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Production of cardio-active substances by plant tissue cultures and their screening for cardiovascular activity

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Semi-purified extracts of six plant tissue cultures were examined for their effects on respiration, heart rate, and blood pressure in anaesthetized rabbits. *Ammi visnaga*, *Cheiranthus cheiri*, *Digitalis lanata*, and *Urginea maritima* evoked pronounced vasodilatation and bradycardia which ultimately resulted in the death of the animal.

DIGITALIS-like glycosides (Medora, Tsao & Albert, 1967), the glycosides scopoline (Fritig, Hirth & Ourisson, 1966) and glucovanillin (Goris, 1965), and some triterpenes (Erhardt, Hirth & Ourisson, 1966) have recently been produced by callus plant tissue cultures. *Digitalis* tissue cultures produce cardenolides (Büchner & Staba, 1964), *Ammi visnaga* tissue cultures produce visnagin (Kaul & Staba, 1967), and *Apocynum cannabinum* tissue cultures produce cymarose and glucose but not the cardiac genins normally present in the plant (Carew, Nylund & Harris, 1964). The identification of the cardenolides or related products in *Digitalis* and *Apocynum* tissue cultures has been based entirely upon chromatographic evidence, and by administering *Digitalis mertonensis* tissue culture extracts to guinea-pig isolated heart preparations (Medora, Kosegarten & others, 1967).

Our aim was to determine if semi-purified extracts of *Ammi visnaga* Lam. callus and suspension cultures, *Cheiranthus cheiri* L. (wallflower) suspension cultures, *Cytisus scoparius* Link (broom tops) callus cultures, *Digitalis lanata* Ehrh. suspension cultures, *Digitalis purpurea* L. suspension cultures, and *Urginea maritima* Baker (squill) callus cultures would affect the respiration, heart rate, and blood pressure when administered intravenously to anaesthetized rabbits. The semi-purified extracts were also examined qualitatively for the presence of cardenolides, desoxysugars, and alkaloids by thin-layer chromatography.

Experimental

TISSUE CULTURES AND EXTRACT PREPARATION

Suspension tissue cultures were grown in Erlenmeyer flasks containing modified Murashige's and Skoog's tobacco medium (Lin & Staba, 1961) with 0·1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) on a reciprocal shaker (80 strokes/min, 5 cm in length) at approximately 27°. Callus tissue cultures were grown on the same basal medium with 1·0 ppm of 2,4-D and 1·0% agar in 1-oz, square glass vials. The growth period of suspension cultures ranged from 2–6 weeks and for callus tissue from 6–12 weeks, after which they were separately collected and frozen. Collections were made over approximately two years.

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C. cheiri tissue cultures contained numerous highly developed root structures, *D. lanata* and *U. maritima* cultures were highly organized, containing numerous fasciations (Staba & Lamba, 1963; Carew & Staba, 1965), and from their external appearance *C. scoparius*, *D. purpurea* and *A. visnaga* were undifferentiated.

Frozen tissue cultures of *C. cheiri* (1·5 kg wet wt cells, 9·7 litres medium), *C. scoparius* (1·4 kg wet wt callus), *D. lanata* (3·0 kg wet wt cells, 10 litres medium), *D. purpurea* (0·5 kg wet wt cell, 2·6 litres medium) and *U. maritima* (0·1 kg wet wt callus) were thawed and extracted according to scheme A. Dried *A. visnaga* tissue cultures (3·0 kg wet wt cells, 13 litres medium) were extracted according to scheme B. Media were reduced in volume by evaporation under vacuum and gentle heat before extraction.

Scheme A: tissue and/or media were macerated for 12 hr with methanol (1 : 1 ratio by wet wt and volume respectively). The macerated cells were homogenized for approximately 3 min and filtered. The homogenized cell filtrate or the methanolic-medium mixture were purified with saturated solution of lead subacetate (excess lead removed with 10% Na_2HPO_4). The two semipurified methanolic preparations were extracted with chloroform. The chloroform fractions were combined, evaporated over warm water *in vacuo* to dryness. The residue was redissolved in 75% ethanol (Extract I). The two remaining methanolic preparations were further extracted with a chloroform–ethanol mixture (2: 1). The chloroform–ethanol fractions were combined, evaporated as previously described, and the residue dissolved in 75% ethanol (Extract II). *U. maritima* Extract III was prepared from media and extracted as described above for Extract I.

Scheme B. This was used for *A. visnaga* Extracts A to D. A dichloroethane extract of dried tissue and media were applied on thick-layer plates (Kaul & Staba, 1967) and the following ultraviolet fluorescent zones removed: visnagin (yellowish green, Rf, 0·53) Band A (bright blue; Rf, 0·25); Band B (deep blue; Rf, 0·63). The remaining adsorbent was eleuted with dichloroethane, evaporated to dryness and redissolved in 75% ethanol (Extract A). The dichloroethane extracted cells and medium were extracted with methanol and purified according to the procedure described for Scheme A (Extract B; trace of visnagin still present). Extract C is similar to Extract B, except that only cells were extracted and all the visnagin removed. Extract D is a chloroform–ethanol extract of the residual methanolic extract from Extract C, and was prepared as described previously for Extract II.

THIN-LAYER CHROMATOGRAPHY

Adsorbosil-1 thin-(200 mμ) and thick-(400 mμ) layer plates were used. The plates were activated for 30 min at 100°. The thin-layer solvent system and spray reagents used are shown in Table 1, and the extract concentrations in Table 2. Approximately 30–50 μl of each extract was applied as a 2 cm band. The plates were examined under ultraviolet light for fluorescent zones and then sprayed with either Raymond, xanthydrol, modified Dragendorff or anisaldehyde reagent.

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF TISSUE CULTURE EXTRACTS

Tissue culture extracts	Rf values*		
	Raymond reagent	Xanthydrol reagent	Modified Dragendorff reagent
C. cheiri			
Ext I	0·80, 0·72, 0·53, 0·46, 0·34	0·47, 0·36	0·15, 0·03, 0·00
Ext II	0·76, 0·42, 0·31	0·31, 0·19	0·15
C. scoparius			
Ext I	0·52	—	0·00
Ext II	0·76	—	0·13, 0·00
D. lanata			
Ext I	0·76, 0·72, 0·65, 0·61, 0·55, 0·46	0·87, 0·76, 0·71, 0·65, 0·48, 0·20	0·50, 0·04, 0·04, 0·00
Ext II	0·69, 0·62, 0·59, 0·53	0·67, 0·58, 0·48, 0·45, 0·21	0·57, 0·48, 0·40, 0·14
D. purpurea			
Ext I	0·65, 0·60, 0·50	0·73, 0·63, 0·30	0·00
Ext II	0·90, 0·85	—	0·05
U. maritima			
Ext I	0·80, 0·70, 0·58	0·75, 0·57, 0·40, 0·23, 0·52, 0·19	—
Ext II	—	0·52, 0·19	0·01, 0·00
A. visnaga			
Ext A	0·67	0·46, 0·64	0·33
Ext B	0·76, 0·72, 0·70, 0·67, 0·53	0·73, 0·52	0·76, 0·66, 0·48, 0·34
Ext C	—	—	0·47
Ext D	0·50, 0·56	0·67, 0·75	0·46, 0·63

**TLC system*: Benzene–ethanol 95% (3: 1); Adsorbent: Silica dioxide (Adsorbosil-1, Applied Science Laboratory, State Park, Pa.).
Raymond reagent: Spray A 10% 1,3-dinitrobenzene in benzene. Heat 60° for 3 min. Spray B 6g NaOH dissolved in 25 ml water and 45 ml methanol. *Xanthydrol reagent*: Spray A 0·125% xanthydrol in glacial acetic acid. Spray B conc. HCl acid. Heat 100° for 5 min. *Modified Dragendorff reagent*: 2·6 g bismuth subcarbonate, 7·0 g NaI, 25 ml glacial acetic acid and boil 3–4 min. Set overnight and filter. Stock solution consists of 4 ml filtrate and 16 ml ethyl acetate. Spray consists of 20 ml stock, 40 ml glacial acetic acid, 120 ml ethyl acetate, and 10 ml distilled water. *Anisaldehyde reagent*: 50 ml glacial acetic acid, 0·5 ml anisaldehyde, and 1·0 ml conc. H_2SO_4. To be freshly prepared.
Rf reference compounds: Digitoxin: Raymond, xanthydrol and Dragendorff positive (Rf: 0·57). (b) Digitoxose: xanthydrol positive (Rf: 0·34). (c) Lanatoside ABC: Raymond positive (Rf: 0·18, 0·22, & 0·34). (d) Spartine sulphate: Dragendorff positive (Rf: 0·00). (e) Visnagin: Raymond and Dragendorff positive (Rf: 0·75).

Raymond reagent gives a blue colour with the butenolide ring; xanthydrol reagent gives a reddish colour with desoxysugars such as digitoxose (Wright, 1960). Modified Dragendorff reagent gives false positive tests with at least sixty-five non-nitrogenous compounds, including digitoxin and khellin; the minimal structural feature to give a positive reaction with this reagent appears to be a conjugated carbonyl (ketone or aldehyde) or a lactone function (Farnsworth, Pilowski & Draus, 1962). Anisaldehyde reagent reacts with phenols, terpenes, sugars and steroids to give a variety of colour reactions (Stahl, 1965).

CARDIOVASCULAR EXPERIMENTS

A comparison of the effects of the tissue culture extracts on the respiration, heart rate, and blood pressure was made with rabbits. Fasted rabbits weighing 2–3 kg were anaesthetized with pentobarbitone sodium (35 mg/kg) and a tracheal cannula inserted. The carotid artery was isolated and cannulated for recording blood pressure. Respiration and the electrocardiogram were recorded using the impedance pneumograph with pin chest electrodes. All injections were made intravenously through the marginal ear vein. All extracts containing undissolved particles were

TABLE 2. COMPARISON OF THE TOXIC EFFECTS OF SEVERAL TISSUE CULTURE EXTRACTS ON RESPIRATION, HEART RATE, AND BLOOD PRESSURE IN ANAESTHETIZED RABBITS

Test solution	Extract equivalent*	Amount extract administered to produce death (ml)	Respiratory rate (increase)	Heart rate (decrease)	Blood pressure (decrease)
Controls					
Ethanol 75% ..		10·0	(t)**	(t)	(t)
Tinct. of digitalis NF		3·5	+(t)	+(t)	+(t)
Visnagin 1·0% ..		4·7	+(t)	Increase	+(t)
Extracts					
C. cheiri					
Ext I	100 g + 650 ml	0·75	+++	+++	+++
Ext II	,, ,,	8·0	(t)	(t)	(t)
C. scoparius					
Ext I	97·6 g	1·5	Decrease	+	++(t)
Ext II	,,	2·25	+	+	+
D. lanata					
Ext I	100 g + 333 ml	1·5	+	+	++
Ext II	,, ,,	5·0	+(t)	+	+(t)
D. purpurea					
Ext I	40·7 g + 194 ml	6·0	(t)	+	+(t)
Ext II	,, ,,	4·0	+	+	+(t)
U. maritima					
Ext I	20 g	2·5	++	+	+(t)
Ext II	,,	0·5	+	++	++++
Ext III	735 ml	4·0	+	+	+(t)
A. visnaga					
Ext A	200 g + 866 ml	1·0	+(t)	++	++(t)
Ext B	,, ,,	1·0	+	+++	+++
Ext C	500 g	?	(t)	(t)	(t)
Ext D	,,	?	(t)	(t)	(t)

* Ext equiv.: that amount of cells (wet weight) and medium represented in each ml of extract administered
**(t) Transitory effect.

filtered through a Swinny Hypodermic Adapter containing a Millipore Type HA filter, pore size 0·45 μ. Controls were done using 75% ethanol, tincture of digitalis N.F. (Eli Lilly & Co., Indianapolis, Indiana), and 1·0% visnagin (Memphis Chemical Co., Cairo, Egypt) in 75% ethanol.

Results

THIN-LAYER CHROMATOGRAPHY

The positive Raymond, xanthydrol and modified Dragendorff zones observed in the extracts are summarized in Table 1. The fluorescence and anisaldehyde zones observed are summarized below.

C. cheiri. In Extract I at least 12 fluorescent and 10 anisaldehyde zones were observed. In Extract II 12 fluorescent and 3 anisaldehyde zones were observed.

C. scoparius. In Extract I at least 8 fluorescent and 3 anisaldehyde zones were observed. In Extract II 12 fluorescent and 2 anisaldehyde zones were noted.

D. lanata. There were at least 11 fluorescent and 22 anisaldehyde zones in Extract I. Eleven fluorescent and 11 anisaldehyde zones were observed in Extract II. The anisaldehyde zones were the stronger in Extract I.

D. purpurea. There were 12 fluorescent and 9 anisaldehyde zones in Extract I, and 6 fluorescent and 5 anisaldehyde zones in Extract II.

U. maritima. There were 9 fluorescent and 9 anisaldehyde zones in Extract I, and 5 fluorescent and 7 anisaldehyde zones in Extract II.

A. visnaga. There were at least 3 fluorescent and 6 anisaldehyde zones in Extract A, and 6 fluorescent and 6 anisaldehyde zones in Extract B. But Extract C and D did not show distinct fluorescent or anisaldehyde zones.

CARDIOVASCULAR EXPERIMENTS

Control experiments using 75% ethanol in 1 ml doses given at 5 min interval produced a transient fall in blood pressure. There was no significant change in the respiration, electrocardiogram, and heart rate after five 1 ml doses. In doses which totalled 10 ml, the blood pressure began to decline with a simultaneous increase in heart rate and respiration. The animal died usually due to respiratory arrest and cardiovascular collapse.

Tincture of digitalis N.F. was administered intravenously in doses of 0·5 ml at intervals of 5–10 min. Each dose produced a transient fall in blood pressure with a decrease in cardiac rate. This was usually accompanied by an increase in respiratory rate and decrease in depth. The electrocardiogram remained essentially normal in pattern with some increase in the QRS complex. After the administration of a total of 3·0 ml of the digitalis tincture the ECG pattern showed an inverted T wave and with the seventh dose of 0·5 ml the heart went into ventricular fibrillation and the animal expired.

A 1% visnagin solution in 75% ethanol given in doses of 1·0 ml at 5–10 min intervals caused a fall in blood pressure after each injection with an increase in both cardiac and respiratory rate. In the final doses, the ECG pattern showed alterations associated with anoxia to the cardiac muscle. A comparison of the toxic effects of the controls and the extracts is shown in Table 2.

C. cheiri Extract I. This was the most potent and toxic of the extracts. After an initial dose of 0·25 ml there was a fall in blood pressure with a concurrent increase in heart rate and an increase in both rate and depth of respiration. The ECG remained normal. A second injection of 0·2 ml produced a drastic fall in blood pressure, a twofold increase in rate and depth of respiration, and an ECG pattern associated with myocardial anoxia. Death occurred within 2 min from cessation of respiration and cardiovascular collapse.

Extract II. This produced no significant effects.

C. scoparius Extracts I and II. Injections of 1·5 ml of I and 3·0 ml of II in three equally divided doses produced a decrease in blood pressure, cardiac rate, and respiration. The ECG showed changes in amplitude of the QRS complex, particularly after II. Death resulted from cardiovascular collapse and respiratory arrest.

D. lanata Extract I. One ml produced a fall in blood pressure of 40 mm

Hg which was sustained throughout the experiment. The respiration increased in both rate and depth. The ECG pattern showed an inverted T wave. Heart rate was slightly reduced. A second dose of 0·5 ml caused respiratory arrest with a concurrent fall in blood pressure and cardiac rate. Artificial respiration did not improve blood pressure.

Extract II. Less toxic than I. II, in doses of 1 ml produced a transitory fall in blood pressure with a rise in blood pressure above normal in early doses. The ECG pattern showed increased amplitude of the QRS complex. The heart rate declined with each dose. The rate and depth of respiration increased for several min after each injection and then returned to normal. The animals died after a total dose of 4–5 ml due to cardiovascular collapse and respiratory arrest.

D. purpurea Extracts I and II. In 1 ml doses, these produced a transitory fall in blood pressure and a reduction in cardiac rate. Towards the end of the experiment doses of 3 to 4 ml had been given, there was an increase in rate and depth of respiration and increasing bradycardia. The ECG remained essentially normal after the early doses, but after doses of 3 ml, the T wave was inverted and characteristic patterns of anoxia occurred.

U. maritima Extract I. This, in a 0·5 ml dose evoked a transitory fall in blood pressure with no change in cardiac or respiratory rate. The ECG changed in pattern 1 min after injection with increased amplitude of the QRS complex. Subsequent doses of 0·5 ml after 8 min produced a prolonged fall in blood pressure, bradycardia, and an increase in respiratory rate and depth. The ECG pattern alternated between a normal QRS complex and one of increased amplitude. A total of 2·5 ml produced an inverted T wave with the blood pressure falling to zero.

Extract II. Doses of 0·25 and 0·5 ml produced immediate toxic effects with a complete loss of blood pressure within 1 min and respiratory arrest after 2 min. The ECG showed the characteristic pattern associated with anoxia. Artificial respiration was attempted with no improvement in blood pressure.

Extract III. This produced a decline in blood pressure followed by a rise slightly above normal. There was a decrease in cardiac rate with an increase in respiratory rate and depth. Respiratory failure occurred after the fourth dose of 1·0 ml. Excessive salivary flow was noted before death.

A. visnaga Extract A. This, in a 0·5 ml dose, produced a transitory fall in blood pressure and then a rise slightly above normal. This extract also caused a transitory increase in rate and depth of respiration, and a decrease in cardiac rate which remained stable at this slower rate. The second injection of 0·5 ml produced a dramatic fall in blood pressure with a simultaneous decline in cardiac rate and changes in the ECG pattern. The latter changes occurred in the S wave deflection associated with anoxia to the myocardium. A final dose of 0·5 ml produced cardiovascular collapse and respiratory arrest within 1 min.

Extract B. This, in a dose of 1·0 ml, produced a drastic fall in blood

pressure with a slowing of heart and cardiac failure after 10 min. The respiration showed a transitory increase and then cessation of respiration.

Extracts C and D. These produced no significant effects.

Conclusions

The plant tissue culture extracts that were particularly active pharmacologically were Extract I of *C. cheiri*, *C. scoparius*, and *D. lanata;* Extract II of *U. maritima*; and Extracts A and B of *A. visnaga*. All of these evoked pronounced vasodilatation and bradycardia, ultimately resulting in the death of the animal. Extract I of *C. cheiri*, *D. lanata* and *U. maritima* contained from three to six Raymond positive compounds, and *D. lanata* and *U. maritima* each contained six xanthydrol positive compounds. Although *D. lanata* Extract I contained some of Raymond and xanthydrol positive compounds, it did not evoke the strongest cardiovascular effect. Extract I of *C. cheiri*, Extract II of *U. maritima*, and Extract B of *A. visnaga* were the most potent and toxic. *C. cheiri*, *D. lanata*, and *U. maritima* were very highly organized tissue cultures, and perhaps this is related to the significant pharmacological effects observed.

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