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USE OF POTATO DISC AND BRINE SHRIMP BIOASSAYS TO DETECT ACTIVITY AND ISOLATE PICEATANNOL AS THE ANTILEUKEMIC PRINCIPLE FROM THE SEEDS OF *EUPHORBIA LAGASCAE*

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ABSTRACT.—EtOH extracts of the seeds of *Euphorbia lagascae* inhibited crown gall tumors on potato discs, were active against 3PS (in vivo) and 9PS (in vitro) mouse leukemia, and were toxic to brine shrimp. Using the potato disc and brine shrimp bioassays to monitor fractionation, piceatannol (3,4,3′,5′-tetrahydroxystilbene) was isolated as the 3PS active principle. Aesculetin (6,7-dihydroxycoumarin) was isolated in large quantity, was weakly cytotoxic in 9KB, but was inactive in the other bioassay systems. Sucrose was also isolated. A combination of the simple crown gall and brine shrimp bioassays can thus be used both to detect and to isolate plant antitumor substances, minimizing the need for extensive antitumor testing in vivo.

The Eagles KB (9KB) cell culture system and the 3PS (P388) in vivo mouse leukemic system have been used extensively by contractors of the National Cancer Institute (NCI) as screens for plant antitumor extracts and monitors of extract fractionation (1, 2). The need for more rapid and less expensive antitumor prescreens is well recognized at NCI; a number of such prescreens have been proposed (3, 4). Perdue (5) has recently advocated the use of 9KB alone as the principal screen for antitumor extracts, largely on the grounds that most of the plant-derived compounds that have gone into clinical testing came from extracts that gave positive results in 9KB. However, consistent correlation between 9KB and 3PS activities in screening extracts is not evident.[2]

Galsky *et al.* (6, 7) have suggested that the inhibition of crown gall tumor initiation and growth, induced on discs of potato tubers by Ti plasmids from *Agrobacterium tumefaciens*, offers a possible solution to this problem. We have modified Galsky's potato disc assay and have screened a number of compounds and extracts; the combined results of our study (8) with those of Galsky *et al.* (6, 7) now reveal agreement between the potato disc assay and 3PS in 37 of 44 separate determinations. The statistical correlation is excellent between the two assays ($p=2 \times 10^{-6}$; κappa=0.69). The statistical agreement between 3PS and the two cytotoxicity assays (9KB and 9PS) in our screening of euphorb extracts is, on the other hand, quite poor (8). Although more extensive data may be needed, it appears that the crown gall potato disc system is fairly accurate in predicting 3PS in vivo antileukemic activity; it provides some false positives but few false negatives. Furthermore, its speed and simplicity promise to expedite both screening

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[2]In our laboratory at NRRC, 62 crude extracts (mainly from seeds) have been reported as confimed actives after screening over 1,100 samples (9) by NCI contractors. Of these, 40% were active in 9KB only, 31% were active in 3PS only, and only 19% were active in both 3PS and 9KB; the remaining 6% were active only in one of the systems since discarded by NCI. Because of changes in NCI protocols over the years, these figures can be no more than rough approximations, but they do suggest that, with 9KB as a single prescreen, a significant number of 3PS positives would be missed as false negatives and a high number of 9KB active/3PS inactive false positives might be expensive to eliminate.

and fractionation of extracts, in house, by obviating much of the slow and cumbersome process of obtaining bioassays from NCI contractors via parcel post.

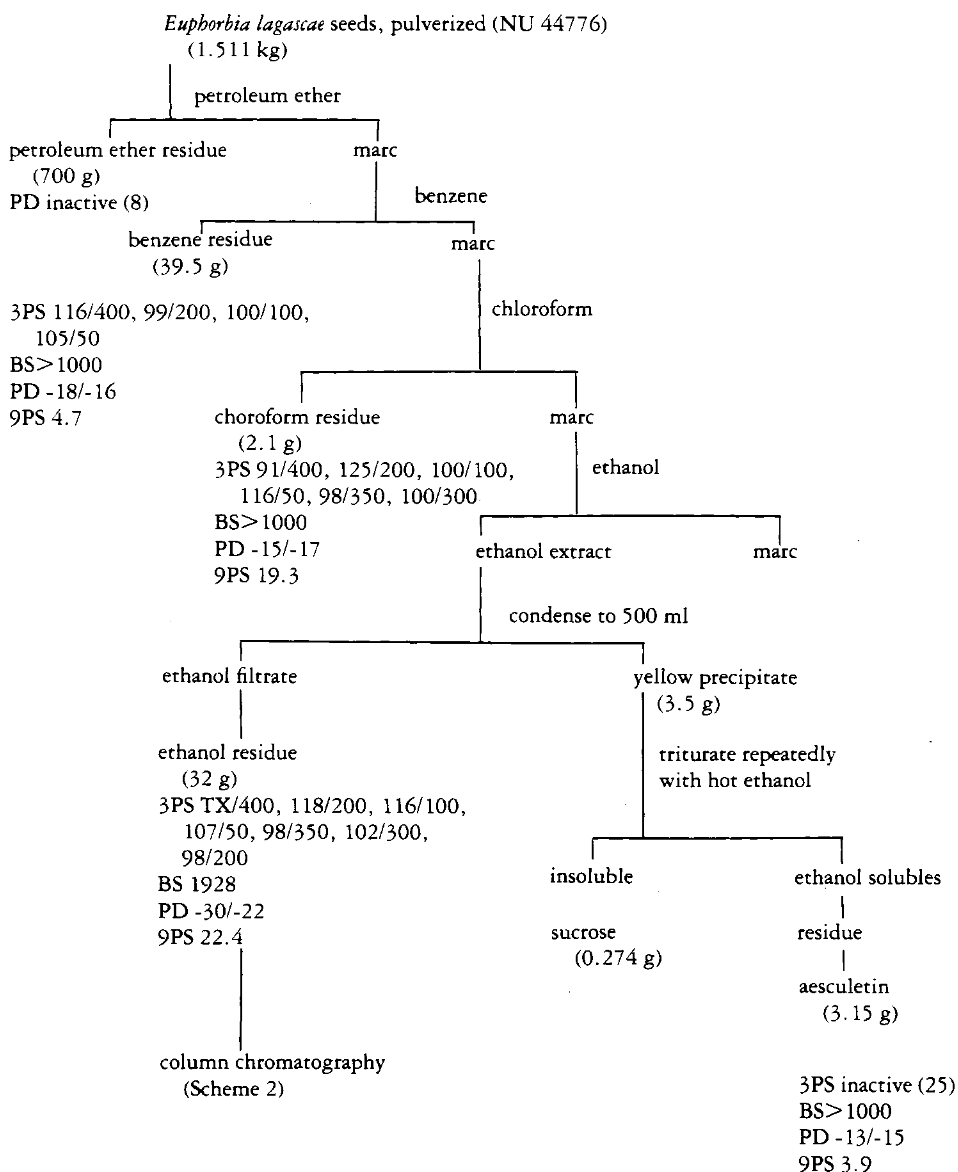
Another system available as a screen for biologically active compounds is the brine shrimp bioassay (10). This system is even faster, simpler, and less expensive than the Galsky potato disc bioassay, although it is a more general assay and is not specific for antitumor activity. Consequently, we undertook the fractionation of an active extract to explore the potential for using the brine shrimp bioassay together with the potato disc bioassay, with more frequent use of the easy and versatile brine shrimp assay for monitoring the numerous pools from column fractionations. Ultimately, compounds thus isolated that appeared active were checked against 3PS.

For this study, we selected extracts of the seeds from *Euphorbia lagascae* Spreng. An EtOH extract of these seeds was previously active in 3PS and 9PS as well as in the potato disc and brine shrimp systems (8, 10). *E. lagascae* seeds were extracted sequentially (Scheme 1) with petroleum ether, C_6H_6, $CHCl_3$, and EtOH. The potato disc inactive petroleum ether extract yielded a considerable amount of oil (56% w/w of dry seeds), which is known to contain a high content of 12,13-epoxyoleic acid (11). The last three extracts were each monitored with four bioassays (3PS, 9PS, brine shrimp, and potato disc). No consistent 3PS activity (Scheme 1) was obtained with any of these extracts; initial activities in the $CHCl_3$ and EtOH extracts were not confirmed. At this stage, the project would have been dropped were it not for significant potato disc activity (30%/22% tumor inhibition) in the EtOH extract. The best 9PS activity (LC_{50} 4.7 μg/ml) was exhibited in the C_6H_6 extract, which also showed hints of 3PS and potato disc activity, but fractionation of this residue led to no concentration of the 9PS activity.

Solvent manipulation of the EtOH extract (Scheme 1) yielded crystalline sucrose and aesculetin (6,7-dihydroxycoumarin), which has been found previously in the seeds of other euphorbs (12); aesculetin was inactive in all systems except 9PS, where cytotoxicity was observed (LC_{50} 3.9 μg/ml). Column chromatography of the remaining EtOH extract (Scheme 2) was performed on silica gel eluted with a gradient of MeOH in $CHCl_3$. Fractions were pooled on the basis of similar tlc patterns, especially with respect to compounds that fluoresced dark-blue under long wavelength uv light. Additional aesculetin was crystallized from the early column fractions (3-4) followed by a pool of fluorescent fractions (5-9) which exhibited brine shrimp activity (LC_{50} 650 μg/ml). A second silica gel column of these active fractions yielded additional aesculetin from fractions 4-6 but concentrated the brine shrimp activity (LC_{50} 320 μg/ml) in a subsequent pool of fluorescent fractions 8-11. A third silica gel column of this active pool yielded a new combination of fluorescent fractions 8-10 which gave a pale-yellow powder of the fluorescent compound; this compound (0.009% yield) was active in all four bioassays: brine shrimp LC_{50} 279 μg/ml; 9PS LC_{50} 2.5 μg/ml; potato discs 20/32% tumor inhibition; and 3PS 123% T/C at 25 mg/kg, confirmed with 131% at 25 mg/kg and 131% at 50 mg/kg. Insufficient material was isolated for 3PS testing at doses above 50 mg/kg.

Spectral analyses (uv, low and high resolution ms, and pmr suggested that the compound was *trans*-3,4,3′,5′-tetrahydroxystilbene (piceatannol; 3-hydroxyresveratrol). Comparisons with an authentic sample confirmed the identification. This known antifungal compound was first isolated from the heartwood of *Vouacapoua* species (13) and has been found in the heartwood of additional Leguminosae (14, 15), as well as elsewhere (16, 17). Piceatannol is a powerful inhibitor of photosynthesis in spinach chloroplasts (18) and may be an inhibitor of plant growth through promotion of indole acetic acid oxidase (19). The presence in seeds of such bioregulatory compounds is likely essential to the proper timing of germination and growth. Piceatannol has been synthesized (20).

SCHEME 1. Extraction of *Euphorbia lagascae* Seeds[a]



[a]3PS activity is assumed when plant extracts increase the life span of leukemic mice by ca. 20% or more (T/C>120%), e.g., 118/200 indicates 118% T/C at 200 mg/kg. (26).

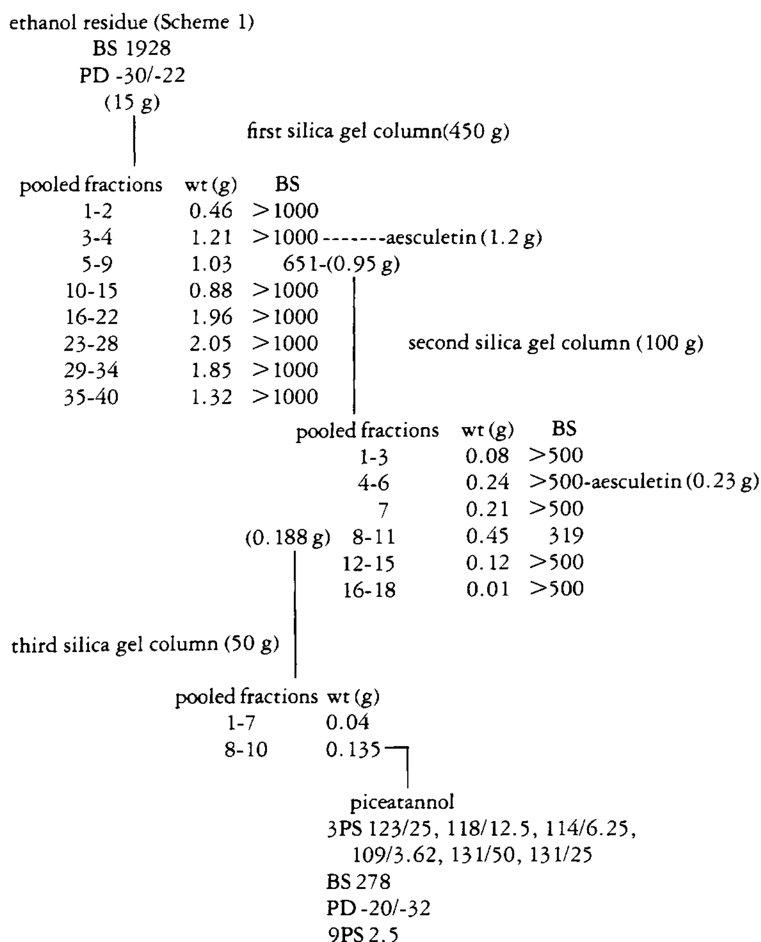
Brine shrimp (BS) activity is assumed when the LC_{50} is <1000 μg/ml (10).

Potato disc (PD) activity is assumed when the no. of crown gall tumors inhibited, vs. controls, is ca. 20% or more in two successive determinations (8), e.g., PD -30/-22 indicates 30% and 22% tumor inhibition in two successive determinations.

9PS activity is assumed when the LC_{50} of crude extracts is <30 μg/ml and <4 μg/ml for pure compounds (26).

While the animal estrogenic/antiestrogenic effects of the synthetic substituted stilbenes are well-known (21), the simple natural hydroxystilbenes, such as piceatannol, have apparently not been previously demonstrated to have in vivo antitumor activity (1-3, 9, 22-25). Thus, the antitumor activity of this class of natural compounds may be

SCHEME 2. Column Chromatographic Fractionation of Ethanol Residue from Seeds of *Euphorbia lagascae* Guided by Brine Shrimp Bioassay (BS) to Isolate Piceatannol[a]



[a]For definitions of bioassays see footnote to Scheme 1.

worthy of future study. This is the first report in which the in-house potato disc and brine shrimp bioassays have been used both to detect animal antitumor activity as a prescreen for 3PS and to monitor the plant fractionation to yield the 3PS active, antileukemic, component.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra were obtained in EtOH on a Perkin-Elmer Double Beam Coleman 124 Spectrometer. Melting points were determined using a Mettler FP5 and are uncorrected. Ir spectra were obtained in KBr pellets on a Beckman IR-33 Spectrophotometer. Pmr (80 MHz) were obtained in DMSO-d_6 on a Varian FT-80 Spectrometer and were referenced to TMS; pmr (470 MHz) were obtained in DMSO-d_6 on a Nicolet NTC-470. Reference aesculetin was purchased from Sigma Chemical Co.; reference piceatannol was kindly supplied by Dr. Mitsugi Kozawa, Osaka College of Pharmacy, Japan. Cims and eims were obtained using a Finnegan 4023; high resolution fab ms were obtained at Eli Lilly and Co. through the courtesy of Dr. John L. Occolowitz. 3PS tests were performed at Illinois Institute of Technology under a contract from the NCI; 9PS tests were performed at the Purdue Cell Culture Laboratory following protocols set by the NCI (26).

PLANT MATERIAL.—Authenticated seeds of *E. lagascae* were available from the seed collection (9) of

the USDA, NRRC, in Peoria. The accession used in the screening (8, 10) was part of collection no. NU 50787. The accession used for large-scale fractionation came from collection no. NU 44776. The seeds were ground with considerable difficulty, due to the high oil content, with a Wiley Mill.

EXTRACTION.—Pulverized plant material (1.511 kg) was extracted in sequence with 30-60° petroleum ether (Soxhlet), C_6H_6 (Soxhlet), $CHCl_3$ (percolation, 5 liters), and EtOH (percolation, 10 liters). Bioassays were performed in four different systems as summarized in Scheme 1.

ISOLATION OF SUCROSE AND AESCULETIN.—The EtOH extract was concentrated under reduced pressure to ca. 500 ml, and a yellow solid (3.5 g) was collected by filtration. The filtrate was condensed to give a brown residue (32 g). The yellow solid was triturated four times with 20-ml portions of hot EtOH, leaving a white insoluble material (0.274 g); this was identified as sucrose: mp 178° (dec.), Lit. mp 160-186° (dec.) (27); ir; pmr; and cochromatography with reference sucrose in five different tlc systems.

Condensing the combined solutions of hot EtOH solubles yielded yellowish crystals of aesculetin, 3.15 g (0.30% total yield from this and subsequent fractions); mp 269-71°, ref. mp 269-70°, Lit. mp 268-70° (27); cims, eims, ir, and pmr spectra were all indistinguishable from those of the reference aesculetin.

ISOLATION OF PICEATANNOL.—Potato disc-active residue of the EtOH extract (15 g) (Scheme 1) was chromatographed over three successive silica gel columns, advancing the brine shrimp-active fractions to the next column, as indicated in Scheme 2. Column fractions were pooled on the basis of similar tlc patterns (10% MeOH in $CHCl_3$ on silica gel), especially with respect to uv fluorescent components. The first column used a gradient of $CHCl_3$-MeOH, 300 ml of each mixture (100:0, 98:2, 96:4, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 50:50, 25:75, and 0:100); fractions of 100 ml each were collected. The second column employed a gradient of $CHCl_3$-MeOH, 100 ml of each mixture (100:0, 95:5, 90:10, 85:15, 80:20, and 50:50) with 100 ml fractions. The third column used a gradient of $CHCl_3$-MeOH, 100 ml of each mixture (100:0, 98:2, 97:3, 95:5, 93:7, 90:10, 87:13, 85:15, 80:20, and 70:30) with 100 ml fractions. Piceatannol (0.135 g, 0.009% yield) precipitated as a pale-yellow powder from a pool of fractions 8-10 from the third column. During the 24 h brine shrimp bioassays, piceatannol blackened the brine solutions, typical of catechol oxidations.

IDENTIFICATION OF PICEATANNOL.—Mp 229°, ref. mp 228°, Lit. mp 229° (13); uv EtOH 221, 240sh, 306sh, 326 nm; pmr (470 MHz, DMSO-d_6): 9.19 (1H, bs, -OH)), 9.10 (1H, bs, -OH), 8.93 (1H, bs. -OH), 8.81 (1H, bs, -OH), (the -OH protons all exchanged with D_2O), 6.96 (1H, d, J=1.96 Hz, H-2), 6.85 (1H, d, J=16 Hz, -C*H*=CH-), 6.83 (1H, dd, J=8.5, 2 Hz, H-6), 6.77 (1H, d, J=8.5 Hz, H-5), 6.70 (1H, d, J=16 Hz, -CH=C*H*-), 6.37 (2H, d, J=2.03, H-2′ and H-6′), 6.12 (1H, t, J=2.01, H-4′); eims (*m/z*, %): M^+ 244 (100), 277 (5), 197 (12); high resolution fab ms 244.07432 (calcd for $C_{14}H_{12}O_4$: 244.07356), assigned NSC 365798.

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