Phytochemistry, 1971, Vol. 10, pp. 2775 to 2780. Pergamon Press. Printed in England.

CHEMICAL COMPONENTS OF CALLUS TISSUES OF PUMPKINS

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(*Received* 26 *October* 1970)

Abstract—Chemical components of callus and other tissues (leaf, stem, root and seed) of pumpkin were investigated to find the specificity of callus tissues. The isolated and identified compounds were squalene, 3 sterols, a triterpene, a fatty alcohol and hydroquinone; the last two being specifically found in the callus. On the other hand, $\Delta^{7,25}$ stigmastadienol and chlorophyll could not be identified in the callus. Fatty acids were detected in all the tissues in a different ratio.

INTRODUCTION

PUMPKINS, one of the most familiar vegetables for mankind, have been studied from various points of view for more than a century, and several papers on their chemical constitution have appeared including carotenoid pigments, amino acids, fatty acids,[1-4] keto acids,[5,6] cucurbitacins,[7] as well as sterols[8-10] and other chemical components. The effects of auxins and kinetins on pumpkins have also been studied and Fadia reported[11] the formation of callus tissues when pumpkins were cultivated in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D).

We have been much interested in the chemical components of various kinds of callus tissues, not only because callus is expected to produce secondary metabolites[12] which are not formed in the original plants, but also because callus might be used as a source to obtain medicinal and useful materials on a relatively large scale. It is also intriguing to elucidate the mechanism of dedifferentiation and redifferentiation of tissue culture from the chemical point of view.

For these purposes, we have studied the conditions to induce both callus tissues (undifferentiated tissue) and their specific chemical components, and compared these with the

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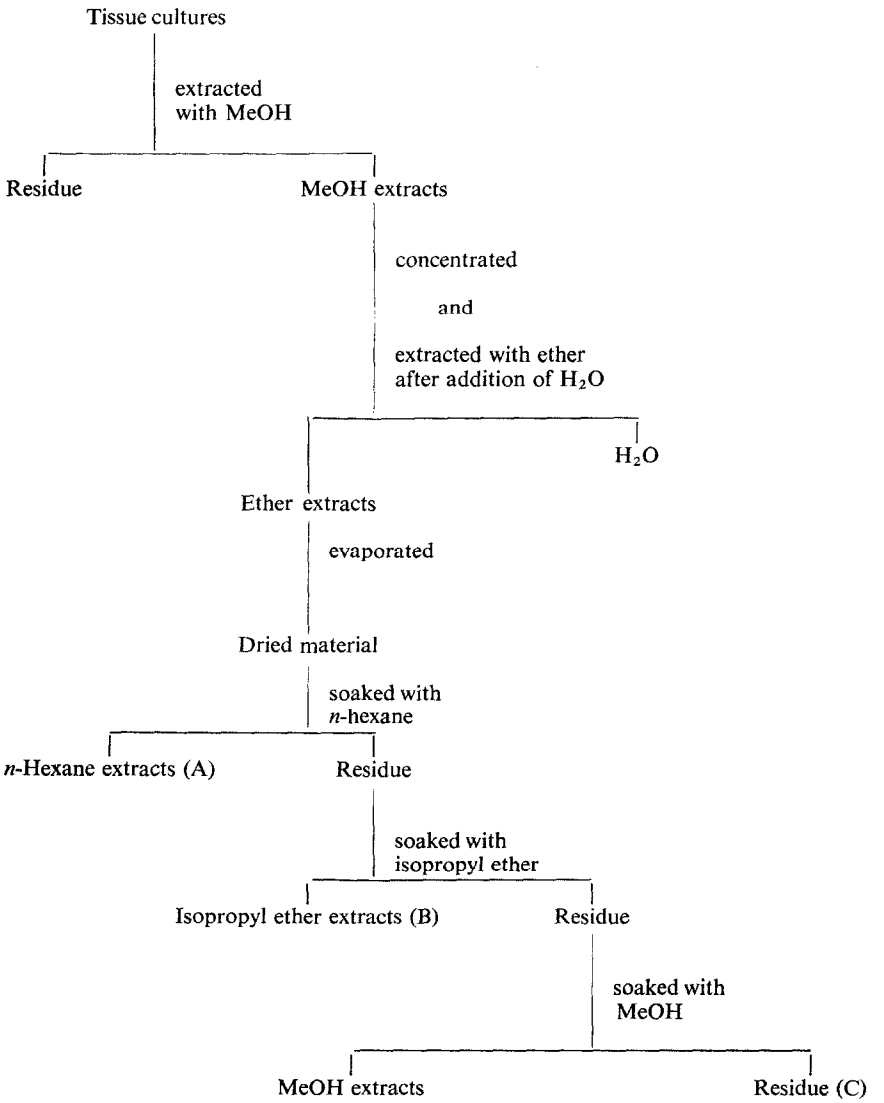
original plant. In this paper we wish to report the results obtained using pumpkins (*Cucurbita maxima* Duch., Japanese name; 'Kurikabochya').

Chemical Components from Tissue Cultures

Callus formation and growth were examined using Murashige and Skoog's basal medium in varying conditions. The effect of the concentration of 2,4-D from 0·1 to 10·0 ppm on the induction of the callus was investigated and it was found that 3 ppm of 2,4-D gave the best result. Yeast extract (YE) and coconut milk (CM) caused an increment of callus growth as did continuous irradiation with light.

Chemical components were extracted and separated from the callus or plant organs by the procedure shown in Scheme 1.



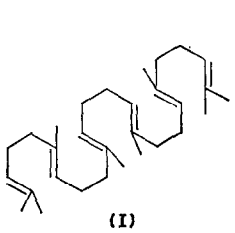
SCHEME 1. EXTRACTION OF CHEMICAL COMPONENTS.

Since IR and NMR spectra of the fraction A (Scheme 1) suggested the presence of a mixture of esters of fatty acids as major components, material from *n*-hexane extracts (A) was hydrolysed with methanolic 2 N-potassium hydroxide and the acidic compounds methylated with diazomethane to give the mixture of methyl esters, which were identified with authentic samples by GLC. The results are shown in Table 1.

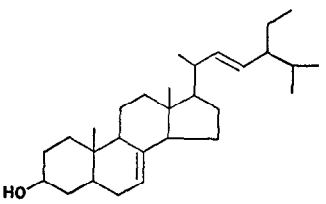
TABLE 1. RELATIVE AMOUNTS OF FATTY ACIDS IN DIFFERENT TISSUES

	Fatty Acid					
Tissue	Lauric	Myristic	Palmitic	Stearic + Oleic	Linolic	Linoleic
Callus	6	6	45	25	6	12
Root	13	4	51	4	4	24
Stem	4	2	34	7	18	35
Leaf	5	2	39	14	12	28
Seed	trace	1	14	37	48	0

The neutral fraction from the above hydrolysis of A obtained from seeds was chromatographed on a silica gel column successively with benzene, and a mixed solvent of benzene–isopropyl ether (30:1). From the benzene eluate was isolated on oily compound (I) and the benzene–isopropyl ether (30:1) eluate afforded a mixture of crystalline compounds (II, III, IV), which were separated by repeated chromatography on both silica gel and silica gel impregnated with silver nitrate columns. Compounds were detected in other tissues by TLC and GLC as well as careful observation of molecular ions in mass spectra. Relative amounts of these compounds were shown in Table 2.



(I)



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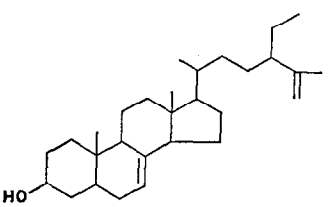

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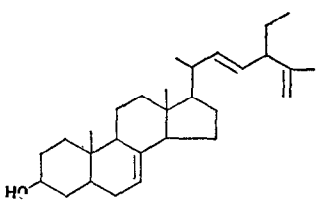

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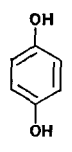

(VI)

TABLE 2. DISTRIBUTION OF CHEMICAL COMPONENTS IN DIFFERENT TISSUES OF PUMPKIN

Component	Tissue					
	Leaf	Stem	Root	Seed Embryo	Seed Endosperm	Callus
Squalene (I)*	–	–	–	500	±	–
α-Spinasterol (II)† + $\Delta^{7,22,25}$-Stigmastatrienol (IV)†	58	62	35	52	53	82
$\Delta^{7,25}$-Stigmastadienol (III)	42	38	65	48	47	0
Higher fatty alcohol (V)†	0	0	0	0	0	18
Triterpene (VII) (unknown)	–	–	+	–	–	+
p-Hydroquinone (VI)	–	–	–	–	–	+
Bitter principles (VIII)‡	+	+	+	–	–	–
Chlorophyll*	537	7·5	7			0

* In mg/100 g fresh wt.
† For compounds II–V, the values are given as percentage distribution.
‡ Detected by taste.

Oily compound (I) was identified as trans squalene by mass spectrum (M^+ 410, mol. wt. of $C_{30}H_{50}$ = 410·70) and direct comparison of IR, NMR and GLC with those of an authentic sample.

Compound II, m.p. 157–158° (lit 157–162°)[13,14] has a molecular formula of $C_{29}H_{48}O$ (M^+ 412·3718, mol. wt. = 412·3704) containing a hydroxyl group [acetate, m.p. 171–172° (lit, 168–173°), M^+ 454·3783, mol. wt. of $C_{31}H_{50}O_2$ = 454·3810], and was identical with an authentic sample of α-spinasterol (II).

Compound III, m.p. 136–140° (lit, 135–142°)[13] M^+ 412, mol. wt. of $C_{29}H_{48}O$ = 412·67, and compound IV, m.p. 156–159° (lit, 157–161°)[13], M^+ 410, mol. wt. of $C_{29}H_{46}O$ = 410·66, were identified as $\Delta^{7,25}$-stigmastadienol (III) and $\Delta^{7,22,25}$-stigmastatrienol (IV), respectively, by comparison of physical data (IR, NMR and GLC) and mixed m.p. with authentic samples.

Compound V was only present in callus tissue and shows a hydroxyl group at 3250 and 1100 cm^{-1} in IR spectrum. Its NMR spectrum is quite simple, exhibiting a straight chain methylene group at 1·25 ppm and a methyl group at 0·83 (bt, J = 5·4). This evidence as well as the fragmentation pattern of the mass spectrum, suggested that compound V is a fatty alcohol. Further investigation is being carried out on its structure.

The isopropyl ether soluble fraction B (Scheme 1) was purified by silica gel chromatography eluted with a mixed solvent of benzene–isopropyl ether (3:1) to give white prisms

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(VI), m.p. 169·5–171·5° (lit, 170–171°), M^+ 110, mol. wt. of $C_6H_6O_2$ = 110.11, which was identical with *p*-hydroquinone by direct comparison with an authentic sample.

From residue (C) was isolated a triterpene (VII), whose methyl ester has m.p. 127–129°, and the molecular formula of $C_{31}H_{50}O_3$ (M^+ 470·3768). Physical evidence suggested that VII is a hydroxylated pentacyclic triterpene with a tertiary carboxyl group and one double bond. The double bond is presumably located at C_{12}-C_{13} position in view of the observation of strong peaks at *m/e* 207 and 262 in the mass spectrum[15] of its methyl ester and the structure of compound VII is under investigation.

Although all the fatty acids found in callus tissue were also detected in other organs of the mother plant, the relative abundance of these fatty acids are different in each tissue. In view of the well established fact that squalene and its 2,3-oxide play an important role[16,17] in the biosyntheses of triterpenes and steroids, it is of interest to note that squalene is only localized in embryo of the seeds (Table 2). The amount of squalene in the endosperm was quite low and other tissues (callus, roots, stems, and leaves) contained no detectable amount.

As shown in Table 2, the relative amounts of compounds II-V are quite different in each tissue, no detectable amount of $\Delta^{7,25}$-stigmastadienol (III) being identified in the callus tissue, while the higher fatty alcohol (V) was only present there. *p*-Hydroquinone (VI) was also only isolated from the callus tissue.

Comparison of existence of the various compounds in different kinds of tissues indicates that the callus is clearly different from those of other organs (differentiated tissue) and produces two specific secondary metabolites, the higher fatty alcohol (V) and *p*-hydroquinone.

EXPERIMENTAL

Tissue cultures from pumpkin. The seeds were sterilized in 70% EtOH for 5 min, followed by immersion in 0·2% $HgCl_2$ solution for 10 min and then washed three times with H_2O. The seeds were placed on Murashige and Skoog's medium supplemented with 0·8% agar, 3 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D), yeast extract (0·2% w/v) and coconut milk (5% v/v). The cultures received continuous white light, and were maintained at 25°. After 1 week, white callus was formed from root tissue. These cultures were transferred to the freshly prepared media every 4–6 weeks and had been maintained continuously for over 3 months prior to use. The cultivation of whole plants was carried out on the same medium but without 2,4-D.

Extraction of material. The callus tissues (636 g) were in a soxhlet with MeOH (2 ml/g) 2 days. This operation was repeated twice, and the combined MeOH extracts concentrated and treated as shown in Scheme 1. Evaporation of *n*-hexane, isopropyl ether and MeOH afforded 2·2 g, 200 mg and 500 mg of crude extract, respectively.

Hydrolysis of n-*hexane soluble fraction* (A). The *n*-hexane soluble fraction (A) was hydrolysed with 2 N KOH in MeOH and worked up as usual into neutral and acidic fractions. Since IR spectra of the acidic part suggested a mixture of fatty acids, it was methylated with diazomethane in the usual way and examined by GLC.

Separation of the neutral part. The neutral part was taken up in benzene and passed through silica gel column, eluted successively with benzene, and benzene–isopropyl ether (30:1). The benzene eluate gave an oily compound (I), which showed IR ν_{max}^{film} cm^{-1} 2980, 2945, 2880, 1670, 992, 838, and 745. NMR ($CDCl_3$) δ 1·46 (s, C=C—Me × 6), 1·50 (s, C=C—Me × 2), 1·85–1·87 (broad s), 5·02 (m, C=C—H × 6) and Mass *m/e* 410 (M^+), mol. wt. of $C_{30}H_{50}$=410·70.

The benzene–isopropyl ether eluate afforded a mixture of II, III, IV, and/or V, which were separated by rechromatography on silica gel impregnated with 5% $AgNO_3$, eluted with benzene–isopropyl ether (30:1).

Compound (II) was recrystallized from MeOH–EtOH, colorless needles, m.p. 157–158°. IR ν_{max}^{KBr} cm^{-1} 3440, 1450, 1040, 970. NMR ($CDCl_3$) δ 0·51 (s, Me), 0·80 (s, Me), 5·00–5·30 (m, 3H). High resolution Mass *m/e* 412·3718 (M^+), mol. wt. of $C_{29}H_{48}O$= 412·3704. On acetylation (AC_2O and NaOAc) it gave the acetate,

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m.p. 171–172°, IR ν_{max}^{KBr} cm^{-1} 1730, 1470, 1370, 1250, 1030, 970, and 898. NMR ($CDCl_3$) δ 0·52 (s, Me), 0·82 (s, Me), 2·00 (s, Me of acetate), 4·55–4·90 (m, CHOAc), 5·00–5·30 (m, 3H). High resolution Mass *m/e* 454·3783 mol. wt. of $C_{31}H_{50}O_2$ = 454·3810.

Compound III was recrystallized from *n*-hexane to give a colorless needles, m.p. 136–140°, IR ν_{max}^{KBr} cm^{-1} 3080, 1645, and 890. NMR ($CDCl_3$) δ 0·53 (s, Me), 0·79 (s, Me), 0·80 (t, Me), 0·90 (d, Me), 1·55 (s, C=C—Me), 3·40–3·60 (m, CHOH), 4·65–4·71 (m, C=CH_2), and 5·05–5·35 (m, =C=CH—). Mass *m/e* 412 (M^+), mol. wt. of $C_{29}H_{48}O$=412·67.

Compound IV was recrystallized from a mixed solvent of MeOH and EtOH, colorless needles, m.p. 156–159°, IR ν_{max}^{KBr} cm^{-1} 3410, 1642, 967, and 892. NMR ($CDCl_3$) δ 0·54 (s, Me), 0·78 (s, Me), 0·83 (t, Me), 1·10 (d, Me), 3·30–3·80 (m, 1H), 4·70–4·80 (m, 2H), 5·00–5·40 (m, 3H). Mass *m/e* 410 (M^+), mol. wt. of $C_{29}H_{46}O$=410·66.

Compound V was recrystallized from *n*-hexane to give a glassy semi-crystalline mass. IR $\nu_{max}^{CCl_4}$ 3250, 2850, 1100, 727 and 717. NMR ($CDCl_3$) δ 1·25(bs. chain —CH_2—). Mass *m/e* 396 (M^+), 378 $(M-18)^+$.

Separation of isopropyl ether soluble fraction (B). Isopropyl ether soluble fraction (B) was chromatographed on silica gel, eluted with benzene–isopropyl ether (3:1) to afford white prisms (VI), m.p. 169–170°, IR ν_{max}^{KBr} 3180, 1515, 1190, 830 and 755. NMR ($CDCl_3$) δ 6·70 (4H).

Separation of residue (C). To a suspension of residue (C) in MeOH was added excess CH_2N_2 in Et_2O and, after usual work up, the methyl ester was chromatographed on silica gel to obtain a white crystal, m.p. 127–129°. IR ν_{max}^{KBr} cm^{-1} 3400, 1725, 1460, 1370, 1140 and 1030. NMR($CDCl_3$) δ 0·78 (s, Me), 0·80 (s, Me), 3·65 (s, COOMe). High resolution Mass *m/e* 470·3768, mol. wt. of $C_{31}H_{50}O_3$ 470·3759.

GLC. Identification of the extracted materials and determination of the methyl ester of fatty acids as well as sterols were carried out with Hitachi Model K-53 gas chromatograph which was fitted with a stainless steel column (1m) detected by flame ionization. For the identifications of the methyl esters of fatty acids and sterols, 5% PDEGS on Diasolid (column temperature 175°) and 1·5% SE-30 on Diasolid (60–100 mesh) (column temperature 240°) were used respectively. The relative amounts of methyl esters of fatty acids (Table 1) and sterols (Table 2) were determined by measurement of GLC-peak area.

TLC. Fractionations of various kinds of compounds were achieved by column chromatography packed with silica gel and silica gel impregnated with $AgNO_3$. The compounds were detected on TLC coated with silica gel (Wakko gel B-100) with or without $AgNO_3$. The layers were dried at 120°. Compounds on the plates were detected by heating after treatment with 10% H_2SO_4, by I_2 staining, or by fluorescence in UV.

Determination of physical parameters. Melting points were taken with a YANAGIMOTO micro melting point apparatus. Nuclear magnetic resonance spectra were taken with a Varian T-60 or A-60 spectrometers and the chemical shifts are in ppm from an internal standard of tetramethylsilane. IR spectra were measured with a Hitachi EPI-S spectrometer. Mass spectra were obtained with Hitachi RMU-60 mass spectrometer employing direct and/or indirect inlets.

α-Spinasterol (II) and $\Delta^{7,22,25}$-stigmastatrienol (IV) were separated by SiO_2–$AgNO_3$ column and detected by GLC/MS.

Acknowledgements—The authors express their sincere thanks to Professor Y. Ueno of Kinki University for his generous gift of the steroids and to Mr. Y. Kato of Hitachi Co. Ltd., for his measurement of high resolution mass spectrometry. After completion of this work, Prof. T. Hidaka of Miyazaki University kindly informed us that methyl esters of stearic and oleic acids could be separated by GLC using 15 per cent PEGS.