suppression of the immune system (Woods and Guiterrez, 1993).

Treatment of skin infected with *T.rubrum* depends on the severity of the infection. Simple forms of *Tinea pedis* can be successfully treated with Tolnaftate, an antiseptic applied as a cream, powder or solution, also known as Tinactin or Tinaderm (Woods and Guiterrez, 1993). The more complex forms, which also involve bacteria, require a combination of both antibiotics and anti-fungal agents, usually neomycin sulfate and anti-fungal agents such as tolnaflate (Woods and Guiterrez, 1993; Prescott *et al.*, 1993).

### 1.3.1 Cancer

Cancer is a major cause of death in Australia, accounting for 28% of deaths in men and 25% of deaths in women (Australian Bureau of Statistics, 1997). The word cancer does not describe one disease, but number of diseases in which abnormal cells proliferate and can spread out of control. Cancer can develop from most types of cells in different parts of the body (Weinberg, 1998). Each different type of cancer has it own growth pattern. Some cancers remain in the body for long periods of time without showing any symptoms, whereas others can grow, invade and spread rapidly. These are usually fatal (Australian Bureau of Statistics, 1997).

Each year, 345,000 new cancer cases are diagnosed in Australia. In 1996, approximately 77,666 new cancer cases and 34,089 deaths due to cancer occurred. The most commonly occurring cancers are cancers of the colon and rectum. However, the type of cancer a person develops is both age and sex dependent. For example, the four most common cancers diagnosed in women are breast cancer, colorectal cancer, melanoma and lung cancer (Australian Bureau of Statistics, 1997). These account for 58% of all cancers in women, whereas the four most common cancers detected in men are prostate cancer, colorectal cancer, melanoma and lung cancer which account for 60% of all cancer in men.

Although a number of cancers share risk factors, most cancers have unique set of factors that are responsible for their onset. For example, some are a result of smoking, dietary

influences, infectious agents or exposure to radiation, whereas others maybe a result of genetic predisposition (Australian Bureau of Statistics, 1997).

Chemotherapy has until recently been the most successful means of combating cancer (Pratt *et al.*, 1994). However, tumour resistance to cytotoxic agents is increasing and becoming a major concern to the medical industry. Such resistance is the main reason for the failure of chemotherapy, and is responsible for the emergence of studies which target this resistance in the hope of discovering novel cytotoxic agents that are either not subject to resistance, or are able to impair resistance mechanisms, therefore establishing sensitivity to existing agents (Clarke, 1990).

## 1.4 Aims of the Study

The aims of this study were to help substantiate some of the reports of the medicinal reports of *Acacia* species in Aboriginal pharmacopoeia. Secondly, it was to determine whether any other biological activity exists in certain *Acacia* species. This was partly determined through the use of the Lettuce Seed germination test for allelopathy.

Eight *Acacia* spp were tested for activity using the following tests:

1. The Brine Shrimp Lethality Test
2. The Crown Gall Anti-Tumour Assay
3. The Disc Diffusion Antibiotic Assay
4. The Lettuce Seed Germination Test

Descriptions of each of these are provided in the chapters that deal with them.

## 1.5 Thesis Organisation

This thesis is organised into seven chapters with an introduction, materials and methods, results and discussion for each chapter. The general introduction describes the background to the study, giving a general outline of traditional medicine and diseases. Chapter two describes the species tested and the collection sites. Chapters 3-6 describe and discuss in detail the screening of several *Acacia* species using a variety of bioassays. Chapter 7 is a general discussion. All references are listed after chapter 7 along with any appendicies.

# CHAPTER 2

# SPECIES AND COLLECTION SITES

## 2.1 Mimosaceae

Mimosaceae are a family of plants comprised of approximately 60 genera and over 300 species, which usually belong to three genera, *Acacia*, *Inga* and *Mimosa*. The members of this family are usually trees and shrubs, but can also exist as lianes or herbs (Cowan, 2001). Many members of this family inhabit a wide range of environments ranging from the tropical and sub-tropical areas to temperate areas, and can be found at altitudes up to 1000 metres. In Australia, the Mimosaceae are represented by 17 genera, 12 of which are endemic to the country.

### 2.1.1 *Acacia*

The *Acacia* genus is considered by many to be the largest genus of vascular plants in Australia. Currently, there are over 900 named species occurring throughout the country (Maslin, 2001). The genus occurs in a wide variety of climatic and soil conditions from the coastal to the sub-alpine and from high rainfall to arid conditions (Elliot and Jones, 1984). Most species of *Acacia* are woody plants that range from prostrate shrubs to small trees. The majority of species in this genus lack true leaves, which if possessed are usually bipinnate, instead the members of this genus possess structures known as phyllodes. However, there are some species that lack both leaves and phyllodes. In such species, the stems act as leaves (Elliot and Jones, 1984). The colour of foliage in this genus varies in colour from dark green to silver-grey.

Flowering in some species is prolific with the foliage being completely covered, e.g. *A. baileyana*. In other species the flower heads are much smaller giving a less abundant appearance. Flowers are usually small, and arranged in either globular heads of two to 60 flowers or in spikes that exist either solitarily or as pairs. The flowers of the genus range in colour from light cream to deep gold, with one species, *A. purpureapetala*, being mauve in colour. Only some of the more promising species used by indigenous people were screened in this study. These will be described in greater detail.

### 2.1.2 *Acacia adsurgens*

*Acacia adsurgens* is a short-lived shrub that grows to four metres in height (Latz, 1995). The phyllodes of *A. adsurgens* are linear, straight of upwardly curved. They are approximately 6-20 cm long and 2-4.5 mm wide. The seed of this species have a relatively soft coat when compared to other species of *Acacia*. They are dorso-ventrally flattened and contained within linear pods (Maslin 2001). This species is widespread throughout Western Australia, the Northern Territory and Queensland between 18°S and 26°S extending to 117°E near Roebourne and east to central Queensland (Fig 2.1). The species also occurs in the far north- eastern corner of South Australia in the Lake Eyre Region. *Acacia adsurgens* grows in sandy and gravely soils, mainly on flat plains and hillsides. The species mainly flowers from May to September, but can flower as early as February (Fig 2.2a and 2.2b).

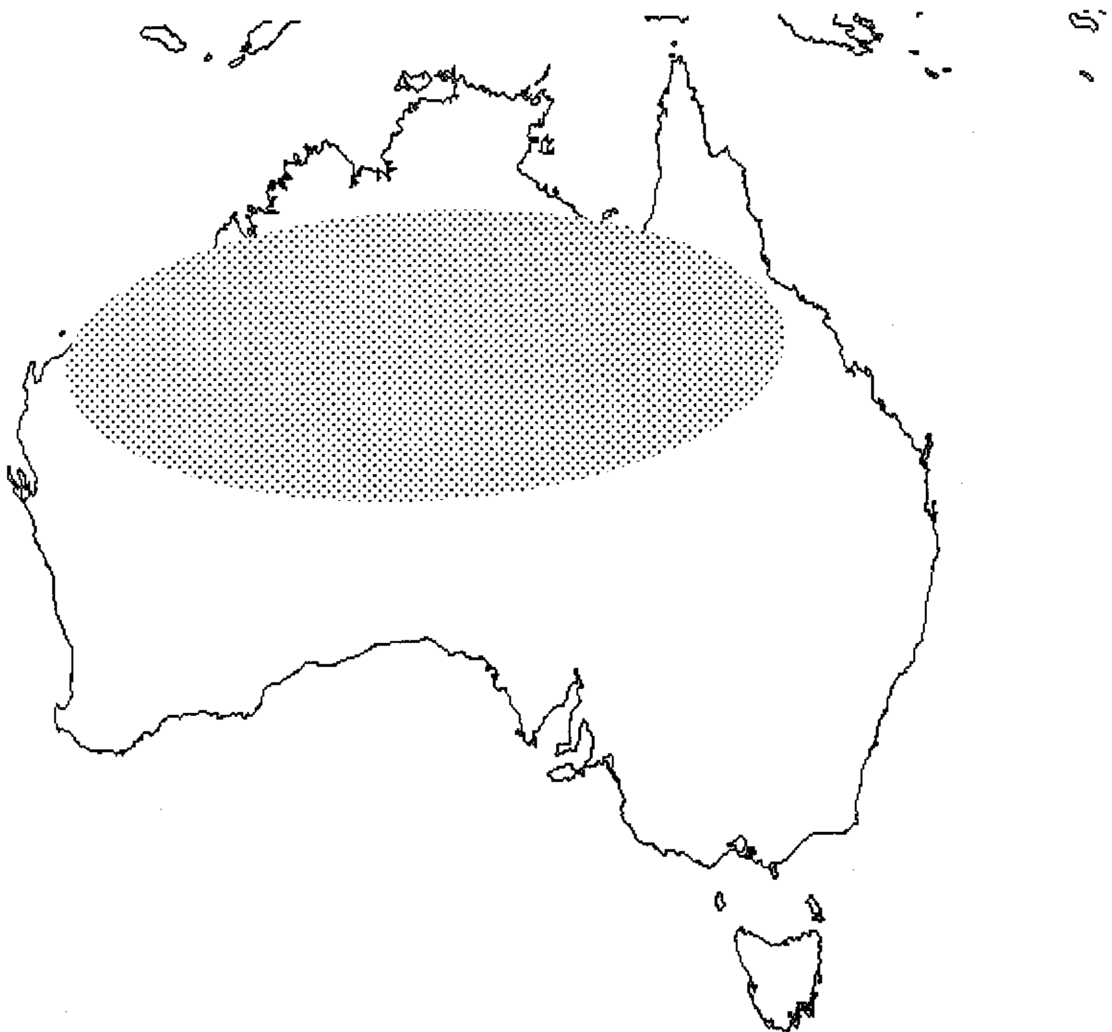

**FIGURE 2.1:** Distribution Map of *Acacia adsurgens* (Courtesy of Bruce Maslin)

**FIGURE 2.2a:** Growth Form of *Acacia adurgens* (Courtesy of Bruce Maslin)

**FIGURE 2.2b:** Flowers of *Acacia adsurgens* (Courtesy of Bruce Maslin).

2.1.3 *Acacia ancistrocarpa*

This species is widespread in the tropical areas of Western Australia, the Northern Territory and Queensland between 17°S and 24°S, and west of 139°E (Fig 2.3) *Acacia ancistrocarpa* usually occurs in mallee-spinifex communities, stony Spinifex grasslands, shrub-steppes and along watercourses in deep red and skeletal soils. *Acacia ancistrocarpa* is a multistemmed, resinous shrub or fastigate tree one to four metres high to four metres in diameter (Fig 2.4a). The bark of this species is smooth on the upper trunk and becoming dark and longitudinally fissured towards the base. The branchlets are angular, often flexuose pale yellowish orange or red-brown in colour and galbrous. The phyllodes *of A. ancistrocarpa* are usually linear or elliptic, straight or slightly falcate, ranging from 4.5 cm to 18 cm long (Fig 2.4b).

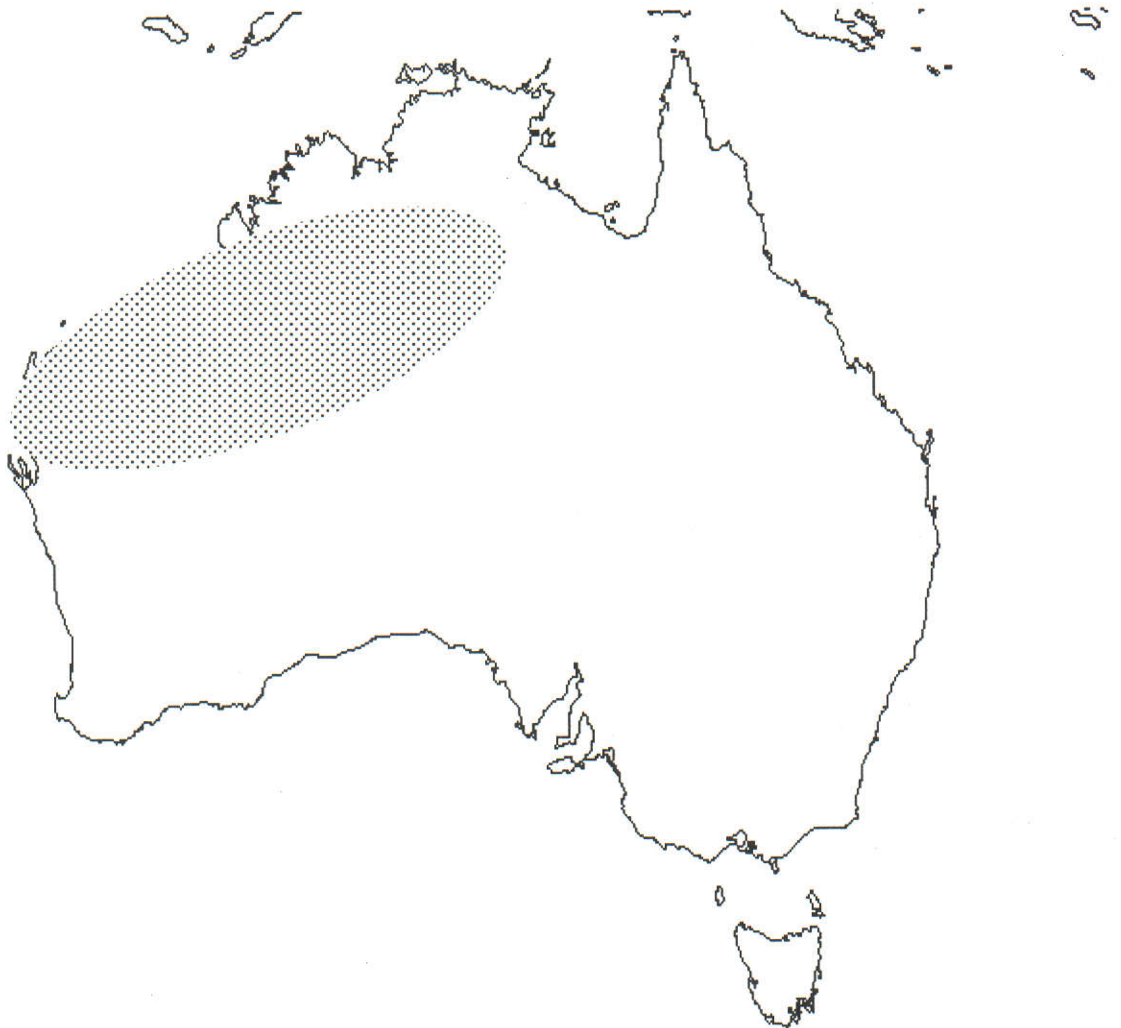

**FIGURE 2.3:** Distribution map of *Acacia ancistrocarpa* (Courtesy of Bruce Maslin).

**FIGURE 2.4a** Growth form of *A.ancistrocarpa* (Courtesy of Bruce Maslin)

**FIGURE 2.4b:** Flowers of *Acacia ancistrocarpa* (Courtesy of Bruce Maslin)

### 2.1.4 *Acacia aneura*

*Acacia aneura* is widely distributed throughout Australia, with the exception of Victoria (Fig 2.5). The species mainly occurs south of the 20° latitude with populations occurring from the Indian Ocean in the west, to the Great Dividing Range in central Queensland and New South Wales. Due its wide distribution, this species inhabits a wide range of habitats. Usually found on loamy or sandy soils in areas of low relief or on shallow rocky soils on hills. *Acacia aneura* often exists as pure stands forming open forest, woodlands and shrublands, or the species exists as prominent vegetation with Eucalypts. Also referred to as the ''Mulga', *A.aneura* exists as a shrub or small tree to 18m tall (Fig 2.6a). The branchlets of this species are a distinguishing character in that they are covered with red-glandular hairs that can harden with age. The phyllodes of the species are terete to flat, up to 9cm long and 0.8 to 9mm in diameter, with longitudinal nerves (Fig 2.6b)

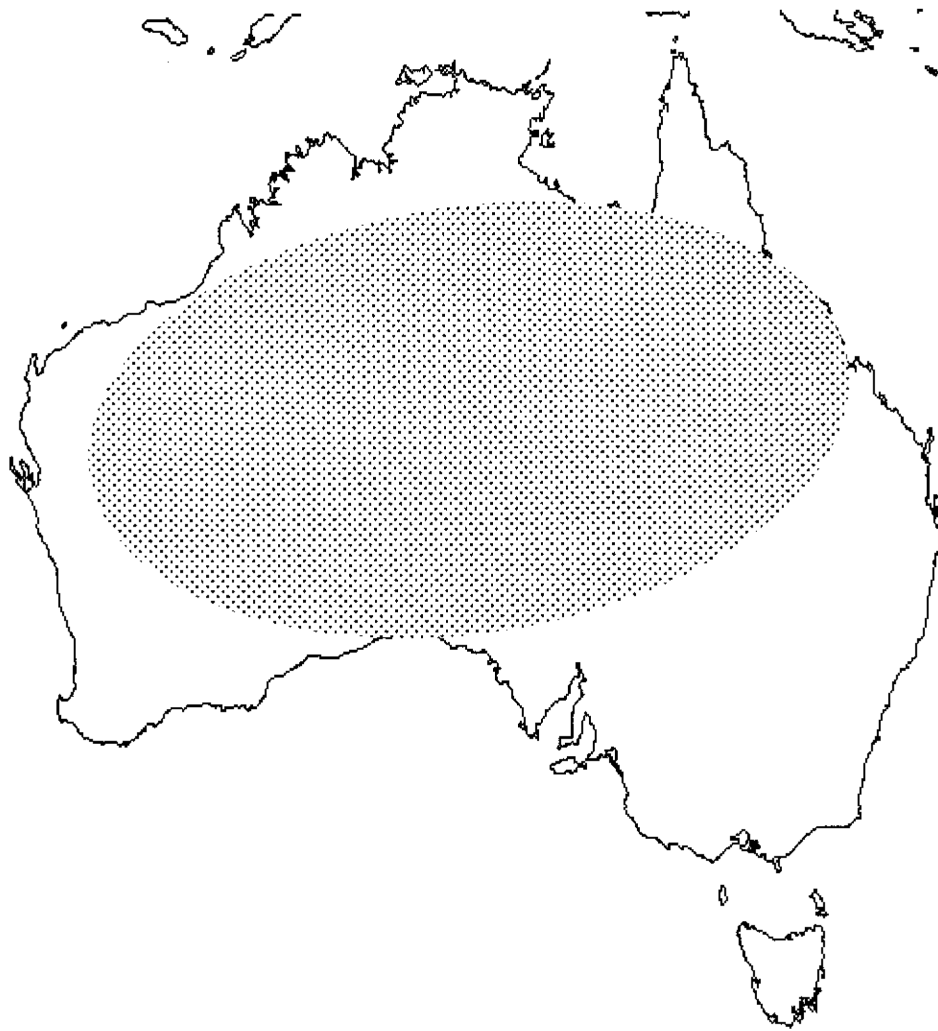

**FIGURE 2.5:** Distibution of *Acacia aneura* (Courtesy of Bruce Maslin)

**FIGURE 2.6a:** Growth form of *Acacia aneura* (Courtesy of Bruce Maslin)

**FIGURE 2.6b:** Flowers of *Acacia aneura* (Courtesy of Bruce Maslin)

### 2.1.5 *Acacia auriculoformis*

*Acacia auriculoformis* is widespread on Northern Cape Penninsula and the Northern part of the Northern Territory extending to New Guinea (Fig 2.7). It grows in well-drained sandy or loamy soils beside watercourses and swamps in closed or low open forests. *Acacia auriculoformis* is a glabrous tree that can grow to 30m in height. The branchlets are flattened towards the apices, the phyllodes of the species are linear to narrowly elliptic falcate and range from 10-20cm long (Fig 2.8a). The phyllodes possess three conspicuous, pale longitudinal veins, which converge with or near the lower margin at the base of the phyllode. The flowers of *A. auriculoformis* are rod-shaped and range from 5 to 8cm long with bright yellow colours (Fig 2.8b).

**FIGURE 2.7:** Distribution map of *Acacia auriculoformis* (Courtesy of Bruce Maslin)

**FIGURE 2.8 a:** flowers of *A.auriculoformis* (Courtesy of Bruce Maslin)

**FIGURE 2.8b:** Growth form of *Acacia auriculoformis* (Courtesy of Bruce Maslin)

### 2.1.6 *Acacia bivenosa*

*Acacia bivenosa* is widespread in the arid zone of Western Australia (Fig 2.9), the Northern Territory and Western Queensland north of 25°S, but also occurs on Dorre Island and in Shark Bay in Western Australia (Elliot and Jones, 1984). The species occurs in a variety of soil and climatic conditions including coastal sands, rocky hillsides and gullies, in shrubland, open shrubland, open woodland and are often associated with Spinifex. Also known as the two-nerved Wattle, *A. bivenosa* is a shrub usually one to three metres high, usually dense and glabrous. The phyllodes of *A. bivenosa* are usually oblong-elliptic to obovate, measuring two to five centimetres long and six to 25mm wide, with a leathery appearance (Fig 2.10a). The flowers of the species are globular, and exist as raceme structures. The flowers are deep yellow in colour, prolific and occur only on the last 20-30 centimetres of the branchlets (Fig 2.10b).

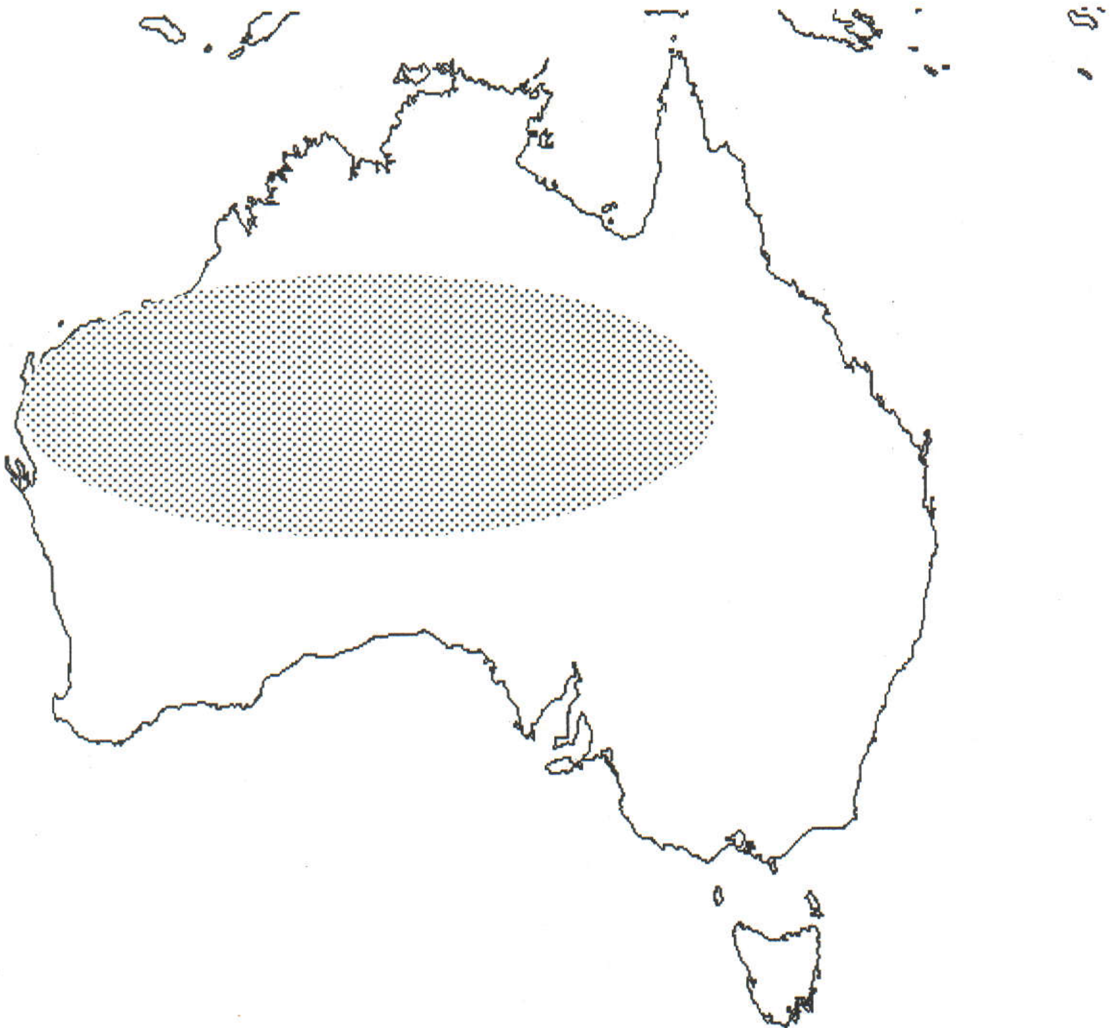

**FIGURE 2.9:** Distribution of *Acacia bivenosa* (Courtesy of Bruce Maslin)

**FIGURE 2.10a:** Growth form of *Acacia bivenosa* (Courtesy of Bruce Maslin)

**FIGURE 2.10b:** Phyllodes and flowers of *Acacia bivenosa* (Courtesy of Bruce Maslin)

### 2.1.7 *Acacia coriacea*

*Acacia coriacea* is a tall shrub to small tree growing to approximately 10m. The phyllodes for these species vary depending on the sub species, there are three, from linear to shallowly arcuate and can be falt to subterete. The phyllodes range in length from 12 to 33cm, and possess numerous closely parallel nerves (Fig 2.11).

The flowers of this species are globular and exist as pairs on the peduncles. They are cream to bright yellow in colour (Elliot and Jones, 1984). A distinguishing feature of this species are the pods are twisted and coiled to being nearly straight before dehiscence, and are bright orange in colour (Cowan, 2001).

The distribution of this species is also dependent on the sub species.
*Acacia coriacea* subsp *coriacea* occurs from Dirk Hartog Island in Shark Bay in Western Australia, to Point Sampson in Northwestern Australia (Fig 2.12).
The species grows on sand dunes and shrubland, but some populations have been found in lateritic soil in open eucalypt woodland.

**FIGURE 2.11:** Growth Form of *Acacia coriacea* (Courtesy of Bruce Maslin)

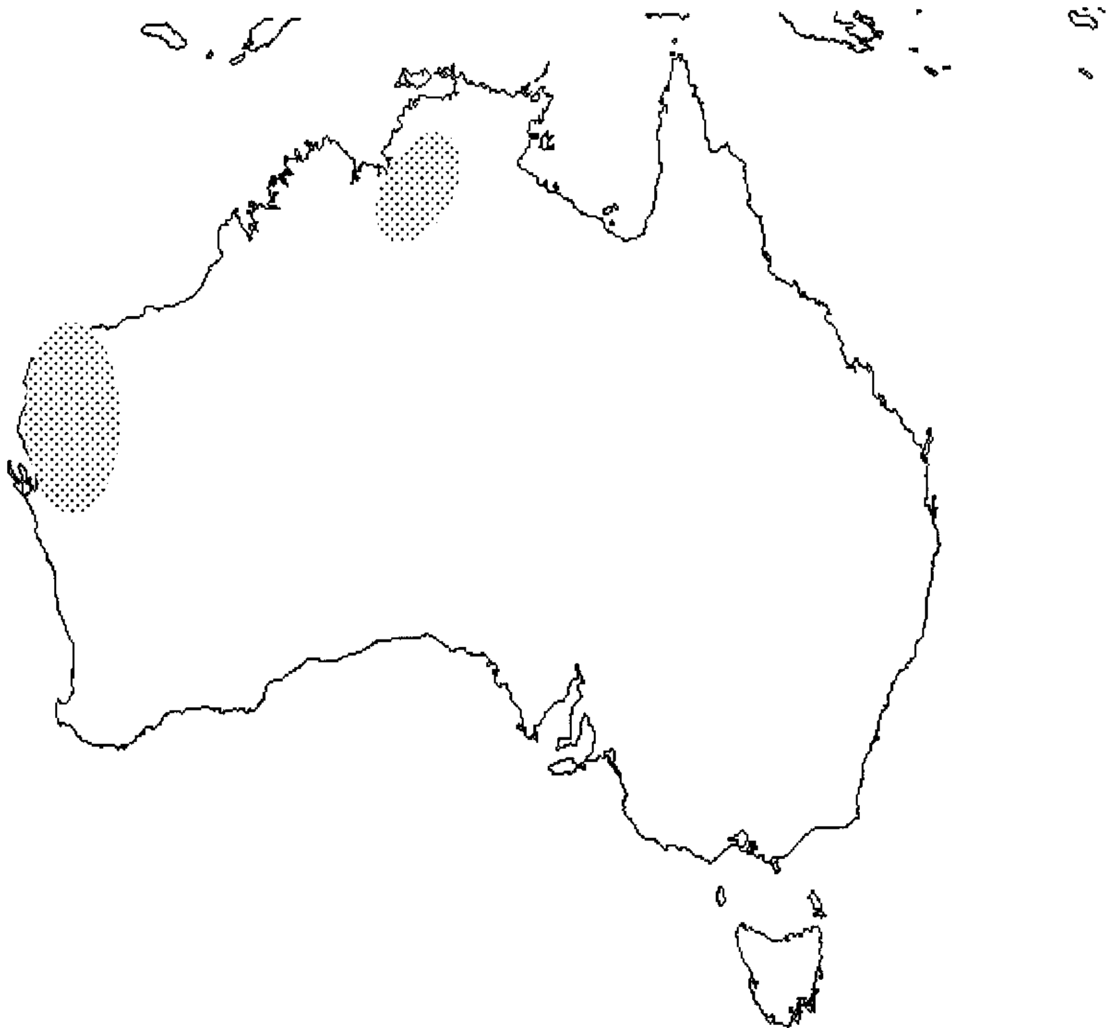

**FIGURE 2.12:** Distribution of *Acacia coriacea* subsp *coriacea* (Courtesy of Bruce Maslin).

*Acacia coriacea* subsp *pendens* predominantly occurs in inland parts of the Pilbara, W.A between the Gascoyne and De Grey Rivers, extending to the coast and islands around Dampier. The species grows along inland watercourses in fringing woodland and on stable coastal dunes (Fig 2.13).

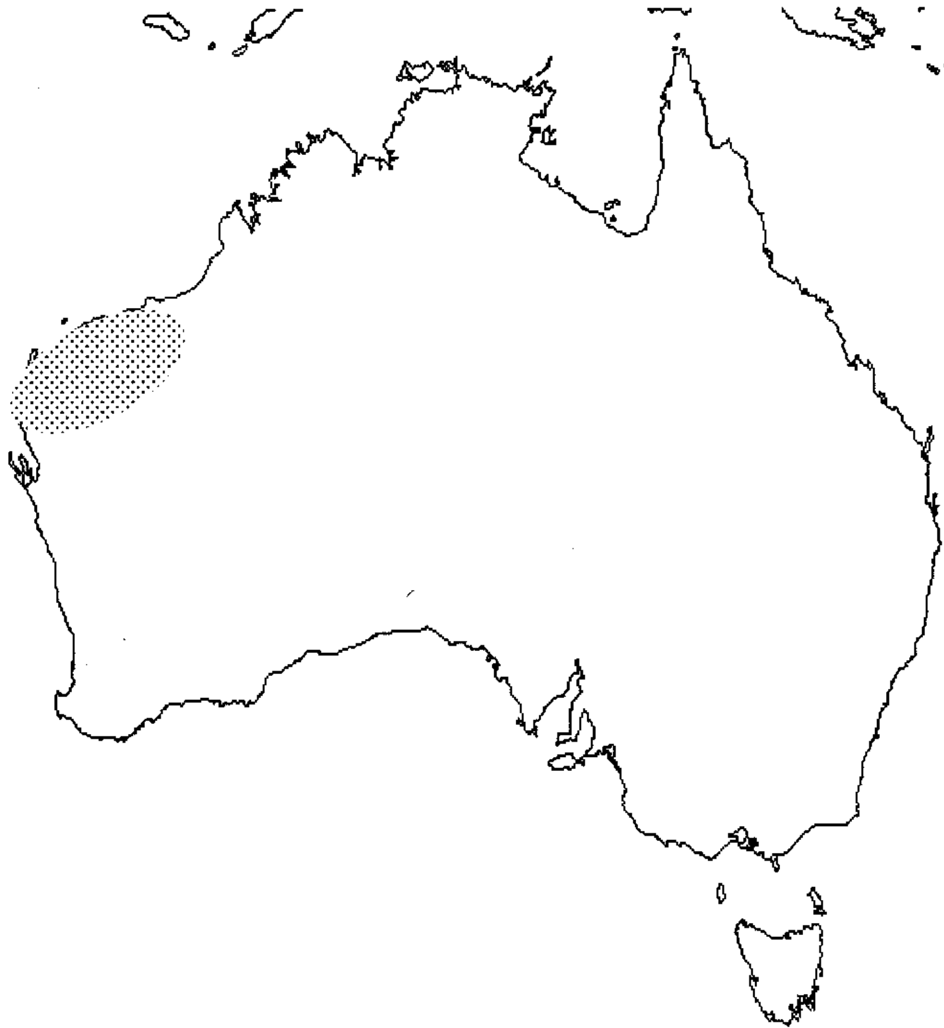

**FIGURE 2.13:** Distribution of *Acacia coriacea* subsp *pendens* (Courtesy of Bruce Maslin).

**FIGURE 2.14:** Phyllodes and Pods of *Acacia coriacea* subsp *sericophylla* (Courtesy of Bruce Maslin)

**FIGURE 2.15:** Flowers of *Acacia coriacea* subsp *sericophylla* (Courtesy of Bruce Maslin)

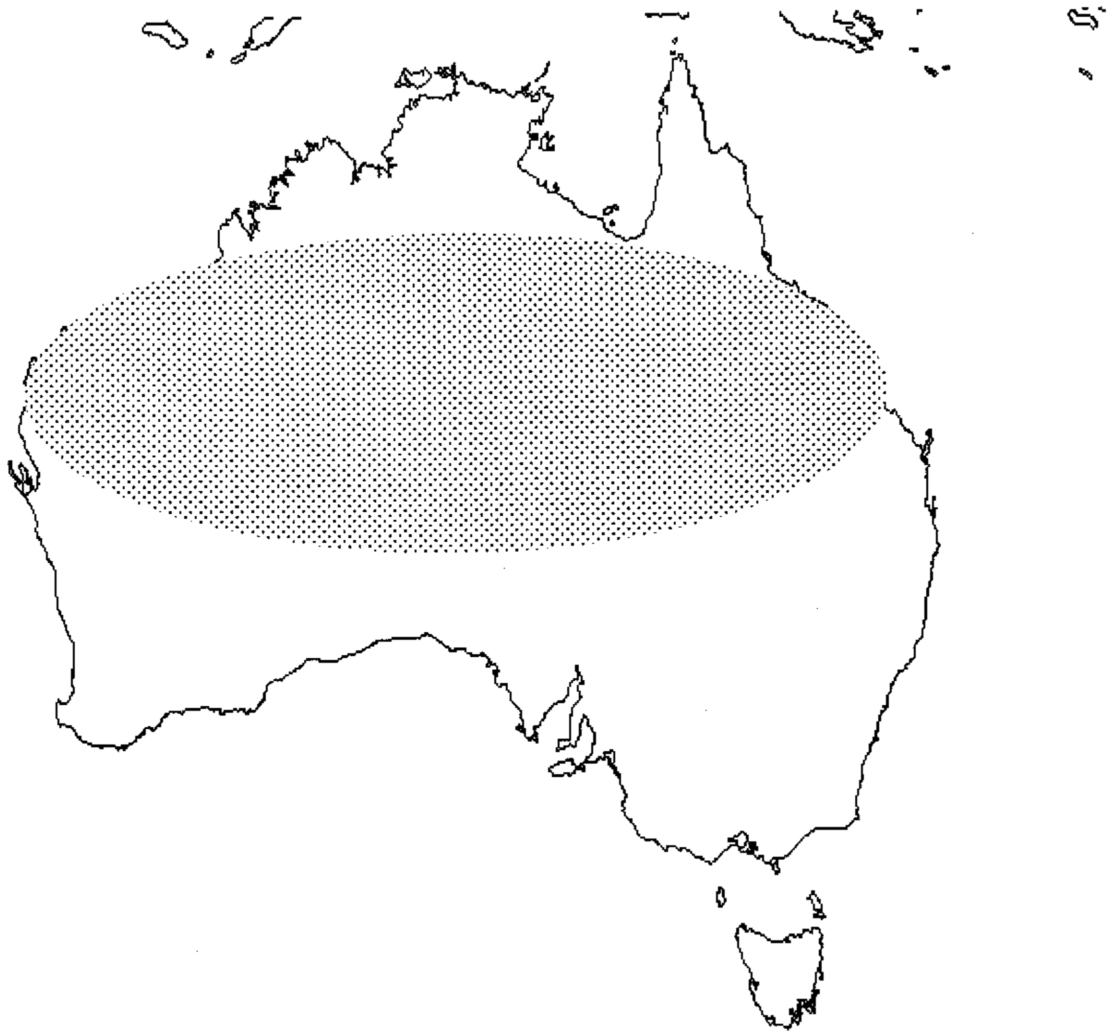

**FIGURE 2.16:** Distribution of *Acacia coriacea* subsp *sericophylla* (Courtesy of Bruce Maslin)

2.1.8 *Acacia dictophleba*

A glabrous shrub one to four metres high, *A.dictophleba* is widespread in the north and central arid zone where the species extends from the Pilbara region in Western Australia, eastwards through southern Northern Territory and north–eastern South Australia to south-west Queensland. *Acacia dictophleba* is particularly common in the Simpson Desert (Fig 2.17)

The species grows mainly in deep red or red-brown siliceous sand on dunes or interdunal areas and occasionally on shallow stony soils (Fig 2.18). The phyllodes are oblanceolate measuring 5 to 7cm long and 10 to 20 mm wide (Elliot and Jones, 1984). The phyllodes have two or three prominent longitudinal nerves, the secondary nerves forming a coarse open reticulum. The flowers of this species occur as either globular heads or obloid in shape, 9 to 13cm in diameter and bright yellow in colour (Elliot and Jones, 1984) (Fig 2.19).

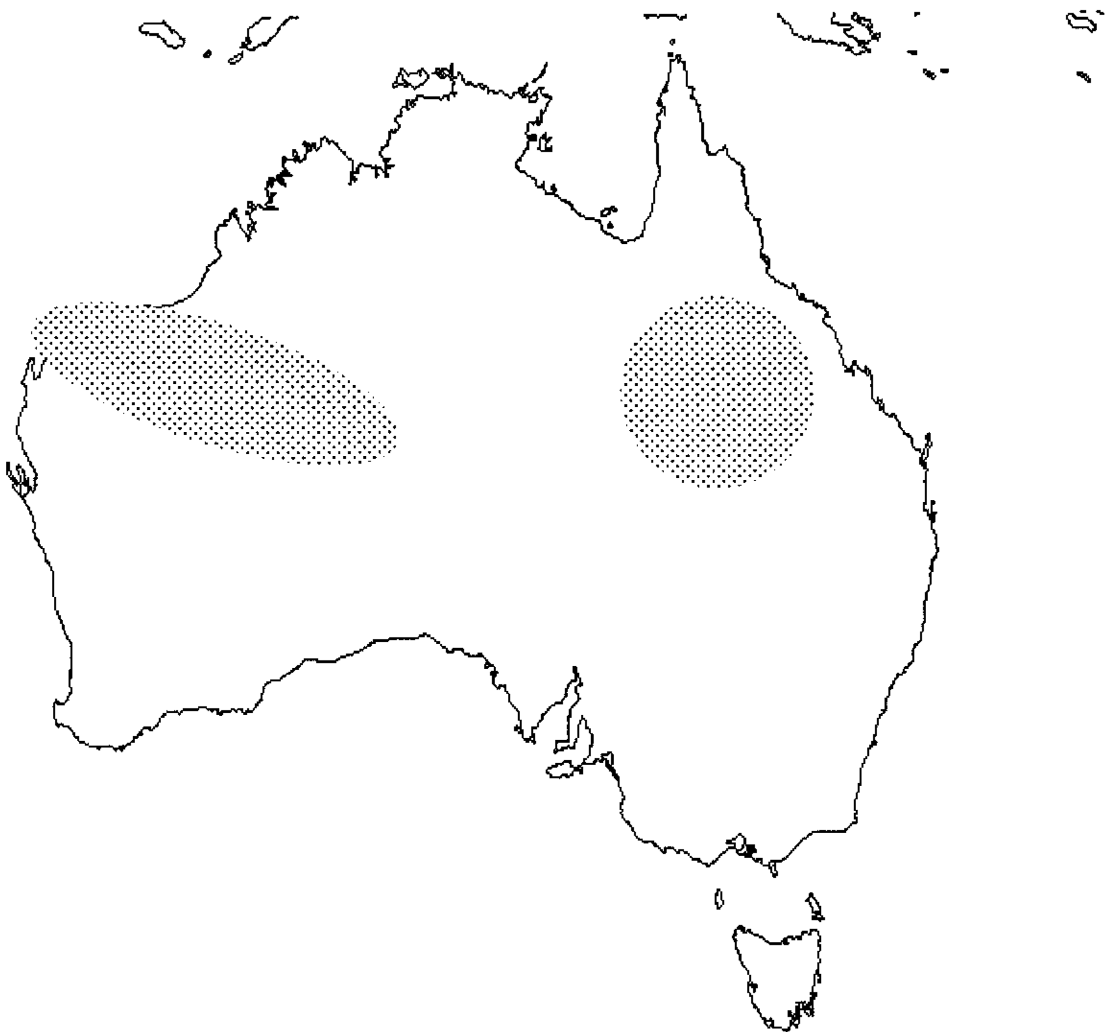

**FIGURE 2.17:** Distribution Map of *Acacia dictophleba* (Courtesy of Bruce Maslin)

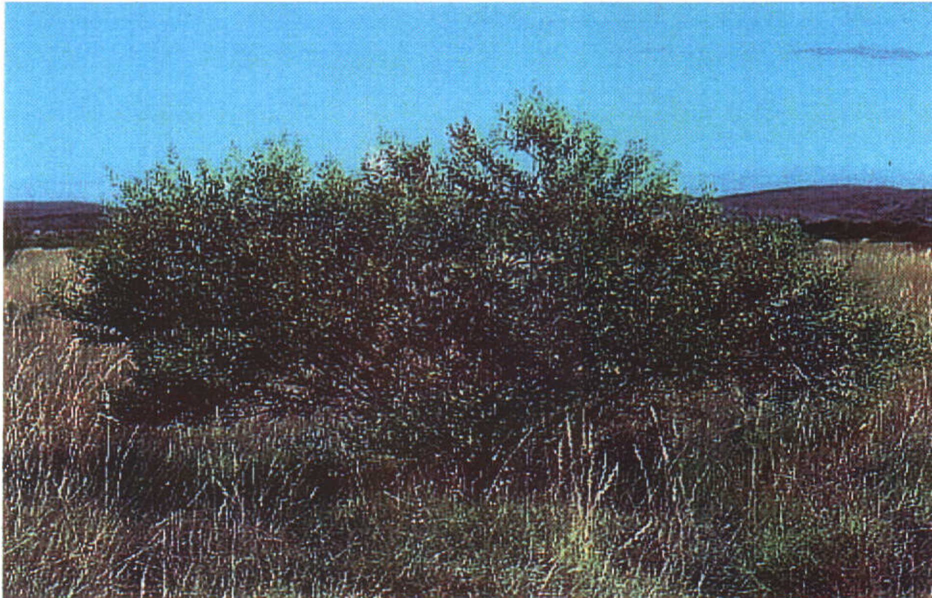

**Figure 2.18:** Growth form of *Acacia dictopleba* (Courtesy of Bruce Maslin)

**Figure 2.19:** Phyllodes and Flowers of *Acacia dictopleba* (Courtesy of Bruce Maslin)

### 2.1.9 *Acacia holosericia*

*Acacia holosericia* is a shrub or tree measuring three to eight metre in height. The phyllodes of this species are obliquely narrowly elliptic, measuring 10-20 centimetres long, and 2 to 5cm wide with an unequal base. The flowers of this species exist as rudimentary racemes with axes 0.5cm long, and are bright golden in colour (Elliot and Jones, 1984)(Fig 2.20). This species is often difficult to distinguish from related species (Maslin pers-comm).

*Acacia holosericia* is widespread in northern Australia occurring from near Derby, Western Australia through the Northern Territory to Rockhampton in Eastern Queensland. The species also occurs in other parts of the region including the Hammersley Ranges national park, parts of central Northern Territory in the South west of Queensland. *Acacia holosericia* grows in gravelly sand or loam and often form communities along watercourses (Cowan, 2001)(Fig 2.21) (Fig 2.22). This is a difficult species to identify due to the variations amongst individuals of the species (Maslin pers-comm).

**FIGURE 2.20:** Pods of *Acacia holosericia* (Courtesy of Bruce Maslin)

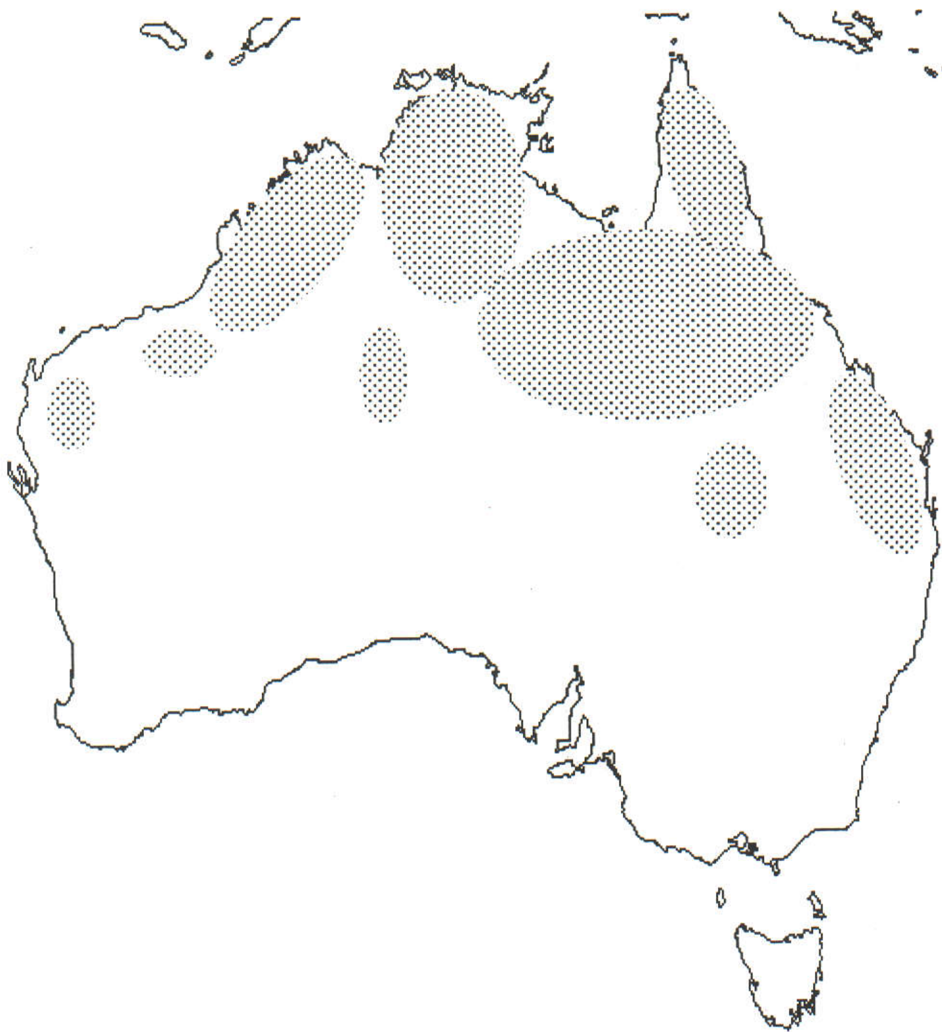

**FIGURE 2.21:** Distribution of *Acacia holosericia* (Courtesy of Bruce Maslin).

**FIGURE 2.22:** Growth form of *Acacia holosericia* (Courtesy of Bruce Maslin)

### 2.1.10 *Acacia inaequilatera*

*Acacia inaequilatera* occurs in the arid areas of Australia, ranging form the North-West cape east to Lake Disappointment, on Nerrima and Luluigui Stations in the Kimberley region (Fig 2.23). The species also occurs from near Balgo Station to the Blackstone ranges, Western Australia to the Davenport ranges in the Northern Territory. *Acacia inaequilatera* grows mainly in sand or sandy loam, often on rocky hills in tall shrub land with Spinifex ground cover.

A glabrous tree, growing to four metres high and sometimes to 8m, *A.inaequilatera* often has a gnarled appearance, due in part to the thick corky bark that this species posses. The phyllodes of the species are inaeqilaterally ovate to elliptic or obovate, sometimes obliquely orbicular and range in length from two to five centimetres (Elliot and Jones, 1984) (Fig 2.24). The flowers of this species are globular and are golden in colour when in bloom and are a distinctive purple when in bud (Fig 2.25).

**FIGURE 2.23:** Distribution map of *Acacia inaequilatera* (Courtesy of Bruce Maslin).

**FIGURE 2.24:** Growth form of *Acacia inaequilatera*

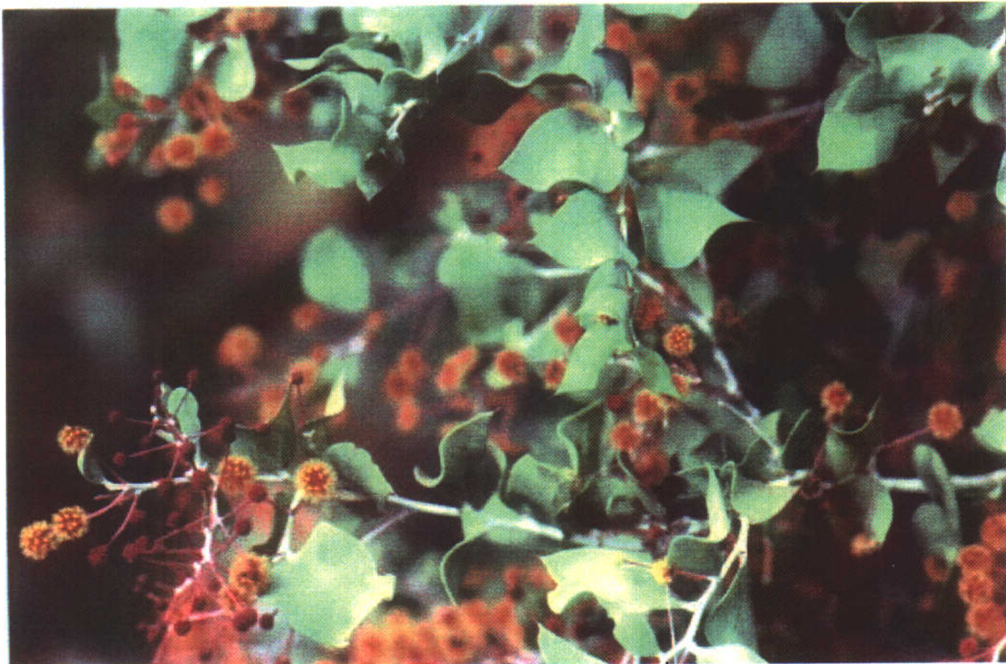

**FIGURE 2.25:** Flowers and phyllodes of *Acacia inaequilatera*

2.1.11 *Acacia pruinocarpa*

*Acacia pruinocarpa* occurs in the arid area of Western Australia and the Northern Territory, being distributed from the south side of the Millstream-Chichester National Park, and east to Tanami and Burt Plain, Northern Territory and the Mann ranges South Australia. The species grows in variety environmental conditions, usually favouring sand or loam, and is associated with *A.aneura* and spinifex (Fig 2.26,Fig 2.27). *Acacia pruinocarpa*, also know as Black Gidgee, is a shrub or tree growing up to 12 metres high (Fig 2.27). The phyllodes of this species normally spread widely and are linear to linear-elliptic, straight or curved, measuring seven to 17 cm long. The midrib and marginal veins are the most prominent in this species with the lateral leave obscure. The flowers of *Acacia pruinocarpa* are globular and exist either pairs or singly and are also bright yellow in colour (Fig 2.28).

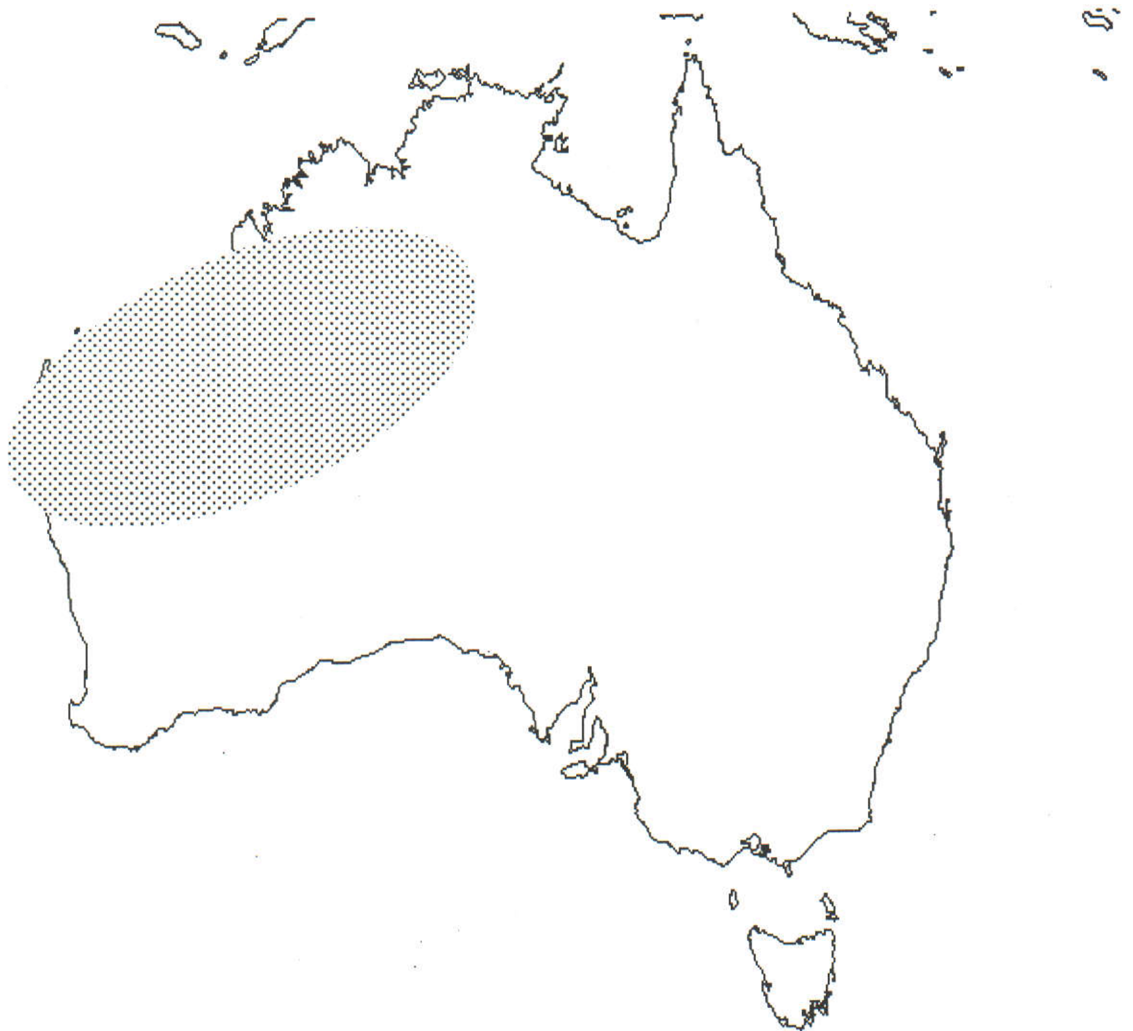

**FIGURE 2.26:** Distribution of *Acacia pruinocarpa* (Courtesy of Bruce Maslin)

**FIGURE 2.27:** Growth form of *Acacia pruinocarpa* (Courtesy of Bruce Maslin)

**FIGURE 2.28:** Flowers of *Acacia pruinocarpa* (Courtesy of Bruce Maslin)

## 2.2 Collection Sites

The species in this study were collected in and around the townsite of Newman (-23.683°S and 119.7314°E), located in the Pilbara Region of Western Australia by the Mulga Research Centre, Curtin University of Technology. *Acacia pruinocarpa* was however, also collected from the Field Trial Area, at Curtin University of Technology, Perth, Western Australia. The species were first identified by Associate Professor John Fox, from the Mulga Research Centre, and then confirmed by Bruce Maslin with the help of the Acacia CD-Rom. Each species were then vouchered, KW1-10 and placed in the Herbarium at the Department of Environmental Biology, Curtin University of Technology.

### 2.2.1 Soil Types

The soils surrounding the townsite of Newman and surrounding areas is mainly associated with the Hamersley and Opthamia Ranges. The soils of the area are mainly stony, shallow earthy loams (Um5.51, Uc5.11). These soils are associated with ranges of banded jaspilite and chert along with shales, dolomites and iron ore formations (Northcote *et al.*, 1968).

The soils surrounding the Curtin University collection site are known as Leached sand soils. These soils are leached sands with a compact layer below the bleach. This layer usually has either bright colours or pale colours below the surface (Northcote, 1968).
The soils of this region all have coarse textures and are usually uniform throughout the profile.

## 2.3 Rainfall And Temperature

The location of Newman, which is on the edge of the Great Sandy Desert in Western Australia, accounts for the large fluctuations in the temperature of the area. The average monthly temperature for Newman is approximately 28.2°C, with the highest average temperature being 46°C during January. The area also experiences seasonal rainfall with the highest rainfall figures being recorded for the warmer months of January and February, which also coincide with the wet season of the Northern Territory. The average yearly rainfall for Newman is approximately 285mm/year.

Curtin University of Technology is located in Bentley, Perth, Western Australia. The South West region of Australia experiences a Mediterranean environment with definite seasonal changes in temperature and rainfall. The rainfall of this collection site is much

higher than that of the previous collection site being 755mm/year. The average monthly temperature is also lower at 23.8°C.

# CHAPTER 3

# BRINE SHRIMP LETHALITY TEST

## 3.1 Introduction

The search for novel biologically active compounds often requires a screening program that employs a variety of bioassays (Colegate and Molyneux, 1993). The screening of medicinal agents is, however, often hampered by the lack of procedures that are simple, rapid and inexpensive. Most are complex and require vast amounts of money as well as specialised technicians. Other factors that can be considered critical to the testing procedure is determining whether or not the bioassays chosen for a particular screening program are appropriate. This is especially true where tests for cytotoxicity are concerned. To eliminate such problems in these sorts of bioassays, the simplest biological response to cytotoxicity is lethality. This is used as an indicator of toxicity because it has only two criteria, dead or alive.

The brine shrimp lethality test (BSLT) is an excellent example of such a screening procedure. This bioassay is simple, rapid and inexpensive to run (Sam, 1993). Furthermore, it tests for general toxicity without specialisation and is therefore essential as a preliminary bioassay in the study of biologically active compounds.

In fact, the BSLT has led to the isolation of a number of different and interesting compounds (Sam, 1993; McLaughlin and Rogers, 1998). The depsipeptide antibiotic, beauvericin, for example, was first isolated from *Beauveria bassiana* using the BSLT as a guide to the fractionation procedure. Beauvericin is a specific cholesterol acyltransferase inhibitor (Logrieco *et al.*, 1998), but has also been shown to cause apoptosis and cytolysis in mammalian and turkey white blood cells (Logrieco *et al.*, 1998). Other compounds that were isolated include some from *Eupatorium odoratum*, which was once considered to be non-toxic. *Cerbera odollam*, an ornamental plant from which cerberin and neriiflolin were isolated. Both of these cardiac glycosides were isolated and shown to have a relatively high toxicity (Sam, 1993). McLaughlin and Rogers (1998) have, reported that the use of the BSLT has led to the discovery and isolation of over 300 novel pesticidal and anti tumour compounds, in particular the annonaceous acetoginins, which is a new class of natural pesticides.

The test organism in the BSLT is the brine shrimp, *Artemia salina*. *Artemia salina* is a crustacean and a member to the sub order Branchipoda of the order Anostraca (Sam, 1993). This species has a worldwide distribution and occurs in bodies of water ranging from the brackish to the ultra-saline. Its high tolerance to salinity makes the organism relatively easy to culture and to study.

There is, however, a large variation in the growth forms of *Artemia salina.* This may result from the wide range of environmental conditions that the organism can tolerate. The life cycle of the species remains the same, however (Sam 1993). By often inhabiting water systems, *Artemia salina* experiences seasonal evaporation causing the organism to adapt through producing diapaused (inactive) eggs that can survive low to zero water levels. As long as the eggs remain dehydrated, they have a high resistance to extreme conditions and can be stored for long periods of time. This is one of the many positive attributes that make this species ideal as a test organism for bioassays of this type.

Once hatched, the eggs absorb water thus initiating embryogenesis. This occurs between 16 to 36 hours after immersion. The embryo emerges from the egg encased in the hatching membrane, but soon develops antennae and mandibles. These enable the organism to break away from the membrane and to develop into a free-swimming nauplii larvae. It is at this stage that it is ready for use in the bioassay.

There are a number of parts to this study. The main aim of the experiment was to determine whether *Acacia* species have the potential to be a new source of cytotoxic agents. The experiment was also to determine the potential of each species to be developed as pesticidal agents.

## 3.2 Materials And Methods

### 3.2.1 Preparation Of Plant Material

Eight different *Acacia* species were collected in 2001 and 2002 from sites around Newman in the Pilbara region of Western Australia and from the Field Trial Area of Curtin University of Technology. The phyllodes of each species were removed from the stems and dried at 50°C for 48 hours. The leaves were then ground into a fine powder using a vegetative grinder (Dietz-Motoren KG, Eleckromotorenfragrik, West Germany, 220V, type WRB 80C120QSIL). The powdered plant material was used to prepare four extracts according to extraction procedures routinely employed by Professor Emilio Ghisalberti at the Chemistry Department of the University of Western Australia. Firstly, hexane, dichloromethane and methanol were used to generate three extracts (Fig 3.1). Each having varying levels of water solubility. Hexane removes the fat-soluble compounds, while dichloromethane removes the partially fat soluble compounds. Methanol, which is polar, removes the water- soluble compounds. All extractions were dispensed in to 5ml vials, after which they were frozen and used as required.

The final extraction was performed with deionised water. For each of the eight species, a total of 50g of leaf powder was dissolved into 50mL of deionised water. This was then filtered using Whatman filter paper, leaving only the water soluble compounds in solution. Since this bioassay requires water- soluble extracts, only the methanol and aqueous extracts were used. When the methanol extracts were used, they were first evaporated to remove the methanol and then redissolved in deionised water.

All eight of the *Acacia* species were screened for chronic toxicity over a 24 hour period.

The values produced in this study were compared to those reported by Sam (1993) for potassium dichromate ($K_2C_2O_7$). This was to establish a relative toxicity for each of the extracts. The extracts were then converted into parts per million (ppm) by following the protocol mentioned by (Sam, 1993).

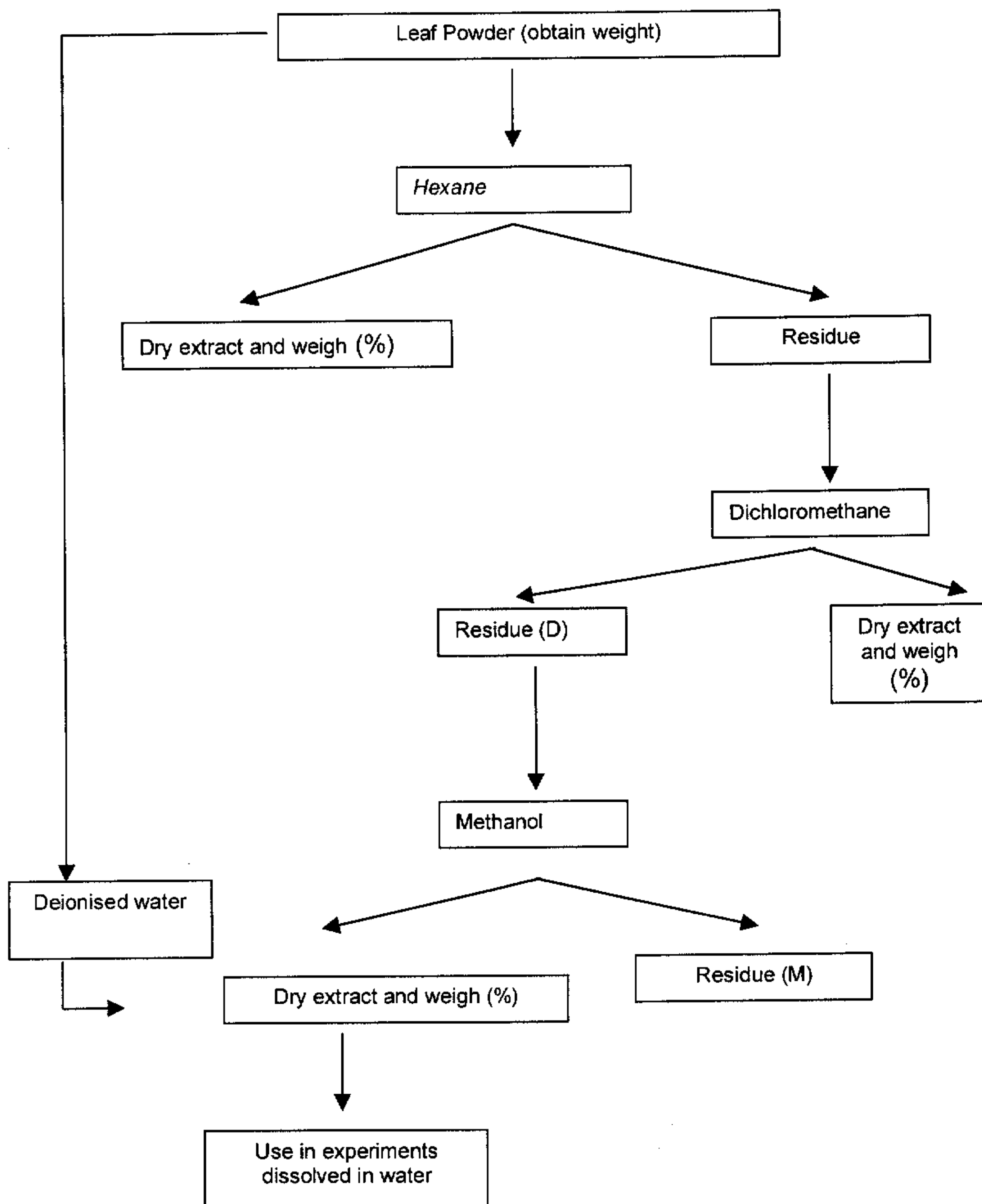


**FIGURE 3.1:** Extraction Procedure of *Acacia* species (recommended by Professor Emilio Ghisalberti, Department of Chemistry, University of Western Australia)

### 3.2.2 Culture Of *Artemia salina*

For this study, the eggs of *Artemia salina* were obtained from Dr Mick Payne and Dr Rob Rippengale, Department of Environmental Biology, Curtin University of Technology, Western Australia. Diapaused eggs were rehydrated in 20% seawater (v/v DI $H_2 0$) at 25°C and were constantly aerated for one hour. These eggs were then immersed into 4% sodium hypochlorite for seven minutes to decapsulate the eggs, followed by a single rinse in water to remove any traces of the bleach. The eggs were then returned to 20% seawater for 24 hours, during which time the majority of organisms had hatched and these were the nauplii larvae were used in this test.

A number of concentrations (dependent an availability) of the aqueous extracts of the eight *Acacia* species were prepared using 20% seawater and then transferred to 5mL plastic vials with six replicates for each concentration. The nauplii of ten brine shrimp were then transferred to each vial and left at 25°C for 24 hours with a control group of only 20% seawater. After 24 hours, the $LC_{50}$ was determined (chronic toxicity) using the Reed-Muench method (Sam, 1993). This is where the accumlated deaths and accumulated survivors are plotted on the same set of axes. The $LC_{50}$ value is calculated as the value where the two lines of the graph intersect. Their mortality, accumulated deaths and accumulated live numbers where also determined (Table 3.1).

## 3.3 Results

### 3.3.1 Preliminary Results

All eight species exhibited some cytotoxicity, but only those that were active below 400ppm were considered worth further evaluation. This is in accordance with other laboratories (Sam, 1993). *Acacia pruinocarpa* was the most toxic species tested. The highest mortality for the compounds from the phyllodes of this species was 4.2% at 3.71ppm, which was significantly lower than that of the other species (Table 3.1). The $LC_{50}$ was 100ppm, which was well below 400ppm (Fig 3.2)(Fig 3.10).

In contrast, *A. inaequilatera* was one of the least toxic species tested, yielding an $LC_{50}$ of 8912 ppm (Fig 3.3). The mortality rate of this species was also low with only the higher doses of 14675 ppm and 7337.5 ppm yielding 100% mortality at 24 hours (Table 3.1).

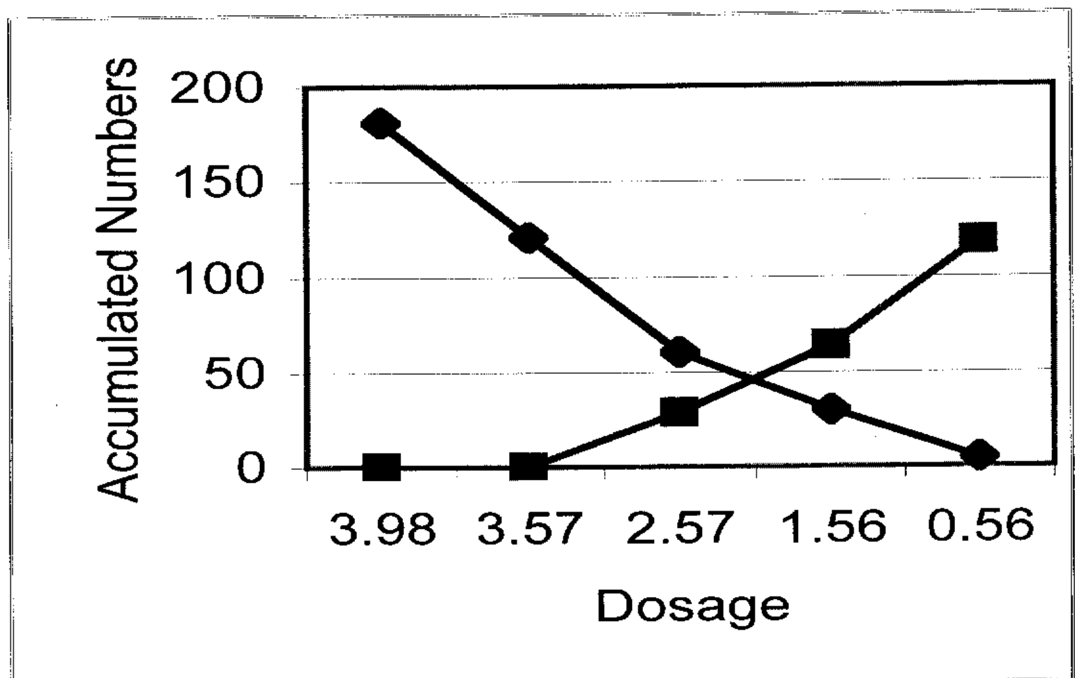


**FIGURE 3.2:** Graphs used to estimate the $LC_{50}$ of *Acacia pruinocarpa*

**TABLE 3.1:** Mortality of *Artemia salina* when exposed to eight *Acacia* species

| Species | Conc (ppm) | Dosage | Dead | Alive | Accumulated Dead | Accumulated Alive | Mortality (%) |
|---|---|---|---|---|---|---|---|
| *A.adsurgens* | 4030 | 3.605 | 60 | 0 | 152 | 0 | 100 |
| | 1612 | 3.207 | 60 | 0 | 92 | 0 | 100 |
| | 161.2 | 2.207 | 25 | 35 | 32 | 35 | 0 |
| | 16.12 | 1.2 | 7 | 53 | 7 | 88 | 0 |
| *A.aneura* | 2000 | 3.3 | 60 | 0 | 263 | 0 | 100 |
| | 1000 | 3 | 60 | 0 | 203 | 0 | 100 |
| | 500 | 2.69 | 60 | 0 | 143 | 0 | 100 |
| | 250 | 2.4 | 48 | 12 | 83 | 12 | 87 |
| | 200 | 2.3 | 35 | 25 | 35 | 37 | 48 |
| | 20 | 1.3 | 0 | 60 | 0 | 97 | 0 |
| *A.auriculformis* | 5100 | 3.7 | 60 | 0 | 65 | 0 | 100 |
| | 2040 | 3.309 | 5 | 45 | 4 | 45 | 8 |
| | 240 | 2.38 | 0 | 60 | 0 | 105 | 0 |
| | 24 | 1.38 | 0 | 60 | 0 | 165 | 0 |
| *A.bivenosa* | 8500 | 3.93 | 50 | 0 | 147 | 0 | 100 |
| | 6200 | 3.79 | 50 | 0 | 97 | 0 | 100 |
| | 4600 | 3.66 | 35 | 15 | 47 | 15 | 75.8 |
| | 3000 | 3.47 | 12 | 38 | 12 | 53 | 18.5 |
| *A.coriacea* | 4000 | 3.6 | 60 | 0 | 138 | 0 | 100 |
| | 2000 | 3.3 | 60 | 0 | 78 | 0 | 100 |
| | 1000 | 3 | 18 | 42 | 18 | 42 | 42.8 |
| | 500 | 2.69 | 0 | 60 | 0 | 102 | 0 |
| *A.dictopleba* | 3700 | 3.57 | 60 | 0 | 135 | 0 | 100 |
| | 370 | 2.56 | 60 | 0 | 75 | 0 | 100 |
| | 37 | 1.56 | 15 | 45 | 15 | 45 | 25 |
| | 3.7 | 0.56 | 0 | 60 | 0 | 105 | 0 |
| *A.inaequilatera* | 14675 | 4.16 | 60 | 0 | 173 | 0 | 100 |
| | 7337.5 | 3.865 | 60 | 0 | 113 | 0 | 100 |
| | 3668 | 3.564 | 25 | 35 | 53 | 35 | 58 |
| | 1834.4 | 3.26 | 28 | 32 | 28 | 67 | 29 |
| | 917.18 | 2.96 | 0 | 60 | 0 | 127 | 0 |
| *A.pruinocarpa* | 9280 | 3.98 | 60 | 0 | 181 | 0 | 100 |
| | 3715 | 3.57 | 60 | 0 | 121 | 0 | 100 |
| | 371.5 | 2.57 | 31 | 29 | 61 | 29 | 67 |
| | 37.15 | 1.56 | 25 | 35 | 30 | 64 | 31.9 |
| | 3.71 | 0.56 | 5 | 55 | 5 | 119 | 4.2 |

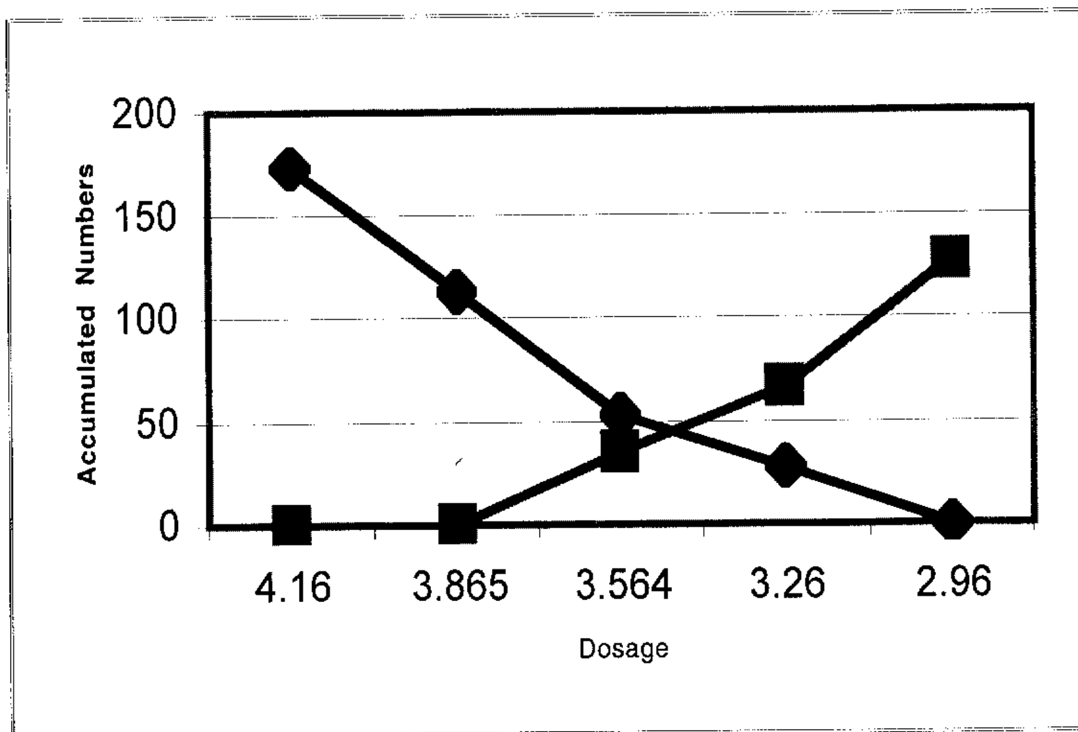


**FIGURE 3.3:** Graphs used to estimate the $LC_{50}$ of *Acacia inaequilatera*

As far as the other species were concerned, the second most promising species was *A.adsurgens*, followed by *A.dictopleba*, *A.aneura*, *A.auriculoformis*, *A.coriacea*, and *A.bivenosa*.

*Acacia adsurgens* produced an effect at concentrations as low as 16.12 ppm (Table 3.1). The mortality of this species was one of the highest also yielding 100% mortality at the higher doses and only showing a decrease at the lower doses. *A.adurgens* yielded an $LC_{50}$ of 251ppm (Fig 3.4).

*Acacia dictophleba* was active at concentrations as low as 37 ppm (Table 3.1). The mortality induced by this species was also high, yielding 100% mortality at concentrations of 3700 ppm and 370 ppm. This decreased to 25% at 37 ppm. This species also resulted in one of the lowest $LC_{50}$ at 158 ppm (Fig 3.5).

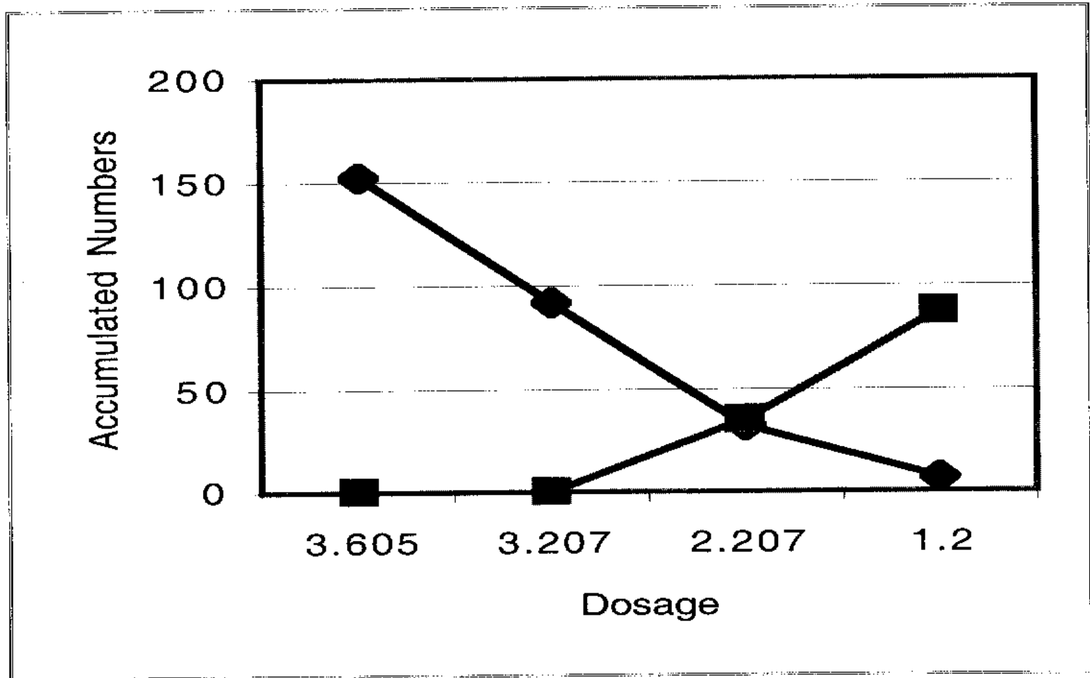


**FIGURE 3.4:** Graphs used to estimate the $LC_{50}$ of *Acacia adsurgens*

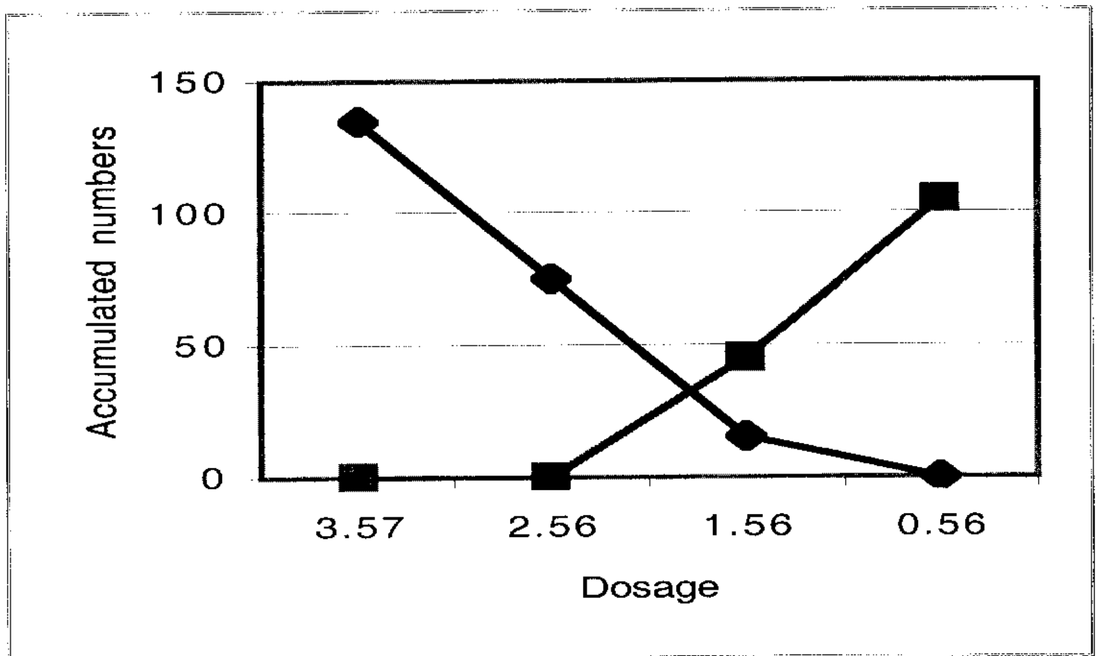


**FIGURE 3.5:** Graphs used to estimate the $LC_{50}$ of *Acacia dictophleba*

Where *Acacia aneura* was concerned, its $LC_{50}$ was 200 ppm (Table 3.1). The mortality of the species was high with the extract yielding 100% mortality at concentrations as high as 2000ppm (Table 3.1). *A. aneura* yielded an $LC_{50}$ of 562 ppm (Fig 3.6).
*Acacia auriculoformis* yielded high mortality only at the higher concentrations (Table 3.1). The mortality followed the same trend as the concentration exhibiting 100% mortality only at the highest concentration (5100 ppm). This decreased to 8% at the next lower concentration. The $LC_{50}$ exhibited by this species was 1259 ppm after 24 hours exposure (Fig 3.7).

*Acacia coriacea* was the second least toxic species. Its $LC_{50}$ value was 2113 ppm (Fig 3.8). The lowest concentration of the extract to produce an effect was 1000 ppm (Table 3.1). As with other extracts, the mortality induced by this species was 100% at the higher concentrations and decreased rapidly with a decrease in the concentration (Table 3.1).

*Acacia bivenosa's* $LC_{50}$ after 24 hours exposure was 3890 ppm (Fig 3.9). The species yielded and effect on the brine shrimp only at concentrations of 3000 ppm or above. The mortality observed in this species was higher than that of *A.bivenosa*, yielding 100% mortality at the higher concentrations. This decreased significantly with a decrease in concentration (Table 3.1).

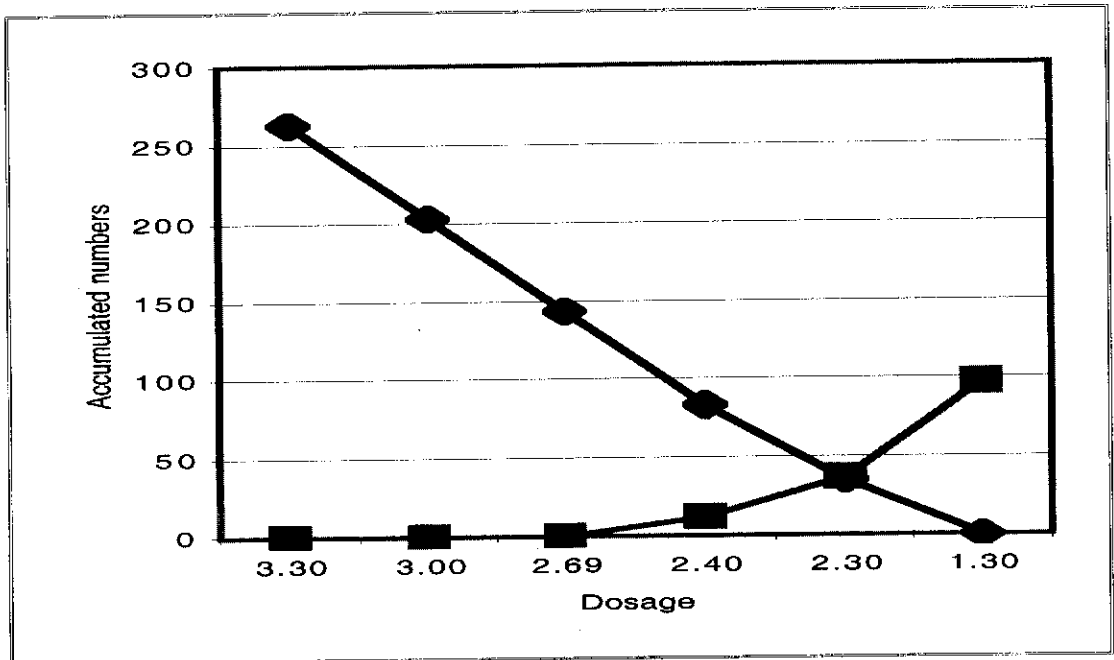


**FIGURE 3.6:** Graphs used to estimate the $LC_{50}$ of *Acacia aneura*

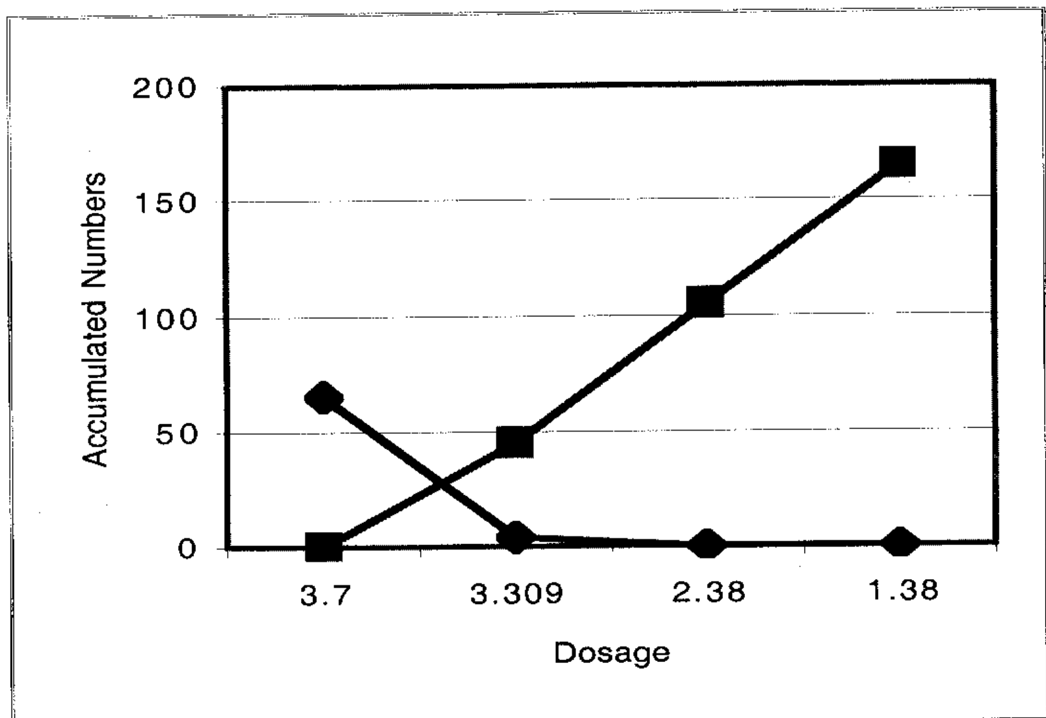


**FIGURE 3.7:** Graphs used to estimate the $LC_{50}$ of *Acacia auriculoformis*

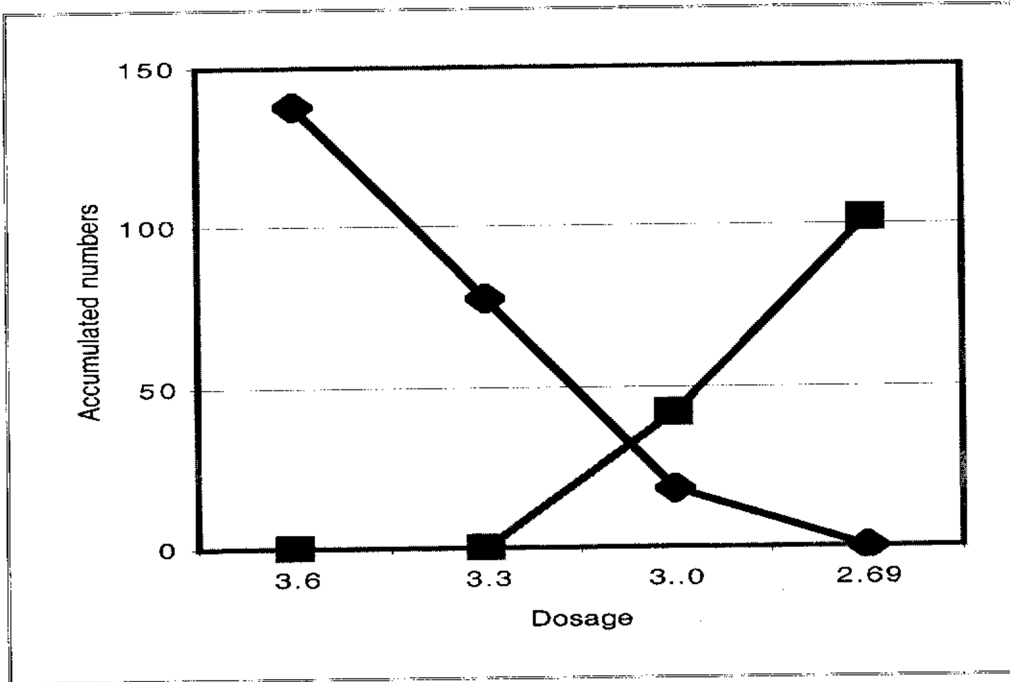


**FIGURE 3.8:** Graphs used to estimate the $LC_{50}$ of *Acacia coriacea.*

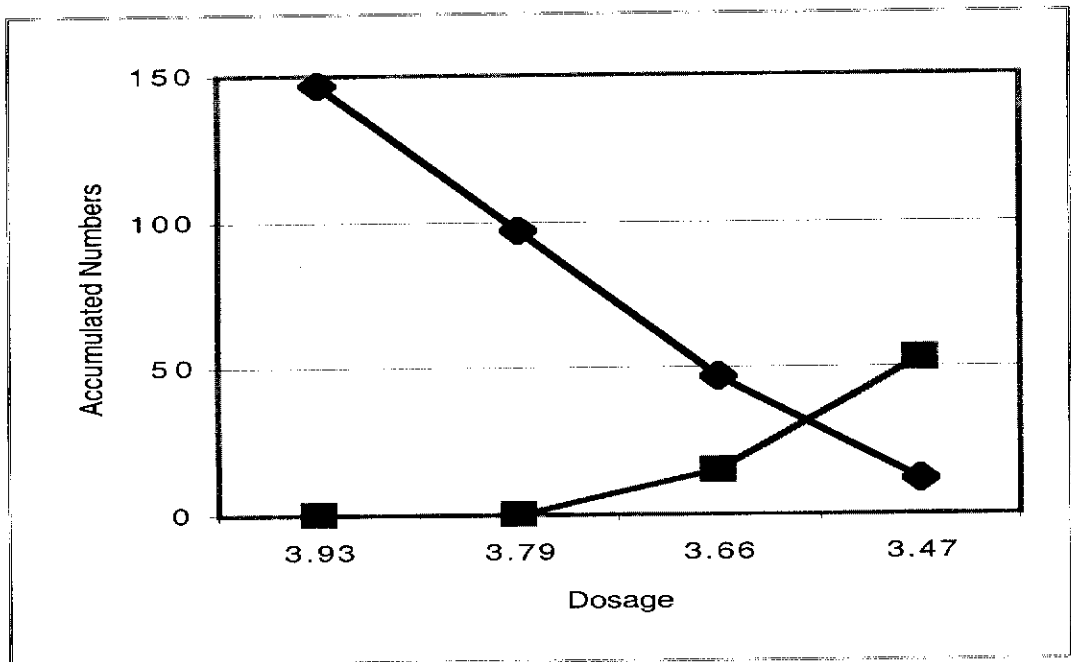


**FIGURE 3.9:** Graphs used to estimate the $LC_{50}$ of *Acacia bivenosa*

Since *A. pruinocarpa* was considered the most cytotoxic, further tests and analysis were perfomed on this species. The median lethal concentration was examined for *A. pruinocarpa*. This estimates the $LC_{50}$, but also allows the calculation of the standard error (Sam, 1993). The median lethal toxicity at 50% was 371ppm while the median lethal toxicity for 25% was 36.3ppm.

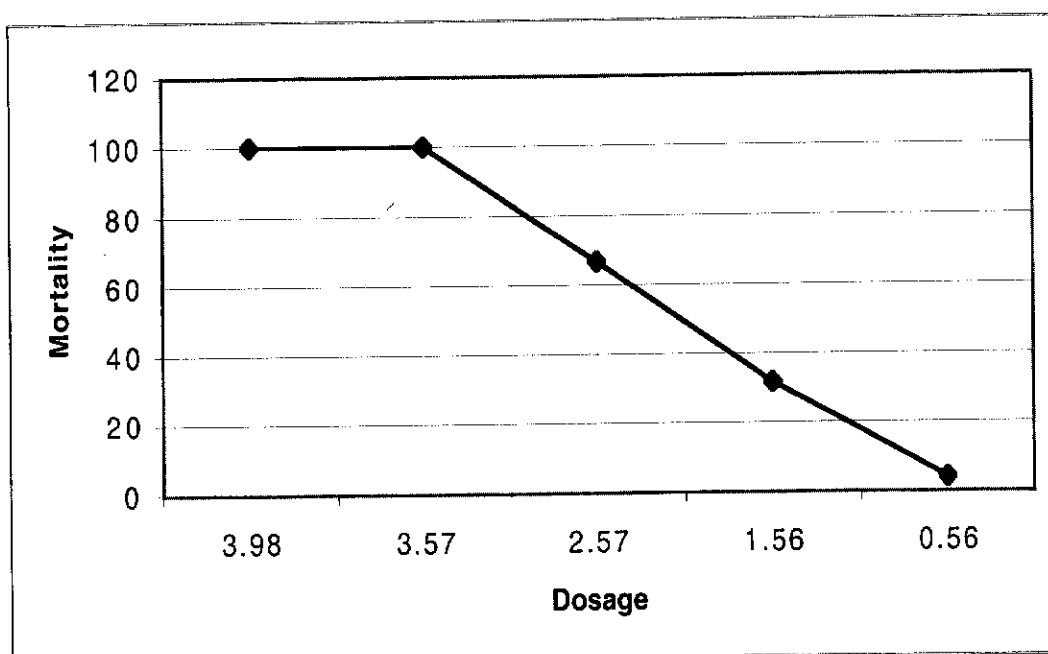


**FIGURE 3.10:** Estimation of the $LC_{50}$ and standard error of *Acacia pruinocarpa* through plotting mortality against dosage. (Median Lethal toxicity 25-75%)

Since tannins are well known in *Acacia* species, it was hypothesised that tannins may have played a role in the cytotoxicity observed. The tannin levels for each of the eight species has either been reported in the literature or was determined in this study. A total of 5g of phyllode powder was obtained from each of the species. The powdered plant material was then boiled with deionised water, filtered using whatman filter paper (No. 1) and made up to 10ml. Folin-Denis reagent was then added to the resulting solution, which was screened in a spectrophotometer set at 760nm to measure the absorbancy. The absorbancy was compared to tannic acid standards, which were prepared using Sigma tannic acid powder and deionised water (Table 3.2).

*Acacia ancistrocarpa* resulted in the highest level of tannins in the species tested in this study. Up to 5% of the dry weight of the phyllode is tannins (Table 3.2). This was followed by *A.holosericia* with 4% then *A.adurgens A.pruinocarpa, A.dictopleba, A.inaequilatera, A.coriacea, A.auriculoformis*. Both *Acacia bivenosa* and *A.aneura* tested negative for tannins (Table 3.2).

**TABLE 3.2:** Tannin levels in the *Acacia* species used in this study (the * denotes tannin levels determined in this study)

| SPECIES | TANNINS (%) | SOURCE |
|---|---|---|
| *Acacia aneura* | 0 | Barr, 1993 |
| *Acacia adsurgens* | 3 | * |
| *Acacia ancistrocarpa* | 5 | * |
| *Acacia auriculoformis* | 1 | Barr, 1993 |
| *Acacia bivenosa* | 0 | * |
| *Acacia coriacea* | 1.2 | * |
| *Acacia dictopleba* | 1.8 | * |
| *Acacia holosericia* | 4 | Barr, 1993 |
| *Acacia inaequilatera* | 1.5 | * |
| *Acacia pruinocarpa* | 2 | * |

### 3.3.2 Polyamide Column Results

A polyamide column was used in this study to remove the tannins from the extracts of *A. pruinocarpa*, since the species had yielded the most promising $LC_{50}$ results of all the species tested, and having a relatively high level of tannins, was fractionated using the polyamide column. This was achieved by placing ICN polamide powder into a measuring cylinder with a one- way valve.

The powdered polyamide was rinsed with analytical reagent grade methanol, after which, the methanol extract of *A.pruinocarpa* was then passed through the powder. The new tannin-free extract was then re-tested using the BSLT. This test produced interesting results, with the toxicity of *A.pruinocarpa* exhibiting a higher $LC_{50}$ than that of the first test (Table 3.3).

**TABLE 3.3:** Mortality of *Artemia salina* when exposed to *Acacia pruinocarpa* following the Polyamide Column.

| DOSE (ppm) | Log Dose | Dead | Alive | Accumulated Dead | Accumulated Alive | Mortality (%) |
|---|---|---|---|---|---|---|
| 532 | 2.72 | 60 | 0 | 68 | 0 | 100 |
| 266 | 2.42 | 4 | 56 | 8 | 56 | 12.5 |
| 133 | 2.12 | 3 | 57 | 4 | 113 | 3.4 |
| 66.5 | 1.82 | 1 | 69 | 1 | 182 | 0.55 |

With the tannins removed, the *A. pruinocarpa* phyllode extract resulted in an $LC_{50}$ value of 478 ppm, which was four times higher than previous tests (Fig 3.11). The average total mortality (100%) was 532 ppm.

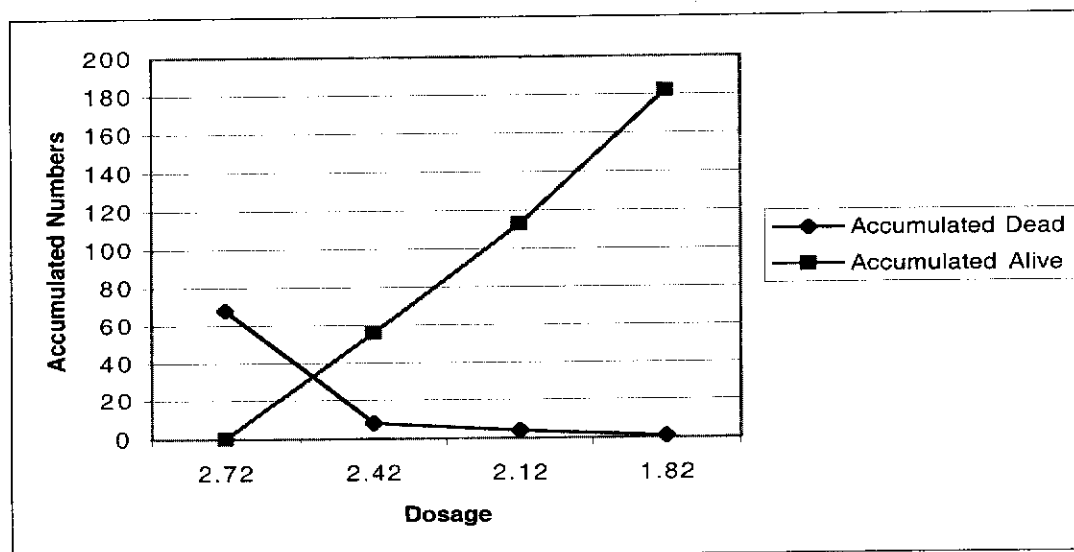


**FIGURE 3.11:** The estimation of the $LC_{50}$ value of *Acacia pruinocarpa* after fractionation with the polyamide column

The median lethal concentration was also determined for tannin-free *A.pruinocarpa* in this test. The median lethal toxicity for 50% was approximately 540ppm, while the median lethal concentration at 25% being 450ppm (fig 3.12)

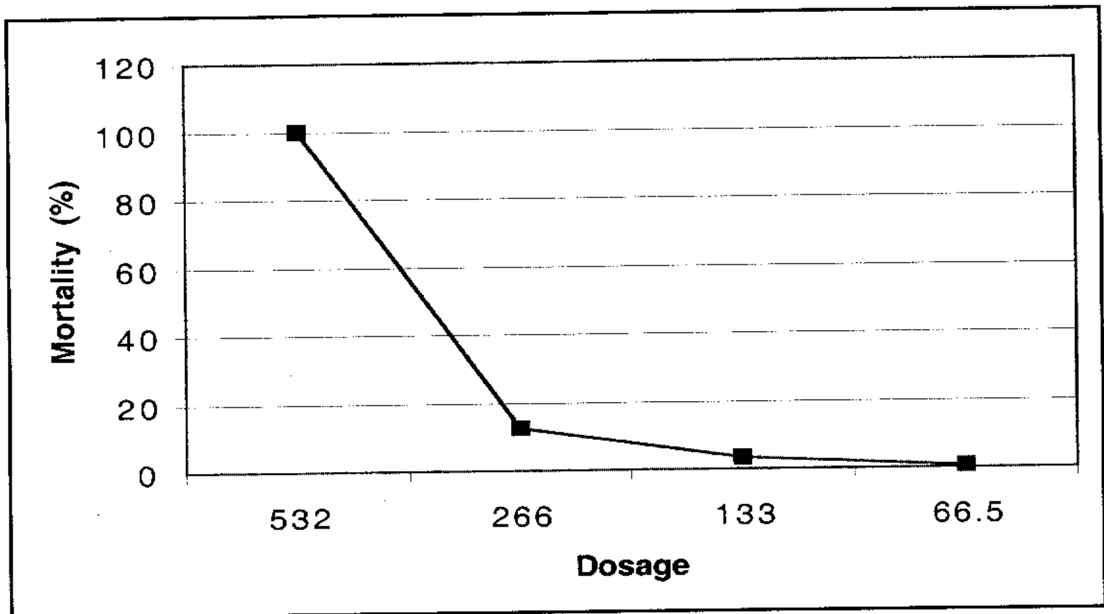


**FIGURE 3.12:** Estimation of the $LC_{50}$ and standard error of *Acacia pruinocarpa* through plotting mortality against dosage. (Median lethal toxicity 25% -75%)

### 3.3.3 Brine Shrimp Lethality Test And Tannic Acid

The effects of tannic acid were also determined using the BSLT. These results corresponded to the same sort of levels of tannins seen in the *Acacia* species. Tannic acid solutions of 0.5%, 1%, 2%, 4% and 8% were prepared using Sigma tannic acid powder dissolved in 100 ml of deionised water.

The brine shrimp were then added to solutions as previously described (Section 3.2.2). All of the preparations were highly toxic with all the organisms were dying within 24 hours.

## 3.4 Discussion

The search for novel biologically active compounds has intensified over the years, with many industries ranging from agriculture to medicine interested in finding chemicals useful to them. This is especially true where resistance to chemicals has become a major concern, eg. resistance to pesticides in agriculture. The BSLT is ideal to screen chemicals for pesticides and cytotoxic agents. It satisfies all the criteria for such a test. It is rapid, inexpensive and requires little specialisation. This test also enables rapid testing where researchers are interested in substantiating claims about plants, such as Indigenous claims regarding medicinal plants.

The BSLT is routinely used in the detection of both cytotoxicity and pesticidal activity. The BSLT is a relatively simple test that is considered to be essential to the preliminary screening of plants for biological activity (Sam, 1993). The BSLT has been in use since 1956 ( Sam, 1993) and has been responsible for the discovery of a number of novel biologically active compounds.

There are a number of advantages to using the BSLT as a preliminary test for biological activity. Aside from fulfilling the criteria of a front-line bioassay, the BSLT has been reported by McLauglin and Rogers (1998) as being superior or equally as accurate as the three in-vitro solid tumour cell lines used in their study. It has also been reported as being the most accurate, giving no false positives and very few false negatives (McLaughlin and Rogers, 1998). There are however a number of disadvantages to using the BSLT. Firstly, the BSLT is not selective by chemical type, it gives general toxicity (McLaughlin and Rogers, 1998). Sam (1993) has reported that there is no single protocol for the determination of lethatlity to *Artemia salina* accepted as a standard, and there is insufficient data to correlate the toxicity of samples to *Artemia salina* with any relevant type of activity such as antimicrobial and cytotoxic activity. Despite these shortcomings, the BSLT is still a very useful test and has resulted in the discovery of novel pharmaceuticals. Moreover, it had been useful in this study, highlighting that *Acacia* species do exhibit biological activity.

All of the *Acacia* species tested exhibited some biological activity when exposed to *Artemia salina*. One species however, demonstrated significant activity, *A.pruinocarpa*. It also appeared that *A.adurgens* and *A.dictopleba* were also useful and worthy of further evaluation.

*Acacia pruinocarpa* exhibited activity at concentrations as low as 3.7 ppm, which when compared to the potassium dichromate standard used by Sam (1993), is considered to be toxic to *Artemia salina*. Initially, this activity was hypothesised to be due to the tannin content of the extract, as tannins are known to exhibit biological activity. For example, Fukuchi *et al* (1989) and Courthout *et al* (1988) have reported that the polyphenolic tannins exhibited activity when tested on the *Herpes simplex* virus. It has also been reported that another group of tannins, the gallo- and ellagitannins have an affect on the HIV reverse transcriptase (Laekman *et al*., 1986). This was supported to some degree after the extract was fractionated through the use of a polyamide column. After the fractionation, *A.pruinocarpa* appeared to have lost potency, which could suggest that tannins were responsible for the activity.

A number of *Acacia* species have been reported by Seigler (2002) and Barr (1993) to contain tannins. The tannin content of *Acacia* species is varied (Barr 1993) and ranges from 0% in *A.aneura* to 12% as in *A.estrophiolata*. The level of tannins in *A. pruinocarpa* is moderate (2%; table 3.2). Tannic acid, when tested on the BSLT, was more toxic. The mortality rate was 100% at concentrations as low as 1.0 ppm. It is, therefore, possible that there is another compound responsible for the cytotoxicity exhibited by *A. pruinocarpa*, which may also have been removed by the polyamide column. This does occur, especially when fractionation is through polyamide columns (Ghisalberti pers-comm).

Seigler (2002) has also reported that *Acacia* species contain other secondary metabolites that have the ability to exhibit biological activity. These include alkaloids, saponins, both of which *A.pruinocarpa* has tested positive for (Appendix 2). The species also produces terpenes and essential oils (Seigler, 2002). It is unlikely that the essential oils would have been responsible for the initial activity, as they would have been removed in the extraction procedure. Saponins, alkaloids and terpenes have the ability to exhibit biological activity. Compounds such as these have not been looked for in *A. pruinocarpa* (Barr 1993). The level of response of these compounds for the initial activity is therefore pure speculation and more research is required to isolate the compound responsible for the activity.

In conclusion, the effects of *A.pruinocarpa* on the BSLT suggests that it has potential as a cytotoxic agent and pesticide. However, more work is required along with more advance bioassays to resolve this issue. It is worthy of further investigation and may yield interesting cytotoxic agents. Since the species is readily available and can be cultivated, it may offer some hope in the fight against chemical resistance and certain cancers.

# CHAPTER 4

# CROWN GALL TUMOUR ASSAY

## 4.1 Introduction

The Crown Gall Tumour assay is an excellent indicator of anti-tumour activity in plant extracts. The test is sensitive enough that it can be used as a preliminary screen for natural products that may be useful in the fight for cancer. This is despite the fact that the test organism is a bacterium that mediates an effect on plants. It is important to note, however, that this test works only with plant species that lack antibiotic activity, otherwise false positives will result (Kerr *et al.*, 1999). These must therefore be first tested against the test organism itself before being used as a bioassay.

The Crown Gall is a neoplastic disease of plants, in which autonomous plant tumour cells are produced from normal wounded plant cells by the bacterium, *Agrobacterium tumefaciens* (Kerr *et al.*, 1999). The disease affects a wide range of Dicotyledons (over 1000 species), including potatoes. As a result of this, *Agrobacterium tumefaciens* is a major concern in agriculture because it affects a large number of crops by reducing yield (Escobar *et al.*, 2003). *Agrobacterium tumefaciens* is a gram negative, non-sporing motile rod-shaped bacterium that is closely related to *Rhizobium*, which forms nitrogen-fixing nodules on legumes. It is also the only known example of a natural vector of genetic exchange between Prokaryotic and Eukaryotic species (Escobar *et al.*, 2003).

Many strains of *A.tumefaciens* exist, all of which are classified into groups known as biovars. Such a classification is based on both the way in which each strain utilises carbohydrates and the type of genes present on its chromosomal DNA. This classification does not, however, have any affect on the pathogenicity of the bacterium. The only exception is biovar three, which has been found to be a pathogen of grapes.

The genes responsible for the Crown Gall disease are not located on the chromosome of *Agrobacterium tumefaciens*, but occur on a large plasmid. This ring of DNA, which is separate from the chromosome, is capable of replicating independently within the cell. It is also capable of being transferred from one bacterial cell to another through conjugation. This is the role of the Ti or tumour inducing plasmid (Escobar *et al.*, 2003).

*Agrobacterium tumefaciens* occurs commonly on and around root surfaces without being pathogenic. Here it survives saprophytically on decaying plant material and root exudates (Escobar *et al.*, 2003). The bacterium only becomes pathogenic when it enters the plant through a lesion. The motile cells of *A.tumefaciens* are attracted to root lesions through chemotaxis, a physiological response to the release of sugars and other root compounds.

The oncognes are responsible for the co-ordination of the infection process by:

- Leading to the production of proteins (permeases) that are inserted in the bacterial membrane for the uptake of compounds that will be produced by the resulting tumours;
- Causing the production of an endonuclease, a restriction enyzme that excises part of the Ti plasmid, the t-DNA.

The t-DNA is released by the bacterium and enters the plant cells through a site of injury. This DNA then integrates itself into the plant chromosomes, therefore directing the function of the cells mainly through the production of hormones such as Cytokinins and Indoleacetic acid, and other plant metabolites such as opines and agrocinopines. The process of DNA transfer is, however, unclear, but does require a conditioning process that is thought to be mediated by bacteria.

The production of hormones such as Cytokinins causes cell proliferation, which leads to the production of galls. These hormones are absent in uninfected plants, which therefore, provides *A.tumefaciens* with a unique food source that other bacteria are unable to use. This is of course provides optimal growth conditions for bacteria (Escobar*et al.*, 2003).

Because of its similarity to tumour growth in other organisms, the crown gall bioassay is ideal as a preliminary screen for anti-tumour agents and anti-cancer agents. Cancer is responsible for 28% of all deaths in male individuals and for 25% of all deaths in females (Australian Bureau of Statistics, 1997). However, the incidence of death amongst the Indigenous Australians is much higher with deaths due to cancer being 40% higher than that of non-indigenous Australians (Australian Bureau of Statistics, 1997).

A number of anti-tumour compounds have been found using this screening technique. Kerr *et al* (1999) has reported that the CGTA has been responsible for the isolation of myricadiol, lupeol, xanthyetin and scaevolal from *Scaevola spinescens*, a plant species native of Australia.
There is no indication that the *Acacia* species used by Indigenous Australians are useful for cancer. Despite this, it was still worthwhile exploring the extracts since they were already available and had shown promise in the BSLT. This would, hopefully, provide further evidence to substantiate some of the results obtained with the BSLT and perhaps lead to new and interesting compounds being found.

## 4.2 Materials And Methods

### 4.2.1 Preparation Of Plant Material

Phyllodes of the more promising species, *A. pruinocarpa* and *A. adsurgens* were ground into powder using a vegetative grinder and extracted using the protocol mentioned in chapter 3. Medium sized Nadine potatoes were used in all of the experiments. The potatoes were surface sterilised by soaking them whole in sodium hypochlorite (bleach) for 10 minutes. The cores from the potatoes were obtained using a sterilised corer and cut to approximately 0.5mm into discs using a sterile blade.

### 4.2.2 Preparation Of Growth Media

Two media were used in this bioassay. The first, Nutrient agar, used to culture the organism for testing was prepared according to instructions provided by the supplier (Oxoid). Glycerol broth, the second media used, was prepared using commercially available glycerol and by dissolving it into deionised water to make up a 15% glycerol broth solution. The nutrient broth was prepared using Nutrient broth powder and made up according to instructions also provided by the supplier (Oxoid).

### 4.2.3 Preparation Of Pathogenic Material

A slope culture of *Agrobacterium tumefaciens* was obtained from NSW Agriculture, Orange Agricultural Institute, Orange NSW, which was was sub-cultured, stored in glycerol broth and frozen. The original culture was stored in the refrigerator (NSW Agriculture, pers comm. 2002).

Before experimentation, fresh cultures were thawed out. A sterile metal loop was used to scrape some colonies from the culture. These were placed in vials with nutrient broth and incubated at 30°C for 24 hours.

### 4.2.4 Experimental Procedure

Four petri dishes with agar were prepared for each extract. Five potato discs were added to each dish. The extracts of both *A.pruinocarpa* and *A.adsurgens* were added to vials of *Agrobacterium tumefaciens*, after which they were shaken. Two drops of this solution were added to each of the potato discs in their respective petri dishes.

Two controls were also used in this bioassay. The first was with potato discs only, which was used as an absolute control to determine the natural growth/decomposition of starch. The other control was with only *Agrobacterium tumefaciens* added to the potato discs. The petri dishes were then incubated at 30°C for 12 days, after which the potato discs were stained using iodine solution to ascertain the level of starch present. The cells lacking starch were then counted using a dissecting micorscope to determine the growth of the pathogen. This, therefore, gave the percentage inhibition of the test extract. Those greater than 20% inhibition were considered useful.

### 4.2.5 Data Analysis

A *t*-test was initially performed on the raw data (inhibition percentages). These data were first transformed using the Arcsine transformation (Ott, 1993), to test for normality. A *t*-test was then performed on the transformed data to determine significance.

## 4.3 Results

The cell count of the controls revealed a higher number of empty cells (fig 4.1), with the average number of empty cells per potato disc was 64.5 compared to 26.8 empty cells for *A.pruinocarpa* and 47.6 empty cells for *A.adsurgens*. The high number of empty cells demonstrates that the bacterium, *Agrobacterium tumefaciens* was utilising the starch content of the cells, therefore producing tumours.

When the cell counts of both species were compared to the cell counts of the controls (Fig 4.1), both *A.pruinocarpa* and *A.adsurgens* demonstrated statistical significance producing $p$ values of $7.65 \times 10^{-7}$ and 0.003, respectively (Table 4.1).

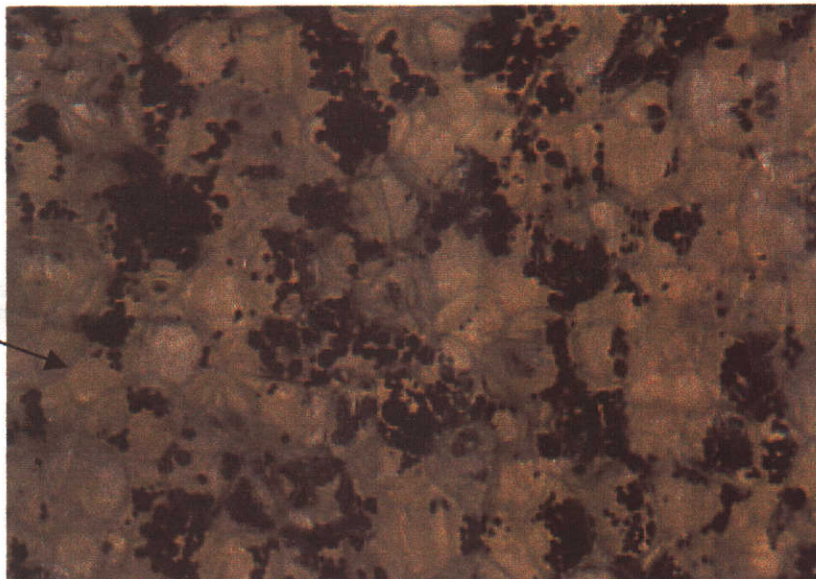

**FIGURE 4.1:** Control group after 12 days exposure to *Agrobacterium tumefaciens* showing starch granules in potato cells

Both *A.pruinocarpa* (Fig 4.2) and *A.adusrgens* extracts exhibited significant inhibition of *Agrobacterium tumefaciens* (Table 4.1).

Both species exhibited a significant inhibition factor with *A.pruinocarpa* producing 31% inhibition and *Acacia adsurgens* producing 37% inhibition.

**TABLE 4.1:** Results of Statistical Testing on the results from the bioassay using *Acacia pruinocarpa* and *Acacia adsurgens* (Based on cell count)(Raw Data).

| **Species** | *Acacia pruinocarpa* | *A.adsurgens* | **Control** |
|---|---|---|---|
| Control | $p = 7.65 \times 10^{-7}$ ** | $p = 0.003$ ** | N/A |

( ** denotes significance)

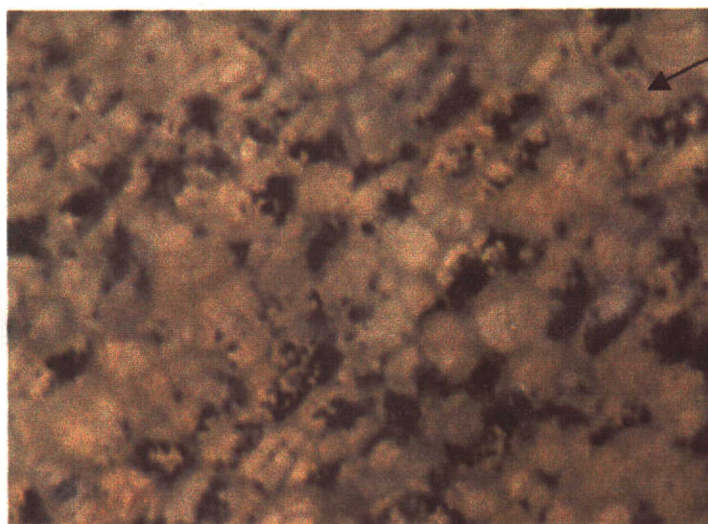

**FIGURE 4.2:** Experimental group after 12 days of exposure to *Agrobacterium tumefaciens* and *Acacia pruinocarpa*. Empty cells are clearly visible.

The results of the *t*-test performed on the transformed data revealed that there was a significant difference between the two species and the control.

**TABLE 4.2:** Results of statistical testing on *A.pruinocarpa* and *A.adsurgens* showing the Arcsine values

| Species | Percentage Inhibition | %inhibition/100 | Arcsine | *p* value |
|---|---|---|---|---|
| *A.pruinocarpa* | 31 | 0.31 | 0.315 | 0.042 |
| | 32 | 0.32 | 0.326 | |
| | 33 | 0.33 | 0.336 | |
| | 29 | 0.29 | 0.294 | |
| | 32 | 0.32 | 0.326 | |
| | 31 | 0.31 | 0.315 | |
| *A.adurgens* | 33 | 0.33 | 0.336 | 0.053 |
| | 34 | 0.34 | 0.347 | |
| | 35 | 0.35 | 0.358 | |
| | 33 | 0.33 | 0.336 | |
| | 33 | 0.33 | 0.336 | |
| | 31 | 0.31 | 0.315 | |

## 4.4 Discussion

Cancer is not a single disease, but rather a range of symptoms in which abnormal cells proliferate and spread out of control. Such cells can develop from most cell types in different parts of the body with each cancer having its own pattern of growth and development. With the incidence of cancer in both indigenous and non-indigenous Australians increasing (Aust Bureau of Statistics, 1997), and the increase in the resistance of such cancers to chemotherapy drugs, the search for novel biologically active compounds that possess anti-tumour activity has intensified (Clarke, 1990).

The Crown Gall tumour bioassay is a preliminary test that allows for the search of compounds that exhibit anti-tumour qualities. The test organism, *Agrobacterium tumefaciens*, affects approximately 1000 species of dicotyledons, ensuring that the test would give a good indication of the anti-tumour activity (Escobar *et al.*, 2003). This has been employed in related studies. For example, Kerr *et al.*, (1999) have reported that the species, *Scaevola spinescens*, exhibited anti-tumour activity when tested on *Agrobacterium tumefaciens*, thus, substantiating claims of the medicinal qualities of the species. It was also reported by Kerr *et al* (1999) and McLaughlin and Rogers (1998), that an inhibition factor of at least 20% was considered to be significant. Both *A.pruinocarpa* and *A.adsurgens* exhibited significant activity when tested on *Agrobacterium tumefaciens* producing inhibition percentages of 31 and 37, respectively.

It is unknown whether *A.pruinocarpa* and *A.adsurgens* possess any compounds similar to those reported by Kerr *et al* (1999) for the anti-tumour activity in *Scaevola spinescens*. As mentioned in previous chapters, it is unlikely that any of the known compounds present in *A.pruinocarpa*, for example, the tannins, are responsible for the activity.

However, it is not known if *A. pruinocarpa* and *A.adsurgens* contains any triterpenes similar to those reported by Kerr *et al* (1999). It is likely, however, that both species do contain similar compounds as Barr (1993) has reported that a number of *Acacia* species contain similar terpenoids.

The Potato disc Assay produces results that correlate very strongly with the 388 (in vivo, murine leukemia) anti-tumour assay (McLaughlin and Rogers, 1998). The crown gall tumour assay fulfils all the requirements of a front-line screening technique as it is simple, requires little specialisation, inexpensive and is considered to be statistically reliable in predicting cytotoxicity (McLaughlin and Rogers, 1998).

In conclusion, it is reasonable to suggest that both *A.pruinocarpa* and *A.adsurgens* contain compounds that exhibit cytotoxicity when tested on the crown gall tumours. However, the nature and composition of such compounds is unknown and that these compounds maybe terpenoids that are known to exhibit cytotoxicity. More testing is required to determine and isolate the type of compound that are responsible for the cytotoxicity exhibited by *A.pruinocarpa* and *A.adsurgens*.

# CHAPTER 5

# DISC DIFFUSION ANTIBIOTIC BIOASSAY

## 5.1 Bacteria And Their Effects On Humans

Prokaryotic organisms are those which lack nuclei. This includes bacteria. As a group, bacteria are considered to be amongst the smallest organisms, ranging from 1 to 10 $\mu$m. These are also amongst the most numerous of all organisms. Bacteria can inhabit a wide variety of environments ranging from the very alkaline to very basic conditions. Individual species are usually classified according to the shape of the organism. For example, the cocci are usually spherical in shape, while the bacilli are rod-shaped. Most species are essential to life on Earth and are responsible, for example, for the decomposition of organic matter in the environment. There are, however, many that cause disease in humans and other organisms. The severity of the disease is related to the structure of the cell wall. The diseases caused by the gram- positive bacteria are less severe than those caused by the gram negative species (Campbell, 1993).

Approximately 200 species of bacteria are pathogenic to humans. Pathogenicity differs greatly amongst the species and is dependent on the virulence of the species and the immunity of the host organism. For example, individuals with compromised immune systems, such as those infected with the Human Immunodeficiency Virus (HIV) and cancer, are usually susceptible to such infections (Prescott *et al.*, 1993). Bacterial infection can exhibit various forms of lesions once an individual is contaminated. These include macules, pustules, wheals, nodules, vesicles/bullae, ulcers, and scars. Generally, bacterial infections of the skin are caused by *Staphylococcus aureus* and *Streptococcus pyogens*.

## 5.2 Fungi And Their Effects On Humans

Fungi, in contrast, are a group of eukaryotic organisms that are usually multicellular and lack flagella. They have cell walls that are composed of chitin, which is a flexible polysaccharide containing nitrogen similar in structure to the chitin found in the exoskeletons of insects (Campbell, 1993). All species of fungi are heterotrophs and therefore obtain their nutrients through absorption. They achieve this by being saprobic decomposers, parasites and mutualists.

Fungi are also responsible for a number of diseases in humans. A number of species also work in association with some bacteria species. The yeast, *Candida albicans*, is responsible for a number of diseases, one of which is thrush.

## 5.3 Antibiotics

Antibiotics are described as chemical substances that have the capacity to inhibit or destroy organisms. This is a process known as antibiosis (Mann, 1991). Although not fully understood, what is now known about the antibiotic effect was first observed in the 19th century by the French chemist, Louis Pasteur. He observed that certain saprophytic bacteria can kill anthrax germs (Mann, 1991). Antibiotics, however, did not come into widespread use until 1910, when the German doctor and chemist, Paul Ehrlich, discovered the drug Salvarson. This was a treatment for syphilis (Mann, 1991).

The mainstream use of antibiotics did not, however, come to the forefront of medicine until 1928, when the antibiotic effects of *Penicillium* was accidentally discovered by Alexander Flemming. This was later synthesized into what is known today as the antibiotic, Penicillin. It was not, however, the first antibiotic to be used in the treatment of diseases, since the purification process was not perfected until the 1940's. The first reported antibiotic to be used was Tyrothricin, which was isolated from soil bacteria (Mann, 1991). This was never used as a general antibiotic because of its high level of toxicity (Mann, 1991).

Antibiotics can be classified in several ways. The most common method of classification is by their mechanism of action against pathogens. They are however, also classified on the basis of their chemical composition. For example, Penicillins and Cephalosporins contain β-Lactam rings in their structure (Figure 5.1), whereas the macrolide antibiotics, such as Erythromycin, have lactone rings. Antibiotics of these types are routinely prescribed for a variety of medical conditions such as malaria, Bubonic plague, Impetigo, pneumonia and various throat infections (Prescott *et al.*,1993; Kagan, 1980).

Antibiotics have been routinely prescribed for a variety of illnesses including malaria, pneumonia, impetigo and throat infections. It is this type of indiscriminate use of antibiotics, along with their over prescription, that has contributed to the emergence of antibiotic resistant pathogens, which is a major concern for medical practitioners and pharmaceutical manufacturers (Mitsuhashi *et al.*, 1971, Langford and Benrimoj, 1996). Penicillin resistant pathogens, such as *Staphylococcus aureus*, are of most concern to the medical community. Penicillin- resistant antibiotics usually produce penicillinases, enzymes that have the capacity to denature the antibiotic (Mann, 1999). It is important to note, however, that many strains of *S. aureus*, along with gram-negative enteric

bacteria, are also resistant to macrolide antibiotics such as Erythromycin and constitute a major group of resistant pathogens (Mitsuhashi *et al.*, 1971).

Both Penicillin and Erythromycin are susceptible to resistance by pathogens. These are therefore, described in this chapter because their mechanisms of action may help to explain any activity, if found, in *Acacia* extracts. These antibiotics are the only two described because they give a good indication of how antibiotics work. Furthermore, they are readily available. There are, however, many more antibiotics available, all of which have similar modes of action.

### 5.3.1 Mechanisms Of Action

#### 5.3.1.1 Penicillin

As one of the first major discoveries in pharmacology, Penicillin is one of the most widely used antibiotic agents in modern medicine (Prescott *et al*,. 1993). Penicillin belongs to a group of antibiotics known as β-Lactam agents. This group also includes the Cephalosporins, a group of antibiotics that are very similar in structure. The members of this group all have a β-Lactam ring in their structure (Figure 5.1), which is essential for antimicrobial activity as it irreversibly blocks the activity of the enzyme responsible for the construction of the cell wall through bonding with the functional end of the enzyme causing structural weakness in the cell wall (Prescott *et al*,. 1993) (Figure 5.1).

A number of different types of penicillin exist, all of which are synthesized by the fungi *Penicillium*. There are a number of methods for classifying this group of antibiotics, but in Australia they are classified according to their individual spectrum of activity.

##### 5.3.1.1.1 Narrow Spectrum Penicillin

The narrow spectrum penicillin group are mainly active against gram-positive bacteria, eg. *Staphylococcus aureus* and *Streptococcus pyogens*. Such antibiotics are inactivated by the beta-lactamase enzymes that are produced by staphylococci and some other bacteria; these enzymes hydrolyse the penicillin rendering them useless against pathogens. Some of the antibiotics in this group are also sensitive to stomach acids and are therefore not administered orally. The antibiotics in this group include Benzylpenicillin, or penicillin G, which is the most commonly administered form of penicillin. Then there are Procaine penicillin and Benzathine penicillin, both of which

are administered intramuscularly, as is Phenoxymethypenicillin. These are not as sensitive to the stomach acids. They can, therefore, also be administered orally.

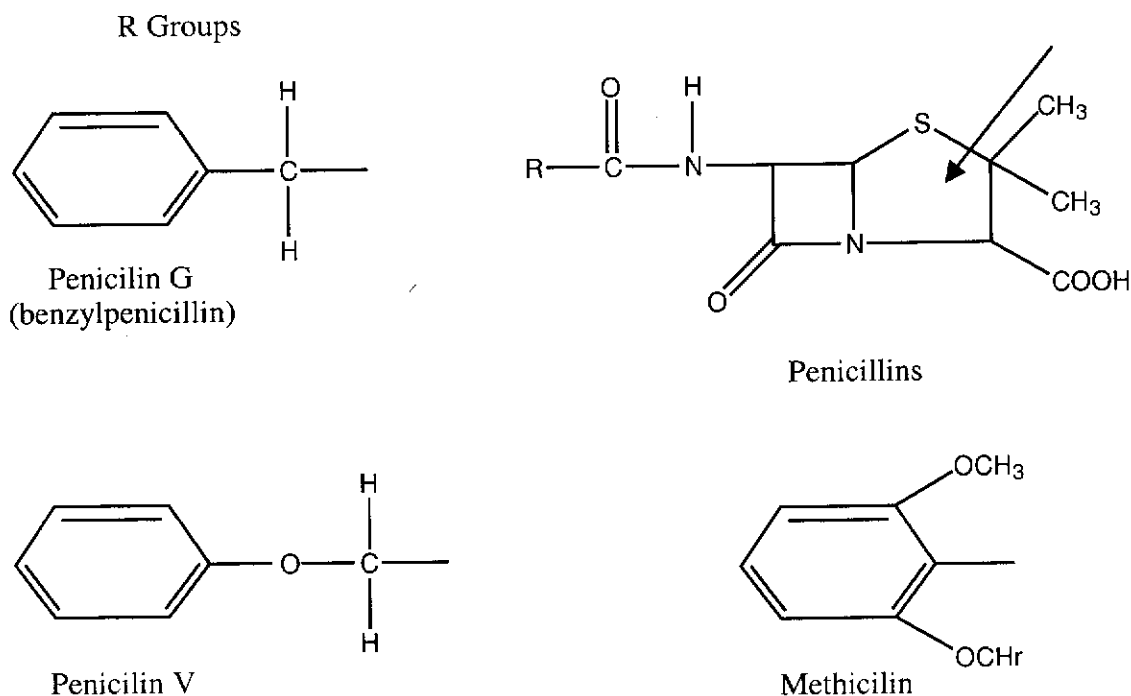


**FIGURE 5.1:** The different structures of the β-Lactam antibiotics. The arrow denotes the β-lactam ring

### 5.3.1.2 Erythromycin

Erythromycin was first discovered in 1952 as a metabolite of a strain of *Streptomyces erythreus*. This antibiotic has routinely been in use for over 40 years and is considered by many as the prototype for the macrolide class of antibiotics (Mitsuhashi *et al.*, 1971). Erythromcyin is sensitive to acids and is degraded very rapidly. Therefore, oral administration requires that the affected individual be given multiple doses of the antibiotic in order to gain any inhibition of the pathogen. Macrolides are characterized by a large 14 or 16 membered lactone ring linked to one of two amino sugars. Erythromycin is a 14 membered macrolide (figure 5.2) (Prescott *et al.*,1993).

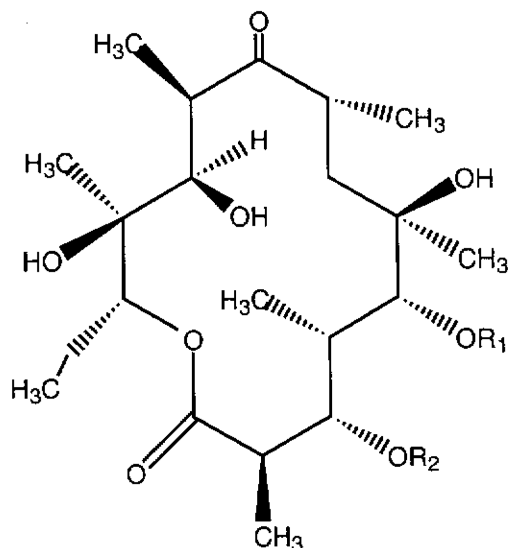


**Figure 5.2:** Structure of the Macrolide antibiotic Erythromycin.

The mode of action of antibiotics, such as erythromycin, differs from that of pencillins. It is thought that erythromycin binds to the 50S subunit of the bacterial ribosomes, causing a dissociation of the tRNA from the ribosomes. This in turn prevents translocation of the peptide chain, which disrupts protein synthesis (Prescott *et al.*, 1993).

Generally, macrolide antibiotics are relatively broad-spectrum and are, therefore, active against both gram-negative and gram-positive bacteria (Prescott *et al.*, 1993). However, when compared to the newer macrolides, such as Azithromycin, Erythromycin has a narrow spectrum, even though it is active against some gram-negative bacteria, eg. *Treponema*. The therapeutic usefulness of this antibiotic is based on its activity against gram-positive bacteria. Therefore, erythromycin is used in the clinical treatment of infections of the skin, bone and soft tissue, respiratory tract and diseases such as Clamydia and Syphilis. Erythromycin and other macrolide antibiotics are also used as a substitute for pencillin in cases where the infected individual has an allergy or hypersensitivity to pencillin.

The main aims of this test areto determine if *Acacia* species possess any antibiotic potential, and whether there is a significant difference in potency between the species and the control if such activity exists.

## 5.4 Materials And Methods

### 5.4.1 Preparation Of Plant Material

The plant material used in this bioassay was prepared using the same protocol as described in chapter 3.

### 5.4.2 Preparation Of Pathogenic Material

#### 5.4.2.1 Media

Mueller Hinton, Sabouraud (Oxoid) and blood agar plates were used in preparing the agar plates for the bioassays used in this study. Acumedia Mueller Hinton agar and Sabouraud agar was prepared by mixing the powdered agar with deionised water according to instructions. Once the solution was prepared, it was autoclaved. The resulting sterile solution was then left to cool to approximately 50°C before being poured into sterile petri dishes. The petri dishes were then cooled in a lamina flow cabinet so that the agar set to a gel with a consistency ideal for growth. To maintain sterility, the petri dishes were then wrapped and refrigerated. The Blood agar plates were obtained pre-prepared from the Microbiology Department of Royal Perth Hospital.

#### 5.4.2.2 Bacteria

Two species of bacteria were used for this aspect of the study. Pure cultures of *Staphylococcus aureus* and *Streptococcus pyogens* were obtained from the Department of Biomedical Sciences at Curtin University of Technology. The cultures were divided so that half the colonies were grown on fresh agar plates while the rest were placed into glycerol broth for cold storage (< 5°C) as a base culture. The colonies cultured on agar plates were incubated at 37°C for 24 hours before being used.

#### 5.4.2.3 Fungi

Cultures of *Candida albicans* and *Trichophyton rubrum* were also obtained from the Department of Biomedical Sciences at Curtin University. Cultures of *Candida albicans* were divided and kept for both immediate use and cold storage as described insection 5.4.2.2. *Tricophyton rubrum* was cultured on Oxoid Sabouraud agar and kept in cold storage (< 5°C) on the agar.

#### 5.4.2.4 Storage Of Pathogens

Pathogens such as those used in this study reproduce at a rapid rate. Mutation can therefore occur frequently. To reduce the incidence of mutation, the pathogens were stored and frozen to slow the rate of reproduction was slowed. All pathogens, with the exception of *Trichophyton rubrum*, were stored in 15% glycerol broth. This solution has a significantly lower level of nutrients than that of both Blood and Mueller Hinton Agar. Each pathogen was isolated using a sterilised metal loop, and was then transferred to the glycerol broth, after which it was shaken gently to evenly distribute the colonies throughout the solution.

#### 5.4.2.5 Sterilization Techniques

When using such pathogens, sterility is important. Being pathogenic to humans, organisms such as *Candida albicans, Staphylococcus aureus, Streptococcus pyogens* and *Trichophyton rubrum* can readily infect us. Therefore, in this study, all equipment used was sterilised using standard aseptic techniques. All surfaces were sterilised using 70% ethanol. Metal loops used in the culturing of the pathogens were heated in a flame until red-hot and were then left to cool before being used. Glass rods were also sterilised through flaming. However, these were dipped into 70% ethanol first before being flamed. All work carried out using pathogens was performed in a lamina flow cabinet with gloves and lab coats worn at all times. All glassware used in this study was also autoclaved before use.

#### 5.4.2.6 Experimental Technique

All pathogens, with the exception of *Trichophyton rubrum*, were prepared in the same way when commencing each series of tests. Colonies of the pathogens were removed using a sterilised metal loop, which was transferred to 0.85% saline solution and shaken until it had emulsified. An aliquot of 0.1ml of the saline/bacteria solution was dispensed drop wise on to the respective media (Mueller-Hinton or Blood Agar), and was then spread across the media using a glass rod.

*Trichophyton rubrum*, which is a spore forming fungus, was prepared in a different manner to the other pathogens. Plates of the pathogen were cultured, then, under aseptic conditions, spores were removed from the cultures using a metal loop and applied directly to the media (Sabouraud Agar).

5.4.2.7 Extract Application

The extracts were applied to the pathogen using two methods. The extract was applied drop-wise to sterilised discs (0.5cm in diameter) of Whatman filter paper until saturation had occurred. These were then left to dry in a sterile environment before inoculation. Prior to application, the filter paper was sterilised in an autoclave at 121°C.

In the second method, wells approximately 1 mm deep and 5mm in diameter, were cut into the agar before inoculation. The pathogen was then applied to the plate using the methods described in section 5.4.2.6. The test extract was then applied drop-wise (0.1mm) into the wells.

The antibiotics were applied using the same method regardless of the method used in the application of the plant extracts. Antibiotic discs (Oxoid) were ordered and applied directly to the pathogen. The discs were applied approximately 0.5cm from the edge of the plate to ensure a more complete inhibition zone should one arise, giving more accurate measurements. Two experimental discs and two control discs were applied to each plate.

5.4.3 Data Analysis

Four replicates of each dose for each of the pathogens were used. The data collected from the tests were quantitatively analysed using two tailed *t*-tests. Probabilities of less than 0.05 were considered to be statistically significant. All results are expressed as mean ± SEM.

## 5.5 Results

The results of the test revealed that none of the extracts, with the exception of *A.bivenosa*, exhibited any antibiotic activity (Table 5.1). *Acacia bivenosa* only demonstrated activity on *Staphylococcus aureus*.

**TABLE 5.1:** Results of tests for Antibiosis. Inhibition is exhibited in mm for all extracts and the controls.

| SPECIES | *Staphylococcus aureus* (mm) | *Streptococcus pyogens* (mm) | *Candida albicans* (mm) |
|---|---|---|---|
| Penicillin G | 42.60 ± 0.645 | 38.63 ± 0.416 | N/A |
| Erythomycin | 27.00 ± 0.556 | 25.31 ± 0.654 | N/A |
| Nystatin | N/A | N/A | 21.42 ± 0.43 |
| *A.adsurgens* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.aneura* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.auriculoformis* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.bivenosa* | 8.30 ± 0.60 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.coriacea* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.dictopleba* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.inaequilatera* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| *A.pruinocarpa* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

Unlike the *Acacia* extracts, all three of the test pathogens were inhibited by the appropriate antibiotics that served as control. Both *Staphylococcus aureus* and *Streptococcus pyogens* were inhibited by penicillin. *Staphylococcus aureus* appeared to be the most susceptible to penicillin, producing an average inhibition zone of 42.6mm ± 0.643 (Table 5.1). When compared to *Streptococcus pyogens*, which produced an inhibition zone of 38.63mm ± 0.416, there was a significant difference between the two pathogens ($p = 0.0002$). This indicates that the tests were correctly performed.

Erythromycin appeared to be less potent than penicillin, although both *S.aureus* and *Streptococcus pyogens* were inhibited to some degree (Table 5.1). The results of the *t*-test revealed that erythromycin also produced no significant difference in the inhibition between the two pathogens ($p = 0.88$).
When the results of the effects of penicillin were compared to those of erythromycin, the *t*-test showed that they were not significantly different. ($p = 1.53$)

## 5.6 Discussion

Much has been written about the need for Indigenous Australians to utilise species that had a wide range of uses (Barr, 1992, Latz, 1995). Species such as *Acacia estrophiolata* and *A.ancistrocarpa* serve as excellent examples (Barr, 1992; Latz, 1995). Interestingly, their uses included combating bacterial and fungal infections of the skin. Taking such reports into account, it was surprising then that only one species, *Acacia bivenosa*, produced any antimicrobial activity when tested on *Staphylococcus aureus*, *Streptococcus pyogens*, *Candida albicans* and *Trichophyton rubrum*. Despite this positive result, this activity was not considered to be significant because it only exhibited activity against one pathogen, *Staphylococcus aureus*. To be considered to be of any value, it needed to be active against at least two species (Barr, 1993). Then why was it active against only one pathogen? Perhaps the concentration of the extract used was quite low, i.e. 2mg (Table 6), to elicit an effect on the more robust *Streptococcus pyogens*. More work is required using greater concentrations of extract to determine whether or not *A.bivenosa* extracts are worth pursuing as a source of antibiotic agents.

Where the other species are concerned, it is not uncommon for them to lack antimicrobial activity. Wickens (2001) reported that *Eremophila longifolia* lacked antibiotic activity even though this was prized as a body wash (Barr, 1993). The lack of anti-microbial activity exhibited by *Acacia* species may be attributed to the lack of specific compounds that are known to exhibit this type of effect. Compounds such as the monoterpenes, cineole and terpineole, which have been isolated from both *Melaleuca* and *Eucalyptus* species, have been shown to exhibit antimicrobial qualities (Markham, 1999). Although work on *Acacia* species is limited, Barr (1993) reported that only two species of the Northern Territory tested positive for these types of substances, *A.kempeana* and *A.oncinocarpa*. All other *Acacia* species, including three from this study, *A.aneura*, *A.auriculoformis* and *A.holosericia* all tested negative (Barr, 1993). It is, therefore, reasonable to suggest that the *Acacia* species used by the Indigenous Australians lack the types of compounds required to inhibit antimicrobial activity. So why were they so highly prized as body washes?

Saponins are one possible class of compounds that may account for this activity of some *Acacia* species. Saponins are glycosides that consist of both triterpenes and sterols (Harbourne and Turner, 1984) (Fig 5.3).

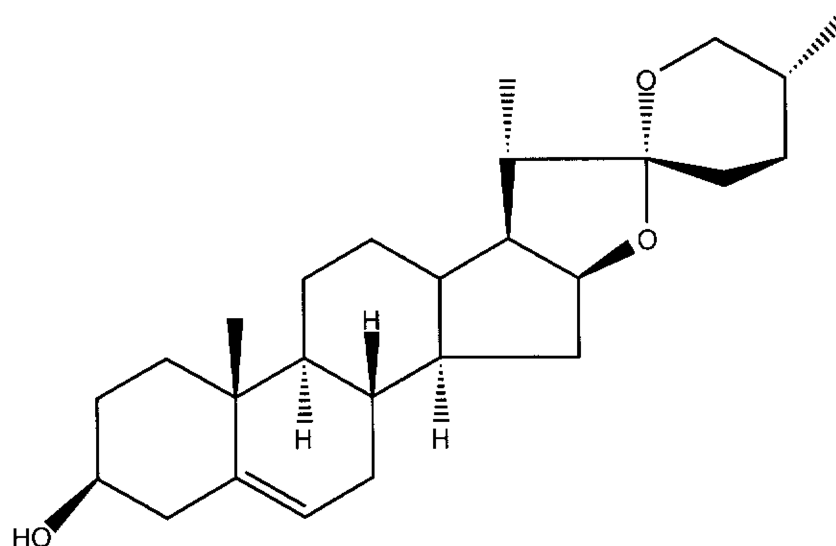


**FIGURE 5.3:** General structure of saponins

These substances are surface- active agents with soap-like properties, which are caused by the fat-soluble and water-soluble elements within the molecule (Taiz and Zeigler, 1991) (figure 5.3). Barr (1993) reported that a number of *Acacia* species, including those used in this study, tested positive for saponins. It is reasonable to suggest, therefore, that it is these types compounds that, when mixed with water, act in the same way as conventional soaps (Piso and Winder, 1991). These may be responsible for washing off the microbes that cause skin diseases in Indigenous and other Australians. The ability of soaps to cleanse skin of both harmful and beneficial microbes is well documented.

Despite only being effective against *Staphylococcus aureus*, one can speculate on its possible mechanism by comparing its activity to those natural products whose mechanism of action is already known, e.g. penicillin and erythromycin.

The use of antibiotics, although indiscriminate, has been widely successful in the treatment of disease. Penicillin, one of the first known antibiotics to come into prominence has been extensively studied. The structure of the antibiotic remains relatively uniform throughout the group of antibiotics, with the different forms being distinguished through the differences that exist in the side chain of the ß-lactam ring (Mann, 1991). The penicillin nucleus is comprised of a five membered ring containing a sulphur atom, which, inturn is attached to the four membered ß -lactam ring (Mann, 1991). The ß -lactam ring is attached to a variety of structures that distinguish each type

of penicillin. For example penicillin F has a six carbon chain whereas penicillin G, featured in this study has one carbon attached to a benzene ring.

In this study, it was demonstrated that penicillin was more potent against pathogens such as *Staphylococcus aureus* and *Streptococcus pyogens* than the macrolide antibiotic erythromycin. Although, there was no significant difference between the two antibiotics, producing inhibition zones that were within the zone of susceptibility for the respective compounds (penicillin > 29mm; erythromycin > 18mm)( $p= 1.58$).

The difference in the susceptibility of pathogens, although not significant, to both penicillin and erythromycin can be attributed to the mode of action of the antibiotic. Penicillin is a cidal antibiotic, i.e. the pathogen is unable to recover even if the antibiotic is removed (Prescott *et al.*, 1993). Penicillin inhibits the growth of a number of pathogens through interfering with cell wall formation through inhibiting the transpepidation enzymes, which are involved in the formation of bacterial peptidoglycan.

Compared to other antibiotics, penicillin has relatively narrow spectrum of activity, used mainly in the treatment of infection caused be gram-positive bacteria. Antibiotics with a narrow spectrum of activity are usually ineffective in the treatment of gram-negative bacteria. This is due in part to the difference in the structure of the cell wall. These antibiotics are unable to penetrate the cell wall of gram-negative bacteria because the structure of the wall is more complex than that of the gram-positive bacteria (Prescott *et al.*, 1993).

Erythromycin, in contrast is a bacteriostatic antibiotic. It inhibits the growth of the pathogen, but only whilst present. Once removed, the pathogen may recover. Bacteriostatic antibiotics, such as erythromycin inhibit pathogen growth through interfering with proteinsynthesis through binding to the bacterial ribosome, the elongation of peptide chains is prevented (Prescott *et al.*, 1993).

Unlike penicillin, erythromycin is a relatively broad-spectrum antibiotic that is effective against gram-positive bacteria, Mycoplasma and several gram-negative bacteria (Prescott *et al.*, 1993). This antibiotic is used predominantly when an allergy to penicillin exisits.

Erythromycin produced no significant difference when tested on both *Staphylococcus aureus* and *Streptococcus pyogens.* Penicillin, in contrast exhibited a significant

difference when tested on the pathogens, appearing to be more effective on *S.aureus* than *S.pyogens*. This difference can be attributed to a number of factors including the lower susceptibility of *Streptococcus pyogens* to penicillin. It can also be spectulated that some resistance has developed in the strain of the pathogens used.

There are a number of reports written about Indigenous use of endemic flora for medicinal purposes. Of these the majority of plants, with the exception of a few *Acacia* species, were used externally as skin and body washes (Barr, 1992; Latz, 1995). The results of this study indicate that activity can probably be attributed to the presence of other compounds such as saponins, for which a number of *Acacia* species tested positive. Whether *A.bivenosa* extracts possess compounds with antibiotic potential is worth pursuing.

# CHAPTER 6

# LETTUCE SEED GERMINATION TEST FOR ALLELOPATHY

## 6.1 Introduction

Secondary metabolites are organic compounds that are not directly involved in the growth and development of plants and other organisms. Unlike primary metabolites, such as lipids, amino acids, nucleotides and carbohydrates, secondary metabolites have no generally recognised roles in assimilation, respiration and transport (Macias *et al.*, 2000). Through a process of overflow metabolism, a number of metabolites are produced, which may improve survival in some organisms.

Secondary metabolites enter the environment via a variety of different routes. In plants, these include release from the foliage, tree resin from both above ground and below ground parts, e.g. the root systems. Once released into the environment, secondary metabolites may reduce competition from other species through inhibiting germination or growth. This is a process known as allelopathy. Allelopathic compounds are usually simple phenolic compounds, e.g caffeic and ferulic acid. These often occur in substantial amounts and inhibit growth and germination in neighbouring plants. In many cases, it even affects members of the same species. Inhibition of germination can be achieved in a number of ways, depending on the mechanism of action of the invasive species. For example, *Centuarea maculosa* exudes Raecemic catechin from the roots, which inturn inhibits germination of neighbouring plants through altering the structure of the cytoplasm in the meristematic zone of the root tip of neighbouring plants (Bais *et al.*, 2003).

Allelopathy is considered to be an emerging branch of applied sciences (Macias *et al.*, 2000). This science has recently come into prominence as the need for alternative herbicides increases, particularly in agriculture where the incidence of chemical resistance to herbicides is also increasing. This is a result of the indiscriminate use of chemicals (Sam, 1993). Resistance to herbicides have the potential to reduce the area available for agricultural practices. In addition, the increased use of herbicides can damage the environment. For example, herbicide resistance in the rice industry of Australia is a major concern because it threatens the sustainability of that industry (Pratley *et al.*,2001). The number of species of weed that are increasingly more resistant to bensulfuron, which is a major herbicide in rice cultivation, is on the rise. The three main species of weed i.e. *Cyprerus difformis*, *Damasonium minus* and *Sagittaria montevidensis* are of particular concern producing 50%, 40% and 35% resistance respectively, to bensulfron.

Allelopathy, in some cases also includes the stimulation of seed germination (Macias *et al.*, 2000). As a result, a number of different species have been tested, both weed species and commercial crops, to determine a possible mode of application for allelochemicals as plant growth regulators (Macias *et al.*, 2000). These would be useful, especially for plant growers and breeders.

Bioassays that target allelopathic substances assess the potency of compounds via their application-induced response to the subject (Macias *et al.*, 2000). These bioassays are usually correlated with phytochemical studies and are therefore often used to evaluate bioactivity through affecting germination and seedling growth.

The most commonly used test species for allelopathic bioassays is *Lactuca sativa* (lettuce). This species is considered to be an ideal screening agent. It is used extensively because it has a fast germination rate and is highly sensitive (Macias *et al*, 2000). *Lactuca sativa* also allows comparison of bioassay results for many different compounds. However, the seed of other vegetables can also be used with similar results (Macias *et al*, 2000).

Since *Acacia* species are known to inhibit germination in some species, and since allelopathic bioassays satisfy all the criteria for front-line bioassays, the eight *Acacia* species were screened for allelopathy. This was done purely as an additional test. It was performed to satisfy one of the aims of the study, which was to determine if any of the *Acacia* species possess biologically active compounds. However, it does not have any bearing on the work with traditional bush medicines.

## 6.2 Materials And Methods

### 6.2.1 Preparation Of Plant Material

Leaves of eight *Acacia* species were dried and ground into powder using a vegetative grinder and was extracted using four solvents, however only the final extract was used in this study (refer to chapter 3). The extraction was then made into four different concentrations for each of the species.

### 6.2.2 Experimental Design

Commercially available seeds of *Lactuca sativa* were used in this bioassay. Twenty-five seeds were arranged into petri dishes lined with filter paper (Whatman, no 1). Each of the petri dishes was prepared with different concentrations of *Acacia* extract (4ml). These concentrations were dependant on the availability of the plant material and differ for each species (Table 6.1). Four replicates of each concentration were used in the study. A group of four petri dishes with 25 seed in each was used as a control group using deionised water only. The petri dishes were then sealed in order to prevent moisture loss and contamination and placed into a dark incubator at 25°C and checked every 24 hours for a total of 72 hours or until all the seed had germinated.

The results were then analysed using the following routinely used measures:

1) Final Germination: expressed as a percentage, is the maximum average percentage of seeds that germinated during the experiment.
2) Mean period of final germination: $\Sigma NiDi/FG$
3) Rate of Germination: $\Sigma NiDi$
4) Percentage of inhibition or stimulation: 100-(FG in aqueous extracts (%)/FG in distilled water (%) x 100) ( 100= stimulation, 0= inhibition).

Where,
N is the daily increase in seed germination
D is the number of days from seed placement (Saxena *et al.* 1996)

A *t*-test was performed on the final germination (FG) to determine whether there was a significant difference between the *Acacia* species tested.

Extracts of the more promising species were then fractionated using a polyamide column to remove any tannins or other compounds that maybe play similar roles in any biological activity (see chapter 3)

## 6.3 Results

### 6.3.1 Preliminary Results

After the initial inoculation of the petri dishes with 4ml of the *Acacia* extracts, some interesting results emerged. All of the *Acacia* species tested, with the exception of *Acacia coriacea*, exhibited some allelopathic activity. These were all compared with the control group, in which deionised water was used. There was 100% germination in the control group seeds, which took 24 hours.

*Acacia pruinocarpa* exhibited the most promising results with 100% inhibition to concentrations as low as 0.925g/L. The only concentration to exhibit any germination was 0.37g/L, which was the lowest concentration tested, and even that only produced a final germination (FG) of 22%. The next promising species was *A.dictopleba*, which exhibited 100% inhibition at concentrations of 20g/L (Table 6.1). At 10g/L, this species produced an FG of 11%, which increased to 100% at 5g/L (Table 6.1).

All of the seeds of *A.coriacea* germinated within 24 hours, like the controls, which also germinated within 24 hours (Table 6.1). The control reached 100% germination within 24 hours, and showed no microbial growth or dessication. *Acacia auriculformis* exhibited 100% inhibition at 200g/L, which decreased to 69% at 20g/L. This species was also one of the more promising species tested.

Despite not showing as much promise as some of the other species, *A.inaequilatera* demonstrated some allelopathic activity when exposed to the lettuce seed. This species yielded an RG of 15.82 at 7 g/L and a FG % of 100% (Table 6.1). *Acacia inaequilatera* yielded an FG of 11% at 28g/L, which increased to 14.9% at 14g/L (Table 6.1).

*Acacia bivenosa* exhibited some inhibition of germination, but none of the concentrations tested completely inhibited germination, as was the case with some of the other species. This species yielded and FG of 68% at 20g/L, which increased to 90% at 10g/L before decreasing to 57% and 69% at 5g/L and 2g/L, respectively (Table 6.1).

*Acacia pruinocarpa, A.adsurgens, A.dictopleba, A.auriculoformis* and *A.aneura* were all significantly different to the control group. *Acacia pruinocarpa* significantly inhibited the germination of the lettuce seeds ($p$= 0.004). *Acacia bivenosa* ($p$=0.005), *A. adsurgens* ($p$= 0.14), *A.auriculoformis* ($p$= 0.02) and *A.dictopleba* ($p$= 0.01) also significantly inhibited the germination of the lettuce seeds. No germination was observed at the higher concentrations of these species, however, *A.adsurgens, A.dictopleba, A.auriculoformis* and *A.aneura* exhibited germination at concentrations between 10g/L (*A.dictopleba*) and 100g/L (*A.auriculoformis*).

As a result of the significant inhibition of the germination of lettuce seeds, *A.pruinocarpa* also produced the lowest rate of germination (RG) and final germination percentage (FG%)(Table 6.1).

*Acacia adsurgens* had the highest RG, 33.919 at 16g/L. The next highest was *A.dictopleba* producing an RG of 29.5 at 2.5g/L (Table 6.1). *Acacia coriacea, A.dictopleba, A.aneura* and *A.adsurgens* produced the highest FG% reaching 100% germination at the lower concentrations.

After the test, the seed, particularly with *A.pruninocarpa* had softened considerably. It was hoped that all the seed could be rinsed and re-tested with deionised water alone. This would have added to the results. The seed that exhibited germination showed some softening, but not to the same extent as *A.pruinocarpa*. The seeds exposed to this extract were so soft that they split open.

The filter paper used as the support for the seed, remained wet throughout the course of the test, providing the seeds with enough moisture to germinate. No growth of moulds or other microbes was apparent in any of the tests. And considering that all of the controls germinated, the results can be treated as reliable.

**TABLE 6.1:** Final germination percentage (FG%), rate of germination (RG), mean period of final germination (MPFG) and the percentage inhibition or stimulation of each of the *Acacia* species.

| Species | Conc (g/l) | Mean | FG% | RG | MPFG | Inhib/Stim |
|---|---|---|---|---|---|---|
| *A.adsurgens* | 161 | 0 | 0 | 0 | 0 | 100 |
| | 80.5 | 25.25 | 100 | 6.042 | 5.1 | 0 |
| | 40.25 | 50.25 | 100 | 18 | 3.9 | 0 |
| | 16.1 | 68 | 100 | 33.919 | 3.2 | 0 |
| *A.pruinocarpa* | 3.7 | 0 | 0 | 0 | 0 | 100 |
| | 1.85 | 0 | 0 | 0 | 0 | 100 |
| | 0.925 | 0 | 0 | 0 | 0 | 100 |
| | 0.37 | 5.5 | 22 | 1.292 | 4.8 | 0 |
| *A.auriculofomis* | 200 | 0 | 0 | 0 | 0 | 100 |
| | 100 | 9.5 | 38 | 2.7 | 4.7 | 0 |
| | 50 | 13 | 52 | 5.25 | 5.4 | 0 |
| | 20 | 17.25 | 69 | 7.125 | 3.6 | 0 |
| *A.bivenosa* | 20 | 17 | 68 | 2.803 | 4.89 | 77 |
| | 10 | 43.75 | 90 | 8.3 | 4.3 | 41.5 |
| | 5 | 14.25 | 57 | 3.75 | 8.9 | 81 |
| | 2.5 | 17.25 | 69 | 1.917 | 5 | 20.69 |
| *A.dictopleba* | 20 | 0 | 0 | 0 | 0 | 100 |
| | 10 | 2.5 | 11 | 0.458 | 6 | 96.33 |
| | 5 | 31.25 | 100 | 12.5 | 3.8 | 58.33 |
| | 2 | 36.5 | 100 | 23.29 | 4 | 0 |
| | 1.25 | 79.5 | 100 | 29.5 | 3.5 | 0 |
| *A.inaequilatera* | 56 | 0 | 0 | 0 | 0 | 100 |
| | 28 | 2.75 | 11 | 0.458 | 6 | 96.33 |
| | 14 | 37.25 | 14.9 | 14.52 | 3.8 | 50.33 |
| | 7 | 44.75 | 100 | 15.83 | 3.9 | 0 |
| *A.aneura* | 100 | 0 | 0 | 0 | 0 | 100 |
| | 50 | 0 | 0 | 0 | 0 | 100 |
| | 25 | 44 | 100 | 16.25 | 3.9 | 41.33 |
| | 10 | 57.75 | 100 | 19.542 | 4 | 0 |
| Control | | 61.5 | 100 | 21.75 | 3.9 | |
| | | 64.5 | 100 | 23.375 | 3.8 | |
| | | 69.75 | 100 | 28 | 3.6 | |

### 6.3.2 Polyamide Results

As mentioned in Chapter 3, tannins are known to be responsible for some biological activity. This is especially true where allelopathy is concerned (see discussion of this chapter). A methanolic extract previously fractionated with a polyamide column was also used in this study to determine whether or not tannins played any role in the observed allelopathy.

After testing lettuce seed with the new *A.pruinocarpa* extract, the species exhibited a significantly lower level of allelopathy ($p$= 5.430 x $10^{-6}$) (Table 6.2). The species also revealed, however, no significant difference to the control after fractionation ($p$ = 4.8) (Figure 6.1).

After fractionation, *A.pruinocarpa* produced values which were much higher than those produced before the polyamide column. After fractionation there was a significant difference between the two extracts. The mean number of germinants was significantly different ($p$= 5.43 x $10^{-6}$). The Final germination % before and after fractionation with the polyamide column were significantly different ($p$= 0.0004). The RG was also significantly different $p$= 4.666 x $10^{-8}$ (Table 6.2).

**TABLE 6.2:** Final germination percentage (FG%), rate of germination (RG), mean period of final germination (MPFG) and the percentage inhibition for *Acacia pruinocarpa* before and after fractionation with the polyamide column.

| Species | Conc (g/l) | Mean | FG% | RG | MPFG | Inhib/Stim |
|---|---|---|---|---|---|---|
| *A.pruinocarpa* | 3.7 | 0 | 0 | 0 | 0 | 100 |
| | 1.85 | 0 | 0 | 0 | 0 | 100 |
| | 0.925 | 0 | 0 | 0 | 0 | 100 |
| | 0.37 | 5.5 | 22 | 1.292 | 4.8 | 0 |
| *A.pruinocarpa* after Polyamide column | 9.033 | 99.75 | 100 | 49.583 | 3.005 | 0.25 |
| | 4.516 | 99.5 | 100 | 49.5 | 3.01 | 0.5 |
| | 2.258 | 99.75 | 100 | 49.75 | 3.005 | 0.25 |
| | 0.903 | 100 | 100 | 50 | 3 | 0 |

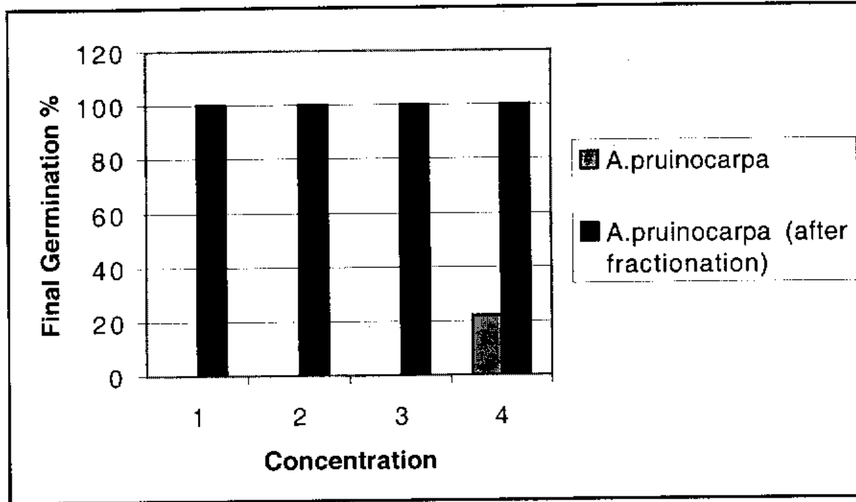


**FIGURE 6.1:** The final germination (%) of the *Acacia pruinocarpa* before and after fractionation

## 6.4 Discussion

The increasing incidence of chemically resistant weed species is becoming a major concern in agricultural systems. This has a major impact on the yield of agricultural crops. Chemical resistance has lent substance to the search for alternative herbicides, which has in turn led to the development of alleopathic studies (Mueller- Dombois and Ellenberg, 1974 Macias *et al.*, 2000).

Allelopathy can be defined as the influence of one plant on another by means of chemical substances. For example, Jefferson and Pennachio (2003) have reported that a number of Chenopod species exhibit allelopathic activity when tested on both lettuce seeds and seeds of other chenopod species. Such allelopathy is not limited to just the Chenopod species. Florentine and Fox (2003) have reported that a number of *Eucalyptus* species, for example *E.microtheca* and *E.camaldulensis* exhibit allelopathy on neighbouring plant species.

There are a number of compounds that are known to be responsible for allelopathic activity in plants. Macias *et al* (1999) have reported that the sesquiterpene lactones, Helivypolide D and Helivypolide E and the bisnorsesquiterpene, Annuionone D, all of which are natural products, are compounds responsible for the allelopathic activity observed in *Helianthus annus*. It has also been previously reported that a number of phenolic compounds, e.g. chlorogenic acid and isochlorogenic acid, both isolated from *Eucalyptus microtheca* (Florentine and Fox, 2003) and tannins exhibit allelopathic activity (Nilsson *et al.*, 2000; Mann, 1987).

In this study, all but one of the *Acacia* species tested exhibited some form of allelopathic activity. It was interesting to note that *A.coriacea* exhibited no activity even though it tested positive for tannins (1.2%)(Appendix 2). Tannins are well known to inhibit germination. *Acacia pruinocarpa, A.adsurgens, A.dictopleba* and *A.aneura* all exhibited significant allelopathic activity when compared to the control group. The most promising species in this study was *A.pruinocarpa*, which was active at concentrations as low as 925ppm. This was significantly lower than any of the other species tested. The tannin level of *A.pruinocarpa* was 2% (chapter 3, appendix 2).

It is not known whether *A.pruinocarpa* possesses any compounds similar to those reported by Macias *et al* (1999). It can therefore be suggested that the allelopathic activity exhibited by this species may be attributed to compounds such as these. It is also reasonable to suggest that such activity can also be attributed to the tannin content of the species. Tannins are considered to be excretion products of many plants, but are

actually involved in the defence mechanism against parasites and grazing animals (Torssell, 1997). Tannins are also known to exhibit allelopathic and other biological activity (Chapter 3) (Seigler, 2002; Courthout *et al.*, 1988). This was supported by the fractionation of the *A.pruinoarpa* extract with the polyamide column. After fractionation, the species appeared to lose potency, exhibiting a significantly lower germination rate, final germination and inhibition/ stimulation percentage. Based on the results of this study, it is likely that the allelopathic activity exhibited by *A.pruinocarpa* is a result of its tannins. However, due to the moderate level of tannins (2%) in this species, the activity could also be attributed to an unknown compound that may have been removed along with the tannins by the polyamide column. As mentioned in chapter 3, this is a possibility.

In allelopathic studies, bioassays are necessary in all stages of the identification and isolation of active compounds. There are a number of inexpensive and simple bioassays that have been in use since the 1940's to detect allelopathic activity (Macias *et al.*, 2000). The most commonly used allelopathic bioassay is seed germination. Macias *et al* (2000) tested a number of different dicotyledon and monocotyledon species to determine the best target species to use as a standard in bioassay such as the one used in this study. *Lactuca sativa* was found to be the most reliable, and combined with its fast germinination and high sensitivity, is considered by many to be a good target species. It is therefore, often used extensively in allelopathic tests.

The Lettuce Seed Germination test is a good example of a front-line bioassay. It fulfils all the criteria of such a bioassay in that it is rapid, inexpensive and requires little specialisation. There is, however, one disadvantage to using this test. At present, there are no established standard procedures for phytotoxic bioassays. Attempts are being made to rectify this problem (Macias *et al.*, 2000).

In conclusion, the lettuce seed germination bioassay is a good indicator of allelopathic activity in plants. *Acacia pruinocarpa* exhibited a significant level of allelopathic activity, suggesting that this species possesses the potential to be a natural herbicide. More advanced testing is required to determine the extent of this potential.

# CHAPTER 7

# GENERAL DISCUSSION AND CONCLUSIONS

## 7.1 Screening For Biological Activity

Little is known about the biological activity of *Acacia* species. Barr (1993), Collins *et al* (1990) and Seigler (2003) have reported that a number of *Acacia* species contain secondary metabolites that are known to exhibit biological activity. For example, *Acacia maidenii* has been reported to stimulate the central nervous system of mice, and *A.ulicifolia* has been reported to have the opposite effect (Collins *et al.*, 1990) No other studies have been attempted to determine the level activity, or if any other biologically active compounds exist in *Acacia* species. This study is the first of its kind to determine if any biological activity, specifically, anti-tumour, antimicrobial, herbicidal and pesticidal, exists in eight *Acacia* species.

The search for novel medicinal and other biologically active compounds of these types has intensified in more recent times. This is in response to the increase in the incidence of chemical resistance, which has become a growing concern in a number of different industries, particularly the agricultural and medical industries. The need for more compounds has contributed to the development of a number of new screening techniques (Sam, 1993). There are two main types: Front-line and advanced bioassays.

Front-line bioassays are tests used as preliminary indicators of biological activity. To be considered useful, a bioassay of this type must fulfil a number of criteria. The test must be inexpensive, simple to perform and require little specialisation. Furthermore, bioassays must be statistically reliable, repeatable, available to a large cross-section of researchers and must be high through-put (McLaughlin and Rogers, 1998; Sam, 1999). A number of bioassays qualify as front-line tests including the brine shrimp lethality test, the crown gall tumour assay, the disc diffusion antimicrobial assay and the lettuce seed germination test. All of these, in addition to fulfilling the criteria of a front-line screening technique, are commonly used as preliminary indicators for biological activity in plant extracts. It was on the basis of this that these tests were selected for this study.

## 7.2 Synthesis Of Results

### 7.2.1 Brine Shrimp Lethality Test

The BSLT is routinely used in the detection of both cytotoxicity and pesticidal activity. It is a relatively simple method of screening plants for biological activity (Sam, 1993). The BSLT has been in use since 1956 (Sam, 1993) and has been responsible for the discovery of a number of novel biologically active compounds.

There are a number of advantages to using the BSLT as a preliminary test for biological activity. McLauglin and Rogers (1998) have reported that the BSLT is superior or equally as accurate as the three in-vitro solid tumour cell lines used in their study. It has also been reported as being the most accurate, giving no false positives and very few false negatives (McLaughlin and Rogers, 1998). There are, however, a number of disadvantages to using the BSLT. Firstly, the BSLT is not selective by chemical type, giving only general toxicity (McLaughlin and Rogers, 1998). Sam (1993) has reported that there is no single protocol for the determination of lethality to *Artemia salina* accepted as a standard, and there is insufficient data to correlate the toxicity of samples to *Artemia salina* with any relevant type of activity such as antimicrobial and cytotoxic activity. Despite these limitations, the BSLT is still a very useful test and has resulted in the discovery of novel pharmaceuticals. Moreover, it has been useful in this study, highlighting that *Acacia* species do exhibit biological activity.

All of the *Acacia* species screened for cytotoxicity in this study exhibited some biological activity when tested on *Artemia salina*. One species, however, demonstrated significant activity, *A.pruinocarpa*. The results for *Acacia adurgens* and *A.dictopleba* were also useful and worthy of further evaluation.

*Acacia pruinocarpa* exhibited activity at concentrations as low as 3.7 ppm, which when compared to the potassium dichromate standard used by Sam (1993), is considered to be toxic to *Artemia salina*. Initially, this activity was thought to be due to the tannin content of the extract. This was supported to some degree by the results of tests using extracts in which the tannins had been removed, which was achieved by fractionating the extract with a polyamide column. After the fractionation, *A.pruinocarpa* appeared to have lost some of its potency. It is reasonable to suggest, therefore, that tannins were responsible for at least some of the activity. However, it is can also be suggested that other compounds may have also played some role.

A number of *Acacia* species have been reported by Seigler (2002) and Barr (1993) to contain tannins. The tannin content of *Acacia* species is varied (Barr 1993) and ranges from 0% in *A.aneura* to 12% as in *A.estrophiolata*. The level of tannins in *A.pruinocarpa* is moderate (2%; Table 3). Tannic acid, when tested on the BSLT, was by far more toxic. The mortality rate was 100% at concentrations as low as 1.0 ppm. It is, therefore possible that there may be at least one other compound responsible for some of the cytotoxicity exhibited by *A.pruinocarpa*. This compound may also have been removed by the polyamide column. This does occur, especially when fractionation is through columns of this type (Ghisalberti pers-comm).

Seigler (2002) has also reported that *Acacia* species contain other secondary metabolites that have the ability to exhibit biological activity. These include alkaloids, saponins, both of which *A.pruinocarpa* has tested positive for (Collins *et al.*, 1990) (Appendix 2). The species also produces terpenes and essential oils (Seigler, 2002). It is unlikely that the essential oils would have been responsible for the initial activity, as they would have been removed in the extraction procedure. Saponins, alkaloids and terpenes have the ability to exhibit biological activity. Compounds such as these have not been looked for in *A. pruinocarpa* (Barr 1993). The level of response of these compounds for the initial activity is therefore pure speculation and more research is required to isolate the compound responsible for the activity. The level of toxicity exhibited by *A.pruinocarpa* extracts would certainly warrant further testing, especially for chemicals with anti-tumour potential.

7.2.2 Crown Gall Tumour Assay

The crown gall tumour assay (CGTA) is also a useful preliminary bioassay the assists with the search for novel anti-tumour compounds. The test organism, *Agrobacterium tumefaciens*, affects approximately 1000 species of dicotyledons ensuring the bioassay is accurate in the indication of anti-tumour activity in plant extracts (Escobar *et al.*, 2003). In this study, the CGTA was used to substantiate some of the results obtained by the brine shrimp lethality test. As with the BSLT, *A.pruinocarpa* and *A.adsurgens* exhibited significant anti-tumour activity by inhibiting tumour formation by of 31 and 37% in tests with *A.pruinocarpa* and *A.adsurgens*, respectively.

Kerr *et al* (1999) reported that anti-tumour activity does exist in some native plants, with *Scaevola spinescens* exhibiting significant activity when tested on *Agrobacterium tumefaciens*. It was also reported that this activity may have also been triterpenes. It is not known whether *A.pruinocarpa* and *A.adsurgens* possess compounds similar to those identified by Kerr *et al* (1999) in *Scaevola spinescens*. It is, however, likely that both *Acacia* species contained similar compounds, as a number of other *Acacia* species contain different forms of triterpenes (Barr, 1993). Whether these same compounds were responsible for the activity seen in the BSLT has yet to be determined.

The CGTA is a bioassay that also satisfactorily fulfils the criteria of a front-line test. In addition it correlates strongly with the 388 (in-vivo, murine leukemia) anti-tumour assay (McLaughlin and Rogers, 1998), which is a standard advance anti-tumour assay. The use of the crown gall assay is usually warranted before the more expensive, time consuming and difficult P388 test is attempted. In fact, it can reduce the wait often

experienced by researchers and having to test their substances in other researchers' laboratories. The test is also considered to be statistically reliable in predicting cytotoxicity in plant extracts.

### 7.2.3 Disc Diffusion Antibiotic Assay

The search for novel antibiotic compounds has come to the forefront of the medical profession with the increasing incidence of pathogen resistance to antibiotics. *Acacia* species have long been regarded as potential sources of novel antibiotic compounds with a number of species, particularly *A.auriculoformis*, *A.estrophiolata*, *A.holosericia*, and *A.inaequilatera*, being employed by Indigenous Australians as skin washes and treatment for skin infections (Barr, 1993; Latz, 1995).

It was surprising then that only one of the *Acacia* species, *A. bivenosa*, tested positive when exposed to the common pathogen, *Staphylococcus aureus*. This was not considered to be significant as the extract demonstrated activity against only one of the test pathogens (Chapter 5), it must be active against at least three pathogens (Barr, 1993). Despite this, *Acacia bivenosa* shows promise as a source for novel antibiotic compounds and would certainly warrant some more additional testing. Perhaps other extracts could be screened using other solvents, e.g. hexane and dichloromethane.

The lack of antimicrobial activity exhibited by the other *Acacia* species tested in this study may be attributed to the lack of compounds such as cineole and Terpineole. These occur in *Eucalyptus* and *Melaleuca* species and are known to mediate antibiotic activity. They may have been so highly prized as skin and body washes due to their high saponin content. A number of *Acacia* species, including the ones used in this study, have tested positive for saponins (Barr, 1993; Appendix 2).

### 7.2.4 Lettuce Seed Germination Bioassay

Allelopathic studies have recently emerged as the agricultural industry struggles to find ways in which to combat weeds and invasive species. This is a result of the increased incidence to resistance to chemical compounds, which, like antibiotics in medicine, was the result of the indiscriminate use of pesticides.

A number of plants have been reported to produce allelopathic compounds. For example, the phenolic compounds, chlorogenic acid and isochlorogenic acid, both of which have been isolated from *Eucalyptus microtheca*, exhibit allelopathy when tested on lettuce seed (Florentine and Fox, 2003). Other natural products that exhibit

allelopathic activity include tannins, Helivypolide D, Helivypolide E and Annuione D (Nilsson *et al.*, 2000; Macias *et al.*, 1999; Mann, 1987). Some of these are now in the advanced stages of testing before being used commercially in agriculture. Again, before such expensive testing is commenced, extracts are usually screened using front-line bioassays.

The Lettuce Seed Germination Bioassay is one such test. It is a good indicator of allelopathic activity in plant extracts. In this study, all but one of the *Acacia* species exhibited some form of activity when exposed to the lettuce seed. It was interesting that *Acacia coriacea* demonstrated no allelopathic activity at all even though it tested positive for tannins (1.2%), which are well known to inhibit germination. Four of the other species tested, *A. pruinocarpa*, *A.adsurgens*, *A.dictopleba* and *A.aneura* all exhibited significant activity when compared to the control group. *Acacia pruinocarpa* was the most promising of all the species tested, exhibiting activity at concentrations as low as 0.925g/L. This was significantly lower than any of the other species tested. Again it was not clear which compounds, or class of compounds, were responsible for this activity.

After fractionation with the polyamide column, *A.pruinocarpa* exhibited a significantly lower level of allelopathy ($p= 0.0004$). It is reasonable to suggest, therefore, that the initial activity exhibited by *A.pruinocarpa* was the result of its tannin content. However, it is not known whether this species possesses any compounds similar to those reported by Macias *et al* (1999). Therefore it is also reasonable to suggest that there may be other compounds responsible for the allelopathic activity, and that these compounds may have been removed from the extract along with the tannins during fractionation with the polyamide column.

## 7.3 Conclusions

*Acacia* species have long been regarded as sources of biological activity (Barr, 1993). This study was guided by the Indigenous use of *Acacia* species as sources of medicine, which inturn led to the use of bioassays. All of the *Acacia* species used in this study exhibited various levels of biological activity, which is not surprising considering their value to the aboriginal people.

*Acacia pruinocarpa* demonstrated the most promise as a source of biologically active compounds, exhibiting activity at very low concentrations. The compounds responsible for the various activities have not been determined. However, it has been suggested that tannins are responsible for eliciting the some of the activity observed in this study.

It was not in the scope of this study to identify the active constituents responsible for the activity observed in *Acacia pruinocarpa* or any of the other species. All of the bioassays used in this study are good examples of front-line bioassays. The criteria listed for a bioassay to be considered front-line are fulfilled by the tests used in this study.

In conclusion, the bioassays used in this study are good indicators of biological activity in plant species and can be used as a guide for further testing.

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# APPENDICIES

## APPENDIX 1

## PUBLICATIONS

The following is a list of publications that have resulted from this MSc project.

Journal Articles

Wickens, K. and Pennacchio, M. (2003) A Search for biologically active compounds in *Acacia pruinocarpa*. *Journal of Ethnopharmacology*. In Preparation

Wickens, K. and Pennacchio, M. (2002) A search for novel biologically active compounds in the phyllodes of *Acacia* species. *Conservation Science Western Australia* 4(3): 139-44

Conference Proceedings

Wickens, K and Pennacchio, M. (2001) A search for biologically active compounds in the phyllodes of *Acacia* (Mimosaceae) species *Acacia Symposium*, Dallwalinu, July 14 – 16, 2001.

Wickens, K and Pennacchio, M (2003) A Search for Biologically Active Compounds from *Acacia* (Mimosaceae) species. Abstract 7. Royal Society of Western Australia Post-Graduate Symposium, March 30, 2003.

**APPENDIX 2:** Secondary Metabolites of *Acacia* species

| Species | Alkaloid Tests Dragondorff's | Mayers | Tannins | Saponins | Essential Oils |
|---|---|---|---|---|---|
| *Acacia aneura* | | | nil | positive | negative |
| *Acacia adsurgens* | | | 3 | | |
| *Acacia ancistrocarpa* | | | 5 | | |
| *Acacia auriculoformis* | positive | positive | 1% | positive | negative |
| *Acacia bivenosa* | | | nil | | |
| *Acacia coriacea* | | | 1.2 | | |
| *Acacia dictopleba* | | | 1.8 | | |
| *Acacia holosericia* | negative | negative | 4% | positive | negative |
| *Acacia inaequilatera* | positive | positive | 1.5 | | |
| *Acacia pruinocarpa* | positive | positive | 2 | positive | negative |