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Preliminary chemical examination of digitalis tissue cultures for cardenolides

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Three *Digitalis* species were grown as callus or suspension tissue cultures, extracted and examined by paper chromatography. Kedde-positive substances (cardenolide glycosides) were found.

REVIEWS of the potential of higher plant tissue cultures for producing useful compounds (Nickell, 1962; Staba, 1963), and procedures for growing digitalis as static (Staba, 1962) or suspension cultures (Staba & Lamba, 1963) have been published. This communication establishes that callus tissues, and the cells and medium from suspension cultures, of three *Digitalis* species contain secondary plant products similar to digitalis cardiac glycosides.

Material

TISSUE CULTURES

Digitalis lanata Ehrh., *Digitalis purpurea* L., and *Digitalis purpurea* var. *gloxinaeflora* Hort. tissue cultures were grown on our modification (Staba & Lamba, 1963) of Murashige's and Skoog's tobacco medium with (Medium A) and without (Medium B) 2,4-dichlorophenoxyacetic acid (2,4-D) 1×10^{-6}. The tissue cultures were established from germinated seeds, and has been subcultures at three 6-week intervals for approximately 2 years. The callus tissues extracted were approximately 6 weeks old, and the suspension cultures extracted were 3 weeks old. *D. lanata* tissue cultures form organised structures when grown in Medium B, but principally unorganised cell aggregates when grown in medium A (Staba & Lamba, 1963).

Callus tissues were grown in 1-oz. wide-mouth prescription vials (18 ml medium) and cell suspensions in 250 ml Erlenmeyer flasks (50 ml medium) at room temperature (about 27°) and light conditions. Suspension cultures were grown on a reciprocal shaker (88 cycles/min; 9 cm stroke).

Experimental

GENERAL PROCEDURES

Methanol (50%) was used for the initial extraction of callus tissue and suspension cells. The liquid medium from suspension cultures was diluted with methanol to a 50% concentration. Methanolic extracts were purified by lead subacetate addition and a counter current technique for cardenolides (Euw, Hess, Speiser & Reichstein, 1951). The extracts obtained were passed through five separatory funnels, the first containing 3 ml distilled water, the next two containing 3 ml 2N sodium carbonate

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solution, and the last two containing 3 ml distilled water. All partially purified solutions were dried with anhydrous sodium sulphate, evaporated, then dried to a constant weight under reduced pressure (Table 1), and examined chromatographically.

TABLE 1. TISSUE QUANTITIES AND EXTRACTS OBTAINED FROM DIGITALIS TISSUE CULTURES

Tissue culture	Medium and material extracted*	Amount wet cells (g) or medium (ml) extracted	Crude methanolic extract (g)†	Partially purified extracts (mg)			
				Chlorof.	Ether	Chlorof.-Ethanol (7:1)	Chlorof.-Ethanol (2:1)
D. lanata ..	A Ca	208·5	2·97	9·0	—	—	52·0
	A SC	99·5	1·90	45·0	—	25·0	37·0
	A SM	720	—	—	—	34·0	77·0
	B SC	51·5	1·43	42·5	—	27·0	28·5
	B SM	850	—	—	—	91·0	96·0
D. purpurea ..	A Ca	156·7	2·53	11·0	—	—	12·0
	A SC	82·5	1·38	3·0	5·0	—	9·0
	A SM	1·010	—	24·0	19·0	—	43·0
D. purpurea var *gloxinaeflora*..	A Ca	77·3	1·50	8·0	10·0	—	16·0
	A SC	56·0	1·58	4·0	8·0	—	18·0
	A SM	795	—	5·5	9·0	—	27·0

* Ca = Callus Cells, SC = Suspension Cells, SM = Suspension Medium
† Weight of dried material contained in 50 ml methanolic extract

D. lanata EXTRACTION

Callus tissue from agar medium A. The callus tissue from 86 vials (208·5 g wet wt) was homogenised with 210 ml of 50% methanol in a Waring blendor and macerated for two weeks. The cells were filtered on to a weighed filter paper, rinsed with 50% methanol and dried at 105° to a constant dry weight (7·95 g).

The methanolic extract (440 ml) of the callus tissue was diluted to 500 ml with 50% methanol and lead subacetate (10%, 70 ml) added. The resulting precipitate was filtered out and the excess lead in solution precipitated by adding dibasic sodium phosphate (10%). The pH of the solution after filtration was 6·0. The solution was concentrated under reduced pressure at 40° to 25 ml and counter-current extracted with chloroform, 6 × 25 ml, chloroform-ethanol (2:1), 6 × 25 ml, and partially purified as described under General Procedures.

Suspension cultures from liquid medium A. Twenty flasks contained 99·5 g wet wt of cells and 720 ml medium. The distilled water (200 ml) used to wash the cells and flasks was combined with the medium.

The cells (99·5 g) were ground in a mortar with methanol (88%, 150 ml). An additional quantity of methanol (50%) was added and the mixture macerated for 2 weeks. After filtration, 675 ml methanolic solution and 5·11 g (dry wt) extracted cells were obtained. The methanolic solution was purified with lead subacetate and concentrated to 20 ml. This concentrate was extracted with chloroform, 6 × 25 ml, chloroform-ethanol (7:1), 6 × 25 ml, chloroform-ethanol (2:1), 6 × 25 ml, and partially purified as described under General Procedures.

The combined medium-rinse water solution (720–200 ml) was diluted with methanol (920 ml), purified with lead subacetate solution and

concentrated to 85 ml. This concentrate was extracted with chloroform-ethanol (7:1), 5 × 100 ml, chloroform-ethanol (2:1), 5 × 100 ml, and partially purified as described under General Procedures.

Suspension cultures from liquid medium B. Twenty flasks contained 51·5 g (wet wt) cells and 850 ml medium. The distilled water (100 ml) used to wash the cells and flasks was combined with the medium.

The cells (51·5 g) were subjected to the same procedures as described for *D. lanata* cells grown in liquid medium A. After extraction and maceration they yielded 600 ml methanolic solution and 3·47 g (dry wt) extracted cells. The methanolic concentrate obtained (20 ml) was solvent partitioned and treated as previously described. The combined medium-rinse water solution (850–100 ml) was diluted with 950 ml methanol and purified with lead subacetate solution. The methanolic concentrate obtained (77 ml) was solvent extracted and also treated as previously described.

D. purpurea EXTRACTION

Callus tissue from agar medium A. The callus tissue from 61 vials (156·7 g wet wt) was extracted and purified as described for *D. lanata* callus and yielded 600 ml methanolic solution and 4·47 g dry extracted cells. The methanolic concentrate obtained (25 ml) was counter-current extracted and partially purified.

Suspension cultures from liquid medium A. Twenty-five flasks contained 72·5 g (wet wt) cells and 1·01 litres medium.

The cells (82·5 g) were extracted and macerated as described for *D. lanata* cells grown in liquid medium A and yielded 300 ml methanolic solution and 4·48 g (dry wt) extracted cells. The methanolic concentrate (20 ml) was counter-current extracted with ether 6 × 25 ml, chloroform 6 × 25 ml, chloroform-ethanol (2:1) 6 × 25 ml, and partially purified as described under General Procedures.

The medium (1·01 litres) was extracted as described for *D. lanata* cells grown in liquid medium A. The methanolic concentrate (100 ml) was counter-current extracted with ether 5 × 100 ml, chloroform 5 × 100 ml, chloroform-ethanol (2:1) 5 × 100 ml, and partially purified as described under General Procedures.

D. purpurea VAR. *gloxinaeflora* EXTRACTION

Callus tissue from agar medium A. The callus tissue from 23 vials (77·3 g wet wt) was extracted as described similarly for *D. lanata* callus and yielded 400 ml methanolic solution and 2·65 g dry extracted cells. The methanolic concentrate obtained (15 ml) was counter-current extracted and partially purified as described for *D. purpurea* cells.

Suspension cultures from liquid medium A. Twenty flasks contained 56·0 g (wet wt) cells and 795 ml medium.

The cells and medium were purified and extracted as described for *D. purpurea* medium and cells. After extraction and maceration, 600 ml of methanolic solution and 2·82 g of dry extracted cells were obtained.

CHROMATOGRAPHY OF DIGITALIS EXTRACTS

Whatman No. 1 paper chromatograms were developed by the decending technique at 16° for water-containing solvent systems, and at room temperature for formamide-containing solvent systems (Table 2). The

TABLE 2. SOLVENT SYSTEMS, AND RF VALUES OF KEDDE-POSITIVE SUBSTANCES FROM *Digitalis Lanata* PARTIALLY PURIFIED EXTRACTS

Chromatography solvent system	Solvent system reference	Rf Value
Formamide saturated		*Chloroform extract*
1. Xylene-methyl ethyl ketone (1 : 1) ..	Kaiser (1955)	0·18, 0·10, 0·04
2. Benzene-methyl ethyl ketone (1 : 1) ..	,,	0·18, 0·12, 0·04
3. Chloroform-methyl ethyl ketone (1 : 1) ..	,,	0·15, 0·13
(3 : 1) ..		0·31, 0·16
Formamide containing		*Chloroform extract*
4. Tetrahydrofuran-chloroform-formamide (50 : 50 : 6·5)	,,	0·24, 0·20, 0·14
5. Chloroform-formamide saturated ..	Schindler & Reichstein (1951)	0·80, 0·75, 0·7, 0·6, 0·48, 0·30, 0·25, 0·20, 0·15, 0·10
Water saturated		*Chlorof.-ethanol extracts (7 : 1) or (2 : 1)*
6. Methyl ethyl ketone	Miystake & others (1957)	0·63, 0·56, 0·41
7. Butanol-toluene (1 : 1)	Schenker & others (1954)	0·85, 0·80, 0·60, 0·50, 0·45, 0·38, 0·28, 0·20, 0·12

papers were impregnated with water (35%) or with formamide (35–40%) before applying the extracts and marker substances. The papers containing water were dried for 15 min, and those containing formamide for 1 hr at 100°, before spraying with Kedde reagent.

Results and discussion

Because of the small tissue quantities available, paper chromatography was used to separate and detect the cardenolides.

Table 2 depicts the results from chromatograms of *D. lanata* tissue culture extracts. The chloroform extracts were best resolved by solvent system 5, and the chloroform-ethanol extracts by solvent system 6. The tissue cultures did not produce lanatosides A, B, or C but did produce other Kedde-positive polar substances. Extracts from medium B contained larger amounts of Kedde-positive substances than extracts from medium A. Also present on chromatograms from medium A were yellow spots which gave a red rather than the usual violet colour with Kedde reagent. Five Kedde-positive (purple reaction) substances were found in *D. purpurea* extracts from callus cells and suspension medium and in *D. purpurea* var *gloxiniaeflora* extracts.

The quantity of Kedde-positive substances in digitalis tissue culture extracts is low as inferred by the quantity of extract applied to chromatograms (0·5–1·0 mg). The detection sensitivity of digitalis glycosides with Kedde reagent on paper chromatograms is approximately 0·01 to 0·05 mg, thus indicating about 0·002% to 0·02% total cardenolides in *D. lanata* grown in liquid medium A. A concentration range of 0·1% to 0·2% lanatosides A, B, and C has been reported for intact plants of *D. lanata* (Ramstad, 1959).

It is concluded that digitalis tissue cultures produce cardenolides different from those in the intact plant, and that these cardenolides are produced in larger amounts from the culture medium lacking 2,4-D than from the medium containing it. Work is in progress to isolate and characterise the cardenolides produced by digitalis tissue cultures.

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References

Euw, J., Hess, H., Speiser, P. & Reichstein, T. (1951). *Helv. chim. Acta*, **34**, 1821–1833.
Kaiser, F. (1955). *Chem. Ber.*, **88**, 556–563.
Miystake, K., Okano, A., Hoji, K. & Miki, T. (1957). *Pharm. Bull.*, **5**, 157–163.
Nickell, L. G. (1962). *Advances in Applied Microbiology*, Vol. 4, pp. 213–236. New York: Academic Press.
Ramstad, E. (1959). *Modern Pharmacognosy*, p. 131. New York: Blakiston Div., McGraw Hill.
Schenker, E., Hunger, A. & Reichstein, T. (1954). *Helv. Chim. Acta*, **37**, 680–685.
Schindler, O. & Reichstein, T. (1951). *Helv. Chim. Acta*, **34**, 108–116.
Staba, E. J. (1962). *J. pharm. Sci.*, **51**, 249–254.
Staba, E. J. (1963). *Developments in Industrial Microbiology*, Vol. 4, pp. 193–198, Washington, D.C.: American Institute Biological Sciences.
Staba, E. J. & Lamba, S. S. (1963). *Lloydia*, **26**, 29–35.