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Identification of a Plant Phytosterol with Toxicity against Arthropod Pests

J.R.M. Thacker

Department of Agronomy, Horticulture, Entomology and Plant Pathology, College of Agriculture, Sultan Qaboos University, P.O. Box 34, Al Khod 123, Sultanate of Oman

تمييز سيترول نباتي ذو خاصية سمية ضد الآفات المفصلية

الملخص: تم إخضاع عصارة نبات خامة أثبتت سميتها لحلم العنكبوتي إلى تحاليل كيميائية مكثفة باستخدام تقنيات الكروماتوغراف والاسبكتروسكوب (أشعة الطيف). بينت التحاليل أن العامل الكيميائي الرئيسي النشط ربما كان بيتاساتيوسيترول – ٣ – غلوكوسايد وهو سيترول نباتي معروف. وأوضحت الدراسات السابقة أن هذا العنصر قد تم اكتشافه في العديد من أصناف النباتات كما تم اختباره للاستخدام في العديد من العلاجات الطبية. إلا أن هذا العنصر لم يتم تحليله حتى الآن للسيطرة على الآفات المفصلية. تشير النتائج إلى أن هذا المركب ربما يكون ذا فائدة في الاستخدام للسيطرة على أنواع الآفات خاصة الحلم العنكبوتي.

ABSTRACT: A crude plant extract that was toxic to spider mites in a leaf dip bioassay was subjected to detailed chemical analyses using chromatographic and spectroscopic techniques. The analyses revealed that the major active chemical was probably β-sitosterol-3-glucoside, a known phytosterol. The literature indicates that this chemical has been identified in a number of plant species and that it has been tested for utility in a number of medical therapies. It has not so far been assayed for the control of arthropod pests. The data indicate that this compound may be of use in the control of pest species, especially spider mites.

Plant extracts have been used as insecticides for at least the last 4000 years (Yang and Tang, 1988). Their earliest documented use occurs in the Rig Veda, the classic book of Hinduism, that was written in India in 2000 BC (Chopra *et al.*, 1949). Since then, at least 700 plant species have been documented as having been used to control arthropod pests of one type or another (Secoy and Smith, 1983). Many of these extracts are still in use today. For example, in Zimbabwe the sap from the bark of *Spirostachys africana* is used as a pesticide in granaries, in South America, indians use the leaves of *Mammea americana* to protect newly planted trees from insect attack, and the almost worldwide use of neem extract from *Azadirachata indica* has now been extensively documented (Ahmed and Grainge, 1986).

In recent years, the use of these plant extracts as pesticides has begun to attract an increasing amount of interest from agrochemical companies, university researchers, and governmental research organisations. The reasons for the upsurge in interest in native uses of plants as insecticides are many and certainly include the following. First, the costs associated with developing new pesticides are slowly becoming increasingly prohibitive because of low rates of discovery of novel synthetic molecules and because of increasingly stringent registration data requirements (McChesney, 1994). Second, conservative estimates suggest that only 10% of plant species worldwide have been screened for extracts that may be of use for pest control (McLaren, 1986). Third, modern pyrethroid and carbamate insecticides were developed as a result of studies on naturally occurring plant-based insecticides. Fourth, there has recently been the successful development and formulation of the extract azadirachtin, a plant-based insecticide derived from the neem tree. Finally, as stated above, it is known that many native cultures continue to use plant extracts for pest control and that the scientific rationale for the use of many of these products remains to be investigated.

Taken together, all of the above reasons explain the upsurge in interest in extracts from plants that may have pesticidal properties. The aim of the research described in this paper was to add to this body of knowledge on insecticidal plant extracts. Our main aim was to identify the chemically active fraction of a crude

plant extract that had already been shown to be toxic to a wide range of arthropod pest species (Hutton *et al.*, 1996). The specific questions that we attempted to address concerned whether the chemical basis for the toxicity of the crude extract was a mixture of chemicals or a single compound and whether the chemical compound or compounds had been previously described in the literature in relation to pest control and/or in relation to any other process. Answers to these questions would provide a starting point for further research concerning the development of novel plant-based chemicals that could be used for pest control. The procedure we followed involved an initial separation of the crude extract using soxhlet apparatus, a more detailed separation using vacuum liquid chromatography, and then a final chemical analyses of our most toxicologically active fraction using nuclear magnetic resonance spectroscopy.

Materials and Methods

CRUDE PLANT MATERIAL: Plant material that had been shown to be toxic to a range of arthropod pests in bioassays with crude extracts in 70% ethanol was supplied by Sarami Research Ltd, UK (Hutton *et al.*, 1996). The plant material was supplied as 0.5 kg of dried leaves and stems. The origin of the plant material was unknown at this time. All experiments were undertaken at room temperature (ca. 20 - 23 °C).

SOXHLET EXTRACTION: Preliminary fractionation of the plant extract was undertaken using soxhlet apparatus (Wells *et al.*, 1993). To carry out the fractionation three soxhlet extraction units were set up in series. A single thickness cellulose extraction thimble was then packed with dried plant material and secured with glass wool. The plant material was then exposed to the solvents petroleum ether, ethyl acetate, and methanol (Coates *et al.*, 1994). Each solvent fraction was then concentrated *in vacuo* using a rotavac (Bocht). This procedure was replicated three times giving a total of nine fractions (three for each solvent) in total. Approximately 150 g of plant material was used for each replicate extraction. Extract yields were greatest for the petroleum ether fraction and lowest with ethyl acetate.

BIOASSAYS: The toxicity of the fractions collected following soxhlet extraction were assayed using leaf dip bioassays and the mite pest species *Tetranychus urticae*. The leaf dip bioassay, which is described in detail by Jepson (1993), essentially comprises the following. Four 20 mm leaf discs were cut from a bean plant (*Vicia fabae*) using a borer. The discs were then dipped in a solution of the extract for 30 seconds and air dried for 60 minutes. All extracts were dissolved in 70% ethanol which also served as a control. The extracts were assayed at a concentration of 0.1mg/ml. After drying, the discs were transferred to moist cotton wool in a petri dish and 10 adult spider mites placed on the discs. A count of the number of dead and live mites was then taken every day until four days after treatment.

DATA ANALYSES: The bioassay data were analysed using one-way analysis of variance on count data. The data were checked to ensure that they met the criteria for homogeneity of variance. The data are presented as the mean percentage mortality recorded at 1,2 and 3 days after treatment.

VACUUM LIQUID CHROMATOGRAPHY: Vacuum liquid chromatography involves a step gradient elution by solvents controlled by vacuum (Coll and Bowden, 1986). It is a technique that has been widely used for the separation of chemicals from plant extracts (Pellitier *et al.*, 1986; Villasenor *et al.*, 1996). A 250 ml sintered glass Buchner filter funnel was packed under vacuum with 200 g silica gel 60H for thin layer chromatography. This gave a layer 5 cm deep. The funnel was attached to a 500 ml Buchner flask. Petroleum spirit was then run through the column four times to ensure the solvent was evenly absorbed. The plant extract in ethyl acetate (3 g extract in 500 ml of ethyl acetate) was then combined with 3 g of silica gel 60H. The solvent was then removed *in vacuo* leaving the extract bound to the silica. This sample was then finely ground and placed in an even layer on the column. Solvents were then passed through the column under vacuum in 500 ml quantities. Each fraction was taken off the column and dried *in vacuo*. The elution was carried out using a stepped gradient of mixtures of petroleum ether, ethyl acetate and methanol. All of the fractions that were separated were assayed for activity using the leaf dip bioassay.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: The fractions obtained from the chromatographic separation that gave the highest level of toxicological activity were then analysed further using proton and carbon^{13} nuclear magnetic resonance spectroscopy. This technique has been widely used in the past to identify chemical compounds within plant extracts (Lumonadio and van haelen, 1986; Pieters and Vlietinck, 1989; Coates *et al.*, 1994).

Results

Table 1 gives the extract yields from the preliminary fractionation carried out with the soxhlet apparatus. Extract yields in the solvents ranged from

TABLE 1

Extract yields following soxhlet extraction of plant material.

Rep.	Dried plant used (g)	Petroleum Spirit Yield	Ethyl Acetate Yield	Methanol Yield
1	149	7.73 g	2.50 g	3.04 g
2	172	5.12 g	2.13 g	4.74 g
3	172	6.35 g	3.53 g	4.23 g

ca. 6 g (petroleum spirit) to ca. 3 g (ethyl acetate). The results of the bioassays that were then carried out with these fractions are shown in Figure 1. At an extract concentration of 0.1mg/ml both the ethyl acetate and methanol extracts were toxic to spider mites. This result was statistically significant ($F = 52.24$, $P < 0.001$). The criteria for significance used was the 5% level and 95% confidence limits were calculated as a means separation test following one-way ANOVA. The petroleum spirit extract was not toxic to mites and the results for this extract are not shown.

The ethyl acetate extract, despite giving a lower level of mortality than the methanol extract, was selected for further fractionation under vacuum. The rationale for this selection was that this fraction was the most suited for vacuum liquid chromatography. The results concerning the toxicity of the resulting fractions are shown in Table 2. Fractions that were toxic to spider mites were detected over the solvent mixture range of 70/30 (petroleum spirit/ethyl acetate) to 30/70. The fraction with the highest acaricidal activity within this range was then analysed using nuclear magnetic spectroscopy.

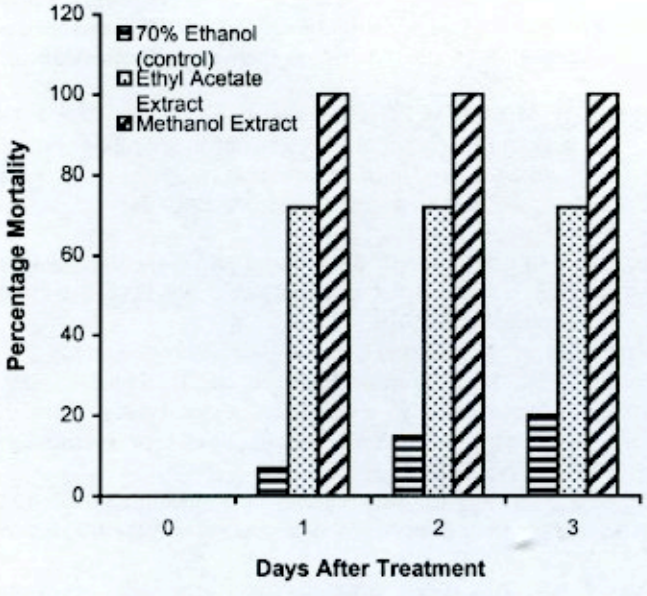

Figure 1. The toxicity of extracts from the soxhlet extraction to the spider mite tetranychus urticae. Statistical analyses of the replicated toxicity tests gave an F-ratio of 52.24 ($P<0.001$).

TABLE 2

Fractions Obtained from Vacuum Liquid Chromatography and their Levels of Activity Against Mites in a Leaf Dip Bioassay.

Extract Fraction (% Petroleum Spirit/Ethyl Acetate)	Statistically Significant Mortality (>60%)
100/0	Not significant
90/10	Not significant
80/20	Not significant
70/30	$P<0.05$
60/40	$P<0.05$
50/50	$P<0.05$
40/60	$P<0.05$
30/70	$P<0.05$
20/80	Not significant
10/90	Not significant
0/100	Not significant

The proton and carbon13 spectra produced by NMR spectroscopy are available from the authors. The spectra showed signals that comprised 11 * C-H2, 8 * C-H, 1 * C=C, and 3 * C-C bonds. The spectra also showed the presence of a glucose molecule. The spectra were the same as that given by Kong *et al.* (1988). The major molecule identified was therefore a phytosterol. Some minor compounds were also present in the spectra and the authors believe that these are probably toxicologically inactive flavinoids. A suggested structure for the major molecule identified is given in Figure 2. This molecule is a phytosterol glucoside. The phytosterol base is sitosterol and the suggested molecular structure shows β-sitosterol-3-glucoside.

Discussion and Conclusions

The compound sitosterol is a major phytosterol that is found in plants and algae (Harwood and Russell, 1984). The major compound in our sample was

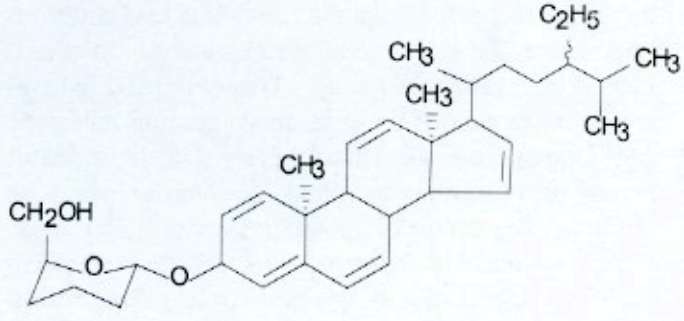

Figure 2. The chemical structure of daucosterol.

TABLE 3

Plants that are known to contain the chemical compound Daucosterol.

Plant Species	Plant part used for extract
Adenophora stenanthina	Roots
Agrimonia pilosa	Roots
Aralia elatia	Wood
Belamcanda globilus	Flowers
Gentiana tibetica	Leaves and Stems
G. macrophylla	Roots
Mirabilis himalicia	Roots
Oplopanax elatus	Stems
Rabdosia angostifolia	Roots
Tribulus terrestris	Leaves
Zea mays	Leaves

probably a sterol glucoside formed by a β-glycosidic linkage between the 3-hydroxyl of sitosterol and the 1-position of glucose. This compound is β-sitosterol-3-glucoside. It has also been called daucosterol. This chemical has been found in many plant species and is not novel. For example, it has been recorded in *Oplopanax elatus* (Zhang *et al.*, 1993), in *Gentiana tibetica* (Zhang and Yang, 1994), in *Eucalyptus Globulus* (Santos *et al.*, 1997), and in *Mirabilis himalacia* (Zhang *et al.*, 1997), to name but a few. A more extensive list of the plants in which daucosterol has been extracted is given in Table 3.

The literature records that the primary use of extracts containing daucosterol have so far been medical. For example, extracts of *A. Pilosa* are cited as being effective in the treatment of cancer (Koshiura *et al.*, 1985), Hsu *et al.* (1987) reports antihaemostatic activity for daucosterol extracts, and other researchers have investigated the usefulness of extracts for the treatment of diabetes (e.g. Molokovskii *et al.*, 1989). To the best of the authors knowledge, references to the use of this compound in relation to the control of arthropod pests do not so far exist.

The data presented in this paper indicate that the plant extracts assayed were toxic to spider mites and that the major chemical constituent of fractionated extracts was the compound daucosterol. It is tempting therefore to conclude that the compound daucosterol is responsible for the toxicological activity observed. Indeed, the chemical structure of daucosterol (Figure 2) is not unlike that of the arthropod moulting hormone ecdysone (Figure 3). This suggests that the mode of action of the compound may be interference with hormonal regulation of cuticular structure. Certainly, eyeball evidence of the dead mites recorded seemed to suggest a degradation of the pest species exoskeleton. Quite clearly further research is required. However, for the time being we conclude that the compound

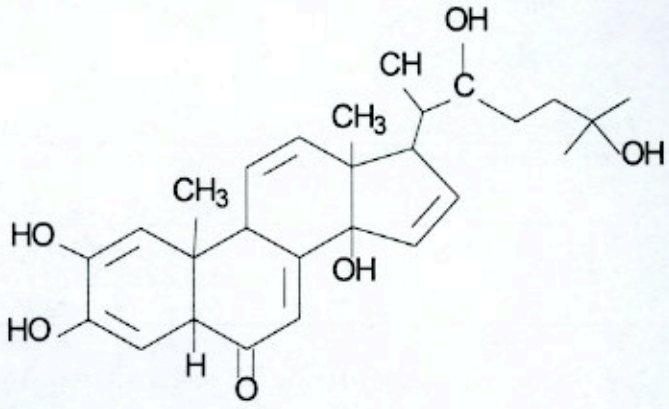

Figure 3. The chemical structure of ecdysone.

daucosterol may have a role to play in the control of arthropod pest species.

References

Ahmed, S. and M. Grainge. 1986. Potential of the neem tree (*Azadirachta indica*) for pest control and rural development. *Economic Botany*40(2):201-209.

Coates, P.A., I.S. Blagbrough, D.J. Hardick, M.G. Rowan, S. Wonnacott, and B.V.L. Potter. 1994. Rapid and effective isolation of the nicotinic receptor antagonist methyllycaconitine from *Delphinium*: assignment of the methylsuccinimide absolute stereochemistry and S. *Tetrahedron Letters* 35(46):8701-8704.

Chopra, R.N., R.L. Bhadwar, and S. Ghosh. 1949. Poisonous plants of India. *Scientific Monograph* No.17, 10-12. Indian Council of Agricultural Research, New Delhi, India.

Coll, J.C. and D.F. Bowden. 1986. The application of vacuum liquid chromatography to the separation of terpene mixtures. *Journal of Natural Products* 49(5):934-936.

Harwood, J.L. and N.J. Russell. 1984. *Lipids in Plants and Microbes*. George Allen & Unwin Publishers, UK.

Hsu, M.F., J.H. Young, J.P. Wang, and C.M. Teng. 1987. Effect of hseien -ho-t'sao (*Agrimonia pilosa*) on experimental thrombosis in mice. *American Journal of Chinese Medicine* 15(1-2):43-51.

Hutton, E.M., J.R.M. Thacker, and C.L. Anand. 1996. The toxicity of four tropical plant extracts to arthropod pests of medical and agricultural importance. In: *Proceedings of the Brighton Crop Protection Conference 1996*, 241-422. BCPC Publications Ltd, UK.

Jepson, P.C. 1993. *Insects, Spiders and Mites*. In: Handbook of Ecotoxicology, P. Calow (Editor) 299-325. Blackwell Scientific Publications, UK.

Kong, L.Y., C.J. Shao, and J.D. Xu. 1988. *Zhaongcaoyao* 19:2-3.

Koshiura, R., K. Miyamoto, Y. Ikeya, and H. Taguchi. 1985. Antitumor activity of methanol extract from roots of *Agrimonia pilosa* Ledeb. *Japanese Journal of Pharmacology* 38(1):9-16.

Lumonadio, L and M. van Haelen. 1986. Indole alkaloids and quassin from *Quassia africana*. *Journal of Natural Products* 49(5):940-942.

McChesney, J.D. 1994. After discovery: the issue of supply strategies in the development of natural products. *Bioregulators for Crop Protection and pest Control*, 144-151. American Chemical Society Symposium Series, ACS, Philadelphia, USA.

McLaren, J.S. 1986. Biologically active substances from higher plants: status and future potential. *Pesticide Science* 17:559-578.

Molokovskii, D.S., V.V. Davydov, and V.V. Tiulenev. 1989. The action of adaptogenic plant preparations in experimental alloxan diabetes. *Problemy Endokrinologii* 35(6):82-87.

Pellitier, S.W., H.P. Chokshi, and H.K. Desae. 1986. Separation of diterpenoid alkaloid mixtures using VLC. *Journal of Natural Products* 49(5):892-900.

Pieters, L.A.C. and A.J. Vlietinck. 1989. Vacuum liquid chromatography and quantitative ^{1}HNMR spectroscopy of tumour-promoting diterpene esters. *Journal of Natural Products* 52(1):186-190.

Santos, G.G., J.C.N. Alves, J.M.L. Rodilla, A.P. Duarte, and A.M. Lithgow. 1997. Terpenoids and other constituents of *Eucalyptus globulus*. *Phytochemistry* 44(7):1309-1312.

Secoy, D.M. and A.E. Smith. 1983. Use of plants in control of agricultural and domestic pests. *Economic Botany* 37:28-57.

Villansenor, I.M., P. Lemon, A. Palileo, and J.B. Bremmer. 1996. Antigenotoxic spinasterol from *Cucurbita maxima* flowers. *Mutation Research* 360:89-93.

Wells, C., W. Bertsch, and M. Perich. 1993. Insecticidal volatiles from the marigold plant (genus *Tagetes*): effects of species and sample manipulation. *Chromatographia* 35(3-4):209-215.

Yang, R.Z. and C.S. Tang. 1988. Plants used for pest control in China: a literature review. *Economic Botany* 42(3):376-406.

Zhang, H.G., G.X. Wu, and Y.M. Zhang. 1993. Chemical constituents from stems of *Oplopanax elatus* Nakai. *Chung Kuo Chung Yao Tsa Chih* 18(2):104-105.

Zhang, Y.Y. and C.R. Yang. 1994. 2 Triterpenoids from *Gentiana tibetica*. *Phytochemistry* 36(4):997-999.

Zhang, G.L., Q.Y. Xing, and M.Z. Zhang. 1997. Glycolipids from *Mirabilis himalacia*. *Phytochemistry* 45(6):1213-1215.