Insect Science (2009) 16, 247–253, DOI 10.1111/j.1744-7917.2009.01255.x

Identification, isolation and characterization of the antifeedant constituent of *Clausena anisata* against *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract Hexane, petroleum ether, chloroform, ethyl acetate, methanol and water extracts of *Clausena anisata* [(Willd.) Hook F. Ex Benth] leaves and roots were evaluated against *Helicoverpa armigera* (Hübner) for antifeedant activities. Antifeedant activity was confirmed, and was found to be higher in root extracts than those of the leaf. Chloroform and petroleum ether extracts of the root showed strongest antifeedant activities (DC_{50}s [concentration (C) causing 50% deterrence compared with the control] 0.014% and 0.016% respectively), and root extracts were fractionated using silica gel column chromatography. One fraction of the chloroform and one of the petroleum ether root extracts was active; and on the basis of mass spectroscopy and ^{1}H and ^{13}C nuclear magnetic resonance spectral data, the active compounds in the two fractions were confirmed to be identical, and identified as osthol [2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)]. The highest concentration of osthol was found in the chloroform root extract. Antifeedant activities of the root extracts, as measured by DC_{50} values, were highly correlated with their osthol contents. Approximately 99% of the variation in bioactivity of the root extracts could be accounted for by variation in osthol content; osthol therefore, appeared to be an antifeedant component of *C. anisata* to *H. armigera*. This may provide a new approach to managing this pest.

Key words active ingredient, antifeedant, *Clausena anisata*, *Helicoverpa armigera*, osthol, root

Introduction

There has been a concerted global effort to develop alternative crop protectants due to the increasing problems associated with the use of toxic synthetic insecticides (Akhtar & Isman, 2004). Although synthetic toxicants have made great contributions to plant protection, they have also had unintended ecological and health effects. Because of these inadequacies and the increasing demand for pesticide-free produce, there is a pressing need to replace synthetic insecticides with cheaper, safer, environmentally friendlier and ecologically more compatible alternatives, including botanical insecticides (Leatemia & Isman, 2004). Plants are rich sources of natural substances that can be utilized in the development of alternative methods of insect control (Sadek, 2003) and over 2 000 plant species belonging to different families and genera have been extensively investigated to discover new sources of botanical insecticides and antifeedants. Among the plant families studied, the Meliaceae, Rutaceae, Asteraceae, Labiaceae, Piperaceae and Annonaceae are perhaps the

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most promising (Akhtar & Isman, 2004). The Rutaceae have received much attention at least partly owing to the presence of triterpenoids (= limonoids) (Connolly, 1983; Akhtar & Isman, 2004). Many of such compounds have been shown to affect insect growth and behavior, acting as antifeedants, toxins and insect growth regulators (Wheeler & Isman, 2001).

Clausena anisata belongs to the family Rutaceae; it is a small tree that grows in the savannah regions of West Africa (Ayensu, 1978). The tree is used in African traditional medicine in the treatment of epilepsy, convulsions, arthritis, rheumatism, hypertension, heart failure, taeniasis, impotence and sterility (Ojewole, 2002), and as an insect repellent (Ayensu, 1978; Ngadjui *et al*., 1989a). Leaves are usually placed on wounds to repel maggots, and the smoke from burning dried plants is used to repel mosquitoes (Okunade & Olaifa, 1987).

Experimental evidence indicated that the oil from *C. anisata* was toxic to the third nymphal instar of grasshoppers (Okunade & Olaifa, 1987), and repellent against a tick, *Ixodes ricimus* (Novak, 1971 cited by Okunade & Olaifa, 1987), and *Callosobruchus maculatus* (Boeke *et al*., 2004). While estragole was identified as the toxic component of the oil to grasshoppers (Okunade & Olaifa, 1987), the coumarins, imperatorin and xanthoxyletin were the antifeedants identified from the bark of the plant against the African armyworm, *Spodoptera exempta* (Gebreyesus & Chapya, 1983). *C. anisata* is a rich source of coumarins, and many investigators have isolated different types of these compounds from the leaves, stem bark and roots of *C. anisata* (Abe & Taylor, 1971; Ngadjui *et al*., 1989a,b, 1991; Okorie, 1975; Mester *et al*., 1977; Lakshmi *et al*., 1984; Chakraborty *et al*., 1995). Despite large amounts of coumarins being isolated from this plant, there are very few reports on biological activities of these plant extracts to insects. Specifically, there is no information regarding biological efficacy of *C. anisata* against the cotton bollworm, *Helicoverpa armigera* Hubner (Lepidoptera: Nuctuidae), which is a major worldwide pest of many crops (Jallow *et al*., 2004; Fakrudin *et al*., 2004b). Here, we investigate bioefficacy of leaf and root extracts of *C. anisata* against *H. armigera*, and then isolated and characterized the active constituents.

Materials and methods

Source and processing of plant material

Fresh mature leaves and roots of *C. anisata* were collected from the Forestry Research Institute (FRIN) Forest Reserve, Olokemeji, via Ibadan, Nigeria (7°23′N, 3°30′E). These specimens were identified by FRIN Herbarium and by the Department of Forestry, University of Ibadan, Ibadan, Nigeria where voucher specimens have been lodged. The samples were shade-dried; the leaves were powdered using an electric blender, while the roots were chopped into small pieces, pounded and powdered. The powdered materials were sealed in a plastic bag and stored at 4°C until needed.

Insects

Larvae of *H. armigera*, were obtained from a laboratory colony reared on artificial diet at 26 ± 1°C, 75% RH and a 16 : 8 h L : D regimen as described by Wang and Dong (2001). The colony was started from insects collected in a cotton field in Zhengzhou, Henan Province, China. Adults were supplied with a 10% solution of honey water. Larvae were reared on artificial diet prepared from the following ingredients: wheat bran (150 g), yeast powder (30 mg), methyl-p-hydrobenzoate (2 g), sibic acid (1 g), L-ascorbic acid (3 g), linoleic acid (1 mL), agar (14 g), and distilled water (744 mL) (Wu & Gong, 1997).

Extraction

Two hundred grams of the leaf or root powder was soaked in petroleum ether, hexane, ethyl acetate, chloroform, methanol and water (500 mL each) for 18 h at room temperature (28 ± 4°C). The extracts obtained were filtered, and the solvents were removed under vacuum (water extracts were freeze-dried). The residues were weighed and stored at 4°C until needed for the experiments.

Test for antifeedant activity

Antifeedant activity of test extracts was assayed by using a leaf disc choice test. Insects to be tested were removed from the culture at the end of the fourth instar in the pre-molt stage. They were placed in isolation without food and starved for 6–8 h after molting into the next instar and then tested. Fresh leaf discs (10 mm diameter) were cut from greenhouse-grown cotton, *Gossypium arboreum* L. cv 'Zhong-12', using a cork borer. Leaf discs were dipped in a 2% concentration of the test substances for 5 sec, while control discs were dipped in the carrier solvent (methanol:dichloromethane at 5:1) and allowed to dry. Four treated and four control discs were arranged in an alternating design in a Petri dish (1.5 cm × 12 cm); a moist sheet of filter paper to prevent desiccation was also provided. A single larva was placed in the center of the

Petri dish and leaf consumption was observed at hourly intervals thereafter. There were 20 replicates for each test extract; Petri dishes were held under evenly distributed fluorescent lights at 27 ± 1°C. When approximately 50% of either substrate in the choice trial had been consumed, larvae were removed from the discs and the disc area consumed was measured using transparency film with a 1-mm^2 grid. A deterrent index (DI) was calculated as (C − T)/(C + T) × 100, where, C and T are the control and treated leaf areas consumed by the insect (Akhtar & Isman, 2004). Bioactive extracts were further tested at 0.2%, 0.1%, 0.05%, 0.02%, 0.01% and 0.005% concentrations, and DI was calculated at each concentration to determine DC_{50}s (concentration (C) causing 50% deterrence compared with the control) for each of the extracts.

Fractionation and isolation procedures

The chloroform or petroleum ether extract of the root (3 g) was dissolved in dichloromethane, column-chromatographed (2 cm × 60 cm) using silica gel 60, 80–200 mesh (Shanghai Chemical Agent Co., Shanghai, China), and eluted with a stepwise gradient of diethyl ether and hexane (0: 100; 10: 90; 20: 80; 40: 60; 60: 40; 100: 0, v/v). A total of 12 fractions were obtained, which were confirmed by thin layer chromatography on 20 × 20-cm plates coated with silica gel containing fluorescent indicator UV_{254}. Each fraction (250 mL) was concentrated using a rotary evaporator and tested for antifeedant activity against *H. armigera* at 2% concentration. Bioactive fractions were further tested at 0.2%, 0.1%, 0.05%, 0.02%, 0.01% and 0.005%.

Analytical procedures

The chemical identity and quantity of the target compound in the active fraction was determined by spectral analysis (mass spectrography [MS], ^{1}H and ^{13}C nuclear magnetic resonance [NMR]). The mass spectrum was obtained using a coupled gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890N-5973N gas chromatography/mass selective detector (GC-MSD). The GC was equipped with a DB-5ms column (60 m × 0.25 mm × 0.15 μm). Helium was used as the carrier gas with a constant flow of 1 mL/min. Two microns of bioactive fraction (VI) was injected and then split with a purge flow of 30 mL/min. The injector temperature was 250°C and the GC-MS transfer line temperature was 280°C, source 230°C, quadrupole 150°C, ionization potential 70 eV (electron-Volt units), and scan range 30–300 *m/z* (mass-to-charge ratio). Following injection, the column temperature was increased from 80 to 220°C at 10°C/min, and held at 220°C for 40 min. Compounds were identified by comparing mass spectra with those of authentic reference compounds and NIST library spectra (Agilent Technologies, Santa Clara, USA). Compounds were quantified by integrating areas of the individual peaks of the chromatogram expressed relative to that of the total eluate.

^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR were recorded on a Bruker DMX-400 MHz NMR spectrophotometer for ^{1}H and ^{13}C (shifts relative to deuterated chloroform used as solvent with tetramethylsilane as an internal standard). Compounds were identified by comparing NMR spectra obtained with those in the literature.

Quantification of osthol in the extracts of C. anisata

The test substances (hexane, petroleum ether, ethyl acetate, chloroform, methanol and water extracts, 200 ng each) were gas-chromatographed as described earlier in order to determine osthol quantities within them. The quantity of osthol in each extract was quantified by integrating areas of the individual peaks of the chromatogram corresponding to osthol expressed relative to that of the total eluate.

Statistics

Percent feeding deterrence values were subjected to probit analysis (Finney, 1971) and DC_{50} values were calculated for each extract. DC_{50} values, determined by probit analysis, were correlated to osthol concentrations of the extracts as determined quantitatively by GC-MS.

Results

Data obtained indicated antifeedant activity in the root extracts of *C. anisata* against the cotton bollworm, *H. armigera*. Compared to leaf extracts (Table 1), antifeedant activity was stronger and more consistent in the chloroform (RC), petroleum ether (RP), methanol (RM) and ethyl acetate (RE) root extracts (Table 1). As extract concentration decreased, deterrent indices also decreased in a dose-dependent manner. While activity was recorded for RC and RP at 0.005% and RE at 0.002%, bioactivity was nil when methanol leaf extract (LM) and RM concentrations were lower than 0.1% (Table 1). Comparing deterrent indices of 50% (DC_{50}) determined from lines of best fit for each root extract (Table 1), RC and RP scored most effectively as antifeedants to *H. armiger*a larvae feeding on

Table 1 Antifeedant effects of crude extracts of *Clausena anisata* leaves and roots on fifth instar *Helicoverpa armigera*.

Extract	df	Leaves			Roots		
		$DC_{50} \pm 95\%$ CI	Slope ± SE	r^2	$DC_{50} \pm 95\%$ CI	Slope ± S.E	r^2
Water	2	–	–	–	–	–	–
Methanol	3	0.452 ± 0.170	0.91 ± 0.547	0.56	0.118 ± 0.148	0.77 ± 0.594	0.63
Ethyl acetate	3	19.154 ± 0.584	0.78 ± 0.429	0.62	0.152 ± 0.012	1.49 ± 0.390	0.83
Chloroform	5	1.334 ± 0.168	0.89 ± 0.335	0.64	0.014 ± 0.007	1.11 ± 0.110	0.95
Hexane	3	3.771 ± 0.574	0.66 ± 0.413	0.46	–	–	–
Petroleum ether	4	1.072 ± 0.378	1.06 ± 0.597	0.51	0.016 ± 0.006	1.82 ± 0.017	0.92

Regression lines were calculated from 4–6 points, $n = 20$ for each point, Treatment df = 6, DC_{50}, effective concentration to reduce feeding by 50% relative to the control in the disc choice bioassay; r^2, coefficient of determination, –, not determined.
χ^2, not significant.
CI, Confidence interval.

leaf discs (DC_{50}s, 0.014% and 0.016% respectively), RE (DC_{50}, 0.25%), RM (DC_{50}, 0.3% and LM (DC_{50}, 0.45%). RC and RP were therefore selected for chromatographic fractionation and isolation of bioactive components.

Open column chromatography and preparative thin layer chromatography yielded six RC and RP fractions, respectively. RCF5 and RPF5 were the only active fractions, and showed feeding deterrence at ≥ 200 ppm; fractions RCF5 and RPF5 were essentially one main peak, at approximately 29 min (retention time), which formed 100.0% and 98.6% of all integrated peaks respectively, from the total eluates.

The spectral data obtained for components RCF5 and RPF5 were the same and are as follows: mass spectra: the electron impact (EI) MS: *m/z* 244, 229, 221, 213, 201, 189, 182, 175, 167, 159, 148, 148, 141, 131, 122, 115, 103, 89, 77, 63, 51, 39, 31. It showed the molecular ion at *m/z* 244. These data are consistent with those reported for osthol [2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)], and with the molecular formula $C_{15}H_{16}O_3$ (Zhou *et al*., 2000; Wei *et al*., 2004; Riviere *et al*., 2006).

Results showed that there also was a significant correlation ($P < 0.05$) between osthol contents of the root extracts and DC_{50}s ($r = -0.99$); bioactivities of root extracts also correlated with their osthol concentration. Although, osthol was not detected in the leaf extracts at the concentration tested, the quantitative analysis of the root extracts indicated that osthol content varied widely between them (methanol, 7.3%; chloroform, 36.2%; petroleum ether, 35.2%; ethyl acetate, 9.7%; hexane, 0.2%; water, not detected). Hexane and water extracts that did not contain detectable levels of osthol were inactive at the tested concentration. Those with least quantities were the least active while the ones with highest osthol quantity were consistently the most active. RCF5 and RPF5, representing osthol in this study, exhibited a similar pattern of activity. The curves obtained for the two were statistically not different ($P < 0.05$); Differences noticed in the DC_{50}s of the two fractions were similarly not significant (0.014% for RCF5 and 0.016% for RPF5) (Fig. 1).

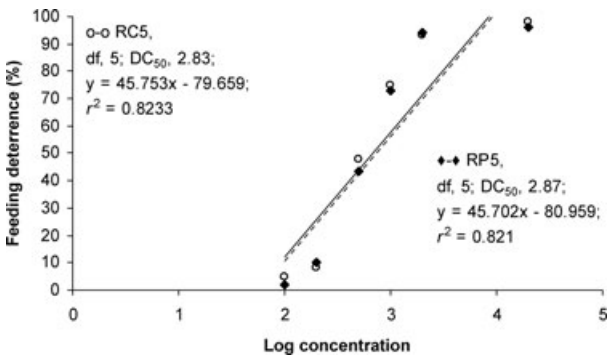

Fig. 1 Deterrency of fractions 5 of chloroform (RC) and petroleum ether (RP) root extracts of *Clausena anisata* in the leaf-disc choice tests using fifth instar *Helicoverpa armigera* larvae.
χ^2 = not significant.
DC_{50} = concentration causing 50% deterrence compared with the control.

Discussion

The antifeedant activity observed in the root extracts of *C. anisata* in this study was attributed to the isolated active

compound, osthol, a 7-hydroxycoumarin derivative. The compound is not new; it was identified from *C. anisata* root earlier (Okorie, 1975) and from many other plants such as *Cnidium monnieri*, *Angelica archangelica* and *Phebalium* spp (Rashid *et al*., 1992; Wei *et al*., 2004). However, antifeedant activity has never been previously reported for osthol. Gebreyesus and Chapya (1983) investigated the petroleum extract of stem bark of *C. anisata* for bioactivity against *S. exempta* and isolated coumarins imperatorin and xanthoxyletin as antifeedants. The variation in the isolated active compounds might be due to differences in the source of plant material, the plant part used, and in the test insects. Many studies have shown that even closely related insect species exhibit widely different susceptibilities to the same plant extract (Isman, 1993) and that responses to antifeedants are species-specific. Similarly, chemical composition of *C. anisata* depends largely on its source (Okorie, 1975; Lakshmi *et al*., 1984; Ekundayo *et al*., 1986).

The strong correlation between osthol contents and bioactivity of the root extracts of *C. anisata* where osthol content accounted for 99% of the variation in bioactivity shows that osthol is an active principle in *C. anisata* against *H. armigera*. However, this does not eliminate the possibility of the occurrence of other substances that may enhance the bioefficacy of osthol; the differences in the dose-dependent relationship curves, which indicated more activity in chloroform and petroleum ether extracts than the isolated compound lends credence to this thought. Such reduction in feeding deterrent response to pure allelochemicals compared to extracts containing a mixture of compounds including pure active ingredients have been similarly reported by Bomford and Isman (1996) and Akhtar and Isman (2004), and the conclusion was that mixtures would likely prevent a decrease in feeding deterrent response, which may result from repeated exposure to a deterrent.

Although osthol has not previously been linked specifically to insect antifeedant activities, its production in *Pastinaca sativa* enhanced resistance in the plant to the webworm, *Depressaria pastinacella* (Zangerl & Berenbaum, 2004). It has also been proven to possess a wide spectrum of fungal inhibition, anti-proliferative, anti-HIV and cytokine release inhibition effects (Zhou *et al*., 2000; Reviere *et al*., 2006). Similarly, many other hydroxycoumarin derivatives have been reported to have antifeedant and/or toxic effects on insects (Dreyer *et al*., 1987; Patton *et al*., 1997). Coumarins are generally considered phytoalexins since they are produced by the plant as a defence mechanism against attack by other organisms (Berenbaum *et al*., 1991; Weinmann, 1997). Although information is still scanty on the mode of action of osthol against insects, in rats, it is reported to interfere with the binding of thyrotropin releasing hormone to its receptor, and thus alters receptor-evoked intracellular signals (Ojala, 2001). Many coumarins caused insect immobilization by inhibition of mitochondria electron transport and neurotoxic action (Nicholson & Zhang, 1995; Zhen *et al*., 1998), while some others caused a reversible or irreversible inhibition of cytochrome P450 activity in *Manduca friday* (L.) (Lepidoptera: Sphingidae) (Neal & Wu, 1994).

The stronger bioactivity observed in the root extract compared to those of the leaf is likely due to the accumulation of higher contents of the active principle in the roots than in the leaves. Although coumarins are manufactured in the leaves, large amounts are concentrated in the roots (Ojala, 2001) and most of the coumarins reported from *C. anisata* have been isolated from the roots (Abe & Taylor, 1971; Ngadjui *et al*., 1989a,b, 1991; Okorie, 1975; Mester *et al*., 1977; Lakshmi *et al*., 1984; Chakraborty *et al*., 1995).

Similarly, chloroform and petroleum ether root extracts appeared to contain more activity and were the most effective against the larvae of *H. armigera* compared to other extracts. The observed differences in the efficacy of the extracts could only be explained in terms of polarity of the solvents used. Previous workers (e.g. Jaglan *et al.*, 1997) who similarly observed higher bioactivities in chloroform extracts of the neem seed kernel concluded that highly polar solvents (such as methanol and water) would have also extracted many inactive polar substances, such as sugars, tannins, and others, thus diluting the active principle in the extracts. However, the lower polarity of chloroform precluded the extraction of these inactive substances, and hence had a broader spectrum of polarity extracting a wider range of active substances, increasing their effectiveness at lower concentrations (Jaglan *et al.*, 1997). Our results support this conclusion.

The level of deterrence observed in this study compared favorably with other extracts and compounds reported against *H. armigera*, for example, condensed tannins from cotton, which reduced feeding at 0.1% (Chan *et al*., 1978b), while *Eucalyptus camaldulensis* and *Tylophora indica* had DC_{50}s of 9.3% and 5.2% for hexane and 6.9% and 2.8% for ethanol extracts respectively (Kathuria & Kaushik, 2005). As with most other antifeedants, activity pales in comparison with the outstanding activity of azadirachtin, with DC_{50} of 0.00125 μg cm^{-2} against *Spodoptera litura* (Isman, 1993). Nonetheless, osthol is a source of useful biological activity in the extracts of *C. anisata* and this adds to our understanding the feeding behavior of *H. armigera* and perhaps to its management.

Acknowledgments

We thank Dr. L. A. Adebisi, Department of Forestry, University of Ibadan, Ibadan, Nigeria for assisting with the collection and identification of *C. anisata* plant materials, Feng Li for insect rearing, Wang Xin Xing for extraction and fractionation procedures, and Adewuyi Sheriff, University of Agriculture, Abeokuta, Nigeria for NMR data interpretation. We are also grateful to Prof. M. B. Isman, The University of British Columbia, Canada for his suggestions on the study; and Prof. Marvin Harris of Texas A&M University, College Station, Texas, USA for his contribution to the write-up. Funds provided by Chinese Academy of Sciences and Third World Academy of Sciences (CAS-TWAS), Public Welfare Project from the Ministry of Agriculture of China (Grant No. 200803006), the National Natural Science Foundation of China (Grant No. 30621003), and the State Key Laboratory of Integrated Management of Pest Insects and Rodents (Grant No. Chinese IPM0704) are gratefully acknowledged.

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Accepted March 5, 2009