Industrial Crops & Products xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Insecticidal activity of *Tithonia diversifolia* and *Vernonia amygdalina*

Paul W.C. Green[a,1], Steven R. Belmain[b], Patrick A. Ndakidemi[c], Iain W. Farrell[a], Philip C. Stevenson[a,b,*]

[a] Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK
[b] Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent, ME4 4TB, UK
[c] School of Life Sciences and Bioengineering, Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Tengeru, Arusha, Tanzania

ARTICLE INFO

Keywords:
Callosobruchus maculatus
Sesquiterpenes
Botanical insecticide
Pesticidal plants
Saponins

ABSTRACT

The diversity of synthetic pesticides has been reduced through regulation especially in the European Union, leading to a resurgence of interest in natural plant products for pest control. Here we investigated two Asteraceae species, *Tithonia diversifolia* and *Vernonia amygdalina* that are used by farmers in Africa in bio-rational pest control to determine the chemical basis of activity against pests of stored legumes and identify plant compounds with commercial potential. The cowpea beetle, *Callosobruchus maculatus*, an ubiquitous pest of African stored grain legumes, was exposed to extracts of both plant species at 10, 1 and 0.1% w/v and fractions of these extracts at representative concentrations. Extracts and fractions were toxic to recently emerged adults, but did not reduce oviposition by those females that survived. The sesquiterpene, tagitinin A, was isolated from one of the active fractions and identified using H^1 and C^{13}-NMR and shown also be toxic to *C. maculatus* and so partially explains the activity of the whole plant. Other compounds in the active fractions were identified, at least to structural class, using high resolution mass spectroscopy (HRESI-MS). Sequiterpenes and flavones were common to fractions from both plants. Stigmostane steroidal saponins were the most abundant secondary metabolites in *V. amygdalina*.

1. Introduction

Legume seeds provide food, are sold for profit and used for sowing subsequent crops, so it is essential for small holder farmers in developing countries to minimise insect damage during periods of seed storage (Sola et al., 2014). *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae) is a significant pest of stored legumes throughout Africa (Abate and Ampofo 1996), the Middle East, India and South America, feeding on and contaminating the stored seeds (Tuda et al., 2006), especially cowpea, *Vigna unguiculata* L. Walp (Leguminosae) (Ehlers and Hall 1997). It is possible to control *C. maculatus* using synthetic pesticides e.g., Hill (1983) but these are expensive and require specialist equipment and training to be safely and effectively applied (Matthews et al., 2014). Synthetic pesticides can be toxic or have sub-lethal effects on the wider invertebrate community of beneficial insects, such as parasitoid wasps that can contribute to the control of bruchid pests (Van Alebeek 1996). A cost-effective and environmentally benign way of protecting crops is to use extracts or powdered plant materials of locally available insecticidal plants, and there are a number of examples of these being successfully employed to kill insects and decrease crop losses (Hagemann et al., 1972; Stevenson et al., 2009; Mwine et al., 2011; Belmain et al., 2012; Stevenson et al., 2012; Amoabeng et al., 2013). More recently, field trials testing *Tithonia diversifolia* (Hemsl.) A.Gray (Asteraceae) and *Vernonia amygdalina* Delile (Asteraceae) against field pests of common beans (*Phaseolus vulgaris* L.) demonstrated that extracts were as effective at controlling pest insects as a synthetic pyrethroid (Mkenda et al., 2015a). Extracts of *T. diversifolia* and *V. amygdalina* consist of a range of insecticidal compounds, especially volatile and non-volatile terpenoids (Ganjian et al., 1983; Ambrósio et al., 2008; Adeniyi et al., 2010; Madkour et al., 2013; Mkenda et al., 2015a). Some data report that polyphenolic compounds in *T. diversifolia* extracts inhibit the glutathione-s-transferases of *C. maculatus* (Kolawole et al., 2011) and could explain the lethal effects of the plant extract. Determining the chemical basis of activity in pesticidal plants can inform methods for optimising their use and identify potential candidate compounds for commercialisation (Stevenson et al., 2016). Furthermore, experimentation with the extraction process can alter the yield of compounds and alter efficacy. As part of continuing work on optimising the use pesticidal plants, here, we determine the plant chemistry underlying the biological activity of *T. diversifolia* and

* Corresponding author at: Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK.
E-mail addresses: p.stevenson@kew.org, p.c.stevenson@gre.ac.uk (P.C. Stevenson).
[1] Present address, Syngenta, Jealott`s Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

http://dx.doi.org/10.1016/j.indcrop.2017.08.021
Received 31 March 2017; Received in revised form 8 August 2017; Accepted 14 August 2017

V. amygdalina and discuss scope for improving application of these species for pest control in Africa.

2. Methods

2.1. Preparation of extracts and fractions

2.1.1. Extraction

Dried leaves from *Vernonia amydalina* and *Tithonia diversifolia* were obtained from the Kilimanjaro Region, northern Tanzania (Latitude 3°13′59.59"S Longitude 37°14′54"E) and voucher specimens were deposited at Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania. Powdered material of each plant was extracted in methanol at a rate of 100 mg of plant material per mL of solvent (10% w/v). Extracts were filtered and the solvent evaporated to yield 2.94 g of dried extract from 25.6 g of *V. amygdalina* and 1.93 g from 24.9 g of *T. diversifolia.* So, each gram (100 mg) of plant material yielded 114.8 mg (11.48 mg) and 77.4 mg (7.74 mg) of dried extract for *V. amygdalina* and *T. diversifolia*, respectively. Samples of dried extract were re-dissolved in methanol to 10% w/v equivalence for bioassay (11.48 and 7.74 mg mL^{-1}) and further diluted to 1% (1.15 and 0.774 mg mL^{-1}) and 0.1% (0.12 and 0.08 mg mL^{-1}) to determine dose effects. Stock-solutions of 100 mg mL^{-1} of dried extract in methanol were used for fractionation.

2.1.2. Fractionation

An HPLC system consisting of a Waters 2695 separations module linked to a 2996 photodiodearray detector (PDAD) was used for fractionation of extracts. Aliquots of *T. diversifolia* extract (200 µL) were injected onto a Phenomenex Luna RP18 column (300 × 10 mm, length × i.d.; 10 µm particle size) and eluted at 4 mL min^{-1} using a linear gradient of 40%A: 10%B: 50%C (t = 0) to 90A: 10B: 0C (t = 20–25 min) returning to the starting conditions (t = 27 min), where A = methanol; B = 1% formic acid in acetonitrile and C = HPLC-water. The fractionation of *V. amygdalina* used a shorter column (150 mm) different initial conditions (A = 30%; B = 10%; C = 60%) and a non-linear gradient (Waters, curve = 7) to enhance the separation of compounds with similar retention times. The quantity of material injected and the proportion of each fraction in a 10% w/v extract was calculated. The fractions contributed from 0.17 (F2) to 0.50 mg mL^{-1} (F4) (*V. amygdalina*) and from 0.08 (F3) to 0.49 mg mL^{-1} (F1) (*T. diversifolia)*

2.2. Experimental

2.2.1. Insects

Callosobruchus maculatus were a wild Ghanaian strain, originally collected in 1995. They were housed in a temperature controlled room (28 ± 1 °C, 55% RH) that was kept in permanent darkness. Adults laid eggs on cowpea seeds *Vigna unguiculata* and 24–28 days later the next generation of adults emerged from the beans. The insects used for bioassays were 3–5 days post-emergence.

2.2.2. Bioassay procedure

Fractions and residue were dissolved to concentrations representing their proportions in 10, 1 and 0.1% w/v extracts of each plant species. Aliquots (75 µL) of compounds, extracts, methanol (negative control) or rotenone (1000 and 100 ppm, positive control) were evaporated onto vials (25 mL, nominal capacity) under a stream of air and with constant rotation of the vial. Insects (N = 5-12) were added to the vials, ensuring a ratio of at least 1:1 (male to female). 5 black eyed beans were added to each vial after 72 h. After a further 72 h mortality was recorded. The numbers of eggs laid on both the vials and the beans were counted and from these data the eggs laid per female were calculated. *ANOVA* followed by *Tukey's HSD* post hoc test (95% C.I.) were used to compare the mortality and eggs laid among and between treatments at equivalent concentrations (XLSTAT version 2015.1.03.16409).

Table 1
Mortality and total eggs laid by *C. maculatus* when exposed to different concentrations of fractions prepared from extracts of *Tithonia diversifolia* and *Vernonia amygdalina* for 6 days.[#]

Treatment [ppm for extracts]	LSD mean Mortality	LSD mean eggs laid, per female
EXTRACTS		
Tith 10% [11,480]	92.611 abcd	23.678 abc
Tith 1% [114.8]	92.424 abcd	20.270 abcde
Tith 0.1% [11.5]	77.027 def	17.507 abcdef
Vern 10% [7740]	90.238 abcd	19.378 abcde
Vern 1% [774]	89.841 abcd	15.735 abcdefg
Vern 0.1% [7.7]	82.567 abcde	20.105 abcde
FRACTIONS		
Tith F1 (Tagitinin A) 488 ppm	60.462 f	23.467 abc
Tith F1 (Tagitinin A) 48.8 ppm	100.000 a	17.027 abcdef
Tith F1 (Tagitinin A) 4.88 ppm	98.750 ab	13.088 bcdefg
Tith F2 95.2 ppm	98.571 ab	2.383 g
Tith F2 9.52 ppm	95.123 abcd	12.520 bcdefg
Tith F2 0.952 ppm	98.000 abc	17.113 abcdef
Tith F3 80.5 ppm	90.059 abcd	11.677 cdefg
Tith F3 8.05 ppm	85.844 abcd	9.233 defg
Tith F3 0.81 ppm	100.000 a	14.960 abcdefg
Tith F4 295.7 ppm	100.000 a	13.428 bcdefg
Tith F4 29.57 ppm	87.071 abcd	20.977 abcd
Tith F4 2.96 ppm	96.333 abcd	16.597 abcdef
Tith F5 260.1 ppm	95.500 abcd	13.292 bcdefg
Tith F5 26 ppm	93.389 abcd	18.543 abcdef
Tith F5 2.6 ppm	95.794 abcd	6.667 efg
Vern F1 265.1 ppm	91.813 abcd	20.183 abcde
Vern F1 26.5 ppm	89.143 abcd	16.793 abcdef
Vern F1 2.65 ppm	93.294 abcd	13.977 abcdefg
Vern F2 173.3 ppm	87.908 abcd	14.985 abcdefg
Vern F2 17.3 ppm	90.806 abcd	20.205 abcde
Vern F2 1.73 ppm	78.063 cdef	19.188 abcdef
Vern F3 179 ppm	84.149 abcde	23.045 abc
Vern F3 17.9 ppm	81.452 abcde	22.340 abcd
Vern F3 1.79 ppm	79.852 abcdef	18.155 abcdef
Vern F4 501.5 ppm	79.159 bcdef	20.692 abcd
Vern F4 50.1 ppm	83.357 abcde	24.185 abc
Vern F4 5.0 ppm	90.060 abcd	20.187 abcde
Vern F5 355.79 ppm	78.927 bcdef	27.630 a
Vern F5 35.58 ppm	86.440 abcd	20.823 abcd
Vern F5 3.56 ppm	65.204 ef	26.203 ab
Rotenone 1000 ppm	97.571 abc	15.020 abcdefg
Rotenone 100 ppm	97.778 abc	5.588 fg
Control	20.453 g	20.562 abcd
SEM	2.485	3.647
$p > F$	0	0
Significant	yes	yes

[#] Values with the same letter are not different Tukey's *post hoc* HSD-test (95% C.I.). Tith = *Tithonia*; Vern = *Vernonia*; F1, F2 etc = fraction number, followed by the concentration of the sample applied to the vials, as a w/v percentage equivalent for extracts, or in parts per million for the compound and fractions.

2.3. Analyses

2.3.1. LC–MS

Accurate mass measurements of compounds detected in the extracts were obtained using an LTQ Orbitrap XL, linear ion trap/orbitrap hybrid mass spectrometer (Thermo Scientific, San Jose, California USA) with an electrospray ionisation source (Ion Max, Thermo Scientific) coupled to an "Acella 1250" UPLC system (Thermo Scientific). Samples were injected onto a Phenomenex Luna C18(2) column (150 × 3 mm i.d., 3 µm particle size) at 400 µL min^{-1} and eluted using a linear gradient of 90:0: 10 (t = 0 min) to 0:90:10 (t = 20–25 min), returning to 90:0:10 (t = 27–30 min). Solvents were water, methanol and 1% formic acid in acetonitrile, respectively. The column was maintained at 30 °C. Samples were scanned, using FTMS, from *m/z* 250–2000 in both