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FREE STEROLS, STERYL ESTERS, GLUCOSIDES, ACYLATED GLUCOSIDES AND WATER-SOLUBLE COMPLEXES IN *CALENDULA OFFICINALIS*

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Key Word Index—*Calendula officinalis*; Compositae; identification of phytosterols; steryl esters; steryl glucosides; acylated steryl glucosides; water-soluble steryl complexes.

Abstract—In 3- and 14-day-old seedlings and in the leaves of *Calendula officinalis* the following sterols were identified: cholestanol, campestanol, stigmastanol, cholest-7-en-3-β-ol, 24-methylcholest-7-en-3β-ol, stigmast-7-en-3β-ol, cholesterol, campesterol, sitosterol, 24-methylcholesta-5,22-dien-3β-ol, 24-methylenecholesterol, stigmasterol and clerosterol. Sitosterol was predominant in young and stigmasterol in old tissues. Young tissues contained relatively more campesterol but in old tissues a $C_{28}\Delta^{5,22}$ diene was present suggesting transformation of campesterol to its $\Delta^{5,22}$ analog, similar to that of sitosterol to stigmasterol. All the identified sterols were present as free compounds and also in the steryl esters, glucosides, acylated glucosides and water-soluble complexes. The variations in the amounts of these fractions in the embryo axes and cotyledons of 3- and 14-day-old seedlings and the distribution of individual sterols among the fractions are discussed.

INTRODUCTION

In earlier work we isolated from the green parts of *Calendula officinalis* sitosterol, stigmasterol and a fraction containing Δ^7 sterols [1–3]. It was found that these sterols occur in free and bound forms. Glycosides of sterols were identified as monoglucosides [4] and the acylated steryl glucosides as a mixture of monoglucosides monoesters with fatty acids, mainly palmitic, stearic, linoleic and linolenic [5]. Similar derivatives of sterols have been identified in many plants [6,7]. Also water-soluble sterol complexes have been identified in the leaves of *Kalanchoe bossfeldiana* [8] and in leaves and roots of corn [9].

In the present work the sterols have been identified in the embryo axes and cotyledons of 3- and 14-day-old seedlings and in the leaves of 70-day-old plants of *C. officinalis*. In the seedlings the sterols were identified separately in the free sterols, steryl esters, glucosides, acylated glucosides and water-soluble complexes. The fatty acid components of the steryl esters were also identified.

RESULTS AND DISCUSSION

In the plant studied 13 sterols were identified belonging to the following types: stanols, Δ^7monoenes, Δ^5monoenes and dienes. The first three types contained C_{27}, C_{28} and C_{29} compounds but the dienes were C_{28} and C_{29} compounds. Table 1 presents the results of the quantitative determinations of the sterol content of the fresh tissues of the embryo axes, cotyledons and leaves. The fr. wt was taken as the reference in order to compare the results obtained for the seedlings and the leaves. Between the third and fourteenth days of growth the fr.wt of both the embryo axes and the cotyledons rose ten-fold (from 8 to 80 mg per embryo axis and 9·2 to 95 mg per cotyledon). In this period the total amount of sterols in 100 g of embryo axes did not change which indicated that sterol biosynthesis accompanied the increase in the fr. wt. In the cotyledons however, which at 3 days contained almost twice as much sterols as the embryo axes of the same age, the increase in the fr. wt was greater than the synthesis of sterols. There-

Table 1. Quantitative determination of the sterols in 100g of embryo axes and cotyledons of 3- and 14-day-old seedlings and of leaves of 70-day-old *Calendula officinalis* plants

Sterol	Embryo axes				Cotyledons				Leaves	
	3 days old		14 days old		3 days old		14 days old			
	μg	% of total sterols	μg	% of total sterols	μg	% of total sterols	μg	% of total sterols	μg	% of total sterols
Cholestanol	23	0·3	44	0·4	21	0·1	38	0·4	0	‡
Campestanol	11	0·1	37	0·3	18	0·1	38	0·4	0	0
Stigmastanol	60	0·6	163	1·7	151	0·7	194	2·2	180	1·0
Cholest-7-en-3β-ol	6	0·1	13	0·1	13	0·1	13	0·1	0	0
24-Methylcholest-7-en-3β-ol	47	0·4	42	0·4	77	0·4	64	0·7	1970	11·2
Stigmast-7-en-3β-ol	313	2·9	149	1·5	587	2·8	101	1·2	53	2·9
Cholesterol	552	4·9	383	3·8	862	4·0	453	5·2	15	0·9
Campesterol	1180	10·2	1023	9·9	1345	6·3	427	4·9	247	1·4
Sitosterol	6277	56·2	4639	44·6	12379	58·7	2464	28·0	3470	19·4
24-Methylcholesta-5,22-dien-3β-ol	0	0	0	0	0	0	0	0	213	1·2
Methylenecholesterol	92	0·8	60	0·7	43	0·2	128	1·5	138	0·8
Stigmasterol	2487	22·8	3710	35·8	5282	25·3	4784	55·0	10396	58·2
Clerosterol	172	1·5	75	0·8	209	1·3	38	1·5	1190	5·8
Total (μg)	11220		10436		21006		8744		17871	

fore the content of sterols per 100 g of 14-day-old cotyledons was lower than in 3-day-old cotyledons. In the leaves of the 70-day-old plants the content of sterols per 100 g of fr. wt was somewhat higher than in the seedlings. The higher content of sterols in the cotyledons in comparison to the embryo axes was observed in the seeds of several plants [10] and most probably results from the accumulation of sterols in the cotyledons during seed maturation.

In the various types of sterols, differing by the number or location of double bonds, C_{29} compounds were the main components with the exception of the Δ^7 sterols from the leaves, where C_{28} sterol predominated. Sitosterol and stigmasterol always constituted about 80% of all sterols, but the proportions between these two sterols were different depending on the age of the tissue. In embryo axes of 3- and 14-day-old seedlings and in the cotyledons of 3-day-old seedlings sitosterol predominate while in 14-day-old cotyledons and leaves stigmasterol was the main sterol. Therefore in *C. officinalis*, as with the seedlings of beans [11]. *Digitalis purpurea* [12] and the leaves of tobacco [13,14], in the young rapidly growing tissues the synthesis of sitosterol is very active but in the ageing tissues its transformation to stigmasterol prevails. The results obtained for *C. officinalis* indicate a similar age-dependent transformation for the C_{28} sterols. The amount of campesterol was lower in both the embryo axes and the cotyledons of 14-day-old seedlings. The smallest amount of campesterol was found in the leaves in which, however, a derivative of campesterol possessing a Δ^{22} double bond was present (24-methylcholesta-5,22-dien-3β-ol, the C_{28} analogue of stigmasterol).

The above results suggest that in *C. officinalis* C_{29} and C_{28} sterols are submitted to similar transformations. In young, growing tissues the Δ^5 monoenes are rapidly synthesized, whereas in the

Table 2. The fatty acids of the triterpenoid esters from embryo axes and cotyledons of 14-day-old *Calendula officinalis* seedlings

Acid	% of total acids	
	Embryo axes	Cotyledons
Decanoic	0·6	0·9
Undecanoic	1·1	1·4
Lauric	1·8	1·7
Tridecanoic	2·2	1·8
Myristic	5·1	5·2
Pentadecanoic	5·8	5·3
Palmitic	20·8	19·5
Palmitoleic	1·6	2·9
Margaric	8·1	8·6
Stearic	13·7	12·0
Oleic	3·5	2·6
Linoleic	7·1	8·6
Linolenic	1·7	4·7
Eicosanoic	7·9	8·3
Eicosatrienoic	7·1	7·7
Arachidic	1·4	1·5
Behenic	10·5	7·3

Table 3. Quantitative determination of the free sterols and sterols from the steryl esters, glucosides, acylated glucosides and water-soluble complexes of the embryo axes (A) and the cotyledons (B) of 3-day-old *Calendula officinalis* seedlings

Sterol	Free	Esters	Glucosides	Acylated glucosides	Water-soluble complexes
(A)					
Cholestanol	1·9	0	0	0	0
Campestanol	0·9	0	0	0	0
Stigmastanol	3·6	0·4	0·2	0·2	0
Cholest-7-en-3β-ol	0·3	0·1	0	0·1	0
24-Methylcholest-7 en-3β-ol	1·5	1·7	0·2	0·4	0
Stigmast-7-en-3β-ol	7·2	17·5	0·2	0·2	0·6
Cholesterol	35·0	5·1	2·4	1·3	0·1
Campesterol	52·9	27·0	11·8	2·8	0·1
Sitosterol	279·0	147·0	79·6	16·6	0·7
Methylenecholesterol	2·9	3·0	0·3	0	0
Stigmasterol	138·3	34·8	21·7	4·3	0·8
Clerosterol	6·6	5·1	2·0	0·2	0
Total in 1000 embryo axes	530·1	231·8	118·6	26·1	2·3
Total in 100 g	6540	2280	1460	318	24
% of total sterols	58	25	14	3	0·3
(B)					
Cholestanol	1·2	0	0	0	0
Campestanol	1·7	0	0	0	0
Stigmastanol	12·7	0·5	0·4	0·6	0
Cholest-7-en-3β-ol	0·7	0·3	0	0·1	0
24-Methylcholest-7-en-3β-ol	5·8	0·9	0·2	0·1	0
Stigmast-7-en-3β-ol	44·4	10·0	0·2	0·2	0
Cholesterol	96·2	16·6	2·4	1·3	1·1
Campesterol	387·3	33·8	11·8	2·8	1·2
Sitosterol	638·0	262·0	79·6	16·6	4·6
Methylenecholesterol	1·6	0	0·3	0	0
Stigmasterol	397·6	49·4	21·8	4·3	0
Clerosterol	15·3	3·3	2·0	0·2	0
Total in 1000 cotyledons	1601·5	376·8	148·8	99·7	6·9
Total in 100 g	15100	3550	1350	946	60
% of total sterols	72	17	7	4	0·3

older tissues the conversion into $\Delta^{5,22}$ dienes was more important.

TLC of the steryl estres from the seedlings permitted the separation of the acetates, which constituted about 30% of the whole steryl ester fraction, from the long-chain fatty acid esters. Since we failed to separate the sterol esters from the methylsterol and triterpene monol esters, the fatty acids were determined in the whole fraction. The results (Table 2) showed that the main components of the esters from both the embryo axes and the cotyledons were palmitic (20%) and stearic (13%) acids. This differs from the fatty acids of the triterpenoid esters of *C. officinalis* flowers where only acetic, lauric, myristic, and palmitic acids were identified [15, 16]. In the seedlings the qualitative and quantitative fatty acid composition was different and rather similar to the fatty acid composition of the acylated steryl glucosides from *C. officinalis* seedlings [5] but there was more of the steryl acetates.

Tables 3 and 4 show the sterol content of all the fractions isolated from the embryo axes and cotyledons of 3- and 14-day-old seedlings. The quantitative determinations of individual sterols are presented for 1000 seedlings and not for 100 g of the fr. wt, because this presentation shows more clearly the changes which occurred during the growth of the seedlings.

Quantitatively the most important fraction was the free sterols which constituted 60–70% of the total sterols, then followed the steryl esters, steryl glucosides, acylated steryl glucosides and finally the water-soluble sterol complexes which were

Table 4. Quantitative determination of the free sterols and sterols from the steryl esters, glucosides, acylated glucosides and water-soluble complexes of the embryo axes (A) and the cotyledons (B) of 14-day-old *Calendula officinalis* seedlings

Sterol	Free	Esters	μg/100 seedlings Glucosides	Acylated glucosides	Water-soluble complexes
(A)					
Cholestanol	6·3	12·9	9·1	6·4	0
Campestanol	8·2	7·8	6·4	5·5	0
Stigmastanol	41·3	32·5	31·6	22·7	0
Cholest-7-en-3β-ol	4·2	2·1	2·1	2·1	0
24-Methylcholest-7-en-3β-ol	23·2	2·7	4·4	2·8	0
Stigmast-7-en-3β-ol	96·0	11·2	6·5	3·2	0
Cholesterol	181·0	96·5	10·2	14·8	11·2
Campesterol	353·0	64·0	111·0	35·6	14·6
Sitosterol	2565·0	291·0	621·0	172·0	33·1
Methylenecholesterol	11·2	4·1	10·0	0	0
Stigmasterol	2770·0	142·0	283·0	120·0	0
Clerosterol	34·5	9·0	8·0	3·5	0
Total of 1000 embryo axes	6093·9	675·8	1103·3	388·1	58·9
Total in 100 g	7690	840	1380	460	66
% of total sterols	73	8	14	5	0·7
(B)					
Cholestanol	9·1	11·9	9·1	7·9	0
Campestanol	8·2	11·9	6·3	12·6	0
Stigmastanol	46·6	53·6	26·6	59·0	0
Cholest-7-en-3β-ol	4·8	4·9	0	2·7	0
24-Methylcholest-7-en-3β-ol	41·8	5·7	8·3	7·2	0
Stigmast-7-en-3β-ol	28·4	10·2	3·2	0	0
Cholesterol	188·0	80·0	14·8	28·0	4·1
Campesterol	233·0	64·5	63·9	62·4	11·0
Sitosterol	1180·0	437·0	410·0	376·0	24·0
Methylenecholesterol	7·0	0·5	0	0	0
Stigmasterol	3770·0	426·0	461·0	299·0	0
Clerosterol	vv25·0	5·0	0	0	0
Total in 1000 cotyledons	5541·9	1109·0	1103·2	854·0	39·1
Total in 100 g	5720	1140	1065	885	40
% of total sterols	65	13	12	10	0·5

always the smallest fraction of the total sterols. During the growth of the seedlings from the 3rd to 14th day the total amount of sterols in each fraction increased but to different extents in the various fractions. During this period the large increase of sterols observed in the embryo axes was caused by the increase in the free sterols (10-fold), steryl glucosides (9-fold) and acylated steryl glucosides (15-fold) but not by the steryl esters which increased only 3-fold. In the cotyledons at 3 days old there were larger amounts of sterols in each fraction, particularly the free sterols (three times) and the acylated glucosides (four times), than found in the embryo axes. However the increases during 11 days were slower in all fraction in the cotyledons (3-fold in the free sterol and ester fractions, 7-fold in glucosides and 8-fold in acylated glucosides). As a result of these transformations in the cotyledons of the 14-day-old seedlings as compared to the embryo axes there was somewhat less of the free sterols, the same amount of glucosides, and somewhat more of the esters and acylated glucosides.

The variations in the distribution of individual sterols among different fractions were also noted. In embryo axes and cotyledons of 14-day-old seedlings stanols were distributed nearly evenly among all fractions but cholesterol was mainly in the free sterol and in the ester fractions. In both the embryo axes and the cotyledons of the 3-day-old seedlings there was twice as much sitosterol than stigmasterol in the free sterols but in the cotyledons both these compounds occurred at double the concentration found in the embryo axes. In other fractions in the embryo axes sitosterol predominates over stigmasterol. The ratio of these two sterols was 4:1 in the embryo axes from the 3-day-old seedlings and 2:1 from 14-day-old seedlings. In cotyledons 3-day-old seedlings the ratio (4:1) of

sitosterol stigmasterol was the same as in the embryo axes of this age but in cotyledons from 14-day-old seedlings it was 1:1.

In the water-soluble sterol complexes only those sterols could be detected which were present in a large amounts in the plant. Most probably this fraction comprises only the sterols actually bound with enzyme systems as was suggested by Rohmer *et al.* [9].

EXPERIMENTAL

Material. The plants of *Calendula officinalis* L. variety Radio studied after 70 days were grown in the conditions described earlier [17]. The seeds of plants studied after 3 and 14 days were germinated on moistened cellulose wool and after 7 days supplied with 10× diluted nutrient solution.

The isolation of free and bound sterols. Ground, fresh tissue of embryo axes, cotyledons or leaves was extracted several times with hot MeOH, Et_2O and then with hot H_2O. The combined MeOH and Et_2O extracts after evaporation were fractionated by TLC on Si gel with $CHCl_3$–MeOH (90:10). The steryl esters (R_f 0·98–1), free sterols (R_f 0·86), acylated steryl glucosides (R_f 0·60) and steryl glucosides (R_f 0·30) were eluted with Et_2O. The steryl esters were separated by TLC with CCl_4–$CHCl_3$ (19:1), or petrol–C_6H_6 (2:1). The steryl acetates (R_f 0·15) and esters of fatty acids (R_f 0·45) were then eluted.

Hydrolysis. The steryl esters were hydrolysed with 10% KOH in MeOH at 90° for 20 hr. The sterols were extracted with Et_2O from the alkaline soln and, after adjusting to pH 5, fatty acids were extracted. Steryl glucosides and acylated steryl glucosides were hydrolysed with 10% H_2SO_4 in MeOH at 90° for 20 hr. The H_2O extracts after evaporation to dryness were hydrolysed for 2 hr on a boiling H_2O bath with 0·5% MeOH soln of pyrogallol–60% KOH–MeOH (3:2:3).

Sterol analysis. The sterols obtained by hydrolysis of the different sterol forms were purified in the TLC system: petrol–$CHCl_3$–MeOH (100:50:5), R_f 0·37, and then acetylated. Steryl acetates were fractionated by TLC on $AgNO_3$–Sil gel with $CHCl_3$ (without MeOH). The compounds were visualised by spraying with Rhodamine 6G [18]. The following steryl acetates were eluted: stanols (R_f 0·84), Δ^7 sterols R_f 0·80), Δ^5 sterols (R_f 0·78), stigmasterol (R_f 0·66), sterol dienes (R_f 0·44 and 0·33). Steryl acetates were separated by GLC at 250° on 1% SE 30. The amounts of the different sterols were calculated from the GLC results. The relative R_f values of the steryl acetates (R_f of cholesteryl acetates = 1) were: cholestanol 1·02; campestanol 1·33; stigmastanol 1·63; 5α-cholest-7-en-3β-ol 1·1; (24*R*)-24-methyl-5α-cholest-7-en-3β-ol 1·39; 5α-stigmast-7-en-3β-ol 1·76; cholesterol 1; campesterol 1·32; sitosterol 1·62; stigmasterol 1·43; (24*R*)-24-methylcholesta-5,22-dien-3β-ol 1·14; methylenecholesterol 1·26; clerosterol and isofucosterol 1·56.

The identification of the last two components was verified by MS. The compound of RR_t 1·26 had a molecular ion at *m/e* 440 (1%) and fragmentation ions at 380 (–Ac, 100%); 365 (–Me–Ac, 10%); 296 (–[C_{23}–C_{27}]–H–Ac, 31%); 281 (–[C_{23}–C_{27}]–H–Ac–Me, 11%); 255 (–side chain(Ac, 1%); 253 (–side chain–Ac–2H, 1.5%); 213 (–side chain–Ac–part of ring *D*, 1·3%). The MS corresponded to methylenecholesterol. The compound of RR_t 1·56 had the following MS: *m/e* 394 (–Ac, 100%); 368 (–C_2H_2–Ac, 0·5%); 365 (–C_2H_5–Ac, 1%); 310 (–[C_{24}–C_{27}]–H–Ac, 2%); 296 (–[C_{23}–C_{27}]–H–Ac, 3%); 255 (–side chain–Ac, 4%); 253 (–side chain–Ac–2H, 3%); 213 (–side chain–Ac-part of ring D, 5%). This MS indicates the presence of clerosterol and not *iso*fucosterol.

The analysis of fatty acids from the steryl esters. The mixture of methylated fatty acids was separated by GLC at 200° on Diotomte CQ impregnated with 19% PEGA.

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