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Bioactive metabolites of *Senna didymobotrya* used as biopesticide against *Acanthoscelides obtectus* in Bungoma, Kenya

J. Mining[1], Z.O. Lagat[2], T. Akenga[3], P. Tarus[3*], M. Imbuga[1], M. K. Tsanuo[4]

[1] Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya. [2] Department of Chemistry, Maasai Mara Univeristy, Narok, Kenya. [3] Department of Chemistry and Biochemistry, University of Eldoret, Eldoret, Kenya. [4] Department of Chemistry, Pwani University, Kilifi, Kenya.

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ABSTRACT

Post-harvest pests are known to cause severe losses of beans in particular the bean weevil, *Acanthoscelides obtectus*, necessitating immediate and long term control measures. Convectional pesticides have been used for decades to protect stored grain from pests, but their harmful environmental impact and pest resistance resulting from the need for increased pesticide use have triggered researchers to search for eco-friendly, biodegradable and potent biopesticides. The use of natural products derived from metabolic activity of plants may constitute a new avenue of pest control. This paper describes phytochemical and biological investigations of leaves, root, and stem bark crude extracts and isolated compounds of selected Kenyan plants used to control the bean weevil. Bioassays tests of the compounds were done against brine shrimp to test their insecticidal activity against the bean weevil. Antibacterial and antifungal activity of the crude extracts of *S. didymobotrya* was also done. The extracts of *S. didymobotrya* showed high toxicity as well as adulticidal activity against bean weevil.

INTRODUCTION

Food quality and security pose a big challenge to man. Although a variety of solutions to this problem exist, one of the more important aspect is to improve efficiency in food production and post harvest practices to ensure that food loses are minimized and food produced is of good quality and safe for human consumption. Kenya, like other tropical countries suffers crop losses due to pests. This is partly attributed to conducive conditions for insect survival (Pertunen, 1972).

Synthetic pesticides have been used against post-harvests pests. However, resistance resulting from increased pesticide use, the cost and availability of these conventional insecticide, and potential health hazard both to the consumers and environment has necessitated a relook in the use of the indigenous plant extracts as biopesticides. Although these botanicals have been in use from ancient time, their efficacy, safety and active principles deserve more attention (Alkofali et al., 1989, Delobel et al., 1987, Boeke et al,. 2001).

* *Corresponding Author*
Email: paultarus@yahoo.co.uk

There has been a worldwide effort aimed at screening plant species for bioactivity against pest species. Plants have been screened for repellency and protectants against the bean weevil (Balandrin and Lee, 1985).Throughout history plants have been successfully exploited as insecticides, insect repellents and insect antifeedants (Nakanishi, 1977). Protection of stored products generally involves mixing grain with protectants made up of plant materials with or without minerals (Agona et al., 2001).

The precise strategy used by different communities varies from region to region and appears to depend partly on the type and efficiency of suitable flora available in different locations. *Senna didymobotrya* belongs to the plant family Caesalpiniaceae. Traditionally *S. didymobotrya* is used as a stupefacient for fishing and also for used as a purgative. *In vitro* cultures of *S. didymobotrya* has compounds that can be converted into low energy sweetners and insecticides. Several species of this genus have been found to contain anthraquinones and flavonoids and they have been found to be active against *Escherichia coli* and *Staphylococcus aureus* (Gizachew, 2004). The survey carried out in Bungoma district, Kenya indicates that local farmers have been using the plant in the control of bean weevil.

MATERIALS AND METHODS

Plant material

The root bark and pods of *Senna didymobotrya* were collected from Bokoli area, Bungoma district in October, 2011. After identification by a plant taxonomist, reference specimens were deposited at Jomo Kenyatta University of Agriculture and Technology Herbarium at the department of Botany. The voucher specimens of *S. didymobotrya* were deposited as JMB/4/06/01.

Extraction, fractionation and isolation

The root and stem bark of *S. didymobotrya* (4.0 kg), was air dried in a well-ventilated room and then ground to powder. The plant material powders were sequentially extracted with hexane (10 liters), DCM (12 liters), EtOAc (13 liters) and MeOH (9 liters), respectively in order of increasing polarity at room temperature for three days and the process repeated three times with occasional shaking. The root bark of *S. didymobotrya* yielded 26 g of hexane and 62 g of DCM extract. The two extract were subjected to Vacuum Liquid Chromatography (VLC) and CC on silica gel, prep.TLC eluting with hexane and DCM yielded compounds (**1**) 3β-sitosterol (20.5 mg (**2**) stigmasterol (18.9 mg), chrysophanol (**3**) (21 mg) and physcion (**4**) (14 mg).

Stigmasterol(**1**){(3S,9S,10R,13R,14S,17R)-17-[E,2R,5S) -5-ethyl-6-methylhept-3en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12, 14,15,17-dodecahydro-1H-cyclopenta[a]phen.anthren-3-ol}.White crystalline solid; m.p 129 ˚C, R_f 0.51, Hexane: DCM, 1:4 v/v), IR ν_{max} cm^{-1} (Kbr disc,) 3422, 2920, 1640, 1463, 1380, 1048, 1021, 723; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ, ppm 0.68 (3H, *s*, H-18), 0.83 (3H, *s*, H-21), 0.85 (3H, *s*, H-26), 1.00 (3H, s, H-19), 1.25 (3H, *s*, H-29), 1.57(3H, *s*, H-27), 1.85(H, *d*, J = 3.3 Hz, H-2) 2.00 (2H, *d*, J = 3.3 Hz, H-7), 2.27 (2H, *d*, J = 1.8Hz), 3.54(H, *m*, H-3), 5.35 (2H, *d*, J = 5.3 Hz, H-5 and H-6) and 5.35 (H, *d*, J = 5.1 Hz, H-22 and H-23); ^{13}C-NMR ($CDCl_3$, δ, 400 MHz), 140.8 (C-5), 121.6 (C-6), 138.2 (C-22), 129.3 (C-23), 11.8 (C-18, CH_3), 19.3 (C-19, CH_3), 18.7 (C-21, CH_3), 19.7 (C-26, CH_3), 19.0 (C-27, CH_3), 11.9 (C-29, CH_3).

3β-sitosterol,(**2**),{17-(4-Ethyl-1,5-dimethyl-hexyl)-10,13, dimethyl-2,3,4,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-ol}.White crystalline solid; m.p. 158-159˚C, R_f 0.49 Hex:DCM, 3:7 v/v), IR ν_{max} cm^{-1}(KBr disc,) 3422, 2920, 1640,1463, 1380, 1021, 723; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ, ppm 0.68 (3H, *s*, H-18), 0.83 (3H, *s*, H-21), 0.85(3H, *s*, H-26), 1.00 (3H, *s*, H-19), 1.25 (3H, *s*, H-29), 1.57 (3H, *s*, H-27), 1.85 (H_{eq}, *d*, J = 3 Hz,H-2), 2.00 (2H, *d*, =3.3 Hz, H-7), 3.35 (H, *m*, H=3), 5.37 (1H, d, J = 5.3 Hz, H-5 and H-6) 5.37 (2H, s, H-22 and H-23); ^{13}C-NMR ($CDCl_3$, δ, 400 MHz), 121.6 (C-6), 140.7 (C-5), 11.8 (3H, C-18), 11.9 (3H, C-29), 18.7 (3H, C-21), 19.0 (3H, C-27) 19.4 (3H, C-19), 19.7 (3H, C-26).

Chrysophanol (**3**), {1,8-dihydroxy-3-methylanthraquinone}. Orange crystals; m.p. 194-195˚C, R_f, 0.52, (Hexane: DCM, 2:4 v/v), IR ν_{max} cm^{-1}(KBr disc,), 3425, 1627, 1479, 1272, 752; ^{1}H-NMR ($CDCl_3$, 300 MHz), δ, ppm, 2.47 (3H, *s*, 3-CH_3), 7.10 (1H, *s*, H-2), 7.31(1H, *dd*, H-7), 7.65 (1H, *s*, H-4), 7.67 (1H *t*, H-6), 7.83 (1H, *dd*, H-5), 12.01 (1H, *s*,1-OH); ^{13}C-NMR($CDCl_3$, δ, 400 MHz), 113.1 (C-8a), 113.9 (C-9a), 119.9 (C-2), 121.3 (C-7), 124.3 (C-4), 124.34 (C-5), 124.5 (C-4), 133.7 (C-4a), 136.9 (C-6), 149.3 (C-3), 162.5 (C-1), 162.8 (C-8), 181.8 (C-10, C=O), 192.6 (C-9, C=O)

Physcion (**4**), {1, 8-dihydroxy-6-methoxy-3-methyl anthraquinone}. Orange crystals; mp 206-207˚C, R_f 0.52 (Hex: DCM, 2:4 v/v), IR V_{max} (KRr disc), 3425, 1627, 1479, 1272 and 752.

^{1}H-NMR ($CDCl_3$, 300 MHz), δ ppm, 2.47 (1H, s, 3-CH_3), 3.95 (3H, s, 6-OCH_3), 6.69 (1H, d, H-7), 7.01 (1H, s, H-2), 7.37 (1H, d, H-5), 7.69 (1H, s, H-4), 12.12 (1H, s, 8- OH), 12.3 (1H, s, 1-OH); ^{13}C-NMR($CDCl_3$, δ, 400 MHz), 22.1 (3-CH_3), 56.05 (6-OCH_3), 108.1 (C-4a), 110.2 (C-8), 113.6 (C-2), 121.2 (C-9a), 124.4 (C-4), 133.2 (C-8a), 135.2 (C-5), 148.4 (C-3), 162.5 (C-1), 165.2 (C-5a), 166.5 (C-7), 181.98 (C-10, C=O) and 190.80 (C-9, C=O).

Melting points were determined on an electro thermal melting point apparatus and expressed in ˚C and uncorrected. IR spectra were taken in chloroform solution and recorded on a Shimadzu (Model FTIR-8400 CE) with absorption given in wave numbers (cm^{-1}). NMR spectra were recorded at room temperature on a Bruker DPX-400 NMR. The spectra were recorded in $CDCl_3$ as the solvent and TMS as the internal standard. The chemical shifts reported in δ (ppm) units relative to TMS signal. TLC was performed on aluminium sheets precoated with silica gel 60F_{254} (Merck) with 0.2 mm layer thickness, preparative TLC was done using normal phase silica gel (20x20 cm) and a layer thickness of 0.25 mm. Spots on chromatograms were examined under UV light (λ254 and 366 nm) and sprayed with anisaldehyde and Dragendorff's visualization reagent. VLC column were packed with thin layer chromatography silica gel 60 (6-35 microns mesh, ASTM) and column chromatography on silica gel 60 (0.040-0.063 mm 230-400 mesh, Merck).

Antimicrobial activity

Microbial test cultures and growth conditions

The Gram-negative, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and Gram positive, *Staphylococcus aureus* (ATCC 22923), *Staphylococcus aureus* bacterial strains were used. *Candida albicans* (ATCC 90028) was used as fungal test microorganisms. Bacterial strains were maintained on nutrient dextrose agar (SDA) in Petri dishes. Antifungal and antibacterial *in-vitro* assays were done using disc diffusion assay test (Hutchinson, 1986). All the procedures were done according to clinical laboratory standard institute standards procedures and quality control. Fresh cultures were obtained by growth the test strains overnight at 37°C for bacteria while fungi were grown at 28ºC for 48 hours.

Media preparation

Mueller Hinton Agar (Oxoid, UK) was used for bacteria bioassay while SDA (Oxoid, UK) was used for fungi as solid media; MHA was prepared by dissolving 19 g in 500 ml of distilled water and brought to boil to completely dissolve the

media. Sterilization was achieved by autoclaving at 121 ºand 15 psi for 15 minutes.

SDA was prepared by dissolving 65 g in 1 litre of distilled sterilized water, brought to boil to ensure complete dissolution and autoclaved at 121°C and 15 psi for 15 minutes to ensured sterilization of the media. The media (20 ml) was dispensed onto the pre-sterilized Petri dishes yielding uniform depths of 4 mm. They were then covered and allowed to cool and harden at room temperature. Filter paper discs (6 mm diameter) were prepared and sterilized.

Antimicrobial activity assay

Disc diffusion method was employed in the preliminary antimicrobial screening of both the crude organic extracts and selected isolated purified compounds. Test strains suspensions of 0.5 ml McFarland were prepared from fresh cultures using normal saline. The plates were aseptically streaked with the test microorganisms using a sterile swab and allowed to dry for a few minutes. Test extracts (100 mg) were dissolved in 1 ml of DMSO. Sterile 6 mm diameter filter paper discs were impregnated with 100 mg/ml of the crude extract material and using sterile forceps the discs placed aseptically on the isolated agar plates. The plates were then incubated for 24 hours at 37ºC for bacteria and at 28ºC for fungi. The experiments were carried out in triplicates. Presence of a clear circular zone around the sample impregnated with test sample was used as an inhibition indicator.

The results (mean values of triplicates) were recorded by measuring these zones diameter using a ruler and a disc impregnated with the solvent (DMSO) was included as controls. For comparative purposes standard drug, gentamycin (10 µg/ disc) was the positive control in the assay for the assays.

Brine shrimp bioassay test

The brine shrimp test to evaluate the bioactivity of crude extracts and pure compounds was carried out using brine shrimp (*Artemia salina*) larvae as the test organism. Artificial sea water prepared by dissolving artificial sea salt (Sigma chemicals Co.UK) in distilled water (ca 38 gl^{-1}) was filled into incubation tank (25 cm in diameter and 3.5 cm in height) divided into two unequal compartments separated by a perforated polystyrene wall at room temperature. Brine shrimp eggs (0.5 g) were sprinkled into the larger compartment, which was darkened by covering with hard paper while the smaller compartment was illuminated with electric bulb. After 48 hours of hatching the phototropic nauplii were collected from the illuminated compartment by using the micropipette (Meyer et al., 1982).

Solutions of 1000, 800, 600, 400, 200 µg/ml were prepared by dissolving 1g of each crude extract in ml of dimethyl sulphoxide (DMSO). Serial dilution for the three plants extracts in different vials each with a volume of 10 ml were made by adding artificial sea water and then 10 brine shrimps were transferred into each of the three vials. The experimental vials were maintained under illumination conditions. Controls were placed in a mixture of artificial sea water and 1 ml of DMSO. The nauplii were counted microscopically in the stem of the pipette against a lit background. Each assay was repeated three times and the average number of survived larvae after 24 hours was recorded.

Lethality concentrations (LC_{50} values) for each assay were calculated by taking average of the four experiments and subjecting data to probit analysis, using SAS program version 8.2.

Adulticidal testing against bean weevil

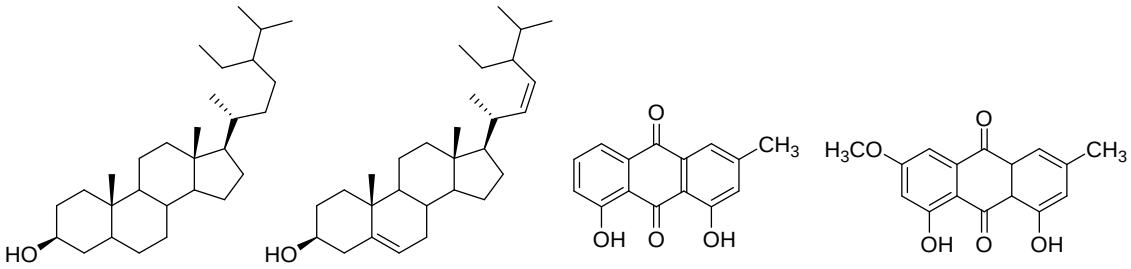
Culturing of bean weevil

Bean weevils infested grains used for the study were obtained locally from Kari, Kisii and Bungoma County in Western Kenya. Dried grains were sieved to remove dirt fine dust and broken or shriveled kernels. One hundred 6 weeks old *Acanthoscelides obtectus* insects, obtained from a laboratory colony reared under ambient conditions with natural photoperiods on untreated (insecticide-free) beans, *A. obtectus* were reared in 1-litre jar containing 50 g uninfected whole bean grain as described by Haines (1991). The jar was labeled, covered and left at 20°C. It was moved into the rearing area for one week for adults to develop. The adult weevils were removed from the medium using 2.0 mm and 0.7 mm opening sieves and discarded. The medium was then returned to the culture jar, covered and allowed to stand for four weeks for adult weevil to emerge. The rearing of the insects was done in the laboratory at room temperature. All equipments used were thoroughly cleaned and sterilized using ethanol

Adulticidal assay

This bioassay test was carried in the laboratory to determine the efficacy of botanicals under different dosage levels. For the crude plant extracts four concentrations (250 ppm, 500 ppm, 750 ppm and 1000 ppm) were admixed with 50 g of uninfected beans. An untreated sample and Actellic super (pirimohos-methyl) treated beans at the recommended rate of 0.5 % (w/w) were used as negative and positive controls, respectively.
The crude extracts were prepared by dissolving 1 g of the test sample in 1 ml of DCM and then mixed with 5 grams of talc ($Mg_3Si_4O_{10}(OH)_2$) and thoroughly mixed and DCM allowed to evaporated in the fume cupboard. These concentrations were admixed with 50 g of beans held in jam jags covered with ventilated lids. The grain was then swirled within the jam until a proper admixture was realized. Each treatment was replicated three times.

The walls of holding trays used for handling the colony were coated with a thin film fluorocarbon resin (Teflon) to prevent individuals from escaping. Twenty randomly selected adults of *Acanthoscelides obtectus* of mixed sexes and age were starved for 24 hours before being introduced into each jam jag. After seven days, the number of dead and live *A. obtectus* in each treatment was recorded from which the mean average mortalities and percentage mean mortality were calculated (Bekele et al., 1996)
The percentage mean mortality result was then subjected to SAS version 8.2 a computer program for analysis to obtain LD_{50}.



(**1**) 3β- sitosterol (**2**) Stigmasterol (**3**) Chrysophanol (**4**) Physcion

RESULTS AND DISCUSSION

The TLC of *S. didymobotyra* revealed the presence of several UV and fluorescing compounds in the crude extracts. Chromatographic separation of the hexane and dichloromethane extracts yielded two terpenoids; (**1**) 3β-sitosterol and (**2**) stigmasterol and two anthraquinones; (**3**) chrysophanol and (**4**) physcion. The structures of the compounds were characterized and identified by their IR, ^{1}H NMR, ^{13}C NMR and comparing with data of authentic samples (Kessler et al., 1988).

The crude extracts (hexane and dichloromethane) of S. didymobotrya were tested for their toxicity against brine shrimp (Artemia salina, leach) lethality assay. The hexane and dichloromethane extracts of S. didymobotyra leaves showed significant toxicity against brine shrimp with LD50 values of 195.0 µg/ml and 345.0 µg/ml, respectively (Table 1). Since brine shrimp is an indicator of toxicity, pharmacological actions and pesticidal effects (Meyer et al, 1982), it was deduced that the DCM extracts of S. didymobotrya had the better bioactivity against brine shrimp.

The hexane extracts of S. didymobotrya showed good activity against S. aureus and B. subtilis (Gram-positive bacterium) with zones of inhibition averaging 23 mm and 12 mm, respectively. It also showed mild activity with inhibition zones of 10.3 mm and 12.0 mm against E. coli and B. subtilis, respectively. Among the dichloromethane extracts the same trend was also observed where the DCM extracts of S. didymobotrya exhibited moderate activity against S. aureus and B. subtilis with inhibition zones of 10 mm for each. The S. didymobotrya DCM extract also registered a moderate activity of 12.3 mm against the yeast, Candida albicans (Table 2).

Among the dichloromethane extracts, the trend was also observed where the DCM extracts of S. didymobotrya exhibited moderate activity against S. aureus and B. subtilis with inhibition zones of 10 mm for each. The S. didymobotrya DCM extract registered a moderate activity of 13.3 mm against C. albicans.

The crude extract of S. didymobotrya was subjected to adulticidal test against the bean weevil (A. obtectus). The effects doses of hexane and DCM on bean weevil after seven days were determined and the mean percentage values computed. The results are as summarized in Table 3. It is evident that adulticidal activities are dose dependent for the extracts. The highest mean adulticidal activity were hexane at almost all doses. DCM extracts

Table 1: The mean LD_{50} values ± S.D for the plant extracts screened against brine shrimp (*Artemia salina*, Leach).

Plant Extract	*SdBH*	*SdBD*	Control
LD_{50}±S.D.(µg/ml)	345.1±0.8	195.0±0.7	0.00

Key: *SdBH* =*Senna didymobotrya* root bark, hexane extract; *Sd*BD=*Senna didymobotrya* root bark, DCM extract.

Table 2: Antibacterial and antifungal activity of hexane extracts of *Senna didymobotrya* root bark, pods and DCM root extract.

Microorganism	Activity expressed in mm of inhibition zones diameter (Mean values=3)			
	***Sd*RH**	***Sd*PH**	***Sd*RD**	**Control**
Escherichia coli	15.6±0.7	10.3±0.6	1.3±1.2	6.0±0.0
S. aureus	18.6±0.6	23.6±0.5	10.0±1.0	6.0±0.0
P. aeruginosa	6.0±1.0	6.0±0.7	6.0±0.8	6.0±0.0
Bacillus subtilis	12.3±0.5	12.0±1.2	10.0±0.1	6.0±0.0
Candida albicans	15.3±0.7	10.3±0.7	13.3±0.1	6.0±0.0

Table 3: Mean percentage (%) adulticidal (±S.D n=3) of hexane and DCM extract from *Senna didymobotrya* against the bean weevil (*Acanthoscelide obtectus*).

Plant extract	250ppm	500ppm	750ppm	1000pm
*Sd*BH	25.0±0.5c	45.0±0.3c	50.0±1.6c	80.0±1.6b
*Sd*BD	25.0±0.5c	75.0±0.3b	95.0±0.0b	100.0±0.0a
Actellic super	100.0±0.0b	100.0±0.0a	60.0±0.3f	100.0±0.0a
Negative control	0.0	0.0	0.0	0.0

Key: *Sd*BH= *Senna didymobotrya*, root bark, hexane extract; *Sd*BD = *Senna didymotrya* root bark, DCM extract; *Sd*RH=*Senna didymobotrya* root bark, hexane extract,*Sd*PH= *Senna didymobotrya* pod, hexane extract.

of S. didymobotrya root bark, showing 100% and 95% adulticidal at 750 and 1000 ppm, respectively, which were comparable to the positive control, Actellic super, a synthetic pesticide at the recommended rate of 0.5% which is in the market.

The crude extracts at high concentration had significantly higher percentage mean adulticidal activity against the bean weevil. This supports the use of this plant as material as grain protectant against destructive pests. This extracts represent an attractive candidate for field evaluation as a protectant of stored bean. The crude extracts can offer suitable and sustainable alternative pesticide to synthetic pesticide.

The brine shrimp and adulticidal activity results for the crude extracts are shown in table 1 and table 2, respectively. The extracts of S. didymobotrya showed higher toxicity as well as adulticidal activity against bean weevil. It was evident that toxicity

against brine shrimp may be a basis of deducing an active adulticidal activity.

These natural plant products may improve efficiency in post harvest practices as a strategy of providing people with sufficient and healthy food in an ecologically sustainable way. Being natural, protectants from plant materials would be easily degraded by biological factors, whereas cases of pollution and poisoning would be reduced. Improving storage would lead to less hunger, improved nutrition for individual, higher standard of living and a sounder economy for the nation.

CONCLUSION

This study has shown that Senna didymobotrya root bark contain anthraquinone and triterpenoids, in addition to other compounds. The results, demonstrate a possible scientific rationale for the incorporation of the root bark of S. didymobotrya into traditional medicine for grain protection.

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