Isolation of seselin from *Clausena anisata* (Rutaceae) leaves and its effects on the feeding and development of *Lucilia cuprina* larvae may explain its use in ethnoveterinary medicine

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Abstract

Ethnopharmacological relevance: The leaves of *Clausena anisata* are used traditionally to expel maggots from wounds of animals in Zimbabwe. We have previously proved in the laboratory that the plant certainly affects the behaviour and growth of blowfly larvae. The objective of this study was to isolate and identify the active compounds responsible for this activity.

Materials and Methods: 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.

Results: The isolated compound was identified by nuclear magnetic resonance (NMR) and mass spectroscopy (MS) as the pyranocoumarin, seselin, chemically known as 2′,2′-dimethylpyranocoumarin . 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) ($p < 0.05$).

Conclusions: This is the first report of the isolation of seselin from the leaves of *C. anisata* and the first report of the compound having an effect against blow fly larvae.

Keywords: seselin; blowfly development; *Clausena anisata*;

Chemical compounds studied in this article: Seselin (PubChem CID: 68229)

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* (Rutaceae) extracts were subjected to bio-assay guided fractionation for isolation of active compounds as this plant demonstrated promising results in the previous assays.

2 Materials and methods

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. The plant species was identified by tree name tags and were authenticated by the Guide at the National Botanical Garden. The voucher specimen of the plant species, numbered PMDN317, is kept at the Medicinal Plant Collection Herbarium of the Department

of Paraclinical Sciences, University of Pretoria, South Africa. Collection, drying and storage guidelines of the plant material followed were as outlined by McGaw and Eloff, 2010.

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

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.

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.

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.

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.

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. ESI-MS were obtained on Waters Synapt HDMS spectrometer.

Compound 2: 2′,2′-dimethylpyranocoumarin also known as seselin: Pale yellow oil; NMR data (Table 1); ESI-MS m/z 228 $[M+H]^+$;

2.3 Bioactivity of Isolated Compound

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.

2.3.2 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.

3 Results

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.

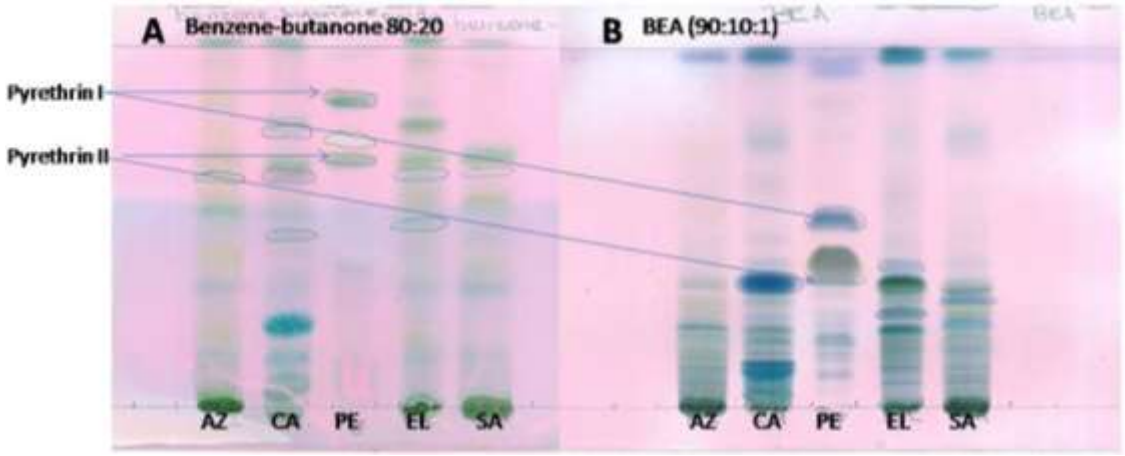

Figure 1: Chromatograms of acetone leaf extracts of *A. zebrina* (AZ), *C. anisata* (CA), *E. lysistemon* (EL) and *S. africanan* (SA) eluted with (A) benzene-butanone (80:20) or with (B) benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] and sprayed with anisaldehyde-sulphuric acid. A commercial pyrethrum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

3.2 Isolation and identification of active compounds from Clausena anisata

3.2.1 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 2′, 2′-dimethylpyranocoumarin. It had a molecular formula of $C_{14}H_{12}O_3$ and a molecular weight of 228 obtained from its ESI-MS spectrum. 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.

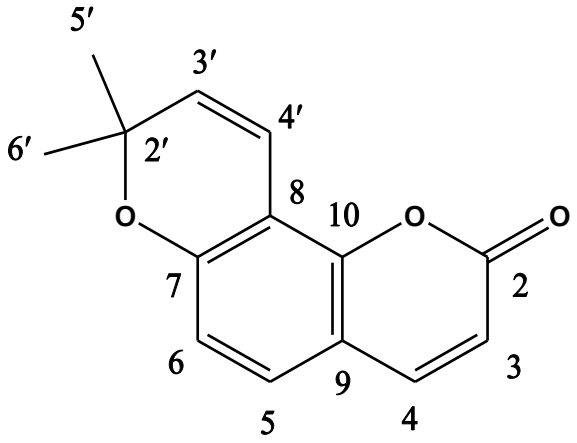

Figure 2: Chemical structure of seselin

Table 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		
2'	77.6		
3'	130.7	5.69 (d, 10.1, 1H)	C-2', 5', 6'
4'	114.9	6.84 (dd, 0.4, 10.1, 1H)	C-2', 7, 8
5'	28.1	1.44 (s, 3H)	-
6'	28.1	1.44 (s, 3H)	-

*HMBC: Heteronuclear multiple-bond correlation

3.2.2 Effect of seselin on blowfly development

Following exposure, all the larvae pupated at the same rate and there was 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 3 & 4). 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 4). 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.

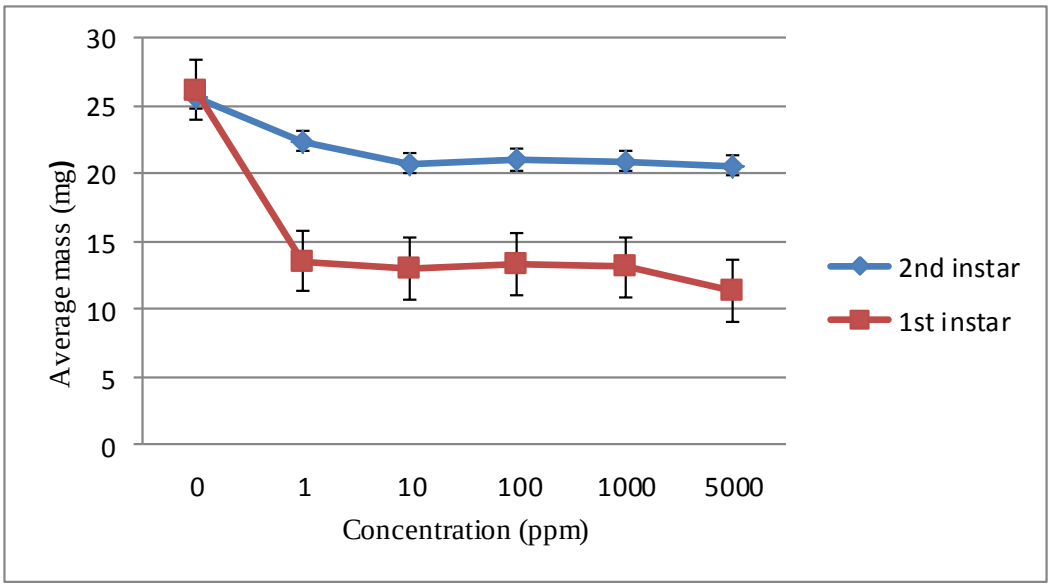

Figure 3: Mass of pupae emerging from larvae exposed to seselin at 1st and 2nd instar stages. The average weight of the control group was 26.19 mg.

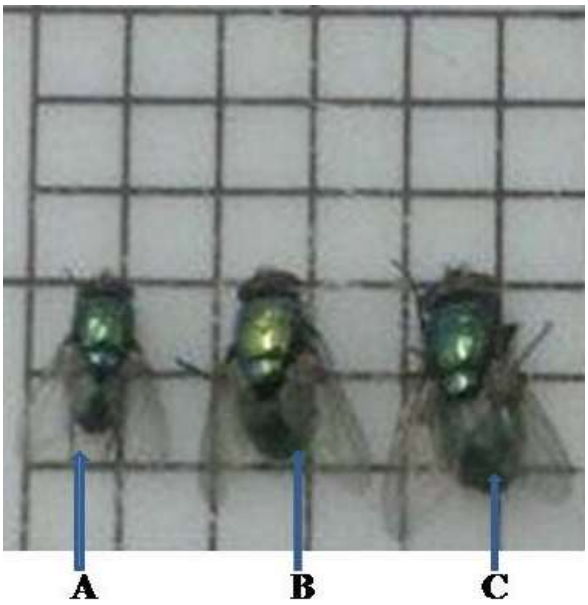

Figure 4: Flies emerging from the larvae exposed to seselin and acetone (one small block = 11.11mm^2)

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)

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, and potentially also an 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 we isolated and characterised, seselin, is a pyranocoumarin (2′,2′-dimethylpyranocoumarin.". 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 was 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 1mg of seselin 153 mg of the acetone extract was needed.

Thus from this ratio, the crude extract which was tested at 150mg/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 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} =0.05ppm) 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.

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.

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