The efficacy of seven southern African ethnoveterinary medicinal plants in the mitigation of cutaneous myiasis

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Lillian Mukandiwa

(MSc. Animal Science, University of Zimbabwe)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Phytomedicine Programme, Department of Paraclinical Sciences,

Faculty of Veterinary Science,

University of Pretoria

Supervisor: Prof. V. Naidoo

Co-Supervisor: Prof. J.N. Eloff

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Declaration

The experimental work and results described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. This thesis has not been submitted in any other form to another University or academic institution for consideration.

I, Lillian Mukandiwa, declare the above statement to be true.

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L. Mukandiwa

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List of Abbreviations

EVM	Ethnoveterinary medicine
NWS	New World Screwworm
OWS	Old World screwworm
PSW	Palaearctic Screwworm
RH	Relative humidity
OP	Organophosphate
AChE	Acetylcholinesterase
Ach	Acetylcholine
NTE	Neuropathy target esterase
OPIDN	Organophosphate-induced delayed neurotoxicity
IGRs	Insect growth regulators
JH	Juvenile hormone
PTTH	Prothoracicotropic hormone
CSIs	Chitin Synthesis Inhibitors
JHAs	Juvenile hormone agonists
GABA	Gamma-aminobutyric acid
CNS	Central nervous system
MIC	Minimum inhibitory concentrations

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Abstract

Myiasis, the infestation of the skin of mammals by larvae of a variety of fly species, has been recognised as a major disease from ancient times. Despite being well known as a disease, it remains poorly controlled in the animal production industry with severe economic losses resulting thereof. The control of the myiasis-causing flies relies heavily on the use of pharmaceutical chemicals such as the organophosphates, pyrethroids or insect growth regulators. Unfortunately these chemicals are characterised by a high potential to cause human or animal toxicity in addition to being environmental contaminants. Another problem resulting from their continued use has been the development of resistance in the treated flies. Newer products need to be discovered. One source of these compounds could be the ethnoveterinary plants already in use by subsistence farmers where insecticides are either unavailable or unaffordable. This study focused on seven plant species used as such in South Africa and Zimbabwe: *Aloe marlothii* A. Berger, *Aloe zebrina* Baker, *Calpurnia aurea* (Aiton) Benth, *Psydrax livida* (Hiern) Bridson (*Canthium huillense*), *Clausena anisata* (Willd), *Erythrina lysistemon* Hutch, and *Spirostachys africana* Sond.

In the first step of evaluation, the selected plant species were screened for their activity against selected wound contaminating bacteria, which are recognised as the inciting factor attracting myiasis-causing flies due to the characteristic odour they produce. Using the microdilution and bioautography methods, all plants had inhibitory activity with the acetone extracts being superior with MICs ranging from 0.04 to 0.6 mg/ml. Using the surface area of a general wound and the density of the extract, it was demonstrated that wounds treated with these extracts could reach these MIC levels. It was concluded that these plants could be beneficial in managing myiasis, in part, by reducing secondary infections and fly attraction.

Two studies were undertaken to determine the *in vitro* effects of these plant species on the behaviour and development of blowfly larvae. For the first study, larvicidal activity of the seven plant species was determined on third instar larvae fed on a combination of

meat (30 g) baited with the acetone leaf extracts (1 ml at 10 mg/ml). *Aloe zebrina*, *C. anisata*, *E. lysistemon* and *S.africana*, induced developmental anomalies in the larvae including paralysis, prolongation of the prepuparium stage, reduced pupation rates, pupae malformation and reduced adult emergence. The results suggested that the plant extracts interfered with the neuroendocrine control in the blowfly. For the second study the four active plant species from the first study were further evaluated at 10, 25, 50, 75, 100 and 150 mg/ml. Larval behaviour, larval development and emergence of adult flies were assessed after exposure to the baited meat. The increasing concentrations of the acetone plant extracts decreased ingestion of the meat by the larvae, pupae mass and adult emergence rates. For *C. anisata* and *S. africana* extracts the increase in the concentration was also associated with larvae circling on top of the testing cups, as far away as possible from the meat, possibly indicating repellency and the emerging adult flies being smaller.

Clausena anisata was selected for field evaluation on populations of blowflies on two farms (one control and one test site) in Mpumalanga (South Africa) over 12 weeks when fly populations were highest. Larvae exposed to liver baits treated with C. *anisata* showed slow development, prolonged larval period, smaller body size, sluggish behaviour, delayed pupation and reduced eclosion rates in comparison to the controls. No significant differences were present between the numbers and sizes of flies on the treated and on the control farm. A difference in fly species was noted on the baits before and after treatment, which may be indicatory of a repellent effect. It is concluded that *C. anisata* could be beneficial in an overall control strategy through its ability to decrease blowfly populations in the long term by reducing the total number of life cycles completed in a year and perhaps as a surface repellent agent.

From the beneficial *in vitro* and in field presence of efficacy, isolation of the active compound(s) from *C. anisata* was attempted. As a first step, due to the potential repellent effect on the farm and the *in vitro* result suggesting a similar effect, the plant was screened for the presence of pyrethrins which are known natural repellents. None of the pyrethrins were present although a terpene compound with an Rf value close to the pyrethrin II compounds was present. Using bio-guided fractionation and column

chromatography, seselin was isolated and identified from the n-hexane fraction which was most active. This study therefore adds support for the use of the selected plants in myiasis and more importantly demonstrates that *C. anisata* may be valuable as a new agent in the control of farm fly populations.

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CHAPTER 1

1.1 Introduction

Myiasis is the infestation of live vertebrate animals with blowfly larvae which, at least for a certain period, feed on the host's dead or living tissue, liquid body substances, or ingested food (Zumpt, 1965). These larvae, known as maggots, use the mentioned body fluids or exudates as their food source for their growth and development, and in the process cause severe tissue damage resulting in discomfort, loss in production, reproduction problems, blindness, lameness and even death (Farkas *et al*., 1997; Snoep *et al*., 2002). Although myiasis has been recognised as a major disease from ancient times (Sherman *et al*., 2000) the disease is still poorly controlled in the animal production industry of many countries in modern times, leading to severe economic losses. Losses that have resulted include abortions, decreased milk production, losses in weight and fertility, poor hide quality, muscle damage and even death from toxicity or secondary infections (Heath *et al*., 1987). In South Africa the direct losses to blowfly attacks and associated treatment is not quantified. One manufacturer of traps estimated an annual loss of R40 million in lost productivity (Anonymous, 2009a).

Species of flies in the families Calliphoridae, Sarcophagidae and Muscidae are the main cause of cutaneous myiasis (Hall, 1991). Of these, the old world screwworm *Chrysomya bezziana* of the Calliphoridae family is the major obligate agent of wound myiasis in most mammals in tropical Africa. In South Africa the parasite is most prevalent in the eastern coastal area, while in Zimbabwe the parasite is distributed across the entire country (Baker *et al*., 1968; Hall, 1991). *Lucilia cuprina,* also of the Calliphoridae family, is the other predominant species inducing ovine cutaneous myiasis (sheep-strike) in many countries worldwide including Australia, New Zealand and South Africa (Dalwitz *et al*., 1984; Heath, 1994; Norris, 1990; Fourie and Horak, 2000).

The control of the myiasis-causing flies relies heavily on the use of pharmaceutical chemicals such as the organophosphates, pyrethroids or insect growth regulators under first world conditions (Phillips, 2009). Unfortunately these chemical compounds are characterised by a low safety index with high potential to cause

human or animal toxicity as well as being contaminants of the environment (Colwell and Dorchies, 2004; Wardhaugh, 2005). Their continued use has also promoted the development of resistance (Hemingway and Ranson, 2000), with the result that newer products need to be developed.

One source of these compounds could be the flora of Africa, in a similar manner in which pyrethrins were identified from the chrysanthemum flower (*Chrysanthemum cinerariaefolium* and *C. coccineum*) (Casida, 1980). To date numerous plants have been identified in numerous surveys for use in the treatment of myiasis. They include: *Aloe marlothii* A. Berger (Van der Merwe *et al*., 2001), *Aloe zebrina* Baker (Luseba and Van der Merwe, 2006), *Calpurnia aurea* (Aiton) Benth (Hutchings *et al*., 1996), *Psydrax livida* (Hiern) Bridson (*Canthium huillense*), *Clausena anisata* (Willd) Hook *(*Chavunduka, 1976), *Erythrina lysistemon* Hutch (Van Wyk *et al*., 1997), and *Spirostachys africana* Sond (Hutchings *et al*., 1996). It was believed that these plants could therefore lead to the discovery of novel structures or the development of active plant extracts for use to protect animals.

1.2 Hypothesis

Medicinal plants used traditionally to treat myiasis have the potential to control myiasis-causing fly larvae.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study was to develop a product based on traditionally used plants that could be used in the treatment of myiasis in animals.

1.3.2 Specific objectives

The specific objectives were to:

a. Evaluate the antibacterial, antioxidant and cytotoxicity activities, *in vitro*, of some plants used to treat cutaneous myiasis in South Africa and Zimbabwe.
b. Evaluate the effect of leaf extracts of selected plants used traditionally to treat cutaneous myiasis in South Africa and Zimbabwe on the development and behaviour of *Lucilia cuprina* and *Chrysomya bezziana in vitro*.

c. Isolate and identify the active compound(s) in the plant extract with most promising activity.
d. Evaluate the value of the most effective extract in decreasing total blowfly populations on farms.

The identified objectives are addressed as published or submitted manuscripts in chapters 3 to 7.

CHAPTER 2

2 Literature Review

2.1 Livestock agriculture

Agriculture forms the backbone of Africa's economy with about 70% of Africans dependent mainly on agriculture for their livelihood (Christiaensen *et al*., 2011). Livestock production contributes about 20-25% of the agricultural gross domestic product with meat and milk being important components in the diet of most people. Livestock also act as a "bank" for provision of cash derived from sales of their products or of the animals themselves in times of crisis, to raise the funds needed to purchase food and meet other family needs. Unfortunately, 25% of livestock in Africa die annually in part from diseases because prevention and treatment is constrained by limited vaccine availability and poor distribution systems of medicinal products (Scoones and Wolmer, 2006). While the financial loss attributed to livestock diseases in sub-Saharan Africa is difficult to quantify, the overall economic losses has been estimated at US$ 2 billion as reflected by reduction in growth, lactation, work output and reproduction are probably of the same magnitude (Anonymous, 2009b).

There are numerous factors that have made it difficult for Africa to deal with the challenge of disease in livestock (Gifford-Gonzalez, 2000; Cheneau, *et al*., 2004; Bonnet *et al*., 2011; GALVmed, 2012). These include:

- Lack of veterinary services: The current state of veterinary services and preparedness levels in developing/transition countries continues to pose a real threat to the ability of preventing and controlling major diseases (GALVmed, 2012). Many African countries have few trained veterinarians to assist in the treatment of animal diseases. In a survey, in 178 OIE Member Countries it was noted that more than half of the countries have fewer than 35 public sector veterinarians per million inhabitants and fewer than 100 private sector veterinarians per million inhabitants in the private sector (Bonnet *et al*., 2011). Resultantly veterinary services in Africa are failing to adequately meet the animal health care needs of poor and smallholder farmers, particularly those living in remote and marginalised areas.

- Lack of access to veterinary medicines: African veterinary services are relatively poorly financed and equipped to deal with disease challenges (GALVmed, 2012). Whilst private practices can be found in nearly all urban and peri-urban areas, they only infrequently reach poor and small farmers in rural areas (Cheneau *et al*., 2004). The great majority of rural poor do not have access to the range and quality of services and products required to support a robust livestock-related livelihood
- Failure of pharmaceutical companies to invest in Africa's diseases: Major pharmaceutical companies regard Africa as a poor market and are unwilling to invest in research and commercialisation of new Africa-specific products (Omamo and d'Ieteren, 2003; GALVmed, 2012). The risk of being undermined by the inability of national authorities to protect their investment from counterfeits, doctoring of the product and generic copies has exacerbated the situation (Omamo and d'Ieteren, 2003)
- Prohibitively high cost of services: Most African countries do not manufacture their own veterinary products and they rely on imports. This results in the prohibitively high cost of the products for the farmers in the region (Vudriko et al., 2011).
- Fraudulent drugs being marketed: Poor quality products are being sold throughout the region and good quality ones are unavailable in many areas (Leonard, 2007). For example, Senegal has 140 veterinary drugs registered but more than 1000 different veterinary drugs marketed (Van Gool, 2008). Throughout the region medicines, vaccines and acaricides are being distributed from a bewilderingly wide variety of manufacturers, have inconsistent dosages, are often past their expiry date, or are sometimes counterfeit. In most countries in Africa formal trade in veterinary pharmaceuticals is subject to import controls by the state and is handled by licensed pharmacies or those working under the supervision of certified animal health personnel. This model is too restrictive, making it very difficult for farmers to obtain the drugs they need legally. And an unmet demand gives special impetus to informal trade, outside of legal channels (Leonard, 2007). As a result, the quality of veterinary medicines reaching smallholder farmers in Africa is poor and getting worse. Analyses of veterinary products in East

and West Africa showed that most did not conform to what was stated to be in the product according to the data sheet supplied and that some contained no active ingredient at all (Leonard, 2007).

- Lack of formal agricultural training amongst informal farmer: Most of the rural and smallholder farmers have no formal training in animal production practices or access to extension agricultural services (Lehloenya *et al*. 2005). This limits their understanding of correct disease diagnosis, use of appropriate dosages and adoption of new technologies in disease control.

The impact of animal diseases, either due to overt disease or disease risk, are all likely to be proportionally greater for the poor because they are exposed to more animal disease risk and have less capacity to cope with that risk than those who are better-off (Anonymous, 2009b). Although veterinary products and services (vaccines, medicinal products, particularly antibiotics and antiparasitics, and veterinary health care) are essential, they are not systematically available to the poor, especially in rural areas. Moreover, they are usually too expensive, diminishing their accessibility to these resource poor farmers. As a result smallholder farmers have had to turn to low cost alternatives. Ethnoveterinary medicine is gaining recognition at the expense of conventional drugs especially because of its greater accessibility, lower costs and apparent effectiveness (Ghirotti, 1996; Wanyama, 1997; Matekaire and Bwakura, 2004; Mwale *et al.,* 2005). Even in commercial farming where the farmers have the resources to access veterinary products and services, there is still a challenge of disease organisms and pests developing resistance (Hemingway and Ranson, 2000). Important opportunities exist in exploring ethnoveterinary medicine, as it may lead to the discovery of novel lead structures to be used in drug production, and also to the development of active plant extracts useful in treating a variety of ailments in animals.

2.2 Ethnoveterinary medicine

The use of ethnoveterinary medicine (EVM) is practised widely for animal health care in many developing countries in Asia and Africa where orthodox veterinary healthcare services are either unavailable or unaffordable (Mathias-Mundy and McCorkle, 1989). In some cases it is the only animal health care intervention available to resource poor farmers (Van der Merwe *et al*., 2001, Abebe, *et al*., 2003;

Matekaire and Bwakura, 2004; Mathias, 2004). It is a complex system of practices made up of various combinations of folk beliefs, skills, knowledge and animal husbandry practices with herbal remedy at the core of the therapy (McCorkle, 1986; Schillhorn van Veen, 1996; Van der Merwe *et al*., 2001). At present, research from different parts of the world indicates that the use of EVM is extensive with farmers, herders, pastoralists and occasionally veterinarians using plants or plant products in the management of a wide spectrum of diseases/conditions (Chavunduka, 1976; Bizimana, 1994; Anonymous, 1994; Hutchings *et al*., 1996; Wanyama, 1997; Martin *et al*., 2001; Viegi *et al*., 2003; Matekaire and Bwakura, 2004; Mwale *et al*., 2005; Luseba and Van der Merwe, 2006; Eloff and McGaw 2006, Lans *et al*., 2007). Examples of diseases and conditions treated with plants include parasitism, traumatic wounds, diarrhoea, dermatitis, ocular disorders, reproductive disorders, poor milk flow, snake bites, bloat and fractures.

While the use of ethnoveterinary medicines is common practice in rural farming areas, it is often questioned for its inherent safety and efficacy by the western world as their use has developed through trial and error and only rarely via deliberate experimentation as expected for the development of modern pharmaceuticals (Katere and Luseba, 2010). As a result EVM has been viewed as less systematic, less formalized, and at times even questioned for its validity. Nonetheless, there is a growing acceptance that EVMs have therapeutic value, and need further evaluation to firstly justify their use, but also as a potential source of newer medications to combat multi-resistant pests and disease organisms (Eloff and McGaw 2006; Lans et al., 2007).

To date the mining of herbal remedies for newer therapies remain small. A recent review by McGaw and Eloff (2008) estimated that only approximately 13% of EVM plants in South Africa have been evaluated for the presence of any biological activity using a valid laboratory model or animal study. While the majority of these studies have focused on potential antibacterial, antifungal and anthelminthic activities, only a few focused on Africa's economically important tick, protozoal, and/or rickettsial diseases (McGaw and Eloff, 2008). As such, much research is required to evaluate plants used to control and treat diseases endemic to Africa, particularly the "neglected diseases" (Anonymous, 2009b). One such "neglected disease" is myiasis, which is an

ectoparasitic disease that results from fly larvae infesting production animals, with only a few reports describing the efficacy of EVMs against it (Morsy *et al*., 1998; Kumarasinghe *et al*., 2002; Khater and Khater 2009). More importantly, to the best of my knowledge no such information is available on the bioactivity of plants, in the southern African region.

2.3 Myiasis

Myiasis is defined as the infestation of living vertebrate animals with dipterous (belonging to the Diptera, a large order of insects that includes the true flies and mosquitoes) larvae which, at least for a certain period, feed on the host's detritus or living tissue, liquid body substances, or ingested food" (Zumpt, 1965). There are two main systems for categorizing myiasis:

- Entomologically, this separates the parasites according to their level of dependence on the host and their preferred food source. Those larvae that feed on only diseased and dead tissue are known as facultative myiasis parasites while those species that feed on the living tissues are known as obligate myiasis parasites. A major difference between these two groups is that the facultative myiasis parasites, which are usually free living, can adapt to a parasitic dependence on the host, while the obligatory myiasis parasites are completely parasitic and die without a host (Zumpt, 1965).
- Anatomically, this separates the parasites by their preferred anatomical location on/in the host. The first attempt in anatomical system of classification was by Zumpt (1965) who simply grouped the myiasis into sanguinivorous, dermal/subdermal (cutaneous), nasopharyngeal, intestinal and urogenital. Of these, cutaneous myiasis is most important, as the invasion of skin tissues by larvae of Diptera create burrows or abscesses in the dermal layers, invade and enlarge existing wounds or form new wounds themselves.
- Stage of infestation: Myiasis can also be divided by the type of larvae involved viz. primary, secondary and tertiary. Primary myiasis occurs when the larvae involved are obligate parasites of living tissues and are able to initiate myiasis. Secondary myiasis is caused by necrophagous (feeding on carrion or corpses) larvae flies, which feed on necrotic tissue. These larvae are unable to initiate

myiasis and will only infest pre-existing wounds. Tertiary myiasis occurs when normally free-living larvae infest an animal when host is near death (Hall, 1991).

2.3.1 Cutaneous myiasis as a major disease

While cutaneous myiasis is a major production limiting disease in Africa occurring in all animals, the species most prone to myiasis are sheep. Cutaneous myiasis results when female flies lay their eggs on the edges of wounds and wool of sheep soiled with urine, faeces or blood (Figure 2-1). The emerging larvae feed on the skin tissues and fluids. Cutaneous myiasis may be benign, as when facultative species confine their activities to diseased and dead tissue, or it may be malignant as when the obligate and/or facultative species attack living tissue (Hall, 1991).

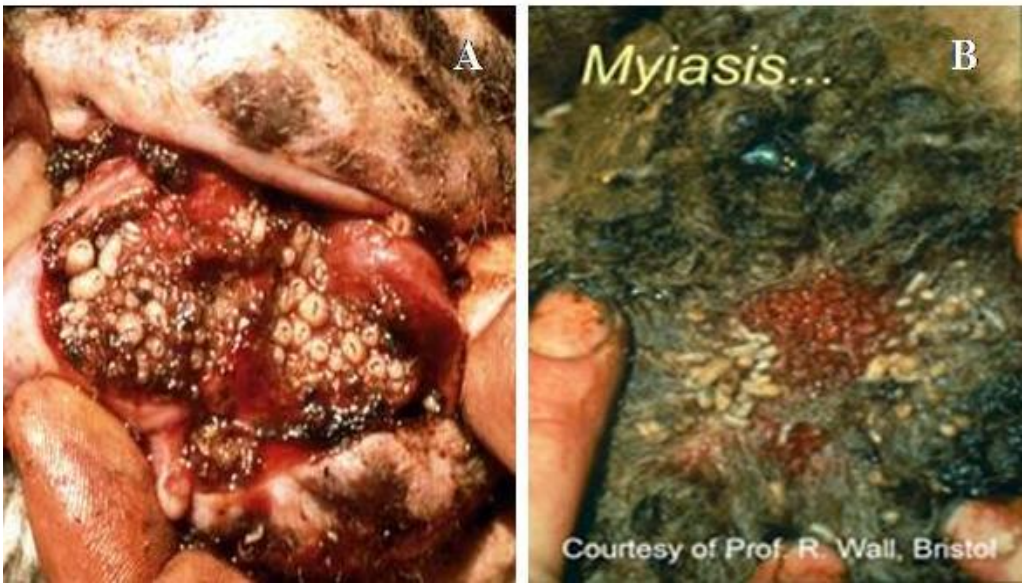


Figure 2-1: Wound myiasis in sheep in the region of the vulva (A) and the rump (B) (Hall, 1997; Wall, 2001)

2.3.2 Agents of Cutaneous Myiasis

There are 18 different genera of flies that may be encountered in cases of cutaneous myiasis. They belong to seven families, Calliphoridae, Sarcophagidae, Muscidae, Phoridae, Cuterebridae, Gasterophilidae and Oestridae (Hall, 1991). Of these, only the first three families are important in wound myiasis and sheep-strike. The three major species of obligate parasites encountered in wound myiasis are *Cochliomyia*

hominivorax, *Chrysomya bezziana*, and *Wohlfahrtia magnifica* (Figure 2-2) (Hall, 1991). Among the facultative parasites, *Lucilia cuprina* and *Lucilia sericata* (Figure 2-2) are the most important. They are the major causative agents for sheep strike in many countries worldwide including Australia (Dalwitz et al., 1984), New Zealand (Heath, 1994) and South Africa (Norris, 1990).

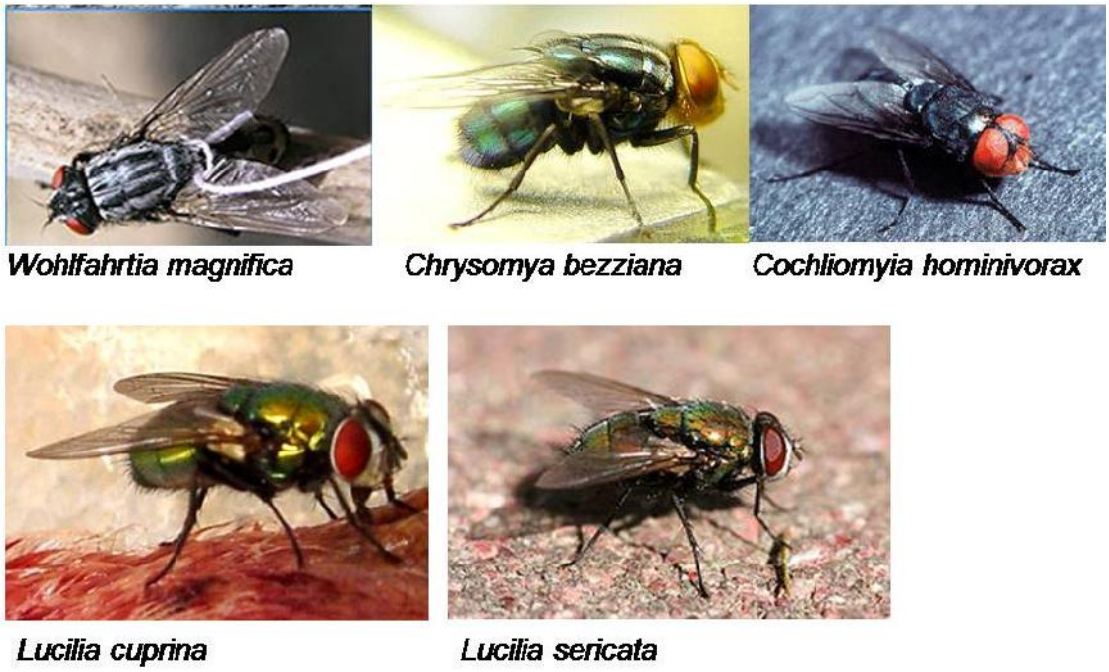


Figure 2-2: The adult flies most commonly associated with myiases

Wohlfahrtia magnifica (Hall, 2010)

Chrysomya bezziana (Screwworm Commission)

Cochliomyia hominivorax (Kucharski, 2003)

Lucilia cuprina (Major, 2009)

Lucilia sericata (Calibas, 2006)

General Sheep Blowfly Life Cycle

Adult flies live for approximately two to three weeks. Blowflies thrive in warm (17ºC-38ºC) and humid conditions (Wilson and Armstrong, 2005). Mature females usually lay two or three batches of eggs with about 200-500 eggs per batch. Within 8–24 hours of the eggs being deposited, the first instar larvae emerge and develop into second instar after 12-18 hours, then further moult into the third instar about 30 hours later. Larvae grow from pin head size to 10-15 mm in length in about 3-4 days, then drop off the sheep to commence pupation a day or two later. They usually fall off at

night or in the early morning when ground temperatures are coolest, and burrow into the soil. This means that a large proportion will pupate and subsequently emerge as blowflies from around sheep camps. Adult flies will normally not travel more than three kilometres from where they hatch during their life span.

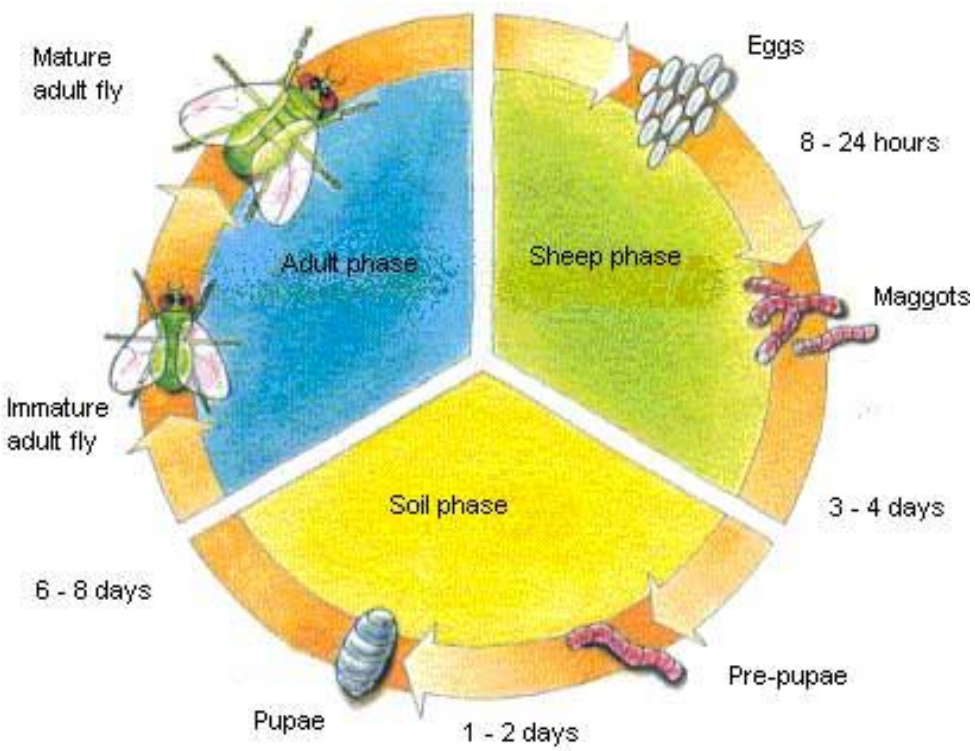


Figure 2-3 : The general Blowfly Life Cycle from egg, larvae, pupae to adult
(Source: Wilson and Armstrong, 2005)

The life-cycle of the myiasis fly is illustrated in Figure 2-3 above (Wilson and Armstrong, 2005). The sheep phase (in green) is about 4 days long during which eggs are laid on live sheep. On hatching the emerging larvae (maggots) feed on sheep tissues and fluids and undergo three moults to reach the mature stage. The soil phase (in yellow) is 7 – 10 days long. After the larvae have had enough food on the sheep they fall off into the ground and burrow into the soil. They pupate in a day or two. Under ideal conditions the formed pupae will hatch in 6- 8 days. The adult phase (in blue) is the period in which the pupae hatch and the immature adult fly develops into a mature fly which begins to mate and prepare for egg laying. This adult phase lasts 2 to 3 weeks.

2.3.2.1 Chrysomya bezziana

Chrysomya bezziana, of the subfamily Chrysomyinae of the dipteran family Calliphoridae (blowflies) is an obligate parasite of mammals including humans and rarely birds (Hall, 1991). It is metallic greenish-blue in colour with a yellow-orange face and reddish-brown eyes (Figure 2-2). On average C. bezziana adults are 10 mm long (Spradbery, 1991). The distribution of C. *bezziana* is confined to the Old World (Africa, Asia and Europe) hence it is called the Old World Screwworm (Anonymous, 2008). It is found throughout much of Africa (from south of the Sahara to northern South Africa), the Indian subcontinent and in Southeast Asia (from southern China through the Malay Peninsula and the Indonesian and Philippine islands to New Guinea). It has also been introduced into several countries on the west coast of the Persian Gulf (Figure 2-4) (Spradbery, 1991).

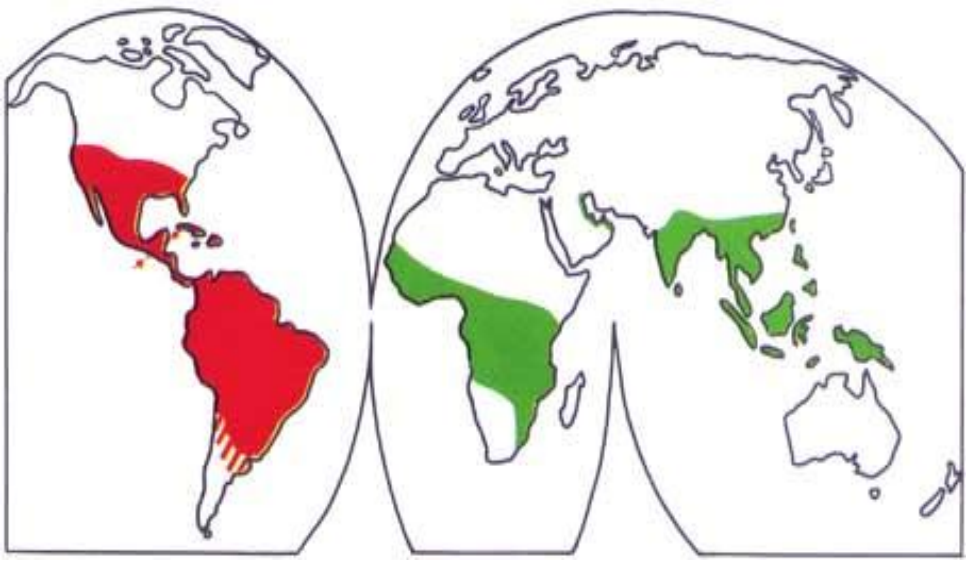


Figure 2-4: Geographic distribution of *Chrysomya bezziana* (green) and *Cochliomyia hominivorax* (red) (Adapted from Hall, 1991).

Chrysomya bezziana lays its eggs at body orifices or wound edges of injured mammals. The numbers of eggs laid per batch; which varies by fly strain, averages in the order of 175 eggs (Spradbery, 1994). The emerging larvae immediately begin to feed on the wound fluids and underlying tissues, burrowing gregariously head-

downwards into the wound. If eggs are deposited on mucous membranes, the larvae can invade undamaged natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears, and genitalia (Anonymous, 2008).

As the larvae feed, tearing the tissue with their hook-like mouthparts (Figure 2-5), the wound is enlarged and deepened, resulting in extensive tissue destruction (Hall, 1991). The feeding activity of the larvae may cause severe tissue damage, resulting in discomfort, loss of production, reproduction problems, blindness, lameness, decreased appetite and low milk production (CFSPH, 2007). Infested wounds often emit a pungent odour, which can be the first indication that at least one animal in a group is infested. Although the odour is not always apparent to humans, it is highly attractive to other gravid females, which lay further batches of eggs adding to the infestation (Hall, 1995). Severe infestations left untreated may result in the death of the host due to toxicity or secondary infections (Anonymous, 2008).

Figure 2-5: Hook-like mouthparts of the screwworms (*C. bezziana* and C. *hominivorax*) and circular hooks around body which allows the larvae to cork-screw into the wound (Kucharski, 2003)

Virtually any wound is attractive to C. *bezziana*, whether natural from fighting, predators, thorns, disease, and/or tick and insect bites or man made from shearing, branding, castrating, de-horning, docking, and/or ear-tagging. Commonly infested natural wounds are the navels of newborn animals and the vulval and perineal regions of their mothers, especially if traumatised. Animal mostly affected include cattle, water-buffalo, sheep, goats, horses, donkeys, dogs, camels, elephants, impala, bushbuck, waterbuck, giraffe, lion, white rhinoceros, eland and black rhinoceros (Hall, 1991). Screwworm myiasis occurs mostly in the wet season when the high temperatures and humidity are conducive for the flies and larval growth e.g. in Zimbabwe *C. bezziana* has been reported to be the second most serious insect pest of cattle after the tsetse fly, *Glossina spp.* (Hall, 1995); particularly during the peak of rainy seasons where it causes myiasis in ears of cattle following heavy infestations with the brown ear tick (*Rhipicephalus appendiculatus*) (Norval *et al*., 1988).

2.3.2.2 Cochliomyia hominivorax

The *C. hominivorax* fly has a deep blue to metallic blue-green colour with a reddish orange head and eyes, and it is 8 -10 mm in length (Figure 2-2)(Spradbery, 1991). It is present in the New World (the Americas) tropics, hence the fly is commonly called the New World Screwworm (NWS). It is distributed throughout the neoarctic and neotropical regions of the western hemisphere (Figure 2-4). Extant populations are found in Central and South America and in certain Caribbean Islands. Historically the range of NWS extended from the southern states of the USA through Mexico, Central America and the Caribbean Islands to northern Chile, Argentina and Uruguay (Hall and Wall 1995) and covered an area of some 23 million km^2. However, this has been largely curtailed through the use of modern fly control practice, which in this case was specifically through the use of a sterile male breeding technique (Hall and Wall 1995). The inability of NWS and OWS to survive low temperatures largely determines their geographic ranges (Spradbery, 1994).

The NWS is an obligate parasite of mammals, rarely birds, and well known for the way in which its larvae eat the living tissue of warm-blooded animals (Hall, 1991). The screw-worm maggots attack healthy tissue. Screw-worm females lay 250-500 eggs in the exposed flesh of warm-blooded animals, including humans, such as in

wounds and the navels of newly-born animals. The larvae hatch and burrow into the surrounding tissue as they feed. The maggots are capable of causing severe tissue damage or even death to the host.

2.3.2.3 Wohlfahrtia magnifica

Wohlfahrtia magnifica, the spotted flesh fly, (Figure 2-2) belongs to the family *Sarcophagidae*. Adults of W. *magnifica* have a striped greyish thorax and an abdomen whose dorsal surface is bright white with a series of contrasting dark black spots, three per tergite (the dorsal sclerite of an abdominal segment of an insect), with the central spots elongated and tending to merge at their anterior edge with the one above. The adults are about 8-14 mm long (Hall *et al*., 2009). It is widely distributed throughout the warmer, southern parts of the Palaearctic region, from the Mediterranean basin, through central and Eastern Europe to northern and central Asia, Mongolia, China and Afghanistan (Hall and Farkas, 2000). *Wohlfahrtia magnifica* is commonly known as the Palaearctic Screwworm (PSW) owing to its geographical distribution. Like most sarcophagids, adult females of *W. magnifica* deposit live larvae, each laying up to about 160, on living warm-blooded animals and they immediately begin to feed on the underlying flesh, moulting twice before reaching maturity, at which point they drop to the ground to pupate. The larvae of PSW burrow deeply into the host's tissues so that only the posterior spiracles are exposed. The adult emerges from the pupa within about one week if not in diapause (period of suspended development) (Hall, 2010). *Wohlfahrtia magnifica* is largely responsible for wound myiasis in southern and eastern Europe, Russia, the Near East and North Africa (Colebrook and Wall, 2004) where it predominately affects sheep and camels, although other hosts such as goats, horses, cattle and pigs are also infested. The genitalia of healthy animals are major infestation sites and that can lead to significant reproductive problems (Sotiraki *et al.,* 2005).

2.3.2.4 Lucilia cuprina

Lucilia cuprina (Figure 2-2) is a facultative ectoparasite of endotherms (Stevens, 2003) particularly the domestic sheep. It is metallic green in colour with reddish eyes.

On average the adult fly is 10 mm long (Wilson and Armstrong, 2005). It is found throughout the world in various warm locations. *Lucilia cuprina* is considered to be the most important cause of 'blowfly strike' in sheep (ovine cutaneous myiasis) in a number of countries including South Africa and Australia. In addition to sheep, other animals attacked include horses, cattle and people (Zumpt, 1965). Female flies deposit their eggs on carcasses, in suppurating wounds or the wool of sheep soiled with urine, faeces or blood. *Lucilia cuprina* female flies lay about 1000 to 3000 eggs in their lifetime of about 21 days. The larvae emerge within 8 hours of being deposited and immediately start feeding on the skin's surface. In the process of feeding the larvae contaminate the wound with bacteria and ammonia carried in the saliva (Tourle *et al*., 2009), which leaves behind a pungent smell which in turn attracts secondary invaders such as *Calliphora croceipalpis Chrysomya albiceps* and *Musca domestica.*

Once infested, sheep commonly show signs of skin irritation characterised by rubbing and biting the affected areas after a first few days from when the larvae start feeding (Farkas *et al*., 1997). The delayed response to clinical signs causes the wool to break in the affected area. After approximately 3 to 4 days, the larva falls off the sheep to commence pupation a day or two later. Under ideal humid circumstances (75% RH, at a temperature of about 25ºC) an adult blowfly can emerge 6 days later and within days can start producing eggs. If it is not treated, severe infestation of sheep by *L. cuprina* larvae induces pathology which can lead to death. The toxin responsible for the pathology and death is ammonium bicarbonate in the excreta of larvae (Guerrini *et al*., 1988a). Ammonium bicarbonate decomposes at 36 to 60°C into ammonia, carbon dioxide and water vapour. The degree to which ammonia forms from ammonium depends on pH. If the pH is low, more ammonia molecules are converted into ammonium ions. If the pH is high, the hydroxide ion abstracts a proton from the ammonium ion, generating ammonia. At the site of infestation the pH ranges between 8.2 and 9.3 which promotes ammonia generation (Guerrini *et al*., 1988b). The larval ammonia from infested sites diffuses into peripheral veins draining infested sites causing rises in systemic ammonia (Guerrini 1997). The ammonia diffuses into the epithelium of the lymphatics, lungs and heart, and eventually results in the vacuolation of the white matter tracts of the central nervous system (Hooper et al., 1972).

Lucilia sericata shares many similar characteristics with *L. cuprina*. These flies are very similar in appearance and morphological characteristics, and are known to cause myiasis (flystrike) in sheep (Stevens and Wall, 1996). Identification between these requires microscopic examination of two main distinguishing characteristics. As opposed to *L. cuprina* which has a metallic green femoral joint in the first pair of legs, *L. sericata* is blue-black and when looking at the occipital setae, *L. sericata* has 6–8 bristles on each side while *L. cuprina* have only one (Bishop, 1991).

2.3.3 Impact of cutaneous myiasis

Flystrike is a serious problem in all the major sheep producing countries of the world, in particular, Australia, New Zealand, South Africa and the United Kingdom. The disease in these sheep is debilitating and results in major production losses and occasionally deaths (Phillips, 2009; Sotiraki and Hall, 2011). The average annual cost of fly strike to the South African sheep industry was estimated at R19.8 million in 1997 (Leipoldt and van der Linde, 1997) and in 2009 one manufacturer of traps estimated an annual loss of R40 million in lost productivity (Anonymous, 2009a). In the Australian sheep industry the annual cost is $A 280 million (Sackett *et al.* 2006). Economic losses associated with flystrike are incurred through direct damage to the animal (most frequently sheep, but occasionally goats and cattle as well), so that growth is slowed, wool is stained, the pelt is damaged, and in extreme cases the animal dies. There is also a considerable expenditure on insecticides applied, mainly as preventive measures, but also used as therapeutic agents. Finally, the time spent in maintaining surveillance over sheep at risk to flystrike, as well as keeping them free of material attractive to flies is a major burden on the labour resources of farmers and can be considered as legitimate costs against flystrike, because these procedures contribute towards prevention of the disease (Heath, 1994).

2.3.4 Control of cutaneous myiasis

2.3.4.1 Chemical control

The majority of registered products used to prevent and treat cutaneous myiasis belong to the following chemical groups:

A. **Organophosphates**

The organophosphate (OP) compounds are a diverse group of chemicals used in both domestic and industrial settings (Katz and Brooks, 2012). Examples of organophosphates include insecticides (malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, ethion), nerve gases (soman, sarin, tabun, VX), ophthalmic agents (echothiophate, isoflurophate), and anthelminthics (trichlorfon). Organophosphates contain a central phosphorus atom with a double bond to either oxygen or sulphur, R1 and R2 groups that are either ethyl or methyl moieties, and a leaving group OR′, which is specific to the individual organophosphate (Figure 2-6) (Gbaruko *et al*., 2009). Diazinon and malathion are the commonly used OPs in myiasis treatment and control. Organophosphates kill most maggots on existing strikes but will only provide very limited protection (two to four weeks) against new strikes (Wilson and Armstrong, 2005).

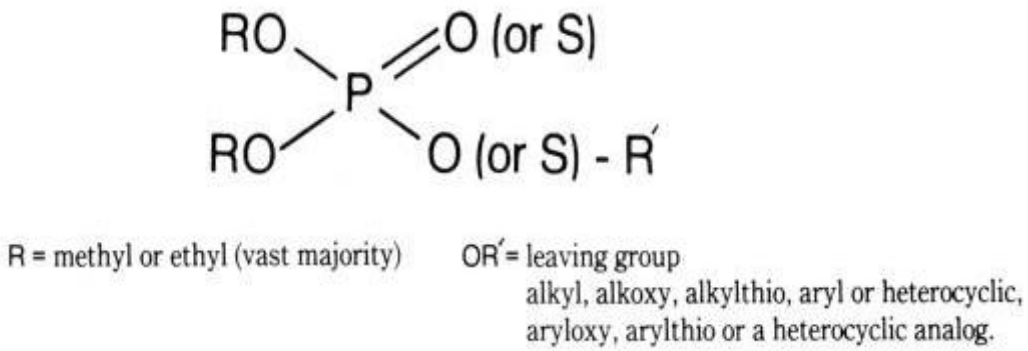


Figure 2-6: The general chemical structure of organophosphates (Gbaruko *et al*., 2009).

The primary mechanism of action of organophosphate pesticides is inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE) (Bajgar, 2004),

which is essential to nerve function in insects, humans, and many other animals. AChE is an enzyme that degrades the neurotransmitter acetylcholine (ACh) into choline and acetic acid. Acetylcholine is found in the central and peripheral nervous system. Organophosphates inactivate AChE by phosphorylating the serine hydroxyl group located at the active site of AChE. The phosphorylation occurs by loss of an organophosphate leaving group and establishment of a covalent bond with AChE. Once AChE has been inactivated, ACh accumulates throughout the nervous system, resulting in overstimulation of muscarinic and nicotinic receptors leading to the death of the targeted insect (Watanabe, 1989).

By virtue of their ability to inactivate AChE non-specifically, the organophosphorus insecticides are highly toxic to mammals and birds with resultant acute toxicity (Gruber and Munn, 1998). Signs of toxicity from ACh accumulation include increased lacrimation and salivation; bronchoconstriction; increased bronchial secretions; meiosis (constriction of the pupil of the eye); gastrointestinal cramps; diarrhoea and vomiting; urination; bradycardia; hypertension; muscle fasciculations (particularly the eyelids and facial muscles); general muscle tremors; weakness or flaccid paralysis; restlessness; emotional lability; lethargy; mental confusion and loss of memory; convulsion; coma; and depression of respiratory centres (Watanabe, 1989; Ecobichon, 1996). Overstimulation may persist for hours to days or even weeks if "aging" has occurred.

Aging involves dealkylation of the bound inhibitor and strengthening of the phosphorus-enzyme bond. The rate of aging varies depending upon the OP compound. Phosphorylated AChE is reactivated by the highly nucleophilic oximes (e.g., pralidoxime); however, aged phosphorylated AChE is not reactivated by oximes (Vale, 1998). In addition to the inhibition of AChE, some OP compounds have additional actions on mammals. They bind to neuropathy target esterase (NTE, or neurotoxic esterase) present in neural tissue (Abou-Donia and Lapadula, 1990). This results in organophosphate-induced delayed neurotoxicity (OPIDN). OPIDN is characterized by ataxia beginning 1 to 3 weeks following exposure, resulting from degeneration of the long axons of neurons in the central and peripheral nervous systems. Signs of OPIDN are an initial weakness of the lower extremities, progressing to the upper extremities, until ataxia and eventually paralysis set in.

Organophosphates can be absorbed rapidly via all routes: respiratory, gastrointestinal, ocular, and dermal. The onset of symptoms is quickest after inhalation (Vale, 1998). Dermal absorption is slower but can result in severe toxicity if exposure is prolonged and can be enhanced if the agent is lipophilic and helped by the solvent and emulsifier used in the formulation of the pesticide. Once absorbed the organophosphate pesticides distribute and accumulate rapidly in fat, liver, and kidney (Vale, 1998). Organophosphate pesticides degrade rapidly by hydrolysis on exposure to sunlight, air, and soil, although small amounts can be detected in food and drinking water (Agrawal and Sharma, 2010). This is a cause-for-concern because of their toxicity, especially in the aquatic environment.

Resistance to OPs was, first reported 14 years after their introduction (Green et al., 1990). Resistance of blowflies to some organophosphate insecticides in South Africa was first reported in the mid 1950s (Fiedler and du Toit, 1956). Resistance is suspected to be present when a reduced protection period is noted. When the OPs were first used for fly prevention, protection for up to 12 weeks was expected. Today many flocks may only receive 2-4 weeks or less protection from flystrike (Evans and Karlsson, 2004). Recent studies have shown that about 90% of the fly population is resistant with the degree of resistance increasing with time (Levot, 1995; Evans and Karlsson, 2004). Resistance to OPs in *Lucilia cuprina* has developed through gene mutations in individual flies which allowed them to survive exposure to an insecticide at a time when normal insects die. *Lucilia cuprina* developed resistance to malathion through a point mutation in the gene that normally produces a carboxylesterase, E3. The mutation improves the ability of carboxylesterase to break down dimethyl OPs, particularly malathion (Newcomb *et al*., 2005). In the case of diazinon, resistance is associated with a separate point mutation that causes the enzyme to lose the carboxylesterase activity and a new OP hydrolase activity is conferred on the enzyme, making it more effective against diethyl OPs such as diazinon (Lightner, 2008).

B. **Pyrethroids**

Pyrethroids are synthetic compounds similar to the natural pyrethrins produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *C. coccineum*) (Lautraite and Sargent, 2009). Pyrethrins are esters of chrysanthemumic acid (R^1 = CH_3) or pyrethric acid (R^1 = CH_3O_2C) (both cyclopropane (three membered ring)

carboxylic acids), with one of three cyclopentanone alcohols (cinerolone, $R^2 = CH_3$; jasomolone, $R^2 = CH_2CH_3$; or pyrethrolone, $R^2 = CHCH_2$) giving six pyrethrin structures (Cage *et al.*, 1998). Despite having good insecticidal activity these natural pyrethrins are rapidly decomposed by light (Casida, 1980). This led to the development of the synthetic analogues, the pyrethroids, with improved stability in outdoor environments, which has increased their marketability in agriculture. They are widely used as agricultural, veterinary and household insecticides (Lautraite and Sargent, 2009). There are two types of pyrethroids, the Type I (non-cyano pyrethroids) and the Type II (α-cyano pyrethroids) (Lautraite and Sargent, 2009). Type I pyrethroids are synthetic pyrethrin analogues with the basic cyclopropane carboxylic ester structure and no cyano group substitution. Examples include allethrin (Figure 2-7A), tetramethrin, resmethrin, d-phenothrin, bioresmethrin, and permethrin. The Type II pyrethroids contain a cyano group at the benzylic carbon atom giving alpha-cyano pyrethroids and the examples include deltamethrin (Figure 2-7B), cypermethrin, cyfluthrin, cyphenothrin, fenvalerate, and fluvalinate (Cage *et al.*, 1998).

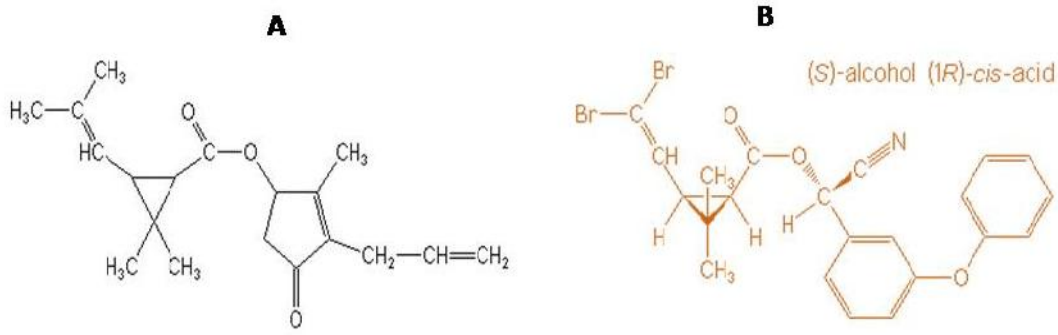


Figure 2-7: The chemical structure of Allethrin (Type I pyrethroid) [A], first pyrethroid, synthesized in 1949 and of Deltamethrin (Type II pyrethroid) [B], the first pyrethroid containing the alpha-cyano-3-phenoxybenzyl moiety developed in 1974

Pyrethroids provide a quick knockdown of insects at low concentrations and have relatively low mammalian toxicity (Sudakin, 2006) because they are quickly deactivated by metabolic processes (Song and Narahashi, 1996). They are effective

against a wide range of insect and mite pests and may be mixed with other pesticides for a broad spectrum of pest control. Both type I and II pyrethroids are axonic poisons, inhibiting the nervous system of insects causing paralysis and death. They act on tiny channels through which sodium is pumped to cause excitation of neurons. They prevent the sodium channels from closing, resulting in continual nerve impulse transmission, tremors, and eventually death of target insect (Soderlund and Bloomquist, 1989). Pyrethroids are usually combined with piperonyl butoxide, an inhibitor of key microsomal oxidase enzymes, which insects use to metabolise the pyrethroids. Piperonyl butoxide therefore prevents the insect's enzymes from clearing the pyrethroid from its body, maximizing the lethality of the pyrethroids through a process of pontentiation (Cage *et al*., 1998).

Pyrethroids also have a high selectivity for insects, being 2250 times more toxic to insects than mammals (Cage *et al*., 1998). This can be explained in terms of differences in the sensitivity of neuronal sodium channels between invertebrates and vertebrates (Song and Narahashi, 1996). The sensitivity of invertebrate neuronal sodium channels to pyrethroids is ten times greater than in mammals (Song and Narahashi, 1996). Furthermore, invertebrates typically have body temperatures 10°C lower than mammals and *in vitro* studies have shown pyrethroids to be more potent at evoking repetitive neuronal discharges at lower temperatures (Song and Narahashi, 1996). It is for the latter reason that pyrethroids are highly toxic to fish and other aquatic organisms (Cage *et al*., 1998). Despite this high selectivity for insects there are still reports of acute toxicity in animals (Wolansky and Harrill, 2008). Signs of pyrethroid intoxication in mammals differ according to type of pyrethroid involved (Verschoyle and Aldridge, 1980). Type 1 pyrethroids toxicity results in hypersensitivity and aggression, general tremor, convulsive twitching, prostration and hyperthermia. Clinical signs of type II toxicity include jerking leg movements and progressive writhing convulsions, salivation, clonic seizures and hypothermia.

Numerous insects have developed high levels of resistance to pyrethroids, these include cockroaches, bedbugs, head lice, and tobacco budworm, pear psylla, fall army-worm, German cockroach, spotted tentiform leafminer, diamondback moth, house fly, stable fly and tobacco budworm (Miller, 1988; Cox, 1998). Nonetheless no recorded resistance to cypermethrin, the commonly used agent to prevent fly strike,

has been reported in Australia, New Zealand and South Africa. In general, site insensitivity and metabolic detoxification are the main resistance mechanisms in insects to pyrethroids insecticides. In the case of site sensitivity, the chemical site of action for the insecticide becomes modified to have reduced sensitivity to the active form of the insecticide. In metabolic resistance the metabolic pathways of the insect become modified in ways that detoxify the insecticide or disallow the metabolism of the applied compound into its toxic forms. The most important forms of metabolic resistance involve multifunction estarases (Miller, 1988).

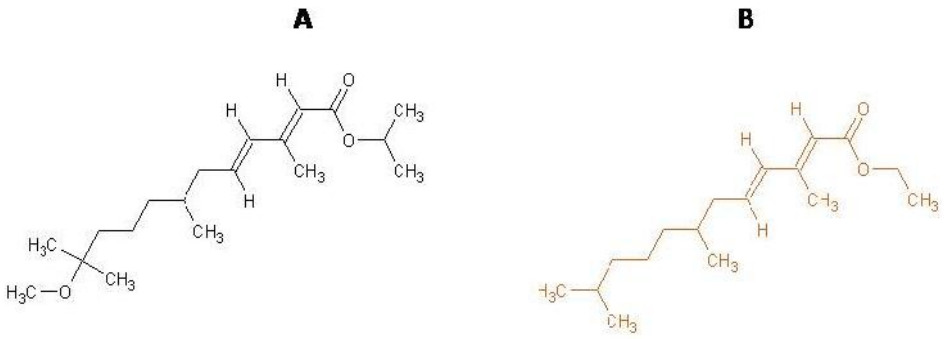
As with the organophosphates, pyrethroids generally decompose quickly in sunlight and air and thus pose little risk in the environment but the more stable pyretroids may be carried by water or air to areas where they may endanger non-target species such as birds, reptiles, fish and plankton (Palmquist *et al*., 2012). Pyrethroids are ubiquitous contaminants of streambed sediment across the United States that occur at levels potentially toxic to invertebrates (Hladik and Kuivila, 2012).

C. Insect Growth Regulators

Insect Growth Regulators (IGRs), also called third-generation insecticides (Williams, 1967a), are pesticides that disrupt the normal activity of the endocrine or hormone system of insects, affecting the development, reproduction, or metamorphosis of the target insect inhibiting its life cycle (Dhadialla *et al*., 2005). They have a much slower mode of action than classic knockdown insecticides and therefore must be a part of a combined insecticide management program (Harris and Waindle, 2006). In the control of myiasis IGRs will not give immediate kill of maggots on existing strikes. However, they do stop maggots from feeding so that they die within two to three days. They also provide long-term protection (up to 14 weeks) against new strikes (Evans and Karlsson, 2004). There are three types of insect growth regulators: hormonal, enzymatic, and chitin synthesis inhibitors (Graf, 1993).

Juvenile Hormones (JH) Analogues: Juvenile hormone (JH) is a sesquiterpenoid involved in a variety of critical functions in insects, including development, reproduction, and morphological differentiation. The major function of JH is the maintenance of the larval status or the so-called juvenilizing effect (Dhadialla *et al*., 2005). A number of chemical analogues have been synthesized which have potent JH

activity as well as the ability to mortally disrupt development of some insect species (Staal, 1975).The major representatives of this class of compounds aremethoprene (Figure 2-8A) and hydroprene (Figure 2-8B) (Graf, 1993).

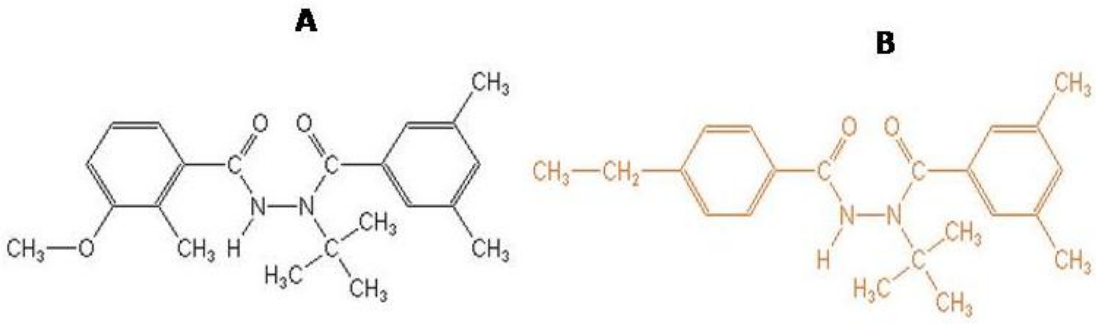


2-8: The chemical structure of juvenile hormone mimics, methoprene (A) and hydroprene (B).

These JH pesticides mimic the JH produced in the insect brain, which forces the insect to remain in a juvenile state. Normally, the production of the JH would ebb as the insect progressed through the nymphal stages until the final moult into the adult stage, when JH production would cease. When exposed to a JH mimic, the insect remains in an immature state, and is rendered unable to successfully moult into the adult stage or become reproductively viable (Staal, 1986). Application of the JH analogues may interfere with the moulting process which causes premature moulting. It also results in deformations of wings and reproductive parts e.g. the ovaries produce infertile eggs (Dhadialla *et al*., 2005). Hormonal IGR's may take as long as one generation, 3-10 days depending on the insect and the weather, to work so they are best utilized early when pest populations are low and are not a good rescue treatment when outbreaks are severe (Harris and Waindle, 2006).

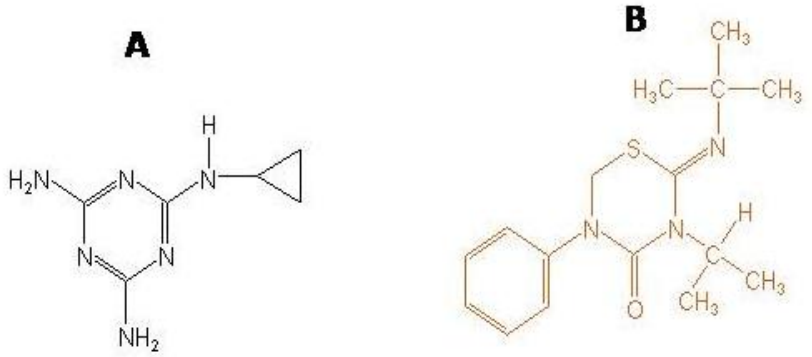
Ecdysone agonists and inhibitors: These interfere with the activity of ecdysone which signals the insect to moult. Ecdysone is a primary moulting hormone that is necessary for insects to go from the larval to pupal stage and from pupal stage to adult stage (Smagghe, 2009). Ecdysone agonists mimic ecdysone and force insects to moult prematurely which results in stoppage of feeding and ultimately death of insect. Other effects of these compounds on insects include increased egg mortality and reduced rates of reproduction (Slama, 1995). Examples include methoxyfenozide (Figure 2-

9A) and tebufenozide (Figure 2-9B). Ecdysone inhibitors inhibit the effects of ecdysone. A common example that has been widely incorporated into the pest management systems is azadiractin from *Azadiractin indica* (Neem). Ecdysone inhibitors antagonise prothoracicotropic hormone (PTTH) production in insects which is the hormone that stimulates ecdysone production (Rybczynski, 2005).



2-9: Structural formula of ecdysone agonists, methoxyfenozide (A) and tebufenozide (B)

Chitin synthesis inhibitors (CSIs): Chitin Synthesis Inhibitors (CSIs) such as cyromazine and buprofezin (Figure 2-10), inhibit the production of chitin, a major component (30–60%) of the insect cuticle (Gijswijt *et al*., 1979). If the insect cuticle, which provides the exoskeletal structure, is disrupted during its formation, it is lethal to the insect. The cuticle needs to be waterproof for protection, soft and flexible to allow movement, extensible in between segments to allow for increase during feeding and growth, and also rigid to provide firm points of attachment for muscle mandibles and claws (Dhadialla *et al*., 2005). Chitin Synthesis Inhibitors alter cuticle composition through reduced chitin levels in the cuticle, resulting in abnormal endocuticular deposition that affects cuticular elasticity and firmness, and causes abortive moulting (Dhadialla *et al*., 2005). Insects treated with CSIs are unable to successfully moult into the next stage.



2-10: Structural formula of chitin synthesis inhibitor, cyromazine(A) and buprofezin (B)

Cyromazine is a potent CSI and it is selective toward dipterous species and fed to poultry or sprayed to control flies on animals, in manure of broiler and egg producing operations. It controls blowfly infesting sheep and persist for up to 13 weeks (O'Brien and Fahey, 1991) after a single pour-on application, or longer if applied by dip or shower. Diflubenzuron is another CSI used in the control of blowflies. Products containing diflubenzuron usually carry a 12-weekflystrike protection claim (Levot and Sales, 2004).

Environmental effects of IGRs: Many IGRs are labelled "reduced risk" by the Environmental Protection Agency, meaning that they target juvenile harmful insect populations while causing less detrimental effects to beneficial insects. Unlike classic insecticides, IGRs do not affect an insect's nervous system and are thus more worker-friendly within closed environments. IGRs are also more compatible with pest management systems that use biological controls.

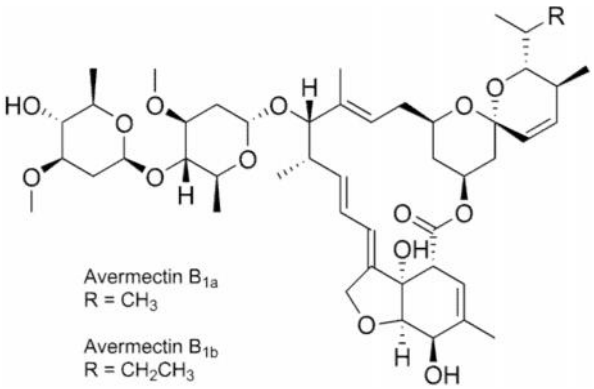
It was originally thought that insects would be unable to develop resistance to molecules that mimic their own hormones (Williams, 1967b), but resistance to all categories of IGRs has developed, including methoprene, hydroprene, kinoprene, pyriproxifen, and diflubenzuron (Shemshedini and Wilson, 1990). Diflubenzuron resistance was detected in blowfly populations in Queensland and New South Wales (Levot and Sales 2002). Resistance of the house fly, *Musca domestica* L., to various JHAs is also widespread (Hammock et al., 1977; Pospischil *et al*., 1996). It has been suggested that insect resistance to JHAs could be due to either degradation of the

artificially applied JHAs in the insect's body before reaching their target sites (Maa 1987; Zhang *et al*. 1998), or modification of the target site resulting in reduced affinity for the JHAs (Wilson and Fabian, 1986; Gruntenko *et al*., 2000). Increased carboxylesterase activity was observed following topical application of hydroprene on the Rutgers strain of M. *domestica* (Maa, 1987). Detoxification mechanisms mediated by cytochrome P450 monoxygenases lead to the metabolism of pyriproxifen and their elimination from the house fly body (Zhang *et al*. 1998) thereby conferring resistance.

D. Macrocyclic lactones

The macrocyclic lactones, which include the avermectins and the milbemycins, possess anthelminthic, insecticidal and acaricidal activity **(**Vercruysse, 2005). The naturally occurring avermectins, a group of 16-membered macrocyclic lactones (Figure 2-11), are all fermentation products from the soil *fungus Streptomyces avermitilis*. Eight different avermectins were isolated which comprise four pairs of homologues, with each pair comprising a major component (the a-component) and a minor one (b-component). They are usually produced in a ratio between 80:20 and 90:10 e.g. avermectin B1 (abamectin) is the mixture of avermectin B1a (>80%) and B1b (<20%) (Pitterna *et al*. 2009). Examples of commercial avermectins include ivermectin, abamectin, doramectin, eprinomectin, and selamectin. Commercially available milbemycins are milbemycin oxime and moxidectin.

The macrocyclic lactones as exemplified by ivermectin, the protype drug from the class, are broad spectrum and are recommended for use in many different animals (dogs, cats, sheep, goats, swine, horses, camels, buffaloes, rodents, birds, fish, reptiles) (Richard-Lenoble *et al*., 2003; Fox, 2006). They are active against many immature nematodes and arthropods. The published literature contains reports of use to treat infections of more than 300 different species of endo- and ectoparasites **(**Vercruysse, 2005). Moreover, a single therapeutic dose can persist in concentrations sufficient to be effective against incumbent nematode infections for prolonged periods after treatment **(**Vercruysse, 2005). In contrast to the other compounds available for parasite control, the major advantage of the macrocyclic lactones, are their prolonged activity against some nematodes for several weeks. Aqueous solutions of ivermectin may be applied by hand jetting to treat or prevent flystrike (Eagleson *et al*., 1993).



2-11: The general chemical structure of the avermectins

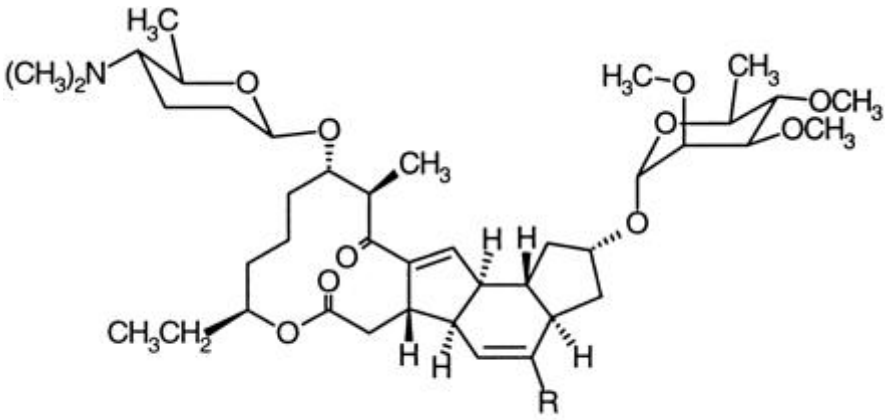
The avermectins block the transmittance of electrical activity in nerves and muscle cells by Ivermectin binds to ligand-gated chloride ion channels including glutamate, glycine, and gamma-aminobutyric acid (GABA) gated chloride ion channels in nerve and muscle cells. This binding to the ion channels stimulates the release and binding of GABA at nerve endings (Bloomquist, 1996, 2003). This in turn causes an influx of chloride ions into the cells leading to hyperpolarisation and subsequent flaccid paralysis of peripheral motor function and death of the parasite (Bloomquist, 1996, 2003). Its selective activity against parasites is due to its high affinity for glutamate-gated chloride ion channels found in the peripheral nervous system of invertebrates (Kane *et al*., 2000). Because ivermectin does not readily cross the mammalian blood-brain barrier (due to the P-glycoprotein pump) to the brain where ligand-gated chloride ion channels are found in mammals, humans are spared from adverse CNS effects of the drug (Cully *et al*., 1994). Nevertheless, within the realm of veterinary medicine there have been reports of severe toxicity of ivermectin affecting collies (herding dogs) (Hopper *et al*., 2002), demonstrating that drug can access the CNS in mammals. Clinical signs of toxicity for ivermectin in animals include mydriasis (pupillary dilation) in dogs, emesis in monkeys and ataxia and depression in most species, stupor and coma in severely affected dogs (Hopper *et al*., 2002).

Macrocyclic lactones rapidly undergo photodegradation yielding terminal polar products that are more than 160 fold less toxic (Halley *et al*., 1993). Although highly toxic to aquatic organisms, tight soil-binding by macrocyclic lactones mitigates against aquatic exposure via run-off or leaching (Halley *et al*., 1989). Synthetic

macrocyclic lactones are also used as additives in livestock feed and are primarily excreted in the faeces, and a broad range of insecticidal activities have been observed against dung-inhabiting insect species such as fly and beetle larvae (Halley *et al*., 1993). Emergence of several insects (e.g. sphaerocerids and sepsids) from manure containing ivermectin residues is reduced; however, the manure does not kill all dung-dwelling insect species (Schmidt, 1983). Overall, the commercially available milbemycins appear to be less harmful to fly and beetle larvae tested than the avermectins (Halley *et al*., 1993).

Widespread macrocyclic lactone resistance has developed in some nematode parasites of sheep, goats and cattle (Loveridge *et al*. 2003; Mejia *et al*. 2003; Kaplan, 2004; Wolstenholme *et al*. 2004; Geary, 2005; Prichard and Roulet, 2007). As a result of intensive ivermectin use and drug selection pressure, mutations of P-glycoprotein encoding genes or of genes encoding glutamate-gated or GABA-gated chloride ion channels may have led to ivermectin resistance in both intestinal helminths and arthropods (Currie *et al*., 2004; Griffin *et al*., 2005; Prichard, 2005). I could not find a reference reporting on ivermectin resistance in blowflies to date.

Spinosad is a broad-spectrum insecticide used against many commercially significant pests that attack livestock, companion animals, humans, crops and other plants (Kirst, 2010). The spectrum of target insects includes many species of Lepidoptera and Diptera along with some members of several other insect orders, such as planthoppers, leafhoppers, spider mites and cockroaches. Spinosad is a mixture of spinosyn A and spinosyn D (Figure 2-12) in a ratio of approximately 5 to 1 (Crouse *et al*., 2001). Spinosyns are actually unique macrocyclic lactones produced by *Saccharopolyspora spinosa*, a soil dwelling microorganism. They are macrolides with a 21-carbon, 12-membered tetracyclic lactones that are attached to two deoxysugars, tri-O-methylrhamnose and forosamine (Huang *et al*., 2009). A commercial product, Extinosad®), containing spinosad as the active ingredient was first registered in Australia in 2001 and then in New Zealand for control of blowfly strike, lice and maggots on sheep (Kirst, 2010) and provides four to six weeks protection (Salgado *et al*. 1998).



Spinosad (Spinosyn A, R=H; Spinosyn D, R=CH_3)

2-12: Spinosad which is a mixture of spinosyn A and spinosyn D (Yano*et al.*, 2002)

The mode of action of spinosad is characterized by rapid excitation of the insect nervous system, leading to involuntary muscle contractions, prostration with tremors, and paralysis (Salgado, 1998; Thompson et al., 2009). These effects are consistent with the activation of the acetylcholine nervous system through nicotinic receptors. Continuous activation of motor neurons eventually leads to the death of the insect from exhaustion within one to two days after ingesting the active ingredient (Salgado *et al*., 1997; Salgado, 1998; Salgado *et al*. 1998). Spinosad also has effects on GABA receptor function that may contribute further to its activity against insects (Thompson *et al*., 2000; Salgado, 1997; Salgado *et al*., 1998).

In contrast to the main macrocyclic lactones, the spinosads must be ingested by the insect, and therefore has little effect on beneficial insect predators and other non-target species when applied onto the animal (DeAmicis *et al*., 1997; Kirst, 2010). Spinosad has low acute mammalian toxicity (Yano *et al*., 2002) and low environmental persistence due to rapid degradation in the environment through a number of diverse degradative and metabolic pathways (Salgado and Sparks, 2005).

Spinosad resistance has been reported in a number of insects such as Danish houseflies, diamond backmoths, armyworm (Baxter *et al*., 2010; Kirst, 2010; Markussen and Kristensen, 2012).It involves alterations of cytochrome P450 gene expression (Markussen and Kristensen, 2012).

E. Carbamates

Carbamates are organic compounds derived from carbamic acid (NH_2COOH) (Adams and Baron 1965). The general chemical structure of carbamates can be seen in Figure 2-13. The carbamate insecticides include aldicarb, carbofuran, carbaryl, and methomyl.

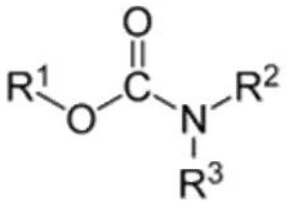


Figure 2-13: The general chemical structure of carbamates

R^{1-3} are hydrogen, alkyl or aryl groups

Carbamates are commonly used as surface sprays or baits in the control of household pests (Ware, 1999). Carbaryl, the first successful carbamate, was introduced in 1956 and is widely used as an insecticide. It is also used in the treatment and control of blowfly strikes (Fraser *et al*., 1967; Mehlhorn and Armstrong, 2001). Methomyl, another carbamate, is often applied as an adult fly bait.

The mode of action of carbamate insecticides is very similar to that of the organophosphate insecticides as they inhibit cholinesterase enzymes. However, they differ in action from the organophosphate compounds in that the inhibitory effect on cholinesterase is reversible (Casida, 1963).The site for inhibition (carbamylation) of the enzyme is the hydroxyl moiety of the serine amino acid. The rate of regeneration of the carbamylated enzyme to AChE is relatively rapid compared with that of an enzyme that has been inhibited (phosphorylated) by an organophosphate pesticide (Reiner and Aldridge, 1967; Reiner, 1971). Carbamates vary in their spectrum of activity, mammalian toxicity and persistence.

In general, the toxicity of carbamates for mammals is low (EHC 64, 1986), with notable exception for aldicarb which by large is considered the most toxic insecticide

(Verster *et al*., 2004).The clinical signs of toxicity in mammals are similar to those outlined above for organophosphates. Carbamates are relatively unstable compounds that break down in the environment within weeks or months with some exceptions, such as dimetilan and carbendazim (EHC 64, 1986). Although carbamates are not very stable under aquatic conditions, some may bioaccumulate in fish due to the slower metabolism with resultant toxicity to fish and other aquatic organisms (EHC 64, 1986). Contamination of groundwater and drinking-water sources by Aldicarb has also been reported (Miller *et al*., 1989). Furthermore on degradation, some carbamates are broken down (oxidation, hydrolysis) into compounds which might be less toxic or even more toxic. For example Carbaryl will be hydrolysed into 1-naphthol, which is just as toxic (Armstrong and Millemann, 1974).

Carbamate resistance has developed in mosquitoes, ticks (Boophilus *microplus*), blowflies, houseflies among others (Roxburgh and Shanahan, 1973; Blackman and Bakker, 1975; Holdsworth, 2005).

Summary of Challenges with Chemical Control

From the discussion above it can be seen that the control of blowflies will always be constrained by the:

- development of resistance in the flies. All of the compounds discussed above act on a very limited number of primary targets or are from a small family of compounds. As a result over reliance on them has resulted in the rapid development and spread of resistance and cross resistance.
- concerns over environmental effects of the chemicals. There has been increasing concern on the damage these compounds cause on environmental and aquatic life forms.
- toxicity of the compounds. Most of the compounds have a low safety index and their activity is not selective against pests only, resulting in toxicity to humans as well as non-target animals.

These factors therefore provide the impetus to study new, more ecologically acceptable methods of pest control. Plant based pest control agents have long been touted as alternatives to synthetic chemicals for integrated pest management. Such

phytochemicals may pose a lower threat to the environment or to human health (Koul, 2008).

2.3.4.2 Alternates to the use of chemicals in the control of myiasis agents

In addition to the chemical control of myiasis flies, other methods are in use by farmers for the control of disease:

A. Mulesing

Mulesing involves the surgical removal of both the wool and skin from around the breech area and is performed on lambs, usually at marking time. The resulting treated area, when healed, is devoid of wrinkles or skin folds and consequently less wool is available for contamination with either urine or faeces and, hence, is less attractive to flies (Tellam and Bowles 1997). Mulesing operations are effective in controlling flystrike but it faced a lot of criticism based on animal welfare concerns. International pressure has resulted in the phasing out of this practice in all wool producing countries (Scholtz *et al*., 2011).

B. Shearing and crutching

Shearing permits the wool to dry more quickly, as short wool is better exposed to the drying elements of sun and wind. Consequently shearing reduces the time available for larval establishment in addition to reducing the local moisture content required for larval growth and survival (larval growth requires 75% relative humidity) (Tellam and Bowles 1997). Shearing also improves the effectiveness of insecticides when applied to sheep for external parasite control (lice and blowflies) by permitting greater penetration of the chemical through the wool and onto the skin (Taylor, 1995). Crutching involves the removal of dags (locks of matted or dung-coated wool) and urine stained wool from around the breech area, thereby reducing the attractiveness of this region to the gravid female blowfly (Phillips, 2009).

C. Early warning systems

Computer models of the population dynamics of *Lucilia* species, using life-cycle and climatic information, have been developed to predict severe fly strike risk periods and the comparative effectiveness of different management practices. Heath (1994) referred to this concept as “early warning of the activity of blowflies before sheep

become at risk." This system requires computer management, automated weather recording stations as well as greater co-operation between neighbouring producers. The detailed information underlying the development of these computer models strongly indicate that the major factor influencing the incidence and development of blowfly strike is the weather, particularly warm and wet conditions (Wardhaugh and Morton, 1990). These climatic conditions are conducive to blowfly strike because they favour the development of fleece rot and dermatitis on sheep skin (Tellam and Bowles, 1997). The models are useful for decisions relating to the use and, particularly, the timing of various control measure. However, these models are not capable of accurate longer-term prediction (> 1 month) of flystrike risk periods (Tellam and Bowles, 1997). In addition, use of algorithms to estimate the necessary data to apply predictive models introduces potential error and complexity. Increased complexity might increase model realism, but also increases the difficulty of implementing and maintaining such systems and reduces their application because complex models are more difficult to explain to the end-user (Ward, 2001)

D. Management to control diarrhoea

One of the main predisposing causes for flystrike is wetting of the breech wool with urine and, to a lesser extent, diarrhoea (Watts and Marchant 1977). Diarrhoea is more prevalent in sheep grazing improved pastures and following rapid grass growth after rains. Providing fibrous supplements can reduce diarrhoea and dags forming on wool and thereby the attractiveness of sheep to flies (Davidson *et al* 2006). Other predisposing factors for diarrhoea in sheep are helminth infestation, low moisture content in pasture grasses, rapid changes in diet, and diseases of the gastrointestinal tract.

E. Selection of naturally resistant sheep

A long-term strategy for reducing the incidence of fly strike is to select sheep resistant to fleece rot and body strike (McGuirk *et al*., 1978). Many studies have been carried out to identify blowfly resistant animals (Greeff and Karlsson, 2009; Scholtz *et al*., 2010). Numerous indicator traits associated with blowfly strike have been identified, namely: the presence of dermatophilosis; low wool colour score; wrinkle-, dag-, urine stain-, breech cover and crutch cover scores (Greeff and Karlsson, 2009; Scholtz *et al*., 2010). These characteristics present indirect selection criteria for the control of

blowfly strike (Scholtz *et al*., 2011). Research has shown that the bare breech trait is heritable that wrinkles and dags are correlated with the incidence of breech strikes (Edwards *et al.*, 2009; Smith *et al.*, 2009). Farmer selection against flystrike also occurs, because when an animal is struck twice or severely struck it is usually culled (Heath, 1994). Although breeding is a long-term solution, it is attractive from an animal welfare, ethical, economic and sustainability perspective unless the flies also adapt.

F. Flytraps

Suppression of blowfly populations as a means of control has long been advocated (Knipling 1979). There is a correlation between the numbers of flies and the incidence of flystrike (Scholtz *et al*., 2011). It has been reported that the incidence of flystrike is related to the logarithm of the density of gravid females in the area during the previous week (Wardhaugh and Morton, 1990). As a result of the logarithmic relationship, a reduction of fly numbers by 70 % would be necessary to reduce flystrike by 50 % (Scholtz *et al*., 2011). One strategy for insect population suppression is the use of attract and kill systems (Cook *et al.* 2007). It has been shown that fly density and strike incidence can be reduced by blowfly trapping in Australia and South Africa. The use of LuciTraps on sheep properties reduced the incidence of blowfly strike in Queensland (Ward and Farrell 2003) and suppressed *L. cuprina* populations in South Africa (Scholtz *et al.* 2000). However, the large numbers of adult females that need to be attracted by traps to achieve effective population management (Broughan and Wall 2001), thereby allowing a noteworthy reduction of pesticide treatment, are seldom achievable (Scholtz *et al*., 2011). As a result the trapping of blowflies is best used as a supplementary measure or in combination with other management systems to reduce flystrike to low levels (Scholtz *et al*., 2011).

2.3.4.3 Plants as biopesticides

Cutaneous myiasis is also a condition managed through the use of plants by livestock keepers in Africa. Examples of the plants used on the Asian continent to expel maggots from wounds include *Carissa opaca* Stapf ex Haines, *Canthium parviflorum* Lam.,*Annona squamosa* Linn., *Ficus bengalensis* Linn., *Prunus persica* (L.) Batsch., *Allium sativum* L., *Tectona grandis* Linn., *Zizyphus rugosa* Lam., *Tamarindus indica* L., *Acalypha indica* Linn. (Handoo, 2006; Anonymous, 1994; Anonymous, 1996).

Likewise in Africa a number of plants are used in the treatment of maggot-infested wounds (Chavunduka, 1976; Hutchings et al., 1996; Luseba and van der Merwe, 2006; van der Merwe *et al*., 2001; Watt and Breyer-Brandwijk, 1962). Usually the leaves of these plants are crushed and packed on to the wound.

2.4 Plants selected for the study

To date many plant species have been identified in numerous surveys for use in the treatment of myiasis. After a study of the literature, seven plant species traditionally used in the treatment of cutaneous myiasis were selected. To promote the sustainable use of plants in case a potential product is identified only species (mainly trees) that are traditionally used as leaves and have a wide distribution in southern Africa were selected. These plant species are *Aloe marlothii* A. Berger (Van der Merwe *et al*., 2001), *Aloe zebrina* Baker (Luseba and van der Merwe, 2006), *Calpurnia aurea* (Aiton) Benth (Hutchings *et al*., 1996), *Psydrax livida* (Hiern) Bridson (*Canthium huillense*), *Clausena anisata* (Willd) Hook (Chavunduka, 1976), *Erythrina lysistemon* Hutch (van Wyk *et al*., 1997), and *Spirostachys africana* Sond (Hutchings et al., 1996). A short description of each is provided.

2.4.1 ***Aloe marlothii*** A.Berger

Family: Asphodelaceae

Common names: Mountain aloe (Eng.), Bergaalwyn (Afr.), Mokgopa (Tswana.); Inhlaba or Umhlaba (Zulu)

Figure 2 14: A flowering Aloe marlothii plant

(Ernst van Jaarsveld, 2007)

*Aloe marlothii*is a perennial, single-stemmed aloe that is usually 2-4 m tall but may grow up to 6 m. Its leaves are large, broad and succulent, light green to greyish green in colour and are covered with spines on upper and lower surfaces (Emms, 2007). Normally, old dried leaves remain on the stem below the upper live leaves. Flowers are held on racemes on a branched candelabra-shaped inflorescence and may be present between May and September. Flower colour varies from the typical orange-red to yellow or bright red. The distinguishing character lies in the slanted inflorescences (Emms, 2007).

Aloe marlothii is a plant of exceptional importance in South Africa. Its leaves are used by communal stock owners in the North West Province to remove maggots from wounds, to reduce tick burdens, to treat and prevent gall sickness (*Anaplasma marginale* infection in cattle), to treat parasitic helminthiasis, diarrhoea, constipation, retained placenta and dystocia (Van der Merwe *et al*., 2001). Leaf decoctions are used to treat African Horse sickness and a combination of the leaf and root decoctions are used in KwaZulu-Natal to treat roundworm infestations in humans (Watt and Breyer-Brandwijk 1962). Both fresh and dried leaves are used in different preparations and in most cases they are mixed with water.

Phytochemical studies of A.*marlothii* show that the plant extracts contain mainly chromones and anthrones (Bistrat *et al*., 2000). These classes of compounds are known to have effects on insects such as mosquitoes (Batista *et al*., 2009). The effects include larvicidal, antifeedant and repellency activities. Therefore, it is possible these compounds could also have on effects on blowfly larvae and could be responsible for the activity reported by communities who use the plant to treat myiasis. *Aloe marlothii* has also been screened for a number of activities including antibacterial (McGaw *et al*., 2000; Naidoo *et al*., 2006, Luseba *et al*., 2007) (MICs provided in Chapter 3), anti-inflammatory, mutagenic (Luseba *et al*., 2007); anti-rickettsial (Naidoo *et al*., 2006); antibabesial (Naidoo *et al*., 2005), anti-tick (Spickett *et al*., 2007) and insecticidal (Pillay *et al*., 2008). In these studies some researchers have confirmed activity whilst others could not. Results obtained in these investigations vary with the solvent types used in the extraction of the plant material.

2.4.2 *Aloe zebrina* Baker

Family: Asphodelaceae

Common names: Zebra Leaf Aloe, Spotted Aloe (Eng),Kanniedood (Afr.), Kgophane (Tswana)

Figure 2-14: Aloe zebrina plants (Philippe Faucon, 2007)

Aloe zebrina is a very small maculated aloe. Plants are stemless and form small groups from offsets at the base. Both surfaces of the leaves have irregular white spots in rows**.** The tips of the leaves dry out in summer forming a curl. The flowers are pale red/pink, striped and relatively small.

Aloe zebrina is used by the Tsonga speaking people of South Africa to treat maggot infested wounds (Luseba and van der Merwe, 2006). The sap and decoctions of the leaves of *Aloe zebrina* were used for bathing sores caused by sexually transmitted infections or taken orally in Botswana. In Namibia, the plant is used to treat urinary and kidney ailments (van Koenen, 2001)

Like most *Aloe* species the yellow leaf sap of *A. zebrina* contains anthrone C-glycosides such as aloin and homonataloin, and the yellow root sap contains anthranoid aglycones such as chrysophanol (a fast orange-brown colorant) and asphodeline. The roots of *Aloe zebrina* further contain aloesaponarin, aloesaponol and related compounds of the 1-methyl-8-hydroxyanthraquinone pathway. Moreover,

isoleutherol is a unique chemical compound found in the roots of spotted aloes (Jansen, 2005). No reports on the efficacy this plant species could be found. However based on the phytochemical constituents it can be effective against blowfly larvae.

2.4.3 *Calpurnia aurea* (Aiton) Benth. subsp. aurea

Family: Fabaceae

Common names: Wild Laburnum (Eng), Wildegeelkeur (Afri.), umKhiphampethu (Zulu)

Figure 2-15: *Calpurnea aurea* leaves, pods and flowers (Wursten, 2004)

Calpurnia aurea is an evergreen, small, multi-stemmed shrub with a height ranging between 2 - 4 m. The flowers are bright yellow and the fruit is a thin pod drying light brown with a papery texture (Notten, 2005). *Calpurnia aurea* is widely distributed in Africa from the Cape Province to Eritrea and also occurs in southern India. In South Africa, *C. aurea* leaves and powdered roots are used to kill lice in humans and ticks in animals and to relieve itches. Unspecified parts are used to destroy maggots and the leaves are used to treat allergic rashes, particularly those caused by caterpillars. In East Africa, leaf sap is used to destroy maggots in wounds. In Nigeria, the seeds are used to treat abscesses. In Ethiopia, it is used to treat stomach complaints, headache, eye diseases, amoebic dysentery and diarrhoea in animals, scabies (skin infection caused by ticks) and as an insecticide (Notten, 2005).

The main pharmacologically active compounds of *C. aurea are* quinolizidine alkaloids. These include 13-hydroxylupanine, calpurnine, virgiline and its pyrrolylcarboxylic acid ester, 10, 13-dihydroxylupanine, calpurmenine and 13-2'-pyrrolylcarboxyl (Radema *et al*., 1979) Alkaloids are known feeding deterrents of lepidopteran larvae, for example 13-hydroxylupanine is highly active against pea aphids (Thayumanavan and Sadasivam, 2003). Although blowflies belong to a different order, the diptera, there is a possibility that these quinolizidine alkaloids will also have an effect on them.

2.4.4 ***Psydrax livida***(Hiern) Bridson **(*Canthium huillense*)**

Family: Rubiaceae

Common names: Green-twigs quar (E), groenboom, groenkwar (Afr.),

Figure 2-16: Psydraxlivida plant (Wursten, 2011**)**

Psydrax livida is a deciduous shrub with branches that are opposite and horizontal. The leaves are held in one plane, ovate, dull green, sometimes with a greyish sheen. The flowers are in axillary clusters and unpleasantly scented. The fruits occur in clusters, are subspherical, and are blackish when ripe (Hyde and Wursten, 2011a).

In Zimbabwe, the leaves of *Psydrax livida* are crushed and packed into a maggot infested wounds in animals order to expel the maggots (Chavunduka, 1976). Different cyanogenic glycosides have been isolated from P. *livida* (Rockenbach *et al*., 1992). These include prunasin, oxyanthin and 5”-benzoate of oxyanthin. A literature search did not yield any reports of efficacy studies on this plant species.

2.4.5 *Clausena anisata* (Willd.) Hook.f. ex Benth.

Family: Rutaceae

Common names: Horsewood, Maggot killer (Eng.), Perdepis (Afr.), Unukambiba(Zulu)

Figure 2-17: *Clausena anisata* shrub (Hyde and Wursten, 2011b**)**

Clausena anisata is a shrub with pinnate compound leaves. The leaves are densely dotted with glands and have a strong scent, when crushed, which has been likened to aniseed. The flowers are small and white with orange-yellow stamens. The inflorescence forms a branched axillary spray (Hyde and Wursten, 2011b)

In South Africa different parts of *Clausena anisata* have been reported in ethnomedicinal surveys to be effective remedies against a number of disease conditions. These include parasitic infections, eye complaints, influenza and other respiratory ailments, heart disorders and hypertension, abdominal cramps, constipation and gastro-enteritis, malaria, fevers and pyrexia, boils, rheumatism, arthritis, and other inflammatory conditions, headaches, body pains, toothaches and swollen gums, convulsions, impotence and sterility, blood tonic and dysentery (Hutchings *et al*., 1996). In West Africa the leaves of *C. anisata* are widely used as insect repellents. In Zimbabwe the leaves are used to expel maggots from wounds of

animals (Chavunduka, 1976).The insecticidal activity and repellency against stored grain pests and mosquitoes (Boeke *et al*., 2004; Ndomo *et al*., 2008; Govindarajan, 2010), antidiabetic (Ojewole, 2002), antimicrobial (Gundidza *et al*., 1994), anti-inflammatory, analgesic and antimalarial properties (Okokon *et al*., 2012) have been confirmed in scientific studies. Efficacy against blowfly larvae has not yet been evaluated.

Different chemical compounds have been isolated from the various parts of the plant. These include terpenoid hydrocarbons, furanocoumarins, lactones, sesquiterpenoids, fatty acids; volatile oil containing phenylpropanoids and an acute toxic principle, estragole; the alkaloids clausanitine and mupamine; as well as more than 20 coumarins (Ojewole, 2002). The presence of some classes of compounds in *C. anisata*, namely terpenoids, coumarins, lactones and alkaloids suggest that it could be effective against maggots. The most potent pest antifeedants are terpenoids. Azadirachtin, derived from the seeds of the neem tree, *Azadiracta indica* A. Juss (Meliaceae), is an example that has already been incorporated in integrative pest management as an antifeedant agent against insects (Kostic *et al*., 2008). Two antifeedant compounds have been isolated from the petroleum ether extract of C. *anisata*, against the larvae of African armyworm (*Spodoptera exempta*), and the compounds have been identified as the coumarins imperatorin and xanthoxyletin (Gebreyesus and Chapya, 1983). Some alkaloids have a similar effect as the organophosphates of blocking the acetylcholinesterase enzyme (Aniszewski, 2007). There are also insecticides on the market based on lactones.

2.4.6 *Erythrina lysistemon* Hutch.

Family:Fabaceae

Common names: Coral-tree (E), Lucky-bean tree (Eng.)), Gewonekoraalboom, Kanniedood (Afr), Mophete (Tswana), uMsinsi (Zulu)

Figure 2-18: *Erythrina lysistemon* tree (Wursten, 2009)

Erythrina lysistemon is a decorative medium-sized tree, up to 12 m high. It is widely distributed in South Africa, from the North West Province up to Messina and then southwards through Mpumalanga, KwaZulu-Natal into the Eastern Cape. It grows in a wide variety of habitats including scrub forest, dry woodland and coastal bush. The tree is leafless for up to 4 or 5 months of the year. The flowers are a brilliant scarlet and are borne in dense racemes while the tree is still leafless (Mbambezeli and Notten, 2002).

Erythrina lysistemon is used traditionally to clear wounds of maggots and for toothache to treat swellings and abscesses. In Madagascar and East Africa, *Erythrina* species are widely used as antimalarials and *E. lysistemon* has antiplasmodial activity (Prozesky *et al*., 2001). The plant has also been screened for antibacterial activity (Rabe and van Staden, 1997) (details in chapter 3). *E. lysistemon* has a high alkaloid content (Juma and Majinda, 2004) and alkaloids are known to affect various insects (Aniszewski, 2007). Alkaloids from *Sophora alopecuroids* influence the larval

feeding, food utilization, larval growth, and adult oviposition of *Clostera anastomosis* (Zhende *et al.*, 2006). It is possible that *E. lysistemon* will have an effect on the blowfly larvae due to the alkaloids.

2.4.7 *Spirostachys africana* Sond.

Family: Euphorbiaceae

Common names: Tamboti (Eng.), Tambotie (Afr.), umThombothi (Zulu), Morukuru (Tswana)

Figure 2-19: *Spirostachys africana* Sond. (Wursten, 2009)

Spirostachys africana Sond is widespread in southern Africa, occurring in the warmer parts of KwaZulu-Natal, Mpumalanga, Northern and North West Provinces, as well as in central Africa (Palgrave, 1990). It is found in deciduous woodlands, often along watercourses in single-species stands. The flowers are borne on the same tree in lateral spikes. The fruit is a smooth capsule consisting of three parts. This three-lobed capsule is a clue to family identification as it is characteristic of the Euphorbiaceae.

The sap of *Spirostachys africana* is used by the Zulu community in South Africa on cattle wounds to kill maggots (Hutchings *et al.*, 1996). The stem bark extract is used traditionally to treat infant's body rashes, diarrhoea, dysentery and stomach pains (Mathabe *et al.*, 2008). The heartwood of the tree is used traditionally as an insect

repellent (Munkombwe *et al*., 1997). The tree is widely known for its fine wood, its toxic properties, and its "jumping beans". The latex is acrid, raising skin blisters on those sensitive to it and causing severe pain and even loss of sight if it enters the eyes (the antidote is said to be cow's milk). The smoke of the burning wood may induce headaches.

Phytochemical studies of the latex from *Spirostachys africana* have shown the presence of beyerene derivatives, diterpenoids, diosphenols, ketols, stachynones and acid metabolites (Baarschers *et al*., 1962; Duri *et al.*, 1992; Munkombwe *et al*., 1997). The terpenoids could account for the antifeedant and insect-repellent properties of *S. africana* and may also be effective against blowfly larvae. Crude extracts from the bark of *Spirostachys africana* have antibacterial activity against diarrhoea-causative microorganisms (*Salmonella typhi*, *Shigella sonnei*, *Shigella dysentery*, *Shigella flexneri*, *Shigella boydii*, *Escherichia coli*.), with minimum inhibitory concentration (MIC) values ranging between 0.156 and 0.625 mg/ml (Mathabe *et al*., 2006). The terpenoids isolated from *Spirostachys Africana* have also been found to exhibit good antibacterial activities and low cytotoxicity (Mathabe *et al*., 2008). Scientific evaluation of *S. africana* against insects has been mainly on mosquitoes where repellency levels ranging between 18 – 48% were reported (Maharaj *et al*. 2010) and stored grain pests (Chikukura *et al*., 2011). As far as could be ascertained this species has not been scientifically evaluated against flies.

2.5 Conclusion

Myiasis costs the livestock industries of many countries large sums through losses in production, stock deaths and the cost of chemicals and labour to manage the parasite. The control of this disease is also constrained by limited access to veterinary products and services in the case of subsistence farming, which has led to the increasing use of plants by the resource-poor livestock producers and pastoralists. While the use of chemical compounds by commercial farmers is widespread, constraints under these circumstances are toxicity, resistance and environmental contamination. There is a need for new safer, cheaper and readily accessible compounds or products to control myiasis. The phytochemical compounds present in plants used traditionally to treat or

control myiasis may be a solution to problems faced by both commercial and subsistence farmers.

2.6 References

Listed in Chapter 10.

CHAPTER 3

Preface

This chapter addresses the first objective of this study which was to evaluate the antibacterial activity, *in vitro*, of some plants used to treat cutaneous myiasis in South Africa and Zimbabwe.

3 In vitro antibacterial activity of seven plants used traditionally to treat wound myiasis in animals in southern Africa

[1]Mukandiwa, L., [2]Naidoo, V. [1]Eloff, J.N.

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Abstract

In the extreme situation of subsistence farming where insecticides and other veterinary medicines are either unavailable or unaffordable, the use of plants in the treatment of wound myiasis in livestock has been reported worldwide. However the exact effect of these plants on myiatic wounds has not been established. This study was therefore undertaken to establish the biological activity of seven species of plants which are used traditionally and are claimed to be effective in the treatment of wound myiasis. Plants that have a wide distribution in southern Africa were selected. This paper focuses on the antibacterial activity of these plants on bacteria known to be among the common contaminants of wounds. It has been shown that bacterial action on wounds produce compounds which have an odour that serve as an attractant of myiasis-causing flies. The antibacterial activity of the plants was investigated using a microdilution assay and bioautography methods. All the tested plants had inhibitory activity against the test bacteria. Inhibiting bacterial activity reduces the attractants of myiasis-causing flies to the wound. Thus inhibiting bacteria action on wounds will interfere with the development of wound myiasis. This could be one of the mechanism through which the plants that are used traditionally in the treatment of wound myiasis work.

Key words: Wound myiasis. Ethnoveterinary medicine**.** Antibacterial activity.

3.1 Introduction

Wound myiasis (infestation of wounds by dipterous larvae) in livestock can be devastating due to production losses, veterinary costs and sometimes death (Anonymous, 2008). The role of bacteria in the attraction of myiasis-causing flies and oviposition has been established in a number of studies (Chaudhury *et al*., 2010). Bacteria such as *Streptococcus pyogenes, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis* and *Klebsiella* spp.found on wounds produce volatile organic, sulphur-containing compounds with an odour that attracts the myiasis-causing flies (Khoga *et al*., 2002). These compounds can also act as ovipository stimuli to the myiasis-causing flies (Emmens and Murray, 1982). Extracts from unsterile sheep fleeces seeded with *Pseudomonas aeruginosa, Proteus mirabilis, Enterobacter cloacae* and *Bacillus subtilis* stimulate oviposition by females of *Luciliacuprina* (Wied.) (Eisemann and Rice, 1987). Wounds already infested with larvae are also more attractive to the gravid females (Hammack and Holt, 1983). The presence of larvae in wounds by themselves is not enough to attract gravid females, but their activity in media contaminated with bacteria increases attractiveness of the wound (Eismann and Rice 1987). As such it is clear that bacterial contamination of wounds is important in the pathogenesis of wound myiasis.

In orthodox veterinary medicine, organophosphate insecticides in conjunction with antibiotics are recommended for the treatment of wound myiasis. The insecticides serve to expel and kill the larvae from the wound (OIE, 2008). The antibiotics deal with the microbial infection on the wound, which promotes wound healing and prevents secondary re-infestation by flies. In the difficult situation of subsistence farming where insecticides and other veterinary medicines are either unavailable or unaffordable, plants have been used in the treatment of wound myiasis in Africa and Asia (Chavunduka, 1976; Van der Merwe *et al*., 2001; Luseba and Van der Merwe, 2006). However, the exact effect of most of these plants on myiatic wounds has not been established. We therefore, undertook a study to establish the biological activity of 7 species of plants which are used traditionally and are claimed to be effective in the treatment of wound myiasis in South Africa and Zimbabwe (Table 1). The study was conducted in an endeavour to validate the traditional use of the plants and

determine those that are highly active. This paper focuses on the antibacterial activity of extracts of these plants on bacteria that are common contaminants of wounds.

3.2 Materials and methods

3.2.1 Plant materials

After a study of the literature, seven plant species traditionally used in the treatment of cutaneous myiasis: *Aloe marlothii* A. Berger (Van der Merwe *et al*., 2001), *Aloe zebrina* Baker (Luseba and Van der Merwe, 2006), *Calpurnia aurea* (Aiton) Benth (Hutchings *et al*., 1996), *Psydrax livida* (Hiern) Bridson (*Canthium huillense*), *Clausena anisata* (Willd) Hook (Chavunduka, 1976), *Erythrina lysistemon* Hutch (Van Wyk *et al*., 1997), and *Spirostachys africana* Sond (Hutchings *et al*., 1996),were selected for further study. More information is provided in Table 3-1.

3.2.2 Plant Collection and Storage

The plant material was collected from the Pretoria National Botanical Garden, South Africa. Voucher specimens and origins of the trees are kept in the garden herbarium. It was dried at room temperature in a well-ventilated room. Collection, drying and storage of plant material guidelines outlined elsewhere were followed (McGaw and Eloff, 2010).

3.2.3 Preparation of plant extracts

Dried leaf material was ground to fine powder using a KIKA-WERKE M20 mill (GMBH & Co., Germany). To obtain the acetone, methanol, dichloromethane and hexane extracts, four separate aliquots of 4 g of the leaf material of each plant were shaken vigorously for 30 min in 40 ml of the respective solvents on an orbital shaker (Labotec®, model 20.2, South Africa). The extracts were allowed to settle, centrifuged at 2000 x g for 10 min and the supernatant filtered through Whatman No. 1 filter paper into pre-weighed glass vials. The extraction process was repeated 3 times for each aliquot of plant material. The extracts were dried in a stream of cold air at room temperature and the mass extracted with each solvent was determined. The dried extracts were reconstituted in acetone to make 10 mg/ml stock extracts which were used for the antibacterial assays. Acetone was used for the reconstitution because of its efficacy in dissolving extracts with a range of polarities (Eloff, 1998a) and its low

toxicity to microorganisms Eloff *et al*., 2007). Twenty-eight extracts were prepared in total.

3.2.4 Antibacterial assay

A serial microplate dilution method (Eloff, 1998b) was used to screen the plant extracts for antibacterial activity. This method allows for the determination of the minimal inhibitory concentration (MIC) of each plant extract against each bacterial species by measuring the reduction of tetrazolium violet. The test organisms in this study included two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213), and *Enterococcus faecalis* (ATCC 29212), and two Gram-negative ones, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). These are some of the most common bacteria known for infecting wounds. The specific strains used are recommended for use in research (NCCLS, 1990). The bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37 °C and a 1% dilution of each culture in fresh MH broth was prepared prior to use in the microdilution assay. Twofold serial dilutions of plant extracts (100 µL) were prepared in 96-well microtitre plates, and 100 µL of bacterial culture were added to each well. The plates were incubated overnight at 37 °C and bacterial growth was detected by adding 40 µL *p*-iodonitrotetrazolium violet (INT) (Sigma) to each well. After incubation at 37 °C for 1 h, INT is reduced to a red formazan by biologically active organisms, in this case, the dividing bacteria. The lowest concentration where there was a reduction of the colour intensity was taken to be the minimal inhibitory concentration (MIC). The MIC values were read at 1 h and 24 h after the addition of INT to differentiate between bacteriostatic and bacteriocidal activities. Acetone and the standard antibiotic gentamicin (Sigma) were included in each experiment as controls.

3.2.5 Bactericidal or Bacteriostatic?

To confirm the bactericidal activity of the plant extracts the method described by Pankey and Sabbath (2004) was used. Only the acetone plant extracts were used in this assay because in most cases in the antibacterial assay they were more effective and potent. Subcultures of samples from clear dilution wells from the MIC assay were made on Müller-Hinton (MH) agar plates by plating 100 µl and subsequently incubating for 24 h at 37°C. The test organisms in this assay were one Gram-negative

bacterium, *Pseudomonas aeruginosa* (ATCC 27853) and one Gram-positive bacterium, *Staphylococcus aureus* (ATCC 29213). A reduction of at least 99.9% of the colony forming units, compared with the culture of the initial inoculum, was regarded as evidence of bactericidal activity.

3.2.6 Bioautography

Bioautography was carried out to confirm the presence and determine number of antibacterial compounds in the plant extracts (Masoko and Eloff, 2005). Thin layer chromatography (TLC) plates (10 x 10 cm aluminium-baked, Merck, F_{254}) were loaded with 100 µg (10 µl of 10 mg/ml) of the extracts and dried before being eluted in three different solvent systems i.e., ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonia hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). The test organisms included, *Staphylococcus aureus* (ATCC 29213), a Gram-positive bacteria and *Pseudomonas aeruginosa* (ATCC 27853) a Gram-negative bacteria. The bacterial cultures, cultured for 14 hours in Muller Hilton (MH) broth were centrifuged at 3500 rpm for 5 minutes and the pellet re-suspended in minimal volume (20 ml) of MH broth. Developed plates were sprayed until damp with the concentrated bacterial cultures in a Bio safety Class II cabinet (Labotec, S.A) and incubated in a humidified chamber (100% relative humidity) overnight at 37^{o}C. The plates were then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) and incubated at 37^{o}C for a further 12 hours. Clear zones against the purple background indicate inhibition of microbial growth by separated plant constituents on the TLC plate.

To detect the separated compounds, a duplicate set of chromatograms developed in the 3 different solvent systems were sprayed with vanillin-sulphuricacid (0.1 g vanillin (Sigma®): 28 methanol: 1ml sulphuric acid) and heated at 110◦C to optimal colour development.

3.2.7 The mass of extract required to inhibit bacterial growth on an average size animal wound

Whatman No 1 filter papers were cut into circles of 4 cm diameter to mimic an average wound size in an animal. The filter paper circles were weighed and then sprayed with the acetone extracts until they were saturated. The filter paper circles were allowed to dry and re-weighed. The mass of extract required to cover the whole circle was calculated and recorded. Mean separation was done using the PDIFF option of SAS (2006). The volume needed to give determined mass values were determined using the concentration of the extracts which was 10 mg/ml. The quantity of extract in mg required to inhibit bacterial growth on wound of 4 cm diameter was calculated as:
Volume of extract X required to saturate the filter paper circle in ml multiplied by MIC value for a particular bacterium obtained from the antibacterial assay for extract X in mg/ml.

3.3 Results

3.3.1 Antibacterial assay

Overall, *E. coli* was the least susceptible bacterium to the plant extracts (Table 3-2). We considered an MIC of 0.16 mg/ml or less to be significant antibacterial activity based on the guidelines in the *Phytomedicine Journal* (Instruction to Authors). Only 4 out of 28 extracts had MIC values equal to or less than 0.16 mg/ml against *E. coli*. Nine of the 28 extracts, 11/28 and 8/28 of the plant extracts had MIC values equal to or less than 0.16 mg/ml against *E. faecalis, P. aeruginosa, and S. aureus* respectively. Most of the plant extracts were active against both Gram-negative and Gram-positive bacteria. In 25/28 analyses (89%) hexane extracts had relatively poor activity (1.25 to 2.5 mg/ml) or no antibacterial activity at the highest concentration tested (2.5 mg/ml). In total, 13 extracts (46%) had MIC ≤ 0.16 mg/ml, of which 6 were acetone extracts, 5 were dichloromethane extracts and 2 were methanol extracts. The antibacterial activity of the plant extracts against both the Gram-positive and Gram-negative bacteria varied with the solvent used to extract the plant material (Table 3-2). As expected, the negative control, acetone, was devoid of any antibacterial activity.

The MICs for each extract type, i.e. methanol, acetone, dichloromethane and hexane, were averaged for the four test organisms. The average activity volumes indicating to

what volume 1 mg of extract from different extractants can be diluted and it would still kill the bacteria were determined by dividing 1 mg by the average MIC for each extract type. Figure 1 shows the average activity volumes of the different extractants. Acetone is clearly the best extractant, followed by dichloromethane, methanol and finally hexane. These results confirm many observations in our laboratory that the most active antimicrobial compounds have an intermediate polarity. On average 1 mg of the acetone extracts can be diluted in 4.2 ml and still kill bacteria whilst those of hexane can only be diluted in 0.8 ml.

To establish the plant species with the highest activity, the total activity of the different plant species was also determined. Total activity indicates the largest volume to which the biologically active compounds in 1 g of plant material can be diluted and still inhibit the growth of bacteria. It is calculated by dividing the quantity of material extracted from 1 g of dried plant material in milligrams by the minimal inhibitory concentration in mg/ml. It is useful to compare the potency of different plants and to detect synergism or loss of activity in bioassay guided fractionation. Figure 2 shows the total activity of the different plant species. *Spirostachys africana* had the highest activity followed by *C. anisata, P. livida* and *E. lysistemon*, respectively. Extracts of 5 of the study plants became less potent with time as shown by the reduced activity volumes after 24h. *Psydrax livid a*nd *C. aurea* were an exception as the extracts seem to get more potent with time.

3.3.2 Bactericidal or Bacteriostatic

All the extracts were bacteriostatic at the determined MICs since growth was observed after plating of the contents of clear wells on MH agar. However they were bactericidal at higher concentrations. All of the tested plant extracts, except *A. zebrina*, were bactericidal against *P. aeruginosa,* at 1.25 mg/ml, with *A. zebrina* being most potent, at 0.625 mg/ml. *Staphylococcus aureus* was most susceptible to the plant extracts, with all the tested plant extracts being bactericidal at 0.625 mg/ml.

3.3.3 Bioautography

Thin layer chromatography (TLC) was used to fingerprint the plant extracts. This allowed for visualization of the different compounds in the plant extracts and identification of biologically active bands on the chromatograms. Bioautography, in

general, showed more than one active band per plant extract (Figures 3-3 – 3-4). Although the hexane extracts had poor antibacterial activity in the microdilution assay bioautography showed that they too contained antibacterial compounds.

3.3.4 The mass of extract required to inhibit bacterial growth on an average size animal wound

Table 3 shows the mass of the acetone extracts of the different plant species required to inhibit bacterial growth on a wound of 4cm diameter. On average the lowest mass of extracts is required when *A. zebrina* is used whilst the highest mass is required when *A. marlothii* is used.

3.4 Discussion

Notably, all the plants in this study had antibacterial activity, albeit some at low and others at high minimum inhibitory concentrations. This observed antibacterial property could be one of the mechanisms through which the plants that are used traditionally in the treatment of wound myiasis work. One has to keep in mind that traditional healers mainly use water extracts. The active plant extracts had broad spectrum antibacterial activity, inhibiting both Gram-negative and Gram-positive bacteria, although the MICs were relatively higher for Gram-negative bacteria. It is known that, in general, the Gram-negative bacteria are less susceptible to antibacterials compared to the Gram-positive ones. This is due to the outer membrane composed of lipopolysaccharides (LPS), phospholipids, and lipoproteins that they possess which is absent in the Gram-positive bacteria. The outer membrane serves as a barrier for the bacterium against the destructive effects of various antibacterial compounds (Hodges, 2002).

Pseudomonas aeruginosa is an opportunistic pathogen and is a common contaminant of wounds. Its action on wounds has been put forward as one of the attractants of myiasis causing flies (Eisemann and Rice, 1987). The fact that the study plants had one or more extracts with activity against *P. aeruginosa* might add validity to their traditional use in the treatment of wound myiasis.

The antibacterial activity of the plant extracts varied with the solvent used for extraction, as expected (Kotze and Eloff, 2002, Eloff *et al*., 2005). This can be explained in terms of the polarity of the compounds being extracted by each solvent and the amount of that compound, in addition to their intrinsic bioactivity. Notably extracts of the same plant had antimicrobial activity against the same microorganism although at varying MIC values. This means that the compound responsible for the antimicrobial activity was present in each extract, as shown by the bioautography, only at different concentrations. The acetone extracts were more effective and potent and this implies that acetone extracted a higher concentration of the antibacterial compound (s) or less of inactive compounds.

Most of the plant extracts became less potent with time. This can be explained if the active component were volatile and being lost from the extract with time. This is unlikely seeing that the hexane extract did not have the highest activity. It is more likely that the active antibacterial compounds may have been broken down or the bacteria were able to overcome the initial inhibitory effects of the antibacterial compounds by metabolizing it. *Psydraxlivida* extracts were an exception to this trend. This could be attributed to some plant compounds within the extract breaking down with time and releasing compounds that have higher antibacterial activity.

Aloe zebrina had the best antibacterial activity against all the bacteria and had the least quantity of extract required to inhibit bacterial growth on an averaged sized wound. However the quantity of extract from 1 g of plant material was relatively low hence its total activity was low. Generally, the bulk of Aloe leaves is water (Koroch *et al*., 2009). The leaves of *A. zebrina* are relatively thin compared to those of other aloes such as *A. marlothii*. As a result, the leaves are easy to dry as a whole and this is how they were used in this study. To determine which plants can be used for further testing and isolation, not only the MIC value is important, but also the total activity. This value indicates the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the bacteria (Eloff, 2000). Extracts with higher values are considered the best to work with. Among the plants that are used to treat cutaneous myiasis, the best plants in inhibiting bacterial growth are *S. africana*, *C. anisata*, *P. livida* and *E. lysistemon*, respectively, based on total activity.

The antibacterial activity of plants observed in this study concurs with previous findings by other researchers. The acetone extract of *Aloe marlothii* was reported to be active against *E.coli, E. faecalis* and *S. aureus* (Naidoo *et al*., 2006).*Calpurnia aurea* was reported to have antibacterial activity against both the Gram-negative bacteria (*Escherichia coli*, *Salmonella pooni*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) and the Gram-positive ones (*Bacillus cereus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus kristinae*, and *Streptococcus pyogenes*) (Adedapo *et al.*, 2008).Two carbazole alkaloids, clausenol and clausenine, isolated from *C. anisata* are active against both Gram-positive and Gram-negative bacteria with MIC values ranging between 1.3 µg ml^{-1} and 40 µg ml^{-1} (Chakraborty *et al*., 1995).The volatile oil from the leaves of *C. anisata* also has significant activity against a number of bacteria and fungi (Gundidza *et al*., 1994). Phytochemically, *E. lysistemon is* rich in flavonoids and alkaloids and over 30 compounds have been isolated from this plant. Three of the isolated compounds have weak activity against the Gram-negative bacteria (*Escherichia coli)* and moderate activity against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) (Juma and Majinda, 2005). According to Pillay *et al*. (2001) the bark of *E. lysistemon* is far more active than the leaves, yielding activity with water, ethanol and ethyl acetate extracts against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtlis.* The main anti-bacterial compound in the *E. lysistemon* bark was isolated and was identified as wighteone. Crude extracts from the bark of *S. africana* have antibacterial activity against diarrhoea-causative microorganisms (*Salmonella typhi*, *Shigella sonnei*, *Shigella dysentery*, *Shigella flexneri*, *Shigella boydii*, *Escherichia coli*.) with MIC values ranging between 0.156 and 0.625 mg/ml (Mathabe *et al*., 2006). Phytochemically, the Euphorbiaceae family to which *S. africana* belongs is rich in alkaloids and terpenoids (Webster, 1986). The inhibitory activity of terpenoids on bacteria has been reported (Drewes *et al*., 2005). One triterpene compound and two diterpenes compounds were isolated from *S. africana* (Mathabe *et al*., 2008) and are active against some of the diarrhoea-causative microorganisms with MIC values ranging between 50 µg ml^{-1} and 200 µg ml^{-1}.

In some cases the observed results differed from previous findings by other researchers. For example, in this study the hexane extract of *A. marlothii* had some antibacterial activity contrary to McGaw *et al*. (2000) who reported that crude

hexanic, ethanolic and aqueous extracts of *A. marlothii* does not have antibacterial activity. The possible reason for this difference in results could be the difference in plant chemical composition due to different times of plant collection and geographical differences. Unfortunately the TLC fingerprint of the plant from the previous research was not available for us to compare with the results from our study to confirm this postulation.

The antibacterial activities of extracts of *A. zebrina* and *P. livida* are being reported for the first time in this paper. Although the antibacterial activity of the other five study plants against some of the microorganisms have been reported against some of the test organisms in this study, in most of the studies the agar diffusion assay methods were used in determining the antimicrobial activity and high minimal inhibitory concentrations of up to 5 mg/ml were reported. In this study the serial microplate dilution method (Eloff, 1998b) was used. This method allows for the determination of the minimal inhibitory concentrations of each plant extract against each bacterial species by measuring the reduction of tetrazolium violet. It is more sensitive and we were able to show that some of the plant species had antibacterial activity at much lower concentrations than previously determined. For example *C. aurea* was reported to have a MIC of 5 mg/ml against *E. coli, P. aeruginosa, S. aureus* (Adedapo *et al.*, 2008) however, in this study we showed that it could still exhibit antibacterial activity against *P. aeruginosa, S. aureus* at 0.156 mg/ml and *E. coli* at 0.625 mg/ml. In addition, although the antibacterial activity of some of the plant species such as *S. africana* have been previously reported against some of the test organisms in this study, this is a first report of their antibacterial activity against *P. aeruginosa,* an important bacteria in the pathogenesis of wound myiasis. In some cases, the findings of this study add to the information on the antibacterial activity of some plant species.Pillay*et al*. (2000) reports that the ethyl acetate, ethanol and water extracts of *E. lysistemon* are ineffective against *E. coli* and *P. aeruginosa* however our results show that extracts from other extractants such as acetone, methanol and dichloromethane have reasonable to good antibacterial activity against these bacteria, with MICs ranging from 0.08 to 0.625 mg/ml.

All the plant extracts in this study were bacteriostatic at the determined MICs and bactericidal at higher concentrations. This is in line with the known fact that the MIC

is simply the concentration of the drug that inhibits the growth of bacteria and inhibition of bacterial growth does not necessarily mean that the bacteria have been killed (Finberg *et al*., 2004). The bactericidal activity of an antimicrobial agent against a particular organism tends to be related to its mechanism of action. In general, agents that disrupt the cell wall or cell membrane, or interfere with essential bacterial enzymes, are likely to be bactericidal, whereas those agents that inhibit ribosome function and protein synthesis tend to be bacteriostatic. The tangible benefit of the extracts to be bactericidal comes in its use in the management of topical infection. While the concentration required to kill the tested micro-organisms is high at 0.6 and 1.25 mg/ml their ability to reach this concentration at the wound site in combination with the poor immune response associated with topical wounds make them beneficial in the clinical management of wounds. If a compound or extract only has bacteriostatic activity it does not mean that it will be ineffective as it may allow the natural defence system of the organism to take control. Many commercial antibiotics have bacteriostatic activity. At higher concentrations they may kill the bacteria. The advantage of controlling topical infections is that much higher concentrations can be used.

Generally small quantities, ranging from 0.006 to 0.147 mg, of acetone extracts are required to inhibit bacterial growth on an average sized wound. This may have to be mixed with a grease to apply to the animals. Traditionally the leaves of the plants are crushed and packed onto a wound.

3.5 Conclusion

The bacteria used in this study are known pathogens of wounds and their inhibition by the plant extracts in this study might validate the traditional use of plants in the treatment of wound myiasis. It has been shown that bacterial action on wounds produce ammonia and volatile organic, sulphur containing compounds which have an odour that serve as an attractant of myiasis-causing flies. Therefore, inhibiting bacterial activity reduces the attractants of myiasis-causing flies to the wound and the stimuli for oviposition. Thus inhibiting bacteria action on wounds will interfere with the development of wound myiasis. This could be one of the mechanism through which the plants that are used traditionally in the treatment of wound myiasis work.

The next step to be addressed is to determine effect of these extracts on larval survival and subsequent development into adult stages.

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Table 3-1: Plants used traditionally to treat wound myiasis in South Africa and Zimbabwe

Scientific name	Family	Plant part used	Distribution	Preparation and administration
Aloe marlothii Berger (Van der Merwe *et al*., 2001)	Asphodelaceae	leaves	Botswana, Mozambique, South Africa (North-West, Gauteng, Limpopo, Mpumalanga, KwaZulu-Natal north of Durban),Swaziland, Zimbabwe.	The leaves are crushed and the juice is applied onto the wounds
Aloe zebrina Baker (Luseba and Van der Merwe, 2006)	Asphodelaceae	leaves	Angola, Botswana, Malawi, Mozambique, Namibia, South Africa (Gauteng, Mpumalanga, Limpopo), Zambia, Zimbabwe.	Succulent fresh leaves are crushed and applied onto the wound
Calpurnia aurea (Ait.) Benth. (Hutchings, 1996)	Fabaceae	leaves	Angola, Mozambique, South Africa, Swaziland, Zimbabwe	Leaf sap is squeezed onto the wound
Psydrax livida (Canthium huillense) (Chavunduka, 1976)	Rubiaceae	leaves	Botswana, Malawi, Mozambique, Zambia, Zimbabwe, Angola, Kenya, Namibia, South Africa(North-West, Limpopo, Mpumalanga)	Leaves crushed and packed into the wound
Clausena anisata (Chavunduka, 1976)	Rutaceae	leaves	Angola, Malawi, Mozambique, Zambia, Zimbabwe, South Africa(Limpopo, Mpumalanga, Eastern Cape, KwaZulu-Natal)	Leaves crushed and packed into the wound
Erythrina lysistemon Hutch (Van Wyk *et al*., 1997)	Fabaceae	leaves	South Africa (North West, Limpopo, Gauteng, Mpumalanga, KwaZulu-Natal, Eastern Cape), Swaziland, Zimbabwe, Botswana, Angola	Leaves crushed and placed on a maggot-infested wound
Spirostachys africana Sond (Hutchings *et al*., 1996)	Euphorbiaceae		Zimbabwe, Mozambique, Swaziland, South Africa (Mpumalanga, KwaZulu-Natal)	The sap is applied onto the maggot infested wound

Table 3-2: Antibacterial activity of 7 plant species used to treat wound myiasis in Southern Africa

Plant species	***Extract***	***Time (h)***	***Antibacterial activity (MIC in mg mL^{-1})***			
			E.coli	*E.feacalis*	*P.aeruginosa*	*S.aureus*
Aloe marlothii	Acetone	1 h	0.313	0.039	0.313	0.313
		24 h	0.313	0.039	0.313	0.078
	Methanol	1 h	1.25	2.5	0.078	0.625
		24 h	0.625	2.5	0.313	0.313
	Dichloromethane	1 h	0.313	0.625	0.313	0.156
		24 h	0.625	0.625	0.625	0.078
	Hexane	1 h	2.5	0.313	0.313	0.625
		24 h	2.5	2.5	2.5	2.5
Aloe zebrina	Acetone	1 h	0.156	0.02	0.156	0.039
		24 h	0.156	0.02	0.156	0.039
	Methanol	1 h	0.313	0.625	0.156	0.078
		24 h	0.156	0.625	0.313	0.156
	Dichloromethane	T1	0.156	0.078	0.156	0.078
		T2	0.156	0.156	0.313	0.039

	Hexane	T1	2.5	0.156	2.5	0.313
		T2	2.5	2.5	2.5	2.5
Calpurnia aurea	Acetone	T1	0.625	0.156	0.156	0.156
		T2	0.625	0.156	0.156	0.156
	Methanol	T1	1.25	1.25	>2.5	>2.5
		T2	1.25	1.25	0.313	
	Dichloromethane	T1	0.625	1.25	0.313	>2.5
		T2	0.625	1.25	0.313	
	Hexane	T1	1.25	2.5	>2.5	>2.5
		T2	1.25	2.5	>2.5	
Clausenaanisata	Acetone	T1	0.625	0.625	0.313	0.313
		T2	0.625	0.625	0.156	0.625
	Methanol	T1	0.625	1.25	0.313	0.625
		T2	0.625	1.25	0.313	0.625
	Dichloromethane	T1	0.313	0.313	0.156	0.313
		T2	0.625	0.313	0.156	0.313
	Hexane	T1	0.625	2.5	0.625	>2.5
		T2	1.25	2.5	1.25	>2.5
Erythrinalysistemon	Acetone	T1	0.313	0.156	0.078	0.313
		T2	0.313	0.156	0.078	0.313

	Methanol	T1	0.625	0.625	0.313	0.313
		T2	0.625	0.625	0.156	0.313
	Dichloromethane	T1	0.625	0.156	0.313	0.313
		T2	0.625	0.625	0.156	0.313
	Hexane	T1	2.5	1.25	0.625	1.25
		T2	2.5	1.25	0.625	1.25
Psydrax livida	Acetone	T1	0.313	0.078	0.313	0.156
		T2	0.313	0.313	0.156	0.078
	Methanol	T1	0.313	1.25	0.313	1.25
		T2	0.313	1.25	0.625	0.625
	Dichloromethane	T1	0.156	0.156	0.313	0.156
		T2	0.313	0.156	0.313	0.078
	Hexane	T2	2.5	0.313	0.313	1.25
			2.5	0.625	0.313	1.25
Spirostachy safricana	Acetone	T1	0.156	0.156	0.156	0.156
		T2	0.156	0.156	0.156	0.156
	Methanol	T1	0.313	0.625	0.313	0.313
		T2	0.313	0.625	1.25	0.313
	Dichloromethane	T1	0.313	0.313	0.313	0.313
		T2	0.313	0.625	0.313	0.313

	Hexane	T1	0.625	2.5	0.313	1.25
		T2	0.625	2.5	0.313	2.5
Gentamycin			1.56×10^{-3}	3.9×10^{-4}	1.56×10^{-3}	7.8×10^{-4}
Acetone			>2.5	>2.5	>2.5	>2.5

Table 3-3: Mass of the acetone extract of different plant species required to inhibit bacterial growth on a wound of 4cm diameter

Plant species	*Average amount of extract sprayed on filter paper* (mg)	*Volume of extract required to cover the 4cm filter paper* (ml)	*Extract required to inhibit bacterial growth on a wound of 4cm diameter* (mg)				
			E.c	***E.f***	***S.a***	***P.a***	**Average**
A. marlothii	4.60 ± 0.5354[c]	0.460	0.144	0.018	0.144	0.144	0.112
A. zebrina	2.75 ± 07937[b]	0.275	0.043	0.006	0.043	0.011	0.026
C. aurea	1.98 ± 0.1260[a]	0.196	0.123	0.031	0.031	0.031	0.054
C. anisata	2.35 ± 0.3416[a]	0.235	0.147	0.147	0.074	0.074	0.110
E. lysistemon	3.10 ± 0.5715[b]	0.310	0.097	0.048	0.024	0.097	0.066
P. livida	2.25 ± 0.6856[a]	0.225	0.070	0.018	0.070	0.035	0.048
S. Africana	2.70 ± 0.7528[b]	0.270	0.042	0.042	0.042	0.042	0.042

Means with same superscripts are not significantly different ($P< 0.05$)

Figures

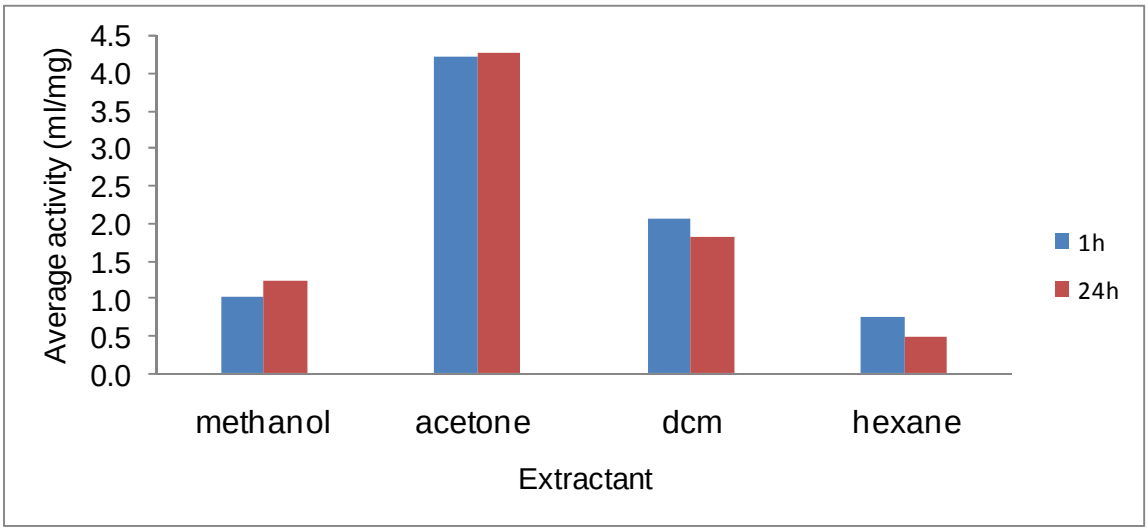

Figure 3-0-1 Average activity volumes indicating to what volume 1 mg of extract from different extractants can be diluted and it would still kill the bacteria

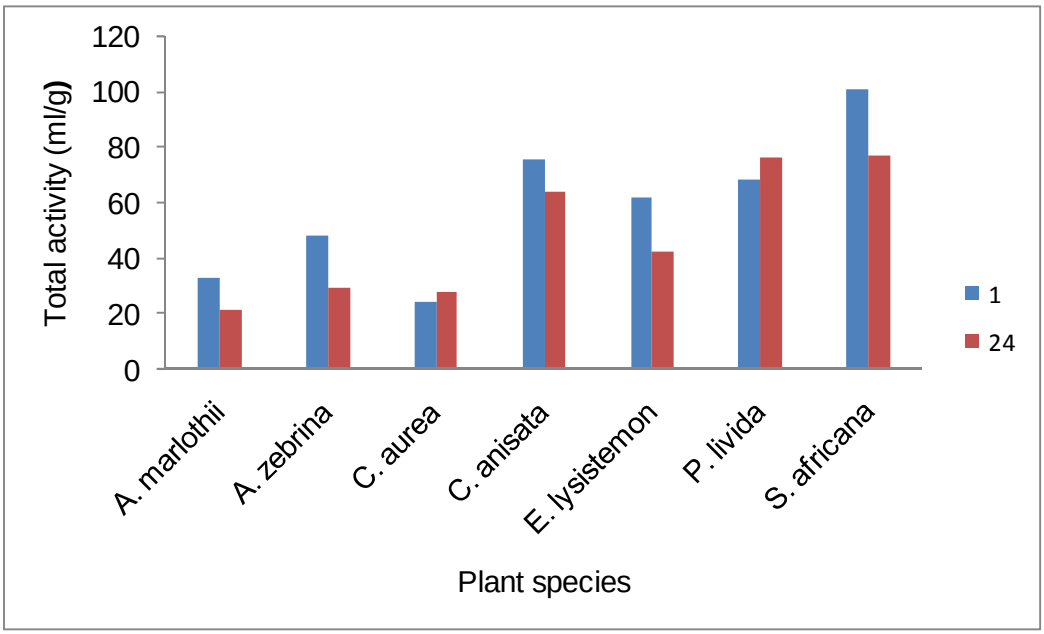

Figure 3-0-2: Total activity of the different plant species indicating the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the bacteria

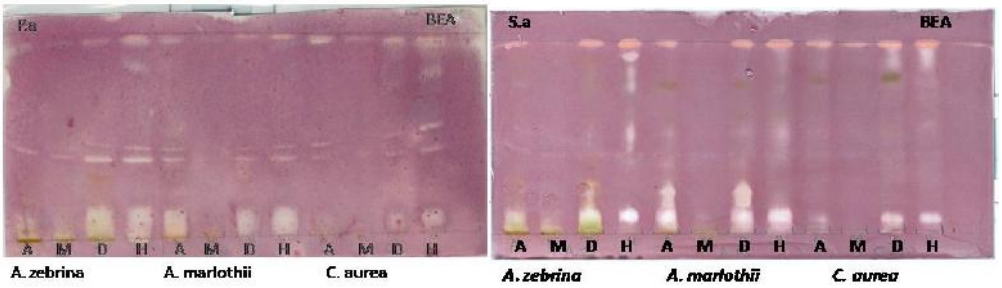

Figure 3-0-3: Chromatograms of acetone, methanol, dichloromethane and hexane leaf extracts of *A. zebrina, A. marlothii, C. aurea* eluted with BEA and sprayed with *P. aeruginosa and S. aureus* respectively. White areas indicate the presence of antibacterial compounds

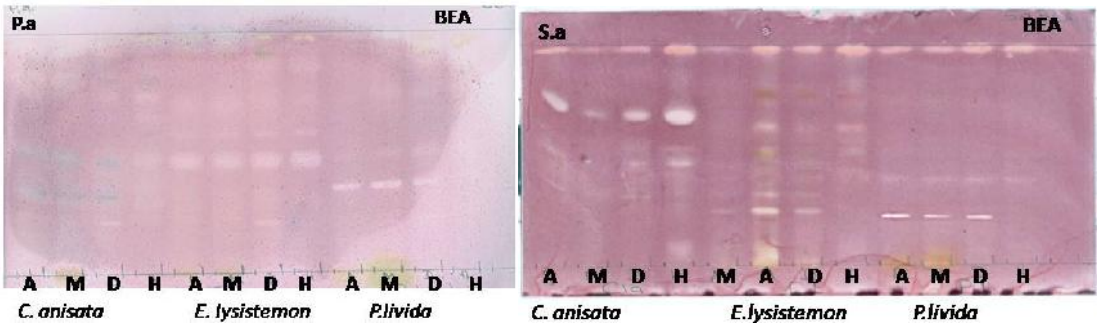

Figure 3-0-4: Chromatograms of acetone, methanol, dichloromethane and hexane leaf extracts of *C. anisata, E. lysistemon, P. livida* eluted with BEA and sprayed with *P. aeruginosa* andS. aureus respectively. White areas indicate the presence of antibacterial compounds

3.7 References

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Postscript

The antibacterial activity of the different extracts may explain the traditional use of *A. malothii, A. zebrina, C. aurea, C. anisata, P. livida E. lysistemon* and *S. africana* to treat wound myiasis. Traditional healers however do not use acetone extracts and some other mechanism may be involved in the activity. In the next chapter the activity of the extracts of these plant species on larval growth and development will be investigated.

CHAPTER 4

Preface

In the previous chapter the antimicrobial activity of the extracts of the 7 selected plant species was determined. The next aspect to investigate would be the activity of the extracts on the growth, development and behaviour of blowfly larvae, *in vitro.*

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4 Evaluation of plant species used traditionally to treat myiasis for activity on the survival and development of Lucilia cuprina and Chrysomya marginalis (Diptera: Calliphoridae)

[1*]Mukandiwa L., [1]Eloff J.N. and [2]Naidoo V.

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Abstract

Myiasis is a common parasitic problem of livestock responsible for severe economic losses in developing and developed countries. There are a number of challenges with the current control strategy, which depends largely on the use of pharmaceutical chemicals. These include inaccessibility, the increasing concern about pesticide accumulation in the environment and potential development of insecticide resistance in the devastating myiasis-causing flies. Consequently the search for alternatives is important. The use of plants in the treatment of wound myiasis in livestock as an alternative to commercial insecticides has been reported in resource poor areas worldwide. We therefore, undertook a study to establish the biological activity of seven plant species used against blowflies in southern Africa. A larvicidal assay was carried out in which third instar larvae of blowfly were fed meat treated with acetone leaf extracts of selected plant species. Four of the species, *Aloe zebrina, Clausena anisata, Erythrina lysistemon* and *Spirostachys africana,* induced developmental anomalies in the blowfly such as paralysis, prolongation of the prepuparium stage, reduced pupation rates, pupal malformations and reduced adult emergence. These results suggest that the plants may contain compounds that interfere with the neuroendocrine control mechanisms in the blowfly.

Keywords: myiasis; blowflies; ethnoveterinary medicine; larvicidal activity; pupal malformation

4.1 Introduction

Myiasis, the infestation of the skin of mammals by larvae of a variety of fly species belonging to the arthropod order Diptera, is a worldwide problem affecting livestock. It has been recorded as one of the most common parasitic problems of livestock in a number of surveys (Snoep, *et al*., 2002; Colebrook and Wall, 2004; Farooq, *et al*., 2008; Chhabra and Pathak, 2009). Although myiasis has been recognised as a major disease since circa the third millennium BC, the disease is still poorly controlled in the animal production industry. As a result, it is responsible for economic losses in developing and developed countries through abortion, reduced milk production, losses in mass and fertility, poor hide quality and cost of pesticides (Heath *et al*., 1987; Farkas *et al*., 1997). In sheep, ewes are mainly affected by blowflies and will not mate easily and will produce less wool of a lower quality (Snoep *et al.*, 2002). There are considerable losses to myiasis in the sheep industry every year. The average annual cost of fly strike to the South African sheep industry is estimated at R40 million a year (BISA, 2009) whilst in the Australian sheep industry it is A$280 million (Sackett *et al*., 2006). Untreated animals may die in 7 to 14 days from toxicity or secondary infections (Anonymous, 2008). If unchecked, screwworm infestations may result in significant economic losses. Before the eradication of the screwworm in USA, annual losses in the livestock sector from screwworms were estimated to be more than US$140 million (Peter *et al*., 2005).

Over the years, the control of myiasis has depended largely on the use of chemical compounds, such as macrocyclic lactones (avermectins), carbamates, pyrethroids and organophosphore insecticides, on the newly hatched larvae, immature forms and adult flies (Colwell and Dorchies, 2004). Insect growth regulators (IGRs) that affect insect growth and development have also been used. This use of chemical compounds has continued despite the potential human and animal toxicity and contamination of the food chain and the environment. The sustainability of this approach to disease control is also questionable as resistance develops. Improper application and frequent use have been the major causes of insecticide resistance in ectoparasites (Wall, 2007).

In addition to the challenges outlined above, the chemical insecticides used in the control and treatment of myiasis are not always accessible either due to high costs or

unavailability on the market. In the extreme situation of subsistence farming the use of plants in the treatment of wound myiasis in livestock as an alternative to the commercial insecticides has been reported worldwide (Bizimana, 1994; Viegi *et al*., 2003; Lans *et al*., 2007), including in southern Africa (Matekaire and Bwakura, 2004; Chavunduka, 1976; Hutchings *et al*., 1996; Luseba and Van der Merwe, 2006). However there have been very few studies to establish the effect of these plants on the myiasis-causing organisms and on possible ways of developing insecticides from these plants.

Faced with the problems of inaccessibility, the increasing concern about pesticide accumulation in the environment and potential development of resistance in the control of the devastating myiasis-causing flies, the search for alternatives becomes of paramount importance. The use of plant extracts and natural compounds for the control of insect vectors of man and animals may be a viable alternative. We therefore, undertook a study to evaluate the biological activity of extracts of seven plant species against blowflies. This contribution is the first report on the activity of extracts of plants used traditionally in the treatment of wound myiasis in South Africa and Zimbabwe on the survival and development of *Lucilia cuprina* and *Chrysomya marginalis*.

4.2 Materials and methods

4.2.1 Plant materials

After a study of the literature, we selected plants that are claimed to be effective in the treatment of wound myiasis in South Africa and Zimbabwe and that have a wide distribution in southern Africa. Detailed information on the traditional use of these species is presented elsewhere (Mukandiwa *et al*., 2012). The selected plants were *Aloe marlothii* A. Berger (Van der Merwe *et al*., 2001), *Aloe zebrine* Baker (Luseba and van der Merwe, 2006), *Calpurnia aurea* (Aiton) Benth (Hutchings *et al*., 1996), *Psydrax livida* (Hiern) Bridson (*Canthium huillense*), *Clausena anisata* (Willd) Hook (Chavunduka, 1976), *Erythrina lysistemon* Hutch (van Wyk *et al*., 1997), and *Spirostachys africana* Sond (Hutchings *et al*., 1996). To facilitate sustainable use of plants only leaves were investigated. The plant material was collected from the Pretoria National Botanical Garden, South Africa from labelled tree species. Voucher specimens are stored in the Herbarium of the Department of Paraclinical Sciences

(Medicinal Plant Collection), University of Pretoria, South Africa. The plant material was dried at room temperature in a well-ventilated room. Collection, drying and storage of plant material guidelines outlined elsewhere were followed (McGaw and Eloff, 2010).

4.2.2 Preparation of plant extracts

Dried leaf material was ground to fine powder (c. 1 mm diameter) using an IKA-WERKE M20 mill (GMBH & Co., Germany). Acetone was used as the extractant because it extracts compounds with a wide range of polarities from plant extracts and is relatively non-toxic to different test organisms (Eloff, 1998). It is also a much more efficient extractant than water and ethanol for metabolites from plant leaves (Kotze and Eloff, 2002). To prepare the acetone extract of each plant, 4 g of the leaf material of each plant were shaken vigorously for 30 min in 40 ml of acetone on an orbital shaker (Labotec®, model 20.2, South Africa). The extracts were allowed to settle, centrifuged at 2000g for 10 min and the supernatant filtered through Whatman No. 1 filter paper into pre-weighed glass vials. The extraction process was repeated 3 times for each aliquot of plant material. The extracts were dried in a stream of cold air at room temperature and the mass extracted with each solvent was determined. The dried extracts were reconstituted in acetone to make 10 mg/ml stock extracts which were used for the larvicidal assays. Acetone was used for the reconstitution because of its efficacy in dissolving compounds with a range of polarities and it is easy to remove from the extracts (Eloff *et al*., 2007). We could show that aqueous extracts had no effect on myiasis larvae (data not shown).

4.2.3 Establishment and maintenance of the fly colony

Blowfly larvae were collected from a vulture restaurant at VulPro which specialises in vulture conservation in the Magaliesberg close to Pretoria. Fresh carcasses are placed on the vulture restaurant every three days. The collected larvae were reared in the laboratory under controlled conditions. After emergence of the flies, the population was identified as a mixture of *Chrysomya marginalis* and *Lucilia cuprina* according to the key described in “Ticks, mites, and insects infesting domestic animals in South Africa” (Howell *et al*., 1978). We worked with the flies as mixed populations to simulate what happens in the field, where animals can have mixed infestations. This emerging population formed the first generation of the laboratory fly colony. The two

species of flies are distinctly different, *L. cuprina* being green in colour whilst *C marginalis* is blue, and on hatching, throughout the whole study, all the groups contained the two species of flies in approximately equal numbers. The flies were maintained at 28°C, 12 h photophase and 60% relative humidity. They were fed with a 1:1 mixture of sugar and powdered milk. Water was also supplied *ad libitum*. Strips of tenderised beef in Petri dishes were supplied for oviposition. The meat pieces with the eggs were transferred into a glass container which was covered with a fine mesh and placed in a humidified chamber at 30 °C, for the eggs to hatch. The emerging larvae were used either for the larvicidal assays or for the continued culture of the fly colony.

4.2.4 Larvicidal assay

The larvicidal assay described by Khater and Khater (2009) with modifications was used to test the effect of plant extracts on the larvae of blowflies. Meat strips (c. 30 g) were placed in Petri dishes (150 mm diameter) and 1 ml of the acetone plant extracts (10 mg/ml) were spread evenly over the different strips of meat. The meat strips in the Petri dishes were left open for 1h for the acetone to evaporate. Twenty late second-instar or early third-instar larvae (day 3-4) of blow fly were placed on each of the treated meat pieces in the Petri dishes. The Petri dishes with larvae were covered with tissue paper, secured with elastic bands then incubated for 24 h in a humidified chamber at 28 ± 2°C. The test for each plant extract was done in triplicate. The experiment was repeated three times. Thus 180 larvae were used for each plant extract. The activity of the larvae was assessed after 24 h and video recordings were made. To assess larval movement a grid was superimposed over the videos and the number of larvae passing through a marked quadrant in 60 seconds was recorded. Counts were done after 45 seconds into the video, at that stage the larvae would have adjusted to the light of the camera. Sustained immobility of the larvae, after exposure to the plant extracts, was considered as death of the larvae. The counts were repeated 3 times. Larval mobility was also assessed in terms of the time taken by 5 selected larvae in each treatment to move from one borderline to the other of a selected quadrant. Forty-eight hours after exposure to the plant extracts (day 6) the larvae were transferred into different beakers and covered with pieces of tissue paper and placed in a humidified incubator and were left there to pupate. After 72 h (day 9) the number of pupae in each beaker and of the larvae that failed to pupate was recorded. The

pupae were placed back in the respective beakers and the beakers were covered with laboratory tissue paper and were left to emerge. After 96 h (day 13) the number of pupae that failed to eclode was recorded. For all assays Ivermectin was used as a positive control (0.1 ml of 1 % Ivermectin) while acetone served as solvent control.

Based on the assessments of larvae movement and development after exposure to the plant extracts, 4 plants were selected for further investigation. The selected plants were further tested against blowfly larvae at 50 mg/ml. From our experiences in assessing biological activities we start at 10 mg/ml and if activity is observed we test lower concentrations. However in this case when we observed paralysis at 10 mg/ml we decided to find out if at higher concentrations these plants species would actually kill the larvae hence we tested them at 50 mg/ml.

4.2.5 Statistical analysis

The data on larval movement, pupal mass, pupation and adult fly emergence rates were analysed using ANOVA (SAS, 2007) to determine if there were any significant differences due to the different plant extracts. Mean separations was done using the PDIFF option of SAS (2007) to determine if the differences across the plant extracts and solvent control were statistically different at P = 0.05.

4.3 Results

In all the treatments, examination of the meat strips showed that the larvae had ingested the treated meat. There was 100% survival of larvae for all tested plant extracts at 10 mg/ml and for the solvent control in the first 24 hours. However in some of the treatments, *A. zebrina*, *C. anisata*, *E. lysistemon* and *S. africana*, partial paralysis of larvae was observed. These larvae failed to move from one point to the next but writhed in one place. In some cases the movement was significantly slower ($p< 0.05$) than normal movement of larvae such as that observed in the control group (Table 1). Larvae in the group exposed to meat treated with acetone only on average took 22.86 ± 1.52 seconds to move across a marked quadrant. Among those exposed to meat treated with *C. anisata* and *S. africana*, none managed to move across the quadrant although they were still alive. Larvae exposed to meat treated with ivermectin also did not move across the marked quadrant and appeared to be dead.

Larvae exposed to *E. lysistemon* and *A. zebrina* took double the time taken by larvae in the control group to move across the quadrant (Table 1). Larvae exposed to the other plant extracts were apparently not affected by the treatments. In terms of the total number of larvae moving across the marked quadrant, in the groups treated with 5 of the plant extracts less than 20% of the larvae moved across the quadrant in 60 seconds (Table 1). In the control group 63% of the larvae moved across the marked quadrant in 60 seconds.

A delay in entering pupation in the groups treated with *A. zebrina, C. anisata, E. lysistemon* and *S. africana* extracts compared with control larvae was also observed. Larvae treated with acetone, *A. marlothii, C. aurea* and, *P. livida* extracts pupated 72 hours after exposure to the treatments (from day 7 of cycle), whereas those treated with extracts of *A. zebrina, E. lysistemon, C. anisata* and *S. africana* extracts pupated from day 8. Fly emergence was complete by Day 12 in the groups of larvae treated with the acetone control treatment, *A. marlothii, C. aurea* and *P. livida* extracts with rates above 90%. In the *A. zebrina, E. lysistemon, C. anisata* and *S. africana* groups pupal mass, pupation and fly emergence rates were significantly reduced ($p< 0.05$) compared to the solvent control group (Table 2).

At the 50 mg/ml concentration there was a marked decrease in the movement of the larvae. Developmental features, such as length of the prepuparium stage, the pupation rates and adult emergence were severely affected. Prolongation of the prepuparium stage was observed in all the treated groups. Larvae from the groups treated with the plant extracts pupated from day 9, whilst those from the control group pupated from day 7. Pupation was reduced to as low as 58% in the *S. africana* treatment and adult emergence was reduced to 78% in the *A. zebrina* (Table 3). Average pupae mass was significantly reduced ($p< 0.05$) in all the four plant extracts but were more pronounced in the *C. anisata* and *S. africana* treatments. Morphological abnormalities of the pupae were pronounced for the *A. zebrina* and *E. lysistemon* extracts. The pupal malformations included small, larviform (larval–pupal intermediate), segmented, and distorted shaped pupae (Figure 1). Some pupae although appearing normal did not eclode, whilst others underwent only partial eclosion.

4.4 Discussion

Lucilia cuprina, one of the test organisms in this study, is the predominant species causing ovine cutaneous myiasis (sheep-strike) in many countries worldwide including Australia (Dalwitz *et al.*, 1984) and South Africa (Norris, 1990). While the other test organism, *Chrysomya marginalis*, is not a primary causative agent of myiasis its sister-fly from the same family, *Chrysomya bezziana*, is however, one of the major obligate parasites encountered in wound myiasis (Hall, 1991). The results obtained in this study are encouraging as they seem to suggest that some of the plants have the potential to be used as control agents against flies commonly implicated in myiasis. In further studies (reported elsewhere) both species of flies were observed to show similar responses to the plant extracts. They both became smaller in size indicating that they were both susceptible to the plant extracts.

The physiological and morphological defects observed in this study have been documented in a number of insects after exposure to some natural plant compounds. A common insecticide azadirachtin, the active principle of *Azadirachtin indica* has a powerful disrupting effect on growth and development in nearly 550 species of insects, inducing effects such as permanent larvae, prolongation of larval/nymphal instars, and inhibition of adult ecdysis (Garcia and Rembold, 1984; Mordue (Luntz) and Nisbet, 2000; Vinuela *et al*., 2000). A number of mechanisms of action have been put forward to explain the physiological and morphological defects caused by natural plant compounds.

The paralysis we observed suggests that the plant extracts interfered with the neuroendocrine control of movement. Most ectoparasiticides in use are neurotoxins, exerting their effect on the nervous system of the target parasite. Examples of such parasiticides are the organophosphate insecticides. Organophosphate pesticides inactivate the acetylcholinesterase enzyme (AChE) by phosphorylating the serine hydroxyl group located at the active site of AChE (Eddleston, *et al*., 2002). This results in disruption of nerve impulses (Coppage and Matthews, 1974), killing the organism or interfering with its ability to carry on normal functions such as movement leading to paralysis. Some alkaloids from plants have a similar effect of blocking the acetylcholinesterase enzyme (Aniszewski, 2007). Two of the study plants that induced

paralysis, *C. anisata* and *E. lysistemon* have a high alkaloid content (Ngadjui *et al*., 1989; Ito *et al*., 2000; Juma and Majinda, 2004).

The prolonged prepuparium stage and morphological deformities we observed indicate that the plant extracts may also have had an effect on the neuroendocrine control of moulting. It is generally accepted that the developmental anomalies induced by plant compounds are due to an interference with the neuroendocrine control of moulting and ecdysis (Rembold, 1989; Schmutterer, 1990). In larval insects three endocrine glands are known to be responsible for releasing neurohormones essential for growth, development and differentiation; the prothoracic gland (PTG), the corpus allatum (CA), and the corpus cardiacum (CC). It has been shown that plant compounds such as azadirachtin A causes progressive degeneration of all these endocrine glands in *L. Cuprina* larvae (Meurant, *et al*., 1994). This morphological degeneration implies a generalised dysfunction of the neuroendocrine system. When the secretion of prothoracicotropin is inhibited, the ecdysteroid production does not increase to the molt-inducing surge hence the prolonged prepuparium stage.

The physiological and morphological defects observed could have been induced by the interference with the juvenile hormone (JH) synthesis and degradation pathways. The insect's normal development is critically dependent upon the level of its own juvenile hormone (JH). When high titres are present only larval moults occur, but when secretion ceases the insect matures to the adult. Some plants have the ability to produce JH mimics **(**Bede and Tobe, 2000**).** The naturally occurring JH mimics include the terpenes, farnesol (VIII), farnesal, and the methyl and ethyl esters of farnesoic acid (Bowers *et al.*1965). The study plants, *C. anisata, S. africana, E. lysistemon,* that induced the developmental anomalies contain high amounts of terpenes (Onusic *et al*., 2002; Mathabe *et al*., 2008; Senthilkumar and Venkatesalu, 2009). These natural compounds may function by occupying juvenile hormone receptors at the synthesis stage, but because many JH analogues have no structural resemblance to natural JH, it is likely that these compounds inhibit the esterase or expoxidase degradation pathways of JH (Russell, 1977). As a result the JH titres remain high which could explain the delayed pupation. Applications of high doses of juvenoids to the last instar larvae of *Ceratitis, Musca, Calliphora,* and Sar*cophaga spp.* affect the formation of puparium **(**Sehnal and Žďárek, 1976). The pupae form in

due time but some retain the elongated larval-like shape and in some cases although the pupae appear perfect in every respect no adults emerge.

Alternatively the observed developmental anomalies could also be attributed to plant chemicals which are similar to the insect moulting hormones. The most common insect moulting hormone produced by plants in relatively large amounts is β-ecdysone (I). This hormone is secreted by the insect's endocrine system and initiates the moults through which the animal grows to maturity (Russell, 1977). Dietary application of ecdysones and their analogues cause mortality during the larval instars, delay in metamorphosis and inhibition of ecdysis in a number of insects (Robbins *et al.*, 1970; Wright and Kaplanis 1970).

4.5 Conclusion

The results indicate that acetone extracts of the leaves of some of the plants used traditionally to control myiasis are active against these blowflies. Although natural products are not necessarily a panacea to pest control, they do give new insights into the development of some insecticides and may be useful to resource poor farmers and for organically produced food. From this preliminary screening it can be concluded that some of these plant species contain compounds that cause paralysis, physiological and morphological defects in blowfly. Thus they have potential as control agents for this livestock pest. Further work is required to isolate and identify the plant compounds responsible for the observed results and to evaluate the mechanism of activity.

4.6 Acknowledgements

The University of Pretoria and the National Research Foundation provided the financial support for this research. The South African National Biodiversity Institute, Pretoria, allowed us to collect plant material in the Pretoria National Botanical Garden. L. Mukandiwa gratefully acknowledges the financial support from German Academic Exchange Service, DAAD, during the period of this study.

4.7 References

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Table 4-1: movement after exposure to acetone plant leaf extracts (10 mg/ml)

Plant Species	***Av. number of larvae passing through a marked quadrant in 60 secs***	***Av. time taken to move across a [1]quadrant (seconds)***
Aloe zebrina	4[a]	44.75 ± 1.20[c]
Aloe marlothii	10.33 ± 1.15[b]	34.02 ± 4.15[b]
Calpurnea aurea	37 ± 1.73[c]	27.05 ± 2.25[a]
Clausena anisata	1[a]	>90*
Erythrina lysistemon	11.67 ± 0.58[b]	39 ± 7.11[b]
Psydrax livida	35[c]	23.5 ± 2.08[a]
Spirostachys africana	4[a]	>90*
Ivermectin	0[a]	>90*
Acetone	37.67 ± 0.58[c]	22.86 ± 1.52[a]

* None moved from one borderline to the other in any of the quadrants for the whole duration of the recording

[1] A grid was superimposed over the videos and the number of larvae passing through a marked quadrant was counted

[a,b,c,] Values with different letters in same column are significantly different ($P < 0.05$)

Table 4-2: Effect of acetone plant extracts at 10 mg/ml on blowfly larvae, on the pupation rate, mass of pupae immediately after pupation and the % of adult fly emergence

Plant species	***Total number of larvae***	***Pupation rate* (%)**	***Average mass of pupae(mg)***	***Adult fly emergence* (%)**
Acetone	180	100 ± 0.00[c]	111.9 ± 10.18[c]	100 ± 0.00[c]
Aloe Marlothii	180	100 ± 0.00[c]	116 ± 3.87[c]	93.4 ± 4.08[b]
Aloe zebrina	180	82.2 ± 2.64[a]	88.7 ± 2.24[a]	74.4 ± 0.88[a]
Calpurnea aurea	180	100 ± 0.00[c]	91.3 ± 1.94[a]	96.8 ± 2.41[c]
Clausena anisata	180	89.4 ± 4.64[b]	86.2 ±1.56[a]	94.8 ± 2.65[b]
Erythrina lysistemon	180	91.1 ± 3.33[b]	85.3 ± 1.41[a]	91.7 ± 2.02[b]
Psydrax livida	180	100 ± 0.00[c]	87 ± 1.00[a]	100 ± 0.00[c]
Spirostachys africana	180	91.1 ± 2.20[b]	96.3 ± 1.5[b]	87.6 ± 1.16[b]
Ivermectin	180	0	N/A	N/A

[a,b,c,] Values with different letters in same column are significantly different ($P < 0.05$)

Table 4-3: Effect of acetone extracts of *A. zebrina, E. lysistemon, C. anisata, S. africana* at 50 mg/ml on blowfly larvae, on the pupation rate, mass of pupae immediately after pupation and the % of adult fly emergence

Plant extract	***No. of larvae***	***Pupation rate (%)***	***Average mass of pupae (mg)***	***Adult emergence (%)***	***Deformed pupae (%)***
Acetone	180	95.7 ± 1.15^{c}	67.3 ± 0.58^{c}	90.7 ± 1.15^{b}	5.3 ± 0.00^{a}
Aloe zebrina	180	70.5 ± 6.72^{b}	50.0 ± 6.93^{b}	78.3 ± 5.88^{a}	49.2 ± 6.97^{b}
Clausena anisata	180	72.2 ± 7.70^{b}	44.0 ± 3.00^{a}	79.8 ± 10.6^{a}	5.3 ± 0.00^{a}
Erythrina lysistemon	180	69.7 ± 6.96^{b}	51.0 ± 12.7^{b}	81.1 ± 2.59^{a}	65.2 ± 13.2^{c}
Spirostachys africana	180	57.6 ± 13.3^{a}	43.0 ± 9.64^{a}	82.8 ± 0.00^{a}	5.3 ± 0.00^{a}
Ivermectin	180	0	N/A	N/A	N/A

[a,b,c,] Values with different letters in same column are significantly different ($P < 0.05$)

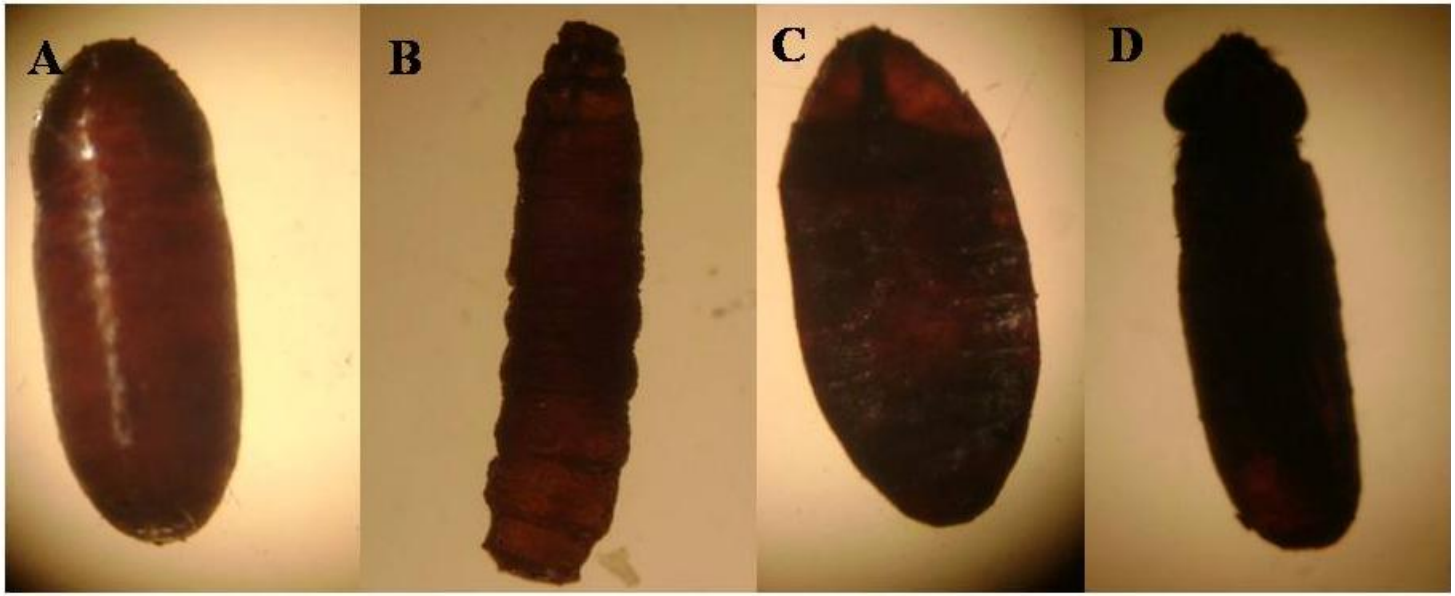

Figure 4-1: Images of the different forms of pupae emerging from larvae exposed to the acetone leaf extracts of *A.zebrina* and *E.lysistemon*

A – Normal shaped pupae with a smooth appearance. Average length 9.2 mm.

B – Larviform, which is an intermediate between pupae and larvae. The pupae maintained the elongated form of the larvae and the tubercles found along the body of the larvae remained visible giving the pupae a ringed appearance. In a normal pupae the tubercles disappear giving the pupae a smooth appearance. Average length 10.5 mm.

C - Distorted shaped pupae. Appeared flat instead of oval and were segmented. Average length 9.2 mm.

D - Pupae that underwent partial eclosion. Only the head emerged whilst the rest of the body remained enclosed and they died in this state.

Postscript

The observed interference of the extracts of *A. zebrina*, *C. anisata*, *E. lysistemon* and *S. africana* with the neuroendocrine control mechanisms in the blowfly further justifies their use in the control of wound myiasis. The next aspect would be to determine the dose-effect relationships of these extracts.

CHAPTER 5

Preface

In the previous chapter, it was established that *Aloe zebrina, C. anisata, E. lysistemon* and *S.africana,* induced developmental anomalies in the larvae including paralysis, prolongation of the prepuparium stage, reduced pupation rates, pupae malformation and reduced adult emergence. The following study evaluates their dose-effect relationships.

5 Extracts of four plant species used traditionally to treat myiasis influence pupation rate, pupal mass and adult blowfly emergence of Lucilia cuprina and Chrysomya marginalis (Diptera: Calliphoridae)

[1*]Mukandiwa L., [1]McGaw L.J., [1]Eloff J.N. and [2]Naidoo V.

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– Annexure 3)

Abstract

Ethnopharmacological relevance: *Aloe zebrina, Clausena anisata, Erythrina lysistemon and Spirostachys africana* are used traditionally in southern Africa to combat and/or treat myiasis, the infestation of the skin of mammals by larvae of a variety of fly species belonging to the arthropod order Diptera.The objective of the study was to establish the *in vitro* effect of extracts of these plant species on blowfly larvae to ascertain the potential use of these extracts and validate the traditional use.

Materials and Methods: *Chrysomya marginalis* and *Lucilia cuprina* larvae cultured in the laboratory under controlled conditions were exposed to meat treated with different concentrations of acetone and aqueous extracts of four different plant species. In addition to solvent controls, the effects of the plant extracts were tested at concentrations of 10, 25, 50, 75, 100 and 150 mg/ml. Larval behaviour, larval development and emergence of adult flies were assessed after exposing the larvae to the treated meat. Ivermectin was used as a positive control while acetone and water served as solvent controls. Cytotoxicity of the extracts was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay against C3A human liver cells, bovine dermis cells and Vero African green monkey kidney cells.

Results: An increase in the concentration of the acetone plant extracts resulted in a decrease in the ingestion of meat by larvae, pupal mass and adult emergence rates. The R^2 values for the correlation between dose and pupal mass was 0.92, 0.74 and 0.65 for *A. zebrina, C. anisata, and E. lysistemon* respectively. For *C. anisata* and *S. africana* extracts the increase in the concentration was also associated with the emerging adult flies being smaller. *C. anisata* was the most toxic plant extract, with an average IC_{50} of 39 µg/ml, while *E. lysistemon* was the least toxic. Vero cells were the most susceptible cell type, with an average IC_{50} of less than 100 µg/ml, and human liver cells were the most resistant to the effect of the plant extracts.

Conclusions: The results suggest that the plants may contain compounds that interfere with larval feeding and the neuroendocrine control mechanisms in the blowfly. It appears that some of these plant species have the potential to deliver a product that can be used to control myiasis.

Keywords: ethnoveterinary; myiasis, blowfly development, sheep strike control

5.1 Introduction

Since ancient times medicinal plants have formed an important part of both human and livestock health care systems (Mukherjee and Wahile, 2006). Preparations derived from plants were the original therapeutic interventions used by man for the control of diseases, including parasites, for improved livestock production (Waller *et al*., 2001). At present extensive documentation exists, worldwide, for plants used traditionally to combat animal diseases (McCorkle, 1986; Fielding, 1998; Viegi *et al*., 2003; McGaw and Eloff, 2008; Katerere and Luseba, 2010). Cutaneous myiasis is one of the conditions commonly managed by farmers using medicinal plants (Farooq *et al*., 2008; González *et al*., 2011; Mukandiwa *et al*., 2012a).

Cutaneous myiasis, caused by blowflies, also known as dermal and subdermal myiasis, remains one of the most common parasitic problems of livestock (Farooq, *et al*., 2008; Chhabra and Pathak, 2009) despite having been recognised as a major challenge in the animal production industry since c. the third millennium BC. The disease is characterised by larvae of blowfly such as *Lucilia cuprina* and *Chrysomya marginalis*, feeding on body tissues and

fluids, after the eggs are laid on livestock on either wounds or areas contaminated with faeces or urine (Hall and Wall, 1995). In southern Africa, *L. cuprina* is a significant cause of myiasis in sheep (Norris, 1990) causing the condition known as sheepstrike. The disease in these sheep is debilitating and results in major production losses and occasionally deaths (Phillips, 2009; Sotiraki and Hall, 2012).

Numerous plants have been documented for use in the treatment of maggot-infested wounds, on the African continent (Chavunduka, 1976; Hutchings *et al*., 1996; Luseba and van der Merwe, 2006; van der Merwe *et al*., 2001; Watt and Breyer-Brandwijk, 1962) as well as on the Asian continent (Anonymous, 1994; Handoo, 2006). In general the leaves of these plants are crushed and packed on to the wound. In some cases additives such as black pepper, palm oil, garlic and salt are crushed together with the leaves into a paste that is then smeared onto the wound. In a few cases fruit sap is applied to the wound.

It is possible that the use of plants to combat myiasis may be related to combating microorganisms that produce volatile compounds that attract female blowflies. The antibacterial activity of seven plant species reported in literature as used traditionally in the treatment of cutaneous myiasis in some communities in South Africa and Zimbabwe was examined (Mukandiwa *et al*., 2012a). Based on the results of that study and a preliminary screening of the plant species to determine the effect of their extracts on larval behaviour and development (Mukandiwa *et al*., 2012b), four plant species were selected for an in-depth study. The selected plants were *Aloe zebrina* Baker (Spotted aloe), *Clausena anisata* (Willd) Hook (Horsewood), *Erythrina lysistemon* Hutch (Coral Tree), and *Spirostachys africana* Sond (Tamboti). These plant species are common throughout the southern Africa region. This study focuses on the *in vitro* effect of leaf extracts of these plant species on blowfly larvae to evaluate the potential use of these extracts.

5.2 Materials and methods

5.2.1 Plant collection and storage

To encourage sustainable use we investigated only leaves of the plant species. The leaves were collected in April from the Pretoria National Botanical Garden, South Africa, dried at room temperature in a well-ventilated room. Samples of the plants were identified by tree name tags and were authenticated by the Guide at the National Botanical Garden. Voucher

specimens of the plant species with specimen numbers PMDN890 for A. *zebrina*, PMDN317 for C. *anisata*, PMDN34 for E. *lysistemon* and PMDN153 for S. *africana* are kept at the Medicinal Plant Collection Herbarium of the Department of Paraclinical Sciences, University of Pretoria, South Africa.

5.2.2 Preparation of plant extracts

Based on our experience with plant extracts used to determine antimicrobial (Eloff, 1998: Kotze and Eloff 2002), antitick (Zorloni *et al*., 2010) and antihelminthic (Bizimenyera *et al*., 2006) activity and our preliminary work (Mukandiwa *et al*., 2012b), acetone was selected as one of the extractants to be used. Dried leaf material was ground to fine powder using an IKA-WERKE M20 mill (GMBH & Co., Germany). To obtain the acetone extract of each plant, 4 g of the finely ground leaf material was shaken vigorously for 30 min in 40 ml of acetone on an orbital shaker (Labotec®, model 20.2, South Africa). The mixtures were allowed to settle, centrifuged at 2000g for 10 min and the supernatant filtered through Whatman No. 1 filter paper into pre-weighed glass vials. The extraction process was repeated 3 times for each aliquot of plant material and the filtrates were pooled together and dried under a stream of cold air at room temperature for mass quantification. The dried extracts were reconstituted in acetone to make the test extracts which were used for the larvicidal assays. Acetone was used for the reconstitution because it does not have an effect on the test organism (Mukandiwa *et al.*, 2012b), it dissolves compounds with a range of polarities and it is easy to remove from the extracts (Eloff *et al*., 2007).

Because traditional healers usually have only water available to extract plants aqueous extracts were also evaluated. To obtain the aqueous extracts, a similar procedure as outlined for the acetone extracts was followed except that distilled water was used as the extractant and that the extracts were dried in a VIRTIS freeze drier (SP Industries, USA). The aqueous extracts were reconstituted in distilled water to make the test extracts. We collected the information on the traditional use of these species from literature. The preparation procedures used by the traditional healers were not specified. In some cases traditional healers may place a paste of the leaves on wounds or make a mixture of the plant material with fat. Under these conditions more non-polar compounds extractable by acetone could have become available. In some cases aqueous extracts are left in the presence of the plant material for long periods. Microbial growth in these, possibly carbohydrate containing mixtures, may also lead to the release of more non-polar compounds from the plant material.

5.2.3 Establishment and maintenance of the fly colony

Blowfly larvae were collected from a vulture restaurant in Hartebeestpoort, South Africa and moved to the laboratory and allowed to pupate. After emergence of the flies, the population was identified as a mixture of *Chrysomya marginalis* and *Lucilia cuprina* (Figure 1). This emerging population formed the first generation of the laboratory fly colony. The flies were maintained at 28°C, 12 h light cycle and 60% relative humidity. They were fed a 1:1 mixture of sugar and powdered milk. Water was also supplied *ad libitum*. Pieces of tenderised beef and liver in Petri dishes were supplied for oviposition. The meat pieces with the eggs were transferred into a glass container which was covered with a mesh and placed in a humidified chamber at 30°C, for the eggs to hatch. The emerging larvae were used either for the larvicidal assays or for the continuation of the fly colony.

5.2.4 Larvicidal assay

The larvicidal assay described by Khater and Khater (2009) was used to test the effect of plant extracts on the larvae of blowflies. The plant extracts were tested at concentrations of 10, 25, 50, 75, 100 and 150 mg/ml. Meat pieces (30 g) with a total surface area of 27.5cm^2 were placed in plastic cups (90mm diameter) and 1 ml aliquots of the plant extracts were spread evenly over the different pieces of meat. The meat pieces in the plastic cups were left open for 1h for the acetone to evaporate. Twenty late second-instar or early third-instar blowfly larvae (day 3-4) were placed on each of the treated meat pieces in the plastic cups. The plastic cups with larvae were covered with tissue paper, secured with elastic bands and then incubated in a humidified chamber at 28 ± 2°C. Forty-eight hours after exposure to the plant extracts (day 6) the larvae were transferred to different plastic cups and covered with pieces of tissue paper and placed in a humidified incubator and were left there to pupate. Seventy-two hours later (day 9) the number of pupae and larvae that failed to pupate was recorded per treatment.

Lucilia cuprina *Chrysomya marginalis*

Figure 5-1: Images of the adult blowflies used in this study. Photographs by John Deeming, Amgueddfa Cymru - National Museum Wales, and R. Major, Australian Museum.

The pupae for each treatment were pooled together and the average mass determined in each experiment. The pupae were placed back in the respective beakers and the beakers were covered with laboratory tissue paper and were left to emerge. After 96 h (day 13) we recorded the number of pupae that failed to eclode. In all assays ivermectin was used as a positive control (0.1 ml of 1 % ivermectin) while acetone and distilled water served as solvent controls. The test for each concentration of plant extract was done in triplicate on three separate occasions (n=9). In total 180 larvae were exposed to each concentration of plant extract.

Larval behaviour (movement and feeding activity) was monitored at 4h, 8h, 24h and 32h after exposure of larvae to the treated meat for durations of 1 hour. Visual assessments of the meat pieces was also undertaken to ascertain if the larvae fed on the treated meat.

5.2.5 Effect of plant extracts on reproduction

The ability of the flies, emerging from larvae exposed to the highest concentration (150 mg/ml) of *C. anisata* and *S. africana* acetone extracts to lay eggs was also assessed. These two plant species were selected for further study due to the significant effect they had in the larvicidal assay. The flies were maintained at 28°C, 12 h light cycle and 60% relative humidity in 2 different cages (30 cm x 30 cm x 48 cm). They were provided with an *ad libitum* mixture of powdered milk and sugar and water. Petri dishes (150 mm diameter) containing meat shreds and ox-liver were placed in the cages for the flies to lay eggs on. The meat shreds were observed for fly eggs every 2 h for 24 h.

5.2.6 Repellency effect of plant extracts

Meat strips treated with acetone extracts (150 mg/ml) of *C. anisata* and *S. africana* in different Petri dishes were placed inside the cages where the flies were housed to determine if the plant extracts could repel the gravid females. The numbers of eggs deposited on the meat shreds were observed every 2 h for 24 h. Meat treated with acetone served as solvent control.

5.2.7 Cytotoxicity

Cytotoxicity was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] assay developed by Mosmann (1983). The extracts were tested against C3A human liver cells, bovine dermis cells and Vero African green monkey kidney cells. The

cells were cultured in minimal essential medium (MEM) Earle's Base (Sigma), supplemented with 2 mM L-glutamine, 0.1% gentamicin (Virbac) and 5% foetal calf serum (Sigma). Suspensions of the cells were seeded into 96-well microtitre plates at a density of 2×10^3 cells per well and incubated for 24h at 37°C in a 5% CO_2 incubator. The medium was aspirated from the cells, and extracts at ten concentrations (ranging from 0.0075 to 1mg/ml), positive control (doxorubicin hydrochloride, Pfizer Laboratories) and negative controls were added and incubated for 5 days. Cell proliferation and viability was quantified by adding 30 µl of a 5 mg/ml solution of MTT in PBS to each well and incubating for a further 4h at 37°C. The medium was removed from the wells without disturbing the MTT crystals and washed with PBS. The PBS was aspirated from the cells and 50 µl of DMSO was added to each well to dissolve the crystallized MTT formazan. The amount of reduced MTT was measured as absorbance at 570 nm using a Versamax microtitre plate reader (Molecular Devices). The results were expressed as a percentage of the control cells and IC_{50} values were calculated from the regression equations.

5.2.8 Data analysis

The data on pupal mass, pupation and adult fly emergence rates were analysed using the general linear model procedures (SAS, 2006) to determine if there were any significant differences due to the different plant extract concentrations, and also to determine the most effective concentrations. Normality was assessed on arcsine transformed data. Mean separations was done using the PDIFF option of SAS (2006) to determine if the differences across concentrations were statistically different at P=0.05. Pupation rate was calculated as the number of pupae formed /the number of larvae. Emergence rate was calculate as the number of flies /the number of pupae formed

5.3 Results

The dried leaves (4g) of E. l*ysystemon*, C.*anisata*, S. *africana* and A. *zebrina* yielded 40, 120, 40 and 8.7mg, respectively, of the acetone extract.

5.3.1 Observations from larvicidal assay

5.3.1.1 Larvae feeding activity

The acetone plant extracts had a clear effect on the feed intake of the larvae with the increase in concentration resulting in a decreased ingestion of meat. The decrease in feeding was not associated with a concurrent decrease in general activity of the larvae, indicating that the effect seen was most likely due to a decrease in palatability of the meat. For *A. zebrina, at* the 10, 25 and 50mg/ml concentrations the meat was well eaten while the higher doses resulted in lower intake. For *C. anisata,* at 10 mg/ml the larvae were on the meat albeit with reduced consumption in comparison to the10 mg/ml of the other plant species. For the higher doses the meat was poorly ingested with most of the larvae circling around the periphery of the plastic cups. For *E. lysistemon,* at concentrations of 10, 25, 50 and 75 mg/ml the meat was well eaten with the higher doses being moderately eaten. For *S. africana,* at 10 and 25 mg/ml the meat was well eaten, moderately eaten at 50 and 75 mg/ml and poorly consumed at the higher doses. The latter was also associated with larvae circling on top of the plastic cups possibly indicating repellency. Larval feeding activity was not affected by any of the aqueous extracts.

5.3.1.2 Average pupal mass

The effect of increasing concentration of the acetone plant extracts on the feeding behaviour of the larvae corresponded with decreased masses of the emerging pupae (Table 1). The R^2 values for the correlation between dose and pupal mass was 0.92, 0.74 and 0.65 for *A. zebrina, C. anisata, and E. lysistemon* respectively. In general there was a better dose response relationship for the higher doses. For *C. anisata and E. lysistemon,* the10 mg/ml had significantly lower average weights compared to the acetone control, while for *A. zebrina* and *S.africana* extracts the pupal mass was significantly different from the control from 75 and 25 mg/ml, respectively. No aqueous extract had any significant effect on the mass of the pupae (results not shown).

Table 5-1: Weight of pupae (g) emerging from the larvae exposed to the different concentrations of the acetone plant extracts expressed as Means ± SD

Concentration (mg/ml)	*A. zebrina*	*C. anisata*	*E. lysistemon*	*S. africana*
Acetone (solvent control)	0.053^{b} ± 0.012	0.053^{c}± 0.012	0.053^{c} ± 0.012	0.053^{c} ± 0.012
10	0.051^{b} ± 0.010	0.043^{b} ± 0.012	0.050^{b} ± 0.011	0.054^{c} ± 0.010
25	0.052^{b} ± 0.009	0.048^{c} ± 0.011	0.053^{c} ± 0.012	0.051^{b} ± 0.013
50	0.048^{b} ± 0.006	0.041^{b} ± 005	0.051^{b} ± 0.010	0.050^{b} ± 0.012
75	0.044^{a} ± 0.010	0.037^{a} ± 0.004	0.043^{a} ± 0.010	0.046^{b} ± 0.008
100	0.043^{a} ± 0.007	0.036^{a} ± 0.008	0.047^{b} ± 0.010	0.036^{a} ± 0.004
150	0.041^{a} ± 0.005	0.035^{a} ± 0.006	0.044^{a} ± 0.015	0.032^{a} ± 0.007

^{a,b,c,d}Values with different letters in same column are significantly different (P< 0.05). Ivermectin results not included as the drug induced 100% mortality in all larvae, thereby preventing pupation

5.3.1.3 Pupation rate

In the larvae exposed to *A. zebrina* and *E. lysistemon* there was an inverse relationship between extract concentration and pupation rates (Table 2). In the larvae exposed to *C. anisata* and *S. africana* the pupation rates decreased as the concentration increased from 10 mg/ml to 50 mg/ml and after that began to rise again such that the pupation rates at 100 mg/ml and above were not significantly different from those of the control (Table 2). In both *C. anisata* and *S. africana* 50 mg/ml appeared to be an optimum value for decreasing pupation rates. However, although there was almost 100% pupation at these high concentrations, the emerging pupae had significantly lower average masses (Table 1). The pupation rates in the larvae exposed to the aqueous extracts were not significantly different from the pupation rates of those exposed to the controls.

5.3.1.4 Deformed pupae

The acetone extracts of *A. zebrina* and *E. lysistemon* caused the pupae to deform in that they still had the larval form. While the pupae from *C. anisata* and *S. africana* were not malformed, the concentration ≥100 mg/ml resulted in the emerging pupae being significantly smaller compared to the control (Table 1).

5.3.1.5 Adult emergence

A significant difference in the rate of adult emergence was seen with an increase in the concentration of the acetone plant extracts (Table 2). For *C. anisata* and *S. africana* extracts the increase in the concentration was also associated with the emerging adult flies being smaller. The emerging flies from larvae that were treated with *C. anisata* and *S. africana* at 150 mg/ml were smaller than the flies emerging from larvae exposed to meat treated with acetone only (Figure 2).

Table 5-2: Pupation rates and adult emergence for larvae exposed to the different concentrations of the acetone plant extracts expressed as means ± SD

Concentration (mg/ml)	*A. zebrina*	*C. anisata*	*E. lysistemon*	*S. africana*
		Pupation (%)		
Acetone (solvent control)	97.77^{d}	97.77^{d}	97.77^{d}	97.77^{d}
10	80.00^{c} ± 5.376	86.88^{c} ± 6.347	88.74^{c} ± 3.988	93.18^{c} ± 6.898
25	72.77^{b} ± 3.784	77.87^{b} ± 1.251	80.16^{c} ± 5.641	88.89^{b} ± 10.184
50	70.48^{b} ± 6.717	72.22^{a} ± 7.698	69.74^{b} ± 6.958	57.58^{a} ± 5.915
75	66.94^{b} ± 3.127	78.87^{b} ± 2.025	59.21^{b} ± 5.228	86.03^{b} ± 12.647
100	61.40^{b} ± 3.039	97.77^{d} ± 3.849	49.51^{a} ± 9.541	100.00^{d} ± 0
150	44.50^{a} ± 1.680	97.77^{d} ± 3.849	41.76^{a} ± 8.081	100.00^{d} ± 0
		Adult emergence (%)		
Acetone (solvent control)	98.89^{c} ± 1.923	98.89^{c} ± 1.923	98.89^{c}± 1.923	98.89^{c} ± 1.923
10	84.16^{a}± 11.031	90.74^{b} ± 8.913	95.23^{c} ± 4.159	98.89^{c} ± 1.923
25	89.84^{b}± 0.277	87.41^{b} ± 10.962	88.73^{bc} ± 1.800	97.78^{c} ± 3.851
50	78.25^{a}± 5.877	79.79^{ab} ± 10.638	81.11^{b} ± 2.594	82.22^{b} ± 1.923
75	77.27^{a}± 4.277	70.00^{a} ± 5.774	83.81^{b} ± 1.255	67.41^{a} ± 4.492
100	82.07^{a}± 2.004	77.94^{a}± 7.584	71.74^{a} ± 11.799	58.89^{a}± 11.705
150	83.71^{a} ± 0.345	95.55^{c} ± 3.851	68.31^{a} ± 9.240	59.19^{a} ± 3.556

^{abcd}Values in the same column with same superscripts, for each data set, are not significantly different. Ivermectin results not included as the drug induced 100% mortality in all larvae, thereby preventing pupation

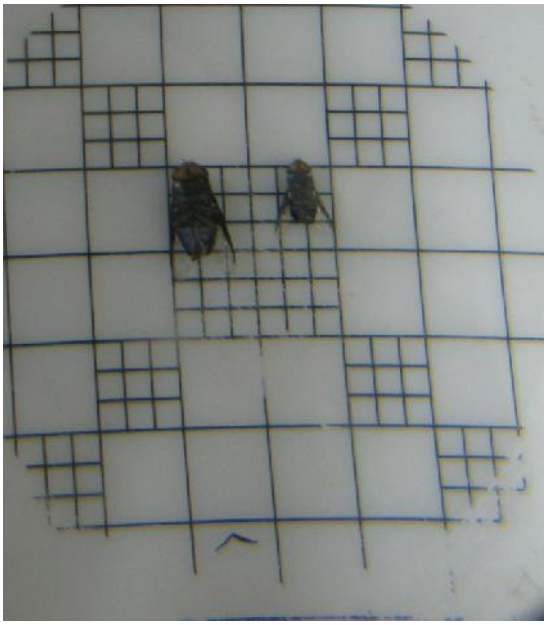

Figure 5-2: Comparison between the emerging C. *marginalis* flies from larvae that were treated with *C. anisata* at 150 mg/ml (smaller) next to the those emerging from larvae exposed to meat treated with acetone only (one small block = 11.11mm^2)

5.3.2 Effect of plant extracts on reproduction

The small-sized adult flies that emerged from the larvae that were exposed to the highest concentration (150 mg/ml) of *C. anisata* and *S. africana* acetone extracts were able to lay eggs at the same time as the control group. The laid eggs hatched within the normal time range for blowflies and gave rise to normal larvae which grew to normal-sized adult larvae and gave rise to normal-weight pupae (0.056 g ± 0.003) and subsequently normal-sized adult flies.

5.3.3 Repellency effect of plant extracts

The blowflies laid eggs on the strips of meat and liver that were treated with the acetone extracts of *C. anisata* and *S. africana*. The plant extracts did not seem to repel the gravid females. The eggs hatched within the normal time range for blowflies and gave rise to normal larvae. The effects on the larvae were comparable to the results obtained following direct placement of larvae onto the treated meat viz. the larvae led to lower mass pupae (0.026g ± 0.005 for *S. africana*, 0.042 ±0.05 for *C. anisata*) compared to the acetone (0.047 ± 0.005) and untreated (0.053 ± 0.005) groups.

5.3.4 Cytotoxicity

The cytotoxicity of the four plant extracts is presented in Table 3. *C. anisata* was the most toxic plant extract, with an average IC_{50} of39 µg/ml, while *E. lysistemon* was the least toxic. Vero cells were the most susceptible cell type, with an average IC_{50} of less than 100µg/ml, and C3A cells were the most resistant to the effect of the plant extracts.

Table 5-3: Cytotoxicity of extracts against Vero, bovine dermis (BD) and C3A liver cells

Species	IC_{50} (µg/ml) ± SD		
	Vero	BD	C3A
A. zebrina	135 ± 96	227 ± 14	259 ± 9
C. anisata	36 ± 4	35 ± 0	46 ± 0
E. lysistemon	124 ± 11	230 ± 26.5	473 ± 41
S. africana	48 ± 16	115 ± 8	193 ± 17
Average IC_{50}	86 ± 51	152 ±95	243 ± 177
Doxorubicin (µM)	17.015 ± 0.096	5.086 ± 0.525	0.524 ± 0.010

5.4 Discussion

The results suggest that some of the plant species that are used traditionally to manage myiasis contain compounds that deter blowfly larvae from feeding. The study plants that were the most potent in deterring the larvae from feeding, *C. anisata* and *S. africana*, contain high amounts of terpenes (Mathabe *et al*., 2008; Senthilkumar and Venkatesalu, 2009). In practice, this deterrence from feeding would fulfil a protective role on an animal. Although the animal may be infested with blowfly larvae the plant extracts would protect the animal from the detrimental effects of the feeding activity of the larvae. The feeding activity of the larvae causes severe damage to hides and underlying muscles, resulting in discomfort, a loss in production, reproduction problems, blindness, lameness and even death (Otranto and Stevens, 2002). The known active plant-based antifeedants/feeding deterrents belong to groups like chromenes, polyacetylenes, saponins, quassinoids, cucurbitacins, cyclopropanoid acids, phenolics, alkaloids, various types of terpenes and their derivatives (Koul, 2008). The most potent antifeedants belong to the terpenoid group, which has the greatest number and diversity of known antifeedants and the most potent example is azadirachtin A. Azadirachtin, derived from

the seeds of the neem tree, *Azadiracta indica* A. Juss (Meliaceae), has already been incorporated in integrative pest management as an antifeedant agent against insects (Kostic *et al*., 2008).

The results of this study indicate a very close correlation between adequacy of larval food, pupal weight, and size of the adult fly as previously postulated by Webber (1955). Restriction of larval food, as is the effect of the plant extracts in this study, resulted in the production of low weight pupae from which small-sized adults emerged. Similar results have been reported for house fly larvae, *Musca domestica* L. (Diptera: Muscidae) exposed to the ethanol crude extract of Chinese star anise fruits, *Illicium verum* Hook. F. (Illiciaceae) (concentrations between $2.5x10^3$ -$10.5x10^3$ mg/l) (Sripongpun, 2008). *Clausena anisata* and *S. africana* at concentrations of ≥ 100 mg/ml led to significantly smaller adult flies that were still able to lay eggs. Similar results of small-sized flies resulting from underfed larvae being able to lay eggs were reported in *Lucilia cuprina* by Webber (1955). The latter study also demonstrated that their fecundity was reduced as the numbers of functional ovarioles, and hence the maximum number of eggs developed in each ovarian cycle, is a linear function of adult size (Mackerras, 1933; Webber, 1955; Foster, et al., 1975, Vogt *et al*., 1985).

All the plant extracts, at least at one concentration, reduced pupation and adult emergence rates. Similar results have been recorded for a number of plant extracts on other species of blowflies. For example, Khater and Khater (2009) reported that the pupation rate was strongly decreased in *Lucilia sericata* after treatment with 16% fenugreek (*Trigonella foenum-graecum*) and celery (*Apium graveolens*). Moreover, adult emergence was suppressed after treatment of larvae with 8% mustard (*Brassica compestris*), 12% radish (*Raphanus sativus*), and 16% fenugreek and celery oils. The effective concentration was in the same order as the values we have found (100 mg/ml = 10%). The Neem seed oil reduces pupation and adult emergence rates in *L. sericata* (El- Khateeb *et al*., 2003). Morsy *et al*. (1998) also reports that pomegranate (*Punica granatum*) reduced pupation rates in C*hrysomya albiceps*. Plant compounds known to have such effects in insects belong to groups like alkaloids and terpenoids (Bowers *et al.*, 1965; Aniszewski, 2007).

Another finding of the study was that in the presence of decreased feeding, at higher concentrations, pupation and adult emergence rates were minimally reduced implying that the active compound(s) required ingestion. In contrast some compounds are transcutaneous exerting their effects via contact without having to be ingested. Reduced feed intake resulted in the production of pupae smaller in size rather than a lower number of pupae hence high pupation and adult emergence rates were still recorded at concentrations of the plant extract where reduced feed intake was observed. Similar results are reported by Webber (1955) and Daniels *et al.* (1991).

Extracts of the plant species used in this study led to a deterrence from feeding, low weight pupae, small adult flies (*C. anisata* and *S. africana*),deformed pupae and reduced pupation and adult fly eclosion (*E. lysistemon* and *A. zebrina*). These effects are similar to what is seen after exposure to insect growth regulators (IGRs). As a group the IGRs act principally on embryonic, larval and nymphal development by interfering with metamorphosis and reproduction. In most cases, IGRs require more time to reduce insect populations than conventional insecticides and will sometimes have to be used in combination with adulticides to achieve an immediate knock-down effect (Graf, 1993).We propose that the plant extracts tested could be effective if used in a control programme and not as single therapeutic agents. Since the plant extracts did not seem to repel gravid females or deter oviposition, they may be useful as fly population control baits i.e. they can be used to modulate the total population numbers via treated baits. Of all the biological parameters associated with growth rate in blowflies, fecundity plays an important role in population dynamics since it determines the population growth potential (Godoy *et al.* 1996, 2001). The fact that the effects on the larvae emerging from eggs laid on treated meat were comparable to the results obtained following direct placement of larvae onto the treated meat viz. the larvae were of lower weight pupae compared to the acetone and untreated groups suggest that the *C. anisata* and *S. africana* extracts are stable for at least 6 days.

In evaluating plant extract safety, Zirihi *et al.* (2005) considered extracts with IC_{50} values of 20 µg/ml and below using *in vitro* cytotoxicity assays to be toxic. Using this criterion,

none of the extracts tested in this study was toxic, although the average cytotoxicity of the *C. anisata* extract was less than 40µg/ml which is cause for concern. The remaining three extracts tested had low levels of toxicity although *in vivo* studies would need to confirm their safety for use. With the topical application the dose to the animal as a whole would probably be safe enough. It is dangerous to correlate cellular toxicity with animal toxicity. The wood of *S. africana* and smoke when wood is burned is very toxic (Watt and Breyer-Brandwijk, 1962), but the leaf extracts had relatively low toxicity to the cell cultures. The cell types selected were used as an overall indication of toxicity. Specifically, the dermal cell cultures were selected as an indicator of dermal toxicity as the product is most commonly applied topically. Another aspect considered was the specific pathophysiology of the larvae as it induces severe dermal ulceration as they feed. As a result, percutaneous absorption and systemic toxicity becomes possible. For this study, the kidney and liver cell cultures were used to indicate systemic toxicity as their high metabolic capacity and preferential blood supply in vivo make them the most susceptible organ systems.

It is noteworthy that the aqueous extracts had no activity in any of these experiments. This is an aspect that needs some more attention to determine exactly how the plant material was used by the traditional healers. If hot water extracts using contaminated water were used microbial action on the leaves may solubilize some non-polar compounds. If a leaf paste was placed directly onto the wound serum may solubilize some of the intermediate polarity compounds soluble in acetone present in the leaves leading to a therapeutic effect. It would be important to evaluate the activity of a plant extract mixed with fat to treat myiasis in animal wounds and bring this information under the notice of rural pastoralists.

5.5 Conclusion

Some of the plant species used traditionally to treat myiasis in southern Africa have deleterious effects on blowflies. These effects can be manifested in several ways including toxicity, anomalies in developmental behaviour, antifeedant and reduction of fecundity and fertility. It appears that some of these plant species have the potential to

deliver a product that can be used to control myiasis. Further work is required to evaluate if the effects observed with these plant extracts in the laboratory would be mirrored in field and clinical studies.

5.6 Acknowledgements

The University of Pretoria and the National Research Foundation provided the financial support for this research. The curator of the Pretoria National Botanical Garden gave us permission to collect the plant material. L. Mukandiwa gratefully acknowledges the financial support from German Academic Exchange Service, DAAD, during the period of this study.

5.7 References

Listed in Chapter 9

Postscript

The observed reduction in the size of adult blowflies by the extract s of *C. anisata* and S. *africana* coupled with the fact that smaller flies lay fewer eggs warrant a field evaluation of the extract.

CHAPTER 6

Preface

In the previous chapter, *Clausena anisata* and *Spirostachys africana* had the most promising activity against the blowflies *Lucilia cuprina* and *Chrysomya marginalis*, with *C anisata* being superior. *Clausena anisata* was selected for further investigation against field populations of blowflies on two farms (one control and one test site) in Mpumalanga (South Africa). The details of the study are presented in the paper below.

6 Field evaluation of acetone extracts of Clausena anisata (Rutaceae) as a blowfly population control product for management of myiasis in livestock

[1*]Mukandiwa, L.,[1]Eloff, J.N., [2]Sibanda, D.R., [3]Naidoo, V.

[1]Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, P. Bag X04, Onderstepoort 0110, South Africa

[2]Mpumalanga Veterinary Services, P Bag X4033 Siyabuswa 0472.South Africa

[3]Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

* Corresponding author Email: lmukandiwa@yahoo.com, kobus.eloff@up.ac.za, Tel: +27 12 529 8525; Fax: +27 12 529 8525

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Abstract

Clausena anisata (Willd) Hook [Family: Rutaceae] is a medicinal plant used traditionally to treat myiasis and as an insect repellent. The effects *C. anisata* extracts on blowflies have been demonstrated in our laboratory. In this study, the impact of C. *anisata* on populations of blowflies on mixed farms in Mpumalanga, South Africa was evaluated by comparing blowfly numbers on two farms, with and without C. *anisata* treated liver, over 12 weeks when fly populations were expected to be high. Flies were exposed to liver baits that were treated with C. *anisata* extracts in a limited manner. Observations were made on the fly behaviour and development, adult sizes and numbers. The *in vitro* effects of C. *anisata* extracts on blowfly behaviour and development were confirmed, i.e., slowed development, prolonged larval period, smaller body size at a given time compared to the control and sluggish behaviour, delayed pupation and a reduced eclosion rate of pupae and adults. No significant differences were evident between the numbers and sizes of flies on the treated and on the control farm, most likely due to the limited nature of the baiting program. We believe that *C. anisata* extract could be valuable in integrated pest management.

Keywords: blowflies; *clausena anisata*; protracted larval stage; pupal malformation

6.1 Introduction

The blowfly *Lucilia cuprina* (Diptera: Calliphoridae) is the major cause of primary blowfly strikes in South Africa especially on wool farms (Howell *et al*., 1978; Scholtz *et al*., 2011). Control of flystrike has largely relied on an integrated approach incorporating the insecticides, husbandry practices (such as shearing, crutching, tail docking) and fly trapping (Tellam & Bowles 1997). However, resistance to many of these insecticides has been reported in some strains of L. *cuprina* (Hughes and McKenzie, 1987; Gleeson *et al*., 1994; Wilson and Heath, 1994) e.g. resistance to the organophosphate insecticides was first reported in the mid 1950's in South Africa (Fiedler and du Toit, 1956). Another concern with these compounds are their low safety index with high potential to cause human or animal toxicity as well as being an environmental intoxicant especially since these chemicals bind to the wool. As a result of the latter there is increasing calls for the removal of certain blow-fly control products from the international market providing the impetus for non-insecticidal control methods (Bates, 2012).

Fly population control method has been advocated as an alternate means of controlling the problem (Knipling, 1979). The advantage of this system is that the control measure decreases the number of adult flies in the environment without having to rely on the exposure of the animal and wool to high concentrations of ectoparasiticides. An example of an effective system is the Lucitrap system (Bioglobal Ltd, Australia) which has been effectively used to reduce fly densities and strike incidence in Australia (Ward, 2001; Ward and Farrell 2003) and South Africa (Scholtz *et al.* 2000, 2001). The LuciTrap system consists of a translucent bucket made from tough ultraviolet-stabilised plastic and a removable lid with a flat surface, entrance cones that allow the sheep blowfly to enter but not leave the trap and a bottle with the chemical attractant (LuciLure) (Levot, 2009). The attractant consists of chemicals designed to mimic the odours of primary food sources of the sheep blowfly - fleece rot, animal carcasses, urine and faeces. In this study we explore the possibility of suppressing populations via attraction and inhibition of life cycle through the use of traps baited with a plant insecticidal extract.

Clausena anisata (Willd) Hook is being used for the treatment of myiasis in some communities in Zimbabwe (Chavunduka, 1976) and as an insect repellent in West Africa. The leaves are crushed and packed onto the wound. This plant species is common in South Africa and throughout the southern African region. From our *in-vitro* studies on the effect of the extracts of *C. anisata* on the blowfly larvae, it was established that extracts of *C. anisata* deterred the second and third instar larvae of blowflies *L. cuprina* and *C. marginalis* from feeding with resultant lower pupae weights, smaller emerging adult flies (Mukandiwa *et al*., 2012) and a net reduction in subsequent fecundity (Mackerras, 1933; Webber, 1955; Foster, et al., 1975, Vogt et al., 1985). This article describes the evaluation of *Clausena anisata* against blowflies in field trials on mixed farms in the Mpumalanga Province, South Africa.

6.2 Materials and methods

6.2.1 Study sites

Blowfly populations were monitored at two farms in KwaMhlanga, 90 km north-east of Pretoria, SA. This area was selected due to a reported high prevalence of myiasis in the summer months, with usually 60 cases occurring per month (Area veterinarian, *Pers. Comm.*). Farm 1 served as a control while farm 2 was baited with *C. anisata* treated liver. The properties were matched with regard to grazing environment; sheep breed, stocking density and flock management and climate (being 4 km apart). On average each farm was 850 ha of predominantly natural pasture, on which 100 sheep, 300 cattle and 80 goats are grazed. Migration of flies between the farms was eliminated as a factor as adult flies will not normally travel more than three kilometres during their life span (Vogt and Woodburn 1979; Gleeson and Heath 1997).

6.2.2 Fly monitoring

Blowfly abundance was monitored between December 2011 and April 2012 using Redtop fly catchers®. Two traps were hung 100 m away from the sheep-night camps, 300 m apart, at 1.2 metres above the ground on trees as per manufacturer’s instructions. The first trapping was done at the beginning of the study to establish the blowfly numbers

on the farms and thereafter, fly trapping was done after every 5 weeks. Each trapping lasted 48 hours and made use of ox-liver as the bait as initial sitting studies showed ox-liver to be a good attractant of the blowflies. Following each trapping, all green and blue-coloured adult Diptera were counted, measured in size and identified.

6.2.3 Preparation of plant extract

Dried and powdered leaves of *Clausena anisata* (437 g) were extracted with acetone (5 L) at room temperature by continuous agitation on an orbital shaker (Labotec®, model 202, South Africa) for 6 h. The mixture was filtered and the solvent removed under reduced pressure at low temperature (40–50^{o}C) with a rotary evaporator. The extraction process was repeated twice and extracts combined to give 37.9 g of dry acetone extract. The dried extract was reconstituted in acetone to make a 150 mg/ml stock extract which was used for the assays. The process was repeated as and when more extract was needed.

6.2.4 Exposing flies to plant extract

After the initial trapping, Farm 2 was exposed for 5 weeks to ox-liver treated with a 150 mg/ml acetone extract of *Clausena anisata*, placed in the Insectivorous Bird Feeders®(Stride Distributors CC, South Africa) (Figure 1). The feeder consists of a bait bucket with a perforated bottom inside a migration bucket, also with a perforated bottom, over a tray. Under normal circumstances the feeding larvae migrate and drop into the feeding tray where they can be eaten by birds. For this study the feeders were modified so that the bottom of the migration bucket was sealed to avoid the crawling out of larvae into the feeding tray of the feeder. The larvae fed on the bait in the smaller bucket until they crawled out, on their own accord, into the migration bucket which had wood shavings to allow for growth and development into subsequent stages.

Figure 6-1: The Insectivorous Bird Feeder®

A mixture of 100 g of blended ox-liver and 5 ml of plant extract (150 mg/ml) were placed in the bait bucket of the feeder (n=10) around the sheep night camps, 1.2 m above the ground. The feeder lids were placed on top of the feeders to keep the rain out. Two holes were made on either side of the feeders to allow gravid female flies to enter and lay eggs on the liver and extract mixture so that the emerging larvae become exposed to the plant extract. The feeders were inspected every 2 days and the insecticide mixture replaced every 6 days. After the 5 weeks of baiting, a trapping was done with the red-top Fly catcher for 48 hours for fly number quantification (as above) prior to another five weeks of exposing flies to the baited ox-liver. Farm 1 which served as the control was exposed to the same system as above with the exception that pure acetone was used in the bait.

At each inspection of the feeders (every 2 days), we checked for the presence of fly eggs on the baits, and larval growth, movement and feeding activity were visually evaluated. For each group of pupae, 50 normal looking pupae were taken to the laboratory to allow for assessment of emerging flies.

6.2.5 Statistical analysis

The fly size data was evaluated by non-parametric t-test for difference to the control group in SPSS 20 (IBM) as the counts were not normally distributed.

6.2.6 Sensory evaluation of the extract

A sensory evaluation of the extract was conducted to determine the acceptability of the extract for use. The participants in this evaluation included farmers, animal health personnel (veterinarians, veterinary nurses and animal health technicians) and researchers in ethnoveterinary studies. The respondents were given a sample of the extract and were asked to comment on the smell. The respondents were asked to rate the extract on a scale of: very bad, bad, neutral, nice and very nice. A total of 50 respondents were used. The reason for undertaking this analysis was to ensure that the extract would be aesthetically acceptable.

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6.3 Results

6.3.1 Fly populations

The flies caught in the red-topped fly catchers were composed of blowflies, *Lucilia* spp (green bottle flies), *Chrysomya* spp (blue bottle flies), the fleshfly, *Sarcophaga haemorrhoidalis* (red-tailed fleshfly) and the housefly, *Musca domestica*. The changes in the populations of flies by species are presented in Table 1 while changes in fly sizes are presented in Table 2. The sizes of *S. haemorrhoidalis* on the treated farm and the control were not significantly different ($p = 0.362$)) at the beginning of the study. However, as the study progressed, the flies in the treated farm were significantly smaller ($p = 0.02$ week 4; $p < 0.00$, week 8; $p = 0.01$, week 12) than those on the control farm, at any given point in time. The other fly species did not show any particular pattern in the changes of fly sizes (Table 2).

Table 6-1: Number of flies over time on both the treated farm and the control farms

	Blowflies		*Fleshfly*		*Housefly*	
Trapping	Treated	Control	Treated	Control	Treated	Control
13/01/2012	560	498	79	51	254	331
18/02/2012	*6000	714	87	35	670	181
23/03/2012	336	402	13	18	87	103
06/04/2012	100	121	5	8	53	74

*Trapping was done 2 weeks after a cow died on the farm and was left in the open for 3 days

Table 6-2: Sizes (mean ± SD) (mm) of the different fly species captured from the treated farm over a 12 week period

		Time (Weeks)			
Fly Type	***Treatment***	***0***	***4***	***8***	***12***
Blue blowfly	Test	12.2 ± 0.5[a]	12.5 ±0.4[b]	11.7 ± 0.5[a]	10.7 ± 0.4[a]
	Control	12.2 ± 0.5[a]	12 ± 0.6[a]	11.8 ± 0.3[a]	11.8 ±0.8[b]
Green blowfly	Test	10.6 ± 0.4[b]	11 ± 0.4[b]	9 ± 0.8[a]	7.8 ± 0.7[a]
	Control	10 ± 0.5[a]	10.5 ± 0.6[a]	9 ± 0.6[a]	9 ± 0.9[b]
Housefly	Test	8 ± 0.3[a]	8.1 ± 0.3[a]	7.8 ± 0.4[a]	7.8 ± 0.5[a]
	Control	8 ± 0.8[a]	8 ± 0.0[a]	8.2 ± 0.4[b]	8 ± 0.5[a]
Flesh fly	Test	14.8 ± 0.3[a]	15 ± 0.0[b]	13 ± 0.6[a]	13.1 ±0.5[a]
	Control	14.6 ± 0.5[b]	14.8 ± 0.3[a]	15 ± 0.2[b]	14.5 ± 0.6[b]

[a,b]For each fly type, at given point in time, the test and control means with different superscripts are significantly different ($p < 0.05$).

6.3.2 Observations

Blowflies were attracted to the baits of both farms and laid eggs. The eggs hatched within the normal period of 8 – 24 hours. However, by day 4 after hatching, the larvae fed to the liver treated with *C. anisata* remained very small, both in length and thickness, compared to the larvae fed to liver treated with acetone only (Figure 2). By day 4, blowfly larvae on the control farm (Farm 1) were very active and began migrating into the bigger bucket containing wood shavings and pupation was observed on day 6. By Day 14, 90% of the pupae had hatched. However on Farm 2, the larvae were less active compared to the control group and most of them failed to crawl into the migration bucket, and pupated in the bait bucket.

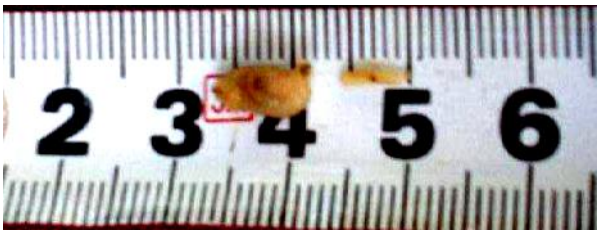

Figure 6-2: Illustration of the different size of 4-day-old larvae from the control farm and treated farm from left to right

The larval stage was also prolonged, with the total time from hatching to pupation lasting 17 to 21 days. The larval stages last 4 to 6 days in the normal life cycles of fly species encountered in this study, under the weather conditions in which this study was undertaken. Larval deaths were also observed in the groups of larvae exposed to *C. anisata* treated liver. A relatively small number of larvae died on the liver. However counts of dead larvae could not made as this could have disturbed the rest of the larvae.

The majority of the emerging pupae (90%) from the treated groups appeared normal and 75% of these pupae hatched after 2 weeks of pupation (Day 35-42). In the control group about 100% of the pupae hatched within 6 -7 days of pupation (Day 13 – 14). Abnormal pupae were observed (Figure 3) on the treated farm. Of the 50 pupae collected for laboratory assessment, 75% from the treated farm ecloded yielding only the fleshfly, *S.*

haemorrhoidalis, while the all the pupae from the control farm hatched giving a mixed population of flies; blowflies (77%), fleshflies (4%) and houseflies (19%).

To ascertain if the absence of blowflies from the treated farm was due to the extract, untreated liver was hung around the sheep camps. The eggs laid were taken to the lab to develop into subsequent stages and 93% of the emerging flies were *Lucilia* and *Chrysomya* spp. When the pupae from Farm 2 that failed to hatch were cracked open, by holding the pupae between the forefinger and thumb and gently applying pressure, half developed flies were found as they had all appendages (head, abdomen and legs) except the wings (Figure 4).

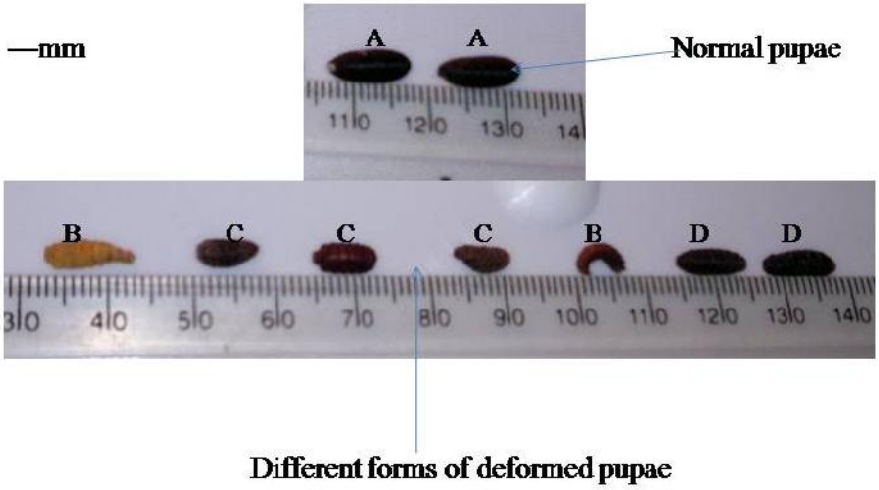

Figure 6-3: Different forms of abnormal pupae emerging from the test farm (B-D) compared to the normal pupae from the control farm (A)

A: Normal pupae

B: Larviform (an intermediate between larvae and pupae)

C: Mal-shaped pupae

D: Segmented pupae

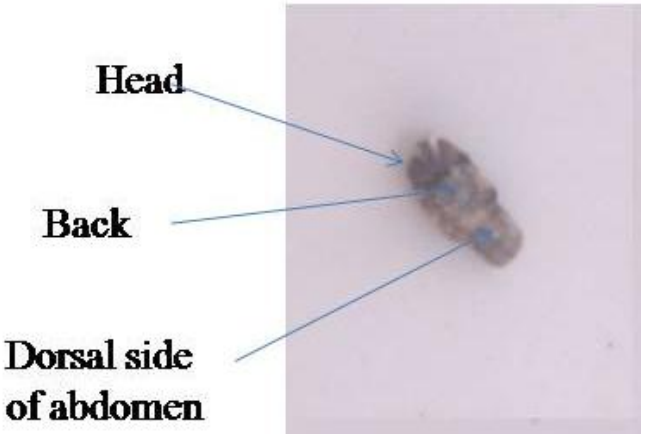

Figure 6-4: Half-developed flies from the pupae that failed to hatch naturally

6.3.3 Sensory evaluation of extract

The majority of the respondents (64%) found the smell of the extract to be neutral (neither bad nor nice) with none of the respondents rating it very bad or very nice. Figure 5 shows the responses of the different groups of respondents.

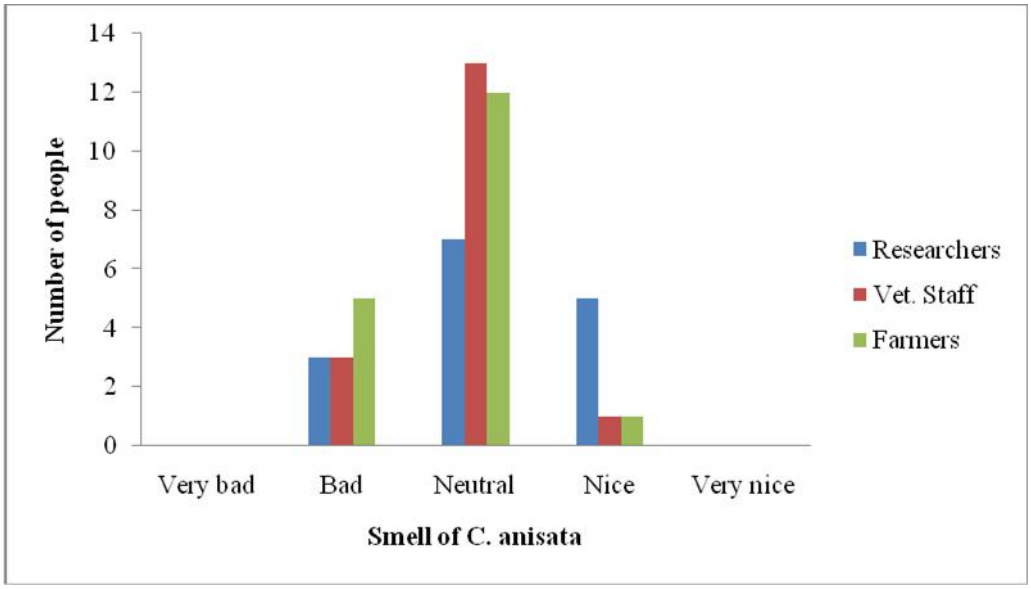

Figure 6-5: Summary of results on the sensory evaluation of the *Clausena anisata* extract

6.4 Discussion

The aim of this study was to ascertain if the effects of the acetone extract of C. *anisata* could be validated in the field to the same extent as observed under laboratory conditions (Mukandiwa *et al*., 2012). One of the major findings of this study was the prolonged time of 21 days taken for the baited larvae to pupate in comparison to the 4-6 days for the control. This is an important finding as the prolongation of the life cycle increases the chance of death in the environment and ultimately would reduce the total environmental population as the total number of life-cycles in one breeding season is reduced.

The data from the monitoring trappings indicated the presence of *Lucilia*, *Chrysomya*, *Sarcophaga* and *Musca* species. Although the fly population was mixed on the farms,

surprisingly, only *S. haemorrhoidalis* emerged from the pupae collected from the treated farm. To ascertain if the absence of blowflies was due to the extract, untreated liver was hung around the sheep camps. The emerging flies from the collected eggs were largely *Lucilia* and *Chrysomya* spp. This, therefore, suggests that either the *Calliphoridae* and *Muscidae* species were more susceptible to the extract and did not survive to the adult stage or that they avoided the baits. The latter is most plausible as the fly numbers and size of adult *Lucilia*, *Chrysomya* and *Muscidae* species did not significantly change over the progression of the study as was observed with S. *haemorrhoidalis*. This therefore suggests the possibility that this extract may have potential benefits as a repellent.

At present the mechanism of the activity of the extracts is unknown. However based on the effect seen, two explanations are plausible with the first being the presence of feeding deterrents and the second the presence of juvenile hormone mimics. Plant-derived compounds have been shown to affect the feeding and diet-selection behaviour of larvae of blowflies (Green *et al*., 2002; Mukandiwa *et al*., 2012). Some are toxic whilst others act as feeding deterrents, for example *Phormia regina* larvae avoided diets containing 100 and 10 ppm azadirachtin and10 ppm pyrethrum extract (Green *et al*., 2004). The alkaloids arecoline, caffeine, quinine, nicotine, among others, have also been shown to reduce food consumption in blowfly larvae resulting in reduced weights of the larvae (Green *et al*., 2002). *Clausena anisata* contains the alkaloids clausanitine and mupamine (Ojewole, 2002). In addition, C. *anisata* contains the coumarins imperatorin and xanthoxyletin which have an antifeedant effect on insects (Gebreyesus and Chapya 1983).

The other plausible and more likely reason for the prolonged life cycle, 21 days in comparison to the 4-6 days of the control, may be more physiological as plants are known to produce insect juvenile hormones (Bede and Tobe, 2000). The major function of juvenile hormone is the maintenance of the larval status or the so-called juvenilizing effect (Dhadialla *et al*., 2005). The plant extract had effects similar to other insect growth regulators (IGRs), which include a slowed development and a prolonged larval period, smaller body size at a given time compared to the control and sluggish behaviour,

delayed pupation and a reduced eclosion rate of pupae and adults The insect growth regulators include the juvenile hormone analogues, ecdysone agonists and inhibitors and chitin synthesis inhibitors. Ultimately IGRs control insect populations (Williams 1956; Mondal *et al*., 2000), albeit in a longer time. Previous studies showed that IGRs caused a decline in populations of the German cockroach (*Blattella germanica* L.) in three to four months after the start of bait or spray treatment, with complete eradication after 12 months (Mosson *et al*., 1995).

The other aim of this study was to ascertain the feasibility of using this plant extract in the field. The two major concerns we had were the possible unpleasant smell of the extract and chemical instability in the environment. For the first characteristic, the plant extract was scored by various persons as being acceptable. This was as an important finding as the leaves of C. *anisata*, from which the extract was derived, are known to be densely dotted with glands and have a strong scent when crushed. The scent is highly unpleasant, characteristic of horse urine as suggested by the common Afrikaans name, *Perdepis* (Horse urine) (Schmidt *et al*., 2002). However for this study the acetone leaf extract was rated by 64 % as having a neutral smell implying that the compounds that give C. *anisata* leaves their characteristic smell are not present in the acetone extract. For the concerns of stability, the extract proved to still be constantly effective despite being changed every 5 days. In the laboratory studies the larvae were exposed to the extract for 48 hours only.

The plant extract failed to decrease the total number of flies in the environment most likely as a result of the failure to remove the other food sources in the environment. This was clearly evident in this study, as the unscheduled death of a cow led to a massive increase in the number of flies trapped 2 weeks later, despite baits still having their desired effect. This therefore suggests that the use of these baits is highly dependent on an integrated control system for best effect.

6.5 Conclusion

Despite the failure of the extract to reduce the total population of flies in the environment, the effect on the larval growth and pupal shape make this extract a candidate for a large study in which the treated bait are used for a longer duration and part of an integral fly control programme.

6.6 Acknowledgements

We are grateful to the farmers who allowed us to conduct our research on their farms. The University of Pretoria and the National Research Foundation provided the financial support for this research. The South African National Biodiversity Institute, Pretoria, allowed us to collect plant material from the Pretoria National Botanical Garden. L. Mukandiwa gratefully acknowledges the financial support from German Academic Exchange Service, DAAD, during the period of this study.

6.7 References

Given in Chapter 9.

Postscript

Clearly the extracts of C. *anisata* are active against blowflies having demonstrated efficacy at both *in vitro* and on farm levels. The next would be to try to isolate and identify the compound(s) responsible for the observed activity.

CHAPTER 7

Preface

In Chapters 4 – 6 it was established that extracts of C *anisata* contain compound(s) that affect blowfly larvae in various ways which include paralysis, inhibition of food intake and growth and repellency. Details of the attempted isolation of these compounds are presented in the paper below.

7 Isolation of seselin from Clausena anisata (Rubiaceae) leaves and its effects on the feeding and development of blowfly larvae may explain its use in ethnoveterinary medicine

[1*]Mukandiwa L.,[1]Ahmed A., [2]Naidoo V. and [1]Eloff J.N.

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Abstract

The acetone extract of *C. anisata* leaf powder was separated by solvent-solvent partition into five fractions. The n-hexane fraction was the most active in the larvicidal assay and therefore subjected to open column chromatography on silica gel. The isolated compound was identified by nuclear magnetic resonance (NMR) and massspectroscopy (MS) as the pyranocoumarin, seselin, (2H,8H-Benzo[1,2-b:3,4-b']dipyran-2-one,8,8-dimethyl). It inhibited feed intake in the first and second instars of blowfly larvae at the minimum concentration tested of 1ppm resulting in significant lower mass pupae (13.5 ± 0.5 mg and 22.4 ± 0.4 mg for the first and second instar larvae respectively) compared to the solvent control group (26.19 ± 0.8 mg) ($p < 0.05$). This is the first report of the isolation of seselin from the leaves of this C. *anisata* and the first report of the compound having an effect against blow fly larvae.

7.1 Introduction

Lucilia cuprina is a member of the blowfly family *Calliphoridae* and is found throughout the world in various warm locations. The larvae of these flies are carnivorous and feed on

the body tissues and fluids of livestock (condition known as myiasis) (Zumpt, 1965) to such an extent that animal production and health is detrimentally affected (Farkas *et al*., 1997; Snoep *et al*., 2002; Sotiraki and Hall, 2012). The condition has been managed by the use of chemical pesticides either in the environment or on the animals themselves (Tellam and Bowles 1997). As a result of the prolonged use and misuse of the products, increasing resistance of blowflies is being reported (Campbell *et al* 1998; Lightner, 2008), resulting in an impetus to find new compounds that could manage these flies. For centuries, medicinal plants have been used to combat parasitism, and in many parts of the world are still used for this purpose (Athanasiadou *et al*., 2007), with specific attention being given to the treatment of myiasis (Chavunduka, 1976; Hutchings *et al*., 1996; Van Wyk *et al*., 1997; Fielding, 1998; Viegi *et al*., 2003; Luseba and Van der Merwe, 2006; McGaw and Eloff, 2008).

These plants therefore constitute an untapped source of lead structures for the control of myiasis. At present, over 119 natural plant compounds that affect insect behaviour and survival have been identified (Boulogne and Petit, 2012). More important a number of these compounds, such as pyrethrins and azadractin, have been very successfully incooperated into pest management systems as either insect antifeedants or repellents (Kostic *et al*., 2008).

We have undertaken several controlled experimental studies in an effort to verify, validate and quantify scientifically the activity of some of the plant species used for the treatment of myiasis in South Africa and Zimbabwe (Mukandiwa *et al*., 2012a, b, c). Extracts of *Aloe zebrina* Baker, *Clausena anisata* (Willd) Hook, *Erythrina lysistemon* Hutch, and *Spirostachys africana* Sond have an effect on larval motility and feeding behaviour, pupation rate, pupal weight and adult fly emergence (Mukandiwa *et al*., 2012 b, c). However, the bioactive compounds in these plant species responsible for these observations have not been identified. For this study *Clausena anisata* extracts were subjected to bio-assay guided fractionation for isolation of active compounds as this plant demonstrated promising results in the previous assays.

7.2 Materials and methods

7.2.1 Plant materials

The leaves of *Clausena anisata* (Wild) Hook. f. ex. Benth were collected in April from the Pretoria National Botanical Garden, South Africa and dried at room temperature in a well-ventilated room. Collection, drying and storage guidelines of the plant material followed were as outlined by McGaw and Eloff, 2010.

7.2.2 Isolation and identification of active compounds from *Clausena anisata*

7.2.2.1 Extraction of Clausena anisata leaves

Dried and powdered leaves of *C. anisata* (437 g) were extracted with acetone (5 L) at room temperature by continuous agitation on an orbital shaker (Labotec®, model 202, South Africa) for 6 h. The mixture was filtered and the solvent removed under reduced pressure at low temperature (40–50^{o}C) with a rotary evaporator. The extraction process was repeated twice and extracts combined to give 37.9 g of dry acetone extract.

7.2.2.2 Pyrethrins detection assay

From the apparent repellent effect from the on-farm and the *in vitro* results the plants were screened for the presence of pyrethrins which are known natural repellents present in plants (Shahba *et al*., 2011), as per the method described by Stahl (1969). Thin layer chromatography (TLC) was used to separate the chemical constituents of the plants. Ten microlitres of each plant acetone extract (100µg/ml) was loaded onto aluminium-backed TLC plates (Merck, silica gel 60 F254) and developed under saturated conditions with benzene-butanone (90:10) (80:20) and benzene/ethanol/ammonia hydroxide (90:10:1) [BEA]. Commercial pyrethrum was used as a standard. The developed chromatograms were viewed under short-wave UV light and then sprayed with *p*-anisaldehyde-sulphuric acid in methanol and subsequently heated until optimal colour development. Under these conditions, the pyrethrin compounds quench fluorescence in short-wave UV light while pyrethrins I and II yield a dark grey colour after heating (Stahl,1969). The method also identifies the cinerins and jasmolins which show up as brown bands.

7.2.2.3 Fractionation by solvent–solvent extraction

The acetone extract was fractionated using the solvent-solvent fractionation procedure. The dried acetone extract (12.7 g) was suspended in 1 L of 30% methanolic solution and extracted with n-hexane (3× 500 mL), dichloromethane (2 × 500 mL), ethyl acetate (2 × 500 mL) and n-butanol saturated with water (2 × 500 mL), respectively. Each fraction was evaporated to dryness under reduced pressure at low temperature (40–50◦C) on a rotary evaporator and remaining H_2O fraction was dried by vaporisation in an oven at 60^{o}C and weighed. This process led to five fractions separated based on solubility characteristics of the constituents.

7.2.2.4 Isolation of an active component from the n-hexane fraction

From the bioactivity assays (see below) of the different polarity fractions, the n-hexane fraction was the most active and therefore subjected to column chromatography. Open column chromatography on silica gel (Kieselgel 60, 70–230 mesh, 0.063–0.200mm, Merck), using gradient solvent of n-hexane:ethyl acetate as mobile phase, with increasing concentration of ethyl acetate was used. The eluent composition ranged from 100:0, 98:2, 95:5, 90:10, 85:15, 80:20 and 70:30(hexane: ethyl acetate). All of the subfractions were combined based on TLC analysis as follows: SFr. 1–4, SFr. 5–7, SFr. 8–11, SFr. 12–13, SFr. 14–18, SFr. 19–23, SFr. 24–29 and SFr. 30–35. Repeated column chromatography of SFr 5-7 using isocratic hexane:ethyl acetate (94:6) yielded Compound 1. Subjecting SFr 12-13 to repeated column chromatography using isocratic hexane:ethyl acetate (92:8) led to the isolation of Compound 2.

7.2.2.5 Structural analysis of isolated active compounds

Spectroscopic techniques,^{1}HNMR, ^{13}C NMR and 2DNMR(HMBC, HSQC, COSY, DEPT), were used for the elucidation of the structures of isolated active compounds using a Bruker ARX-400 nuclear magnetic resonance (NMR) spectrometer (in deuterated chloroform ($CDCl_3$)).Chemical shifts were reported with reference to the respective residual solvents or deuterated solvent peaks. Structures of isolated compounds were confirmed by comparison of their NMR data with those in literature.

7.2.3 Bioactivity of Isolated Compound

7.2.3.1 Effect on blowfly development

The larvae used in this study were collected from a farm outside Pretoria. Ox-liver in containers was placed near the sheep night-camps. Once the fly eggs were observed, the liver was taken to the laboratory. The larvicidal assay described by Khater and Khater (2009) was used to test the effect of the isolated compounds on the larvae of blowflies. The compound was tested at 1, 10, 100, 1000, 5000ppm. One (1) ml aliquots of the compound were mixed with 10g of ground liver and fed to the larvae. The compound was tested against first-instar and second-instar of larvae separately. Either 20 first-instar or early second-instar blowfly larvae (day 3 - 4) were placed on treated liver in plastic cups. Each concentration was tested in duplicate. The experiment was only undertaken once due to the limited quantity of the isolated compound available. The plastic cups with larvae were placed in larger containers containing wood shavings and covered with mesh and secured with elastic. This allowed the larvae to follow their normal behaviour, to migrate, burrow into the wood shavings and pupate. The containers were then incubated in a humidified chamber at 28 ± 2°C. Eight days after exposure of larvae to extract the number of pupae and larvae that failed to pupate was recorded per treatment. The pupae for each concentration were pooled together and the average mass determined. The pupae were placed back in the respective containers which were covered with laboratory tissue paper and were left to emerge. Seven days later we recorded the number of pupae that failed to eclode. For all assays ivermectin was used as a positive control (0.1 ml of 10 000ppm ivermectin) while acetone a solvent control. The *C. anisata* crude extract was also tested to allow for comparison with the isolated compound.

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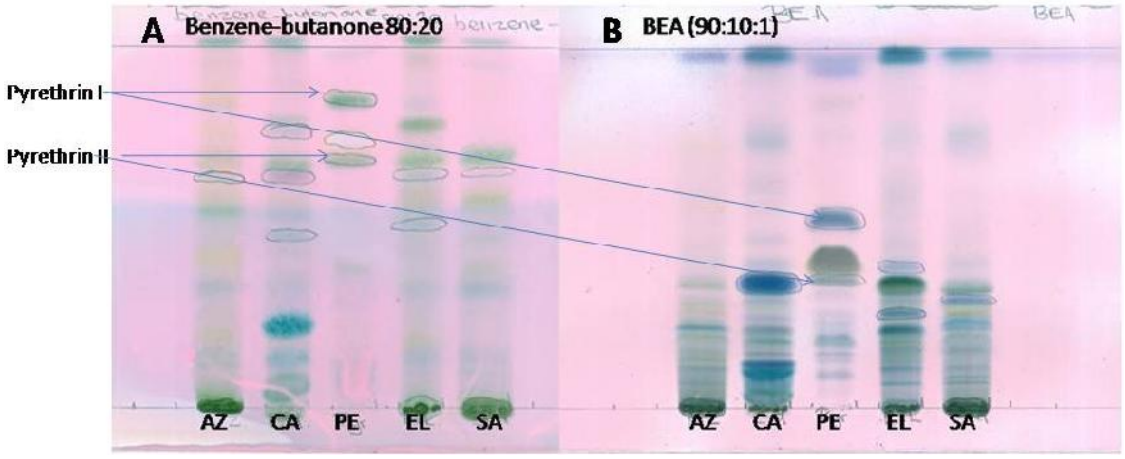
7.2.4 Statistical analysis

The data on pupal mass were analysed using the general linear model procedures (SAS, 2006) to determine if there were any significant differences due to the different concentrations of seselin, and also to determine the most effective concentrations. Mean separations was done using the PDIFF option of SAS (2006) to determine if the differences across concentrations were statistically different at P= 0.05.

7.3 Results

7.3.1 Pyrethrins detection assay

No pyrethrins could be detected in any extract of the four plant species examined. However, following development with anisaldehyde, *C. anisata* had one band with the expected grey colour ascribed to the pyrethrins. However, unlike the pyrethrin compounds the compound fluoresced under short-wave UV light instead of quenching fluorescence. In later isolation, this compound was identified as seselin (details below). *Aloe zebrina*, *E. lysistemon* and *S. africana* also had terpene or tepernoid compounds with RF values close that of the pyretrin II spot (Figure 1- Chromatogram A). It therefore does not appear that any of the species contain pyrethrins.



7-7-1: Chromatograms of acetone leaf extracts of *A. zebrina* (AZ), *C. anisata* (CA), *E. lysistemon* (EL) and *S. africanan* (SA) eluted with (A) benzene-butanone (80:20) or with (B) benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] and sprayed with anisaldehyde-sulphuric acid. A commercial pyrethrum formulation (PE) was used as a standard and the two grey bends indicate the pyrethrins I and II

7.3.2 Isolation and identification of active compounds from *Clausena anisata*

Structure elucidation of compounds

Compound 1 was not sufficiently pure to allow characterization, but from the NMR data it appeared that it could contain lupeol.

The structure of Compound **2** (Figure 2) was elucidated by NMR and MS as the pyranocoumarin, seselin, chemically called 2H,8H-Benzo[1,2-b:3,4-b']dipyran-2-one,8,8-dimethyl. It had a molecular formula of $C_{14}H_{12}O_3$ and a molecular weight of 228.24. The compound had a pale yellow colour and was oily in nature. The spectroscopic data is summarised in Table 1 and is in close agreement with the data of Patra and Mitra (1981). NMR spectra are supplied as supplementary material. Acetone extracted 8.7% (37.9g) of the starting leaf material of *C. anisata* 12.7 g of the extract yielded 83mg of seselin.

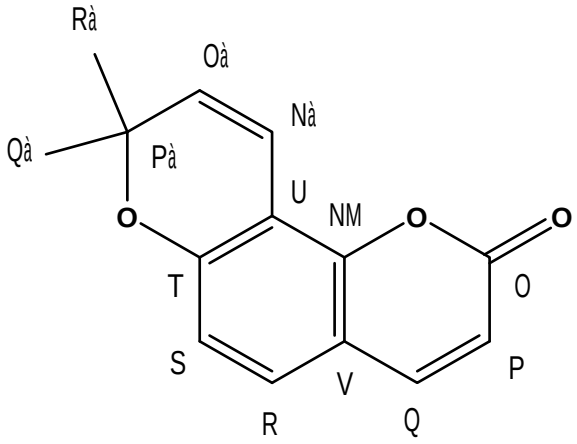


Figure 7--7-2: Chemical structure of seselin

Table 7-1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of seselin (Lillian Cpd2) in ($CDCl_3$ [δ(ppm), *J* (Hz)]

N°	*^{13}C*	*^{1}H*	**HMBC*
2	170.0		
3	112.6	6.19 (d, 9.5, 1H)	C-2, 9
4	143.9	7.56 (d, 9.5, 1H)	C-2, 5, 10
5	127.7	7.17 (d, 8.5, 1H)	C-4, 7, 10
6	113.5	6.68 (dd, 0.4, 8.5, 1H)	C-7, 8, 9
7	156.3		
8	109.3		
9	112.8		
10	150.1		
1'	114.9	6.84 (dd, 0.4, 10.1, 1H)	C-3', 6, 7
2'	130.7	5.69 (d, 10.1, 1H)	C-3', 4', 5', 9
3'	77.6		
4'	28.1	1.44 (s, 3H)	-
5'	28.1	1.44 (s, 3H)	-

*HMBC: Heteronuclear multiple-bond correlation

7.3.3 Effect of seselin on blowfly development

Following exposure, all the larvae pupated at the same rate with complete eclosion. Nonetheless, the pupae from larvae exposed to seselin were of lower mass (p <0.05) and also yielded smaller adult flies in comparison to the control group (Figure 1). While a minor dose response relationship was evident, a plateau effect was achieved as early as the third concentration evaluated. The first-instar larvae were more affected compared to the second-instar larvae, with their emerging flies being the smallest (Figure 2). The EC_{50} value against the first-instar larvae was 0.1 ppm. The EC_{50} value against the second instar larvae could not be computed. The average masses of pupae emerging from the first-instar and second-instar larvae exposed to the *C. anisata* crude extract were 13.93 ± 0.6 mg and 23.71 ± 0.5 mg, respectively while that for the control was 26.19 ± 0.8 mg. All the larvae exposed to ivermectin died.

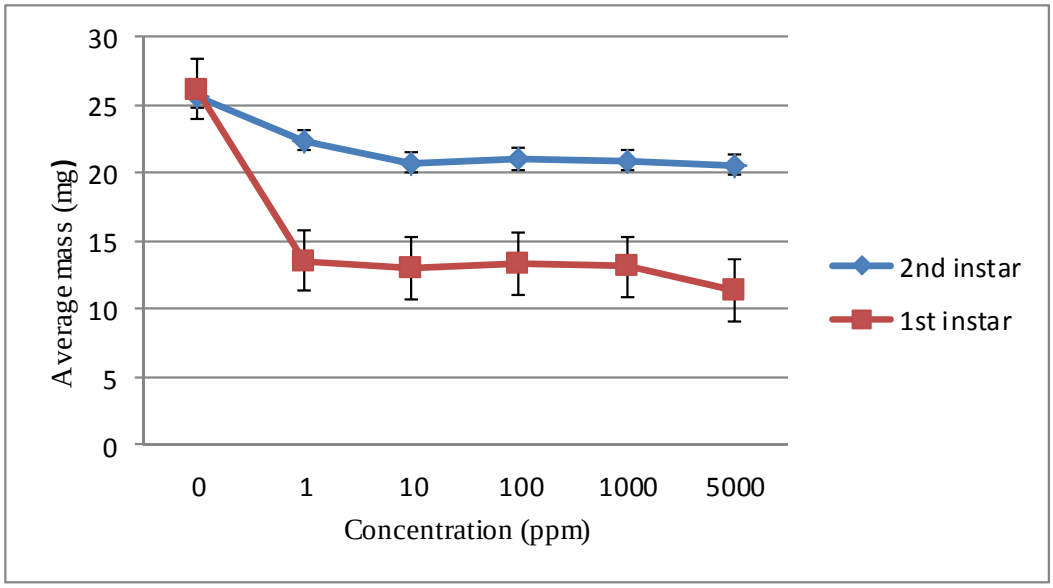


Figure 7-7-3: Mass of pupae emerging from larvae exposed to seselin at 1st and 2nd instar stages. The average weight of the control group was 26.19 mg.

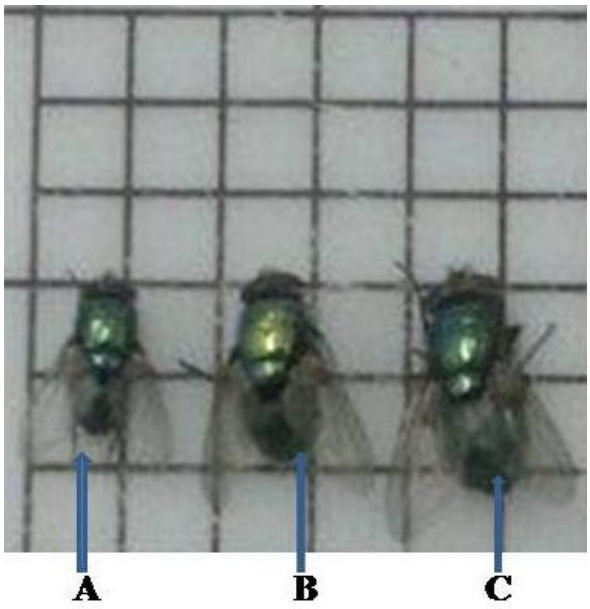


Figure 7-4: Flies emerging from the larvae exposed to seselin and acetone (one small block = 11.11mm^2)

A: Fly from larvae exposed to seselin at 1st instar stage, **B**: Fly from larvae exposed to seselin at 2nd instar stage, **C**: Fly from larvae exposed to acetone (solvent control)

7.4 Discussion

In the previous *in vitro* and field studies, *C. anisata* had a repellent effect and also interfered with the growth and development of the larvae (Mukandiwa *et al*., 2012 b, c). As a result it was suspected that the plant extract could have contained a pyrethrin, which is a known herbal fly repellant, that also acts as insect growth regulator. In the first step in isolating the active agent, compounds present in extracts of *C. anisata* and other plant species were separated by TLC to investigate the presence of pyrethrins. Although the extracts separated did not contain pyrethrins they all contained compounds with the same colour reaction as terpenoids with Rf values close to the pyrethrin II compounds. These compounds may be responsible for the insecticidal activity of the study plants but require further study.The most potent known active plant-based insecticidal and antifeedants/feeding deterrents belong to the various types of terpenes and their derivatives, with examples being the pyrethrins and azadirachtin A (Koul, 2008).

The compound isolated and characterised, seselin, is a pyranocoumarin. Previous research has shown that seselin has good antifungal activity (Bandara *et al*., 1991; Cardenas-Ortega *et al*., 2007). Seselin also has ovicidal activity against *Tetranynchus urticae* (red spider mite) (Tanaka *et al*., 1985) and weak to moderate cytotoxicity (Gunatilaka *et al*, 1994). It also has peripheral anti-inflammatory and antinociceptive properties (Lima *et al*., 2006) with other researchers suggesting that it is more potent than aspirin (Guo *et al*. 2008). Seselin also inhibits phytohemagglutinin-stimulated cell proliferation in human blood mononuclear cells (Tsai *et al*., 2008). In plants, seselin has inhibitory activity in both indole acetic acid oxidase and peroxidase enzyme systems, and inhibits radical growth in seedlings of cucumber, lettuce, radish and wheat (Goren and Tomer, 1971). While *C. anisata* has been widely reported to contain a significant number of coumarins, (especially scopoletin, chalepin, helietin, osthole, coumarrayin, xanthoxyletin, heliettin, imperatorin, furanocoumarin derivatives) (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; Ojewole, 2002), this is the first reported occurrence of seselin in the plant. *Clausena anisata* belongs to the citrus family and seselin has been isolated from the roots of other citrus trees (Shamouti orange, sour

orange, Palestine sweet lime, and Marsh seedless grapefruit) (Tomer *et al.*, 1969). Seselin in the roots of citrus trees has been implicated in autotoxicity, whereby if released into the environment it inhibits germination and growth of same plant species (Singh *et al*, 1999).

This study is also the first to describe the effect of seselin on fly larvae, further validating the successful use of *C. anisata* for treating maggot-infested wounds (Chavhunduka, 1976). Natural coumarins appear to play important roles as natural protective agents in plants against generalist herbivores acting as feeding deterrents to certain insects (Gray and Waterman, 1978). Previous research reveals that natural coumarins have a wide range of toxic effects and suggest that the ingestion of, or even prolonged external contact with plants containing significant quantities of coumarins would have a detrimental effect on an organism not adapted to their detoxification (Gray and Waterman, 1978). Two coumarins contained in *C. anisata* extracts, imperatorin and xanthoxyletin have antifeedant activity against the larvae of African armyworm (*Spodoptera exempta*) (Gebreyesus and Chapya, 1983). This effect was confirmed in this study as the larvae were of lower weights which we ascribe to a lower food intake.

The first-instar larvae were more sensitive to seselin than the second instar. This suggests the compound may be better used as a preventative agent similar to diflubenzuron, an insect growth regulator (Holdsworth, 2005), as it targets the insect life cycle early on before it has a chance to induce severe pathology Unlike in previous studies where the larvae seemed to avoid the meat treated with the crude extract of *C.anisata*, in this study the larvae did not avoid the meat. This suggests seselin is a feeding deterrent (anorexogenic agent) which requires initial consumption for a defined physiological effect i.e., inhibition by gustatory responses (Koul, 2008), as opposed to the repellent type of effects seen with the pyrthroids.

Based on the isolation of 83 mg of seselin from 12,7g of the acetone extract, the isolation efficiency was 0.67%. This percentage yield is comparable to that of pyrethrins which are commercially isolated to produce insecticides. Pyrethrin contents of 0.9 to 1.3% by

weight of dried flowers have been reported (Kolak *et al*., 1999; Casida and Quistad, 1995). Our calculations show that to obtain 1 mg of seselin 153 mg of the acetone extract was needed. Thus from this ratio, the crude extract which was tested at 150 mg/ml would translate to 980 ppm in terms of seselin content. Interestingly, the larvae exposed to seselin concentrations lower than 980 ppm (1, 10 and 100 ppm) had lower mass than the larvae exposed to the crude extract. The poor dose related effect, which in itself is difficult to explain, makes comparisons difficult, but it does appear that seselin is indeed the compound responsible for the antifeedant activity against larvae. The good activity observed at low concentrations (EC_{50} = 0.1 ppm) is comparable to that of azadirachtin which has both primary and secondary antifeedant activity against a large number of insect species including blowflies (Schmutterer, 1990). The desert locust *Schistocerca gregaria* and many species of Lepidoptera, are among the most sensitive to azadirachtin, being deterred by as little as 0.007 ppm in diets (EC_{50} =0.05 ppm) whereas the Hemiptera and Coleoptera are much less sensitive with EC_{50} values of around 100 ppm or more (Isman, 1994, Mordue (Luntz) and Blackwell, 1993).

Seselin was only effective in interfering with pupal mass and adult fly sizes. This was in contrast to the effect of the crude extract, where other pathological effects such as prolonged larval stage and deformed pupae were noted in the developing fly in addition to the repellent activity (Mukandiwa *et al*., 2012b, c). In addition, unlike in our previous studies (Mukandiwa *et al*., 2012 c) where the crude extract of *C. anisata* repelled the larvae as most of them were found circling the periphery of the testing cups, the larvae did not seem to be repelled by seselin. This therefore suggests that other active compounds with repellent and different physiological effects may be present within this plant, and further elucidation may yet reveal other active compound(s). Based on these observations we support the use of the crude extract as it seems to contain various compounds that act at various levels to treat and control myiasis.

7.5 Conclusion

Clausena anisata extracts have potential use in the management and treatment of myiasis in livestock. This may in part be because of the phenolic compounds such as seselin that

have feeding deterrence properties. Further work should be directed towards isolation of the other compounds in *C.anisata,* and these can be combined with seselin and evaluated against larvae as combinations.

7.6 Acknowledgements

The University of Pretoria and the National Research Foundation provided the financial support for this research. The South African National Biodiversity Institute, Pretoria, allowed us to collect plant material in the Pretoria National Botanical Garden. The University of Pretoria Chemistry department ran the NMR spectra. A special thank you goes to Dr Maurice D. Awouafack for his assistance in interpreting the NMR and MS, data. L. Mukandiwa gratefully acknowledges the financial support from German Academic Exchange Service, DAAD, during the period of this study.

7.7 References

References listed in Chapter 9

Postscript

The presence of compounds such as seselin in *C. anisata* which have feeding deterrence activity against insects validates the reported successful use of *C anisata* in the treatment of wound myiasis. However this compound did not show the repellency exhibited by the crude extract suggesting that the *C. anisata* extract contains more than one compound that acts against the blowflies.

CHAPTER 8

8 General Discussion Conclusions and Future Research

8.1 General Discussion

Different medicinal plants, from different families, have been used world-wide to treat maggot-infested wounds (wound myiasis) for decades (Chavunduka, 1976; Hutchings *et al*., 1996; Van Wyk*et al*., 1997; Fielding, 1998; Viegi*et al*., 2003; Luseba and Van der Merwe, 2006; McGaw and Eloff, 2008). The successful manifestation of myiasis in livestock depends on the presence of fly larvae (maggots) on a predisposed site such as a wound, the presence of bacteria on the site, and the conversion of the larval ammonium bicarbonate to toxic ammonia gas. The plants used traditionally to treat myiasis could potentially work at different levels to bring about their therapeutic effect. They could be fly repellents, larvicides, antibacterials and/or antioxidants which are known to promote wound healing by preventing larval contamination of open wounds. The following study was designed to investigate if selected ethnoveterinary medicines could reduce larval wound contamination by either acting as antibacterials, fly repellents, or larvicidals. In addition to validating the traditional use of the selected medicines, the secondary aim of the study was to isolate potentially active component(s) within the plant. Finding active extracts is important as a new organic myiasis control extracts could help reduce the over-reliance on synthetic chemicals which have had severe environmental destructive effects. Isolation of a bioactive compound could lead to a new class of compounds to control flies and can also be used in quality control of extracts.

The bacteria used in this study are common wound contaminants. The presence of bacteria is imperative in the manifestation of myiasis, due to their production of the chemical odour that attracts the myiasis-causing flies (Eismann and Rice 1987; Chaudhury *et al*., 2010). The antibacterial activity of the plants was investigated using serial microdilution (Eloff, 1998) and bioautography methods (Chapter 3). All the evaluated plants had inhibitory activity against the test bacterial strains as previously reported in literature (Gundidza *et al*., 1994; Pillay *et al*., 2001; Mathabe *et al*., 2006;

Naidoo *et al*., 2006; *Adepapo et al., 2008*). The additional findings from these studies was that based on the density of the plant extracts, it is possible for MIC levels to be ascertained at the wound.

Four of the plant species, *Aloe zebrina, Clausena anisata, Erythrina lysistemon* and *Spirostachys africana* had significant detrimental effects on the behaviour and development of blowflies at concentrations between 50 and 150 mg/ml. The higher concentrations of C. *anisata* and *S. africana* extracts appeared to have a repellent effect as the larvae constantly migrated to the periphery of the testing chamber. The latter could possibly be beneficial in repelling larvae from infested wounds and validate the traditional use which reports these plants as agents effective in expelling maggots from wounds. *Clausena anisata* had the highest inhibitory effect on blowfly larvae development *in vitro hence* it was further evaluated for its effect against larvae under field conditions around sheep night-camps. The C. *anisata* extract had a species specific effect. It led to sluggish larval behaviour, retarded larval development of *S. haemorrhoidalis* as well as delayed pupation, reduced eclosion rate of pupae and smaller body sized adults. The extract had a direct repellent effect on blowfly species as none of these flies laid eggs on the baited food, despite the non-baited foods still attracting them. As a result it was concluded that C. *anisata* had a mixed effect of interfering with insect growth regulating activity in addition to being a repellent as seen in the *in vitro* assays.

However while the extract could be effective in the treatment of topical infections, the potential toxicity of the extract to the host animals needed further clarification. To gain a better understanding of the potential for toxicity following cutaneous use, to either the local tissue or for systemic toxicity involving the liver and kidney, the *in vitro* toxicity of extracts of *A. zebrina*, *C. anisata*, *E. lysistemon* and *S. africana* was determined using fibroblast, hepatocytes and renal cell cultures. Based on the criterion that an IC_{50} less than 20µg/ml would induce toxicity only, the *C. anisata* extract had potential toxicity. The ability of the extract to achieve an antibacterial and larvicidal effects in the absence of toxicity validates the ethnoveterinary use of the extracts as a means of reducing the potential to larval wound contamination.

In an attempt to identify the active ingredient, the extracts of C *anisata* were evaluated for the presence of pyrethrin. Based on R_f values on TLC chromatograms using different solvent systems, pyrethrins I and II were not present therein. However, based on the colour reaction with the spray reagent the plant extract may have contained terpenes. Some of the terpenoid compounds inhibit feed intake, reduce pupation and reduce adult emergence rates (Bowers *et al.*, 1965; Aniszewski, 2007; Kostic *et al*., 2008). After bioassay guided fractionation using open column chromatography, seselin a pyranocoumarin, was isolated and identified from *C. anisata* extracts. Seselin had a feeding deterrence effect on second and third instars of blowfly larvae in the absence of a repellent effect and is probably responsible for some of the activities of the plant extract.

8.2 General Conclusion

The aim of this study was to develop a product based on traditionally used plants that could be used in the treatment of myiasis in animals. Based on the *in vitro* and *in vivo* results, this study validates the use of selected medicinal plants in the control of myiasis causing larvae. It appears that some of these plant species have the potential to deliver a product that can be used to control myiasis. The development of a commercially useful product was beyond the scope of this study.

8.3 Future research priorities

- The activity of *Clausena anisata* against other flies and mosquitoes could also be investigated.
- A product could be formulated from the crude extracts and the activity on animals suffering from myiasis could be determined under well controlled experimental conditions.
- Production of a low cost plant extract with good activity may be useful to rural pastoralists in treating myiasis in rural communities.

- In depth investigation of the most promising plant species listed here may lead to the identification of new useful compounds.

CHAPTER 9

9 References

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