Journal of Chromatography, 447 (1988) 277–283
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 535

Note

Isolation and purification of salannin from neem seeds and its quantification in neem and chinaberry seeds and leaves

R. BRYAN YAMASAKI, THOMAS G. RITLAND, MARK A. BARNBY and JAMES A. KLOCKE*
NPI, University of Utah Research Park, 417 Wakara Way, Salt Lake City, UT 84108 (U.S.A.)
(Received April 8th, 1988)

Salannin ($C_{34}H_{44}O_9$) is a biologically active tetranortriterpenoid found in at least four species of plants in the family Meliaceae, including *Azadirachta indica* A. Juss. (neem)[1,2], *Melia azedarach* L. (chinaberry)[3], *M. dubia* Cav.[4] and *M. volkensii* Gurke[5]. The biological activities of salannin include insect feeding deterrency against *Musca domestica* L. (house fly)[6], *Acalymma vittatum* (F.) (striped cucumber beetle), *Diabrotica undecimpunctata howardi* Barber (spotted cucumber beetle)[7], *Spodoptera littoralis* (Boisd.) (Egyptian cotton leafworm), *Earias insulana* (Boisd.) (spiny bollworm)[8], *Aonidiella aurantii* (Maskell) (California red scale) and *Locusta* sp. (locust)[9]. In addition, salannin is an insect growth inhibitor against *Heliothis virescens* (F.) (tobacco budworm)[10].

Salannin has been isolated from crude plant extracts using a variety of techniques, either singly or in combination, including recrystallization[3], column chromatography[4,5,10,11], preparative normal-phase thin-layer chromatography (TLC)[12], and preparative reversed-phase high-performance liquid chromatography (HPLC)[6]. In this paper, we report on the preparative isolation of salannin of single-peak purity from high salannin-yielding neem seeds utilizing flash chromatography combined with HPLC. In addition, we report on the HPLC analysis of samples of neem and chinaberry seeds and leaves from different geographic regions in order to quantify their content of salannin.

MATERIALS AND METHODS

Extraction of salannin from neem seeds

A suspension of 29 kg of ground neem seeds (obtained from Senegal by Vikwood, Sheboygan, WI, U.S.A.) in 35 l of *n*-hexane was agitated occasionally at room temperature for 24 h. The hexane extract was decanted and the process was repeated with fresh *n*-hexane five more times. The hexane extracts were pooled and concentrated *in vacuo* to yield 8.1 kg of a brown oil.

Thin-layer chromatography

Normal-phase analytical TLC was performed on 20 × 20 cm prescored silica gel GHLF plates (0.25 mm, Analtech), using one of four solvent systems listed in Table I. Reversed-phase analytical TLC was performed on 1 × 3 in. $MKC_{18}F$ plates

0021-9673/88/$03.50

TABLE I

R_F VALUES OF SALANNIN IN VARIOUS SOLVENT SYSTEMS ON SILICA GEL AND OCTADECYLSILYLSILICA GEL (ODS) ANALYTICAL TLC PLATES

Solvent system (v/v)	R_F	*TLC plate*
Dichloromethane	0	Silica gel
Diethyl ether	0.32	Silica gel
Diethyl ether–methanol (99:1)	0.45	Silica gel
Dichloromethane–methanol (19:1)	0.57	Silica gel
Methanol–water (4:1)	0.37	ODS

(0.20 mm, Whatman) using methanol–water (4:1) (Table I). Visualization for analytical TLC was accomplished under shortwave UV light, followed by spraying with a vanillin–sulfuric acid–ethanol (3:1.5:100, w/v/v) spray reagent and heating with a hot air gun.

Flash column chromatography

A 2.0-l flash column (Aldrich) was packed with silica gel (40-μm particle size, 18 × 7.0 cm I.D., J. T. Baker) and equilibrated with dichloromethane. The brown oil from the hexane extract described above was diluted with an equal volume of dichloromethane and applied in 1-kg batches onto the top of the column. After flushing the column at 20 ml/min with 1.5 l of dichloromethane, salannin was eluted with diethyl ether at 20 ml/min into fractions of 20 ml. Fractions containing salannin, as determined by TLC (Table I), were pooled and rotary evaporated *in vacuo* yielding 127 g total of a dark brown tarry material. This material was dissolved in 500 ml of methanol, filtered, diluted with 500 ml of methanol–water (3:2), and applied in 100-ml batches onto the top of a second flash column (2.0 l, Aldrich) packed with octadecylsilylsilica gel (ODS) (40-μm particle size, 18 × 7.0 cm I.D., Regis) in methanol–water (4:1). The column was eluted with methanol–water (4:1) at 20 ml/min into fractions of 20 ml. Fractions containing salannin, as determined by TLC, were pooled and rotary evaporated *in vacuo* to yield 34.3 g total of a pale yellow solid.

High-performance liquid chromatography

Preparative HPLC was carried out with a Hewlett-Packard Model 1081B liquid chromatograph. Samples were dissolved in their respective solvents and injected onto the column using a Negretti and Zambra injector. Effluents were detected at 214 nm using a Pharmacia single-path monitor UV-1/214 optical unit and UV-1 control unit. Detected peaks and retention times (t_R) were recorded using a Hewlett-Packard 3388A integrator.

The first of two preparative HPLC steps was done with a Phenomenex silica gel (5-μm particle size) stainless-steel column (25 × 2.0 cm I.D.) eluted isocratically with isopropanol–*n*-hexane (1:19) at a flow-rate of 5.0 ml/min and an average pressure of 630 p.s.i. The column was protected with a Whatman stainless-steel guard column (5.0 × 0.46 cm I.D.) packed with Whatman pellicular silica gel. The second preparative HPLC step was performed on a Phenomenex phenyl (5-μm particle size) stainless-steel column (25 × 2.25 cm I.D.) eluted isocratically with acetonitrile–water (2:3) at a flow-rate of 5.0 ml/min and an average pressure of 1500 p.s.i. This column

was protected with a Whatman stainless-steel guard column (5.0 × 0.46 cm I.D.) packed with Alltech pellicular phenyl.

Analytical HPLC was carried out on a reversed-phase Phenomenex phenyl (5-μm particle size) stainless-steel column (25 × 0.46 cm I.D.) using the same liquid chromatograph, corresponding guard column, detector, and injector system as described above. The solvent system consisted of acetonitrile–water (2:3) run isocratically at a flow-rate of 1.5 ml/min and an average pressure of 2100 p.s.i. Detected peaks were integrated (valley to valley) and retention times were recorded using a Hewlett-Packard 3388A integrator set at a peak width of 0.3, threshold of 0, attenuation of 2, and chart speed of 0.25 cm/min.

A second analytical HPLC method was carried out on a normal-phase Alltech silica gel (5-μm particle size) stainless-steel column (25 × 0.46 cm I.D.) eluted isocratically with isopropanol–*n*-hexane (1:9) at a flow-rate of 1.5 ml/min and an average pressure of 725 p.s.i.

Quantification

Between 2 and 100 g of ground neem seeds (obtained from Senegal, Benin, or Haiti by Vikwood, or from India by Tree Seeds International, Chevy Chase, MD, U.S.A.), neem leaves (obtained from plants grown in the greenhouse from Indian neem seeds), chinaberry seeds (collected in Hurricane, UT, U.S.A. in July and November, 1985), or chinaberry leaves (obtained from plants grown in the greenhouse from Utah chinaberry seeds) were exhaustively extracted in excess *n*-hexane at ambient temperatures. The hexane extracts were decanted and the process was repeated with fresh *n*-hexane three more times. The pooled hexane extracts were concentrated *in vacuo*. Following dilution with 10–100 ml of dichloromethane, 400 μl of the extracts were chromatographed on 20 × 20 cm prescored silica gel GHLF TLC plates (250 μm, Analtech) using dichloromethane–methanol (19:1) as the mobile phase. Visualization under UV light revealed the salannin standard that was run alongside the extracts. The band corresponding to the standard was cut from the plates, eluted with excess methanol, and injected onto an analytical Phenomenex phenyl (5 μm particle size) stainless-steel column (25 × 0.46 cm I.D.) using the same liquid chromatograph, corresponding guard column, detector, injector system, and solvent system as described above. Salannin was quantified by comparing its HPLC peak height with a linear standard curve generated from pure authentic salannin.

Identification of salannin

Salannin isolated from neem seeds was identified by spectral comparison (IR, ^{1}H NMR, electron impact mass spectrometry) and co-chromatography (TLC, HPLC) with an authentic sample.

RESULTS AND DISCUSSION

Similar to previously reported extraction methodologies[4,13], we used *n*-hexane to extract salannin from the neem and chinaberry samples (Fig. 1). Following exhaustive extraction of the samples with *n*-hexane, we detected only trace (<0.5%) amounts of salannin remaining in the marc. The hexane-defatted marc was utilized for the subsequent isolation of azadirachtin, another biologically active tetranortriterpenoid of academic and industrial interests[14].

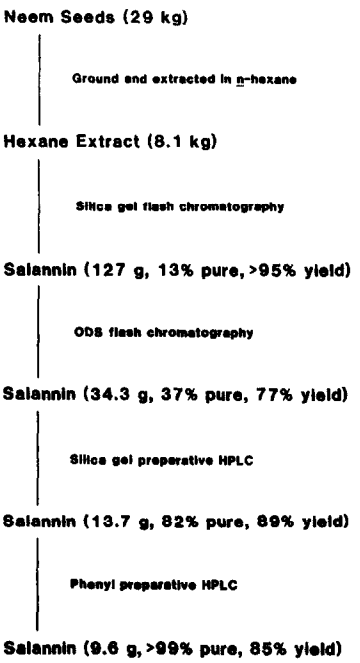

Fig. 1. Flow diagram of the isolation of salannin from neem seeds.

The majority of the components in the hexane extract consisted of fatty materials and other non-polar substances. These fatty materials may be of use in soap manufacturing[15].

Salannin could be easily separated from the fatty materials by silica gel flash chromatography using dichloromethane as the mobile phase. Under these conditions, salannin has an R_F value of zero (Table I), whereas the fatty material is virtually unretarded. This allowed for as much as a kilogram of hexane extract to be loaded onto the 2.0-l flash column at a time. Following elution of the fatty material with dichloromethane, the column was eluted with diethyl ether and the fractions containing salannin were combined, rotary evaporated to dryness *in vacuo*, and subjected to ODS flash chromatography using methanol–water (4:1) as the mobile phase. The diethyl ether and the methanol–water (4:1) solvent systems were chosen as the mobile phases on silica gel and ODS flash chromatography, respectively, since the R_F values of salannin in these solvent systems on analytical TLC are 0.32 and 0.37, respectively. The optimum R_F for separation of a compound using flash chromatography is 0.35[16].

Other TLC solvent systems in which the R_F of salannin approximated 0.50 were used to monitor its presence in the flash chromatography eluent. For example, the R_F values of salannin on silica gel TLC developed in 1% methanol in diethyl ether and 5% methanol in dichloromethane were 0.45 and 0.57, respectively. A vanillin spray reagent was used to visualize salannin on the TLC plates since it consistently gave a characteristic red color reaction that rapidly changed to purple.

From a complex mixture, the two flash chromatography steps concentrated salannin to 37% purity in preparation for the final purification by HPLC (Figs. 1

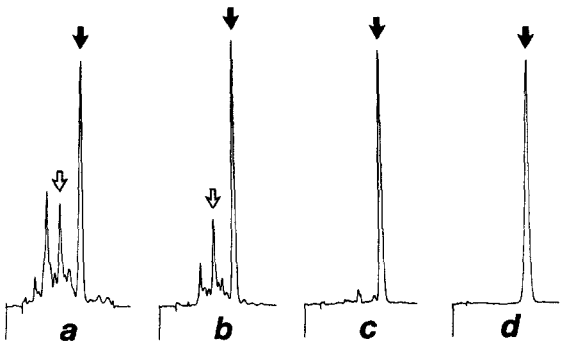

Fig. 2. Analytical HPLC of salannin (solid arrows) recovered after (a) silica gel flash chromatography; (b) ODS flash chromatography; (c) silica gel preparative HPLC; (d) phenyl preparative HPLC. Hollow arrows indicate 3-deacetylsalannin. Analytical HPLC was performed with a phenyl column (25 × 0.46 cm I.D., 5-μm particle size) eluted isocratically with acetonitrile–water (2:3) at 1.5 ml/min.

and 2). We were able to further concentrate salannin to 82% purity by silica gel preparative HPLC (Figs. 1 and 2). We found this HPLC step necessary to remove an impurity not well separated from salannin by phenyl preparative HPLC. In addition, we were able to isolate a total of 1.5 g of 3-deacetylsalannin at this step (Fig. 2). Typically, each 0.9-ml injection, containing 0.35 g of 37% pure salannin in isopropanol, yielded 0.14 g of 82% pure salannin. The appproximate t_R for salannin in this normal-phase system was 46 min.

Salannin of single peak purity could be obtained by further chromatography on phenyl preparative HPLC (Fig. 1). Typically, each 0.7-ml injection, containing 0.25 g of 82% pure salannin in acetonitrile, yielded 0.19 g of 98% pure salannin. In our hands, rechromatography by phenyl preparative HPLC was necessary to purify salannin to single-peak purity. The approximate t_R for salannin in this reversed-phase system was 80 min.

Instead of TLC, the greater resolving power of analytical HPLC was used to determine which fractions from preparative HPLC were appropriate for pooling. We used normal- and reversed-phase analytical HPLC, modifications of our previously reported isolation method for azadirachtin[14], to detect salannin, the t_R values of which were 10.9 and 10.0 min, respectively.

We chose flash chromatography for our prepurification technique, before the final purification by preparative HPLC, since it is a rapid, inexpensive, and easily performed technique with a large sample capacity[14,16]. Flash chromatography combined with phenyl preparative HPLC can generate salannin of >90% purity, adequate for certain investigations. For example, in our structure–activity relationship studies, we use salannin of 90% purity since we purify the chemically modified derivatives from their reaction mixtures.

Absolutely pure salannin was difficult to obtain in our hands, due to the complexity-in-structure of the chemicals found in neem seeds[5,10,13,17–19]. We found silica gel preparative HPLC necessary to remove some contaminants which co-chromatographed with salannin on phenyl HPLC.

In our quantification method, we prepurified the plant hexane extracts by silica

TABLE II

YIELD OF SALANNIN FROM NEEM AND CHINABERRY SEEDS AND LEAVES

Plant material (source)	*Yield (g kg^{-1})*
Neem seed (Benin)	1.3
Neem seed (Senegal)	1.2
Neem seed (India)	0.7
Neem seed (Haiti)	0.4
Neem leaves (India; greenhouse)	Trace
Chinaberry seed (Utah, U.S.A.; July collection)	Trace
Chinaberry seed (Utah, U.S.A.; November collection)	Trace
Chinaberry leaves (Utah, U.S.A.; greenhouse)	Trace

gel TLC in preparation for phenyl analytical HPLC. Known amounts of pure salannin were similarly chromatographed by TLC and the total amount was subsequently detected by phenyl HPLC. The lower limit of sensitivity of our quantification method was 0.5 μg and the linearity range was 0.5-10.0 μg.

We found that neem seeds from the African countries of Benin and Senegal were the two highest salannin-yielding sources (1.3 and 1.2 g kg^{-1}, respectively) that were available to us (Table II). Our neem seeds obtained from India and Haiti contained less salannin (0.7 and 0.4 g kg^{-1}, respectively). The leaves excised from neem plants grown in the greenhouse from our Indian neem seed yielded only trace amounts of salannin (*i.e.*, <0.02 g kg^{-1}). Additionally, two samples of chinaberry seed collected during different times of the year in Utah, U.S.A. and leaves excised from plants grown in the greenhouse from our Utah chinaberry yielded only trace amounts of salannin. Therefore, we chose the Senegalese neem seed for our isolation scheme detailed in this paper since it was a high salannin-yielding source available to us in sufficient quantity.

We are currently preparing derivatives of our purified salannin for testing in insect bioassays. Several of our derivatives have shown significantly enhanced activity compared to salannin. The results will be published elsewhere.

ACKNOWLEDGEMENTS

We thank Prof. I. Kubo (University of California, Berkeley, CA, U.S.A.) for an authentic sample of salannin, R. O. Larson (Vikwood, Sheboygan, WI, U.S.A.) and B. Ross (Tree Seeds International, Chevy Chase, MD, U.S.A.) for the neem seeds used in this study, M. V. Darlington for technical assistance, and B. Gandy for typing the manuscript. This work was supported by a grant awarded by the U.S. Department of Agriculture (87-SBIR-8-0148).

REFERENCES

1 R. Henderson, R. McCrindle, K. H. Overton and A. Melera, *Tetrahedron Lett.*, (1964) 3969.
2 R. Henderson, R. McCrindle, A. Melera and K. H. Overton, *Tetrahedron*, 24 (1968) 1525.
3 S. D. Srivastava, *J. Nat. Prod.*, 49 (1986) 56.
4 L. B. de Silva, W. Stöcklin and T. A. Geissman, *Phytochemistry*, 8 (1969) 1817.
5 M. S. Rajab, M. D. Bentley, A. R. Alford and M. J. Mendel, *J. Nat. Prod.*, 51 (1988) 168.

6 J. D. Warthen, Jr., E. C. Uebel, S. R. Dutky, W. R. Lusby and H. Finegold, *U.S. Dept. Agric., Sci. Educ. Admin., Agric. Res. Results*, Northeast. Ser., No. 2, Beltsville, MD, 1978, 11 p.
7 D. K. Reed, J. D. Warthen, Jr., E. C. Uebel and G. L. Reed, *J. Econ. Entomol.*, 75 (1982) 1109.
8 J. Meisner, K. R. S. Ascher, R. Aly and J. D. Warthen, Jr., *Phytoparasitica*, 9 (1981) 27.
9 J. D. Warthen, Jr., *U.S. Dept. Agric., Sci. Educ. Admin., Agric. Rev. Manuals*, Northeast. Ser., No. 4, Beltsville, MD, 1979, 21 pp.
10 I. Kubo, A. Matsumoto, T. Matsumoto and J. A. Klocke, *Tetrahedron.*, 42 (1986) 489.
11 M. Harris, R. Henderson, R. McCrindle, K. H. Overton and D. W. Turner, *Tetrahedron*, 24 (1968) 1517.
12 K. Nakanishi, *Rec. Adv. Phytochem.*, 9 (1975) 283.
13 K. K. Purushothaman, K. Duraiswamy and J. D. Connolly, *Phytochemistry*, 23 (1984) 135.
14 R. B. Yamasaki, J. A. Klocke, S. M. Lee, G. A. Stone and M. V. Darlington, *J. Chromatogr.*, 356 (1986) 220.
15 C. M. Ketkar, *Utilization of Neem (Azadirachta indica juss) and its Bye-Products*, Directorate of Nonedible Oils and Soap Industry, Khadi & Village Industries Commission, Bombay, 1976, p. 5.
16 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 43 (1978) 2923.
17 D. A. H. Taylor, *Prog. Chem. Org. Nat. Prod.*, 45 (1984) 1.
18 W. Kraus, in Atta-ur-Rahman and P. W. LeQuesne (Editors), *New Trends in Natural Products Chemistry*, Elsevier, Amsterdam, 1986, p. 237.
19 S. Siddiqui, B. S. Siddiqui, S. Faizi and T. Mahmood, *J. Nat. Prod.*, 51 (1988) 30.