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Producción de saponinas triterpénicas en cultivos *in vitro* de *Centella asiatica*

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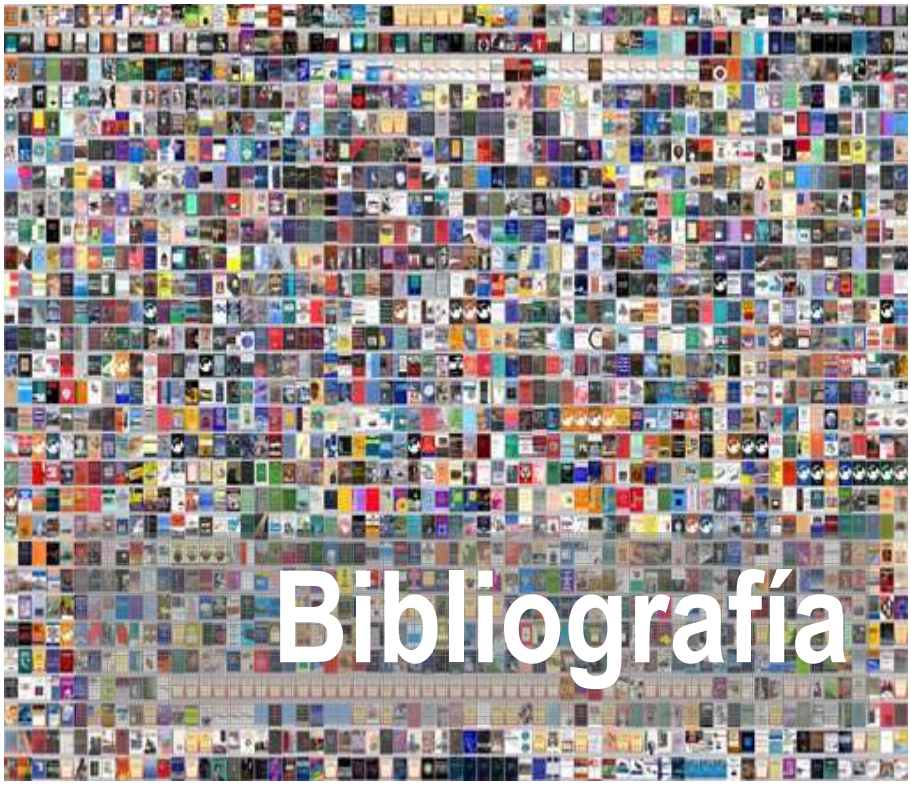
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Anexo de análisis estadístico

Anexo de análisis estadístico

Anexo tabla 1-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas entre los tratamientos para la germinación. En la tabla "a" demuestra que hay diferencias entre los tratamientos puesto que el P-valor<0,05, mientras que en la tabla "b" permite determinar cuales medias de los tratamientos son diferentes.

A1-. Análisis de Varianza para germinación en semillas naturales - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tratamiento	476,25	3	158,75	198,04	***0,0000***
RESIDUOS	6,4128	8	0,8016		
TOTAL (CORREGIDO)	482,663	11			

A2-. Pruebas de Múltiple Rangos para germinación en semillas naturales en cada tratamiento

Método: 95,0 porcentajes Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	3	0,0	0,516914	X
Giberelina	3	7,0	0,516914	X
Escarificación	3	14,0	0,516914	X
Aplastamiento	3	16,0	0,516914	X

B1-.Análisis de Varianza para valores de germinación en semillas artificiales - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:SH/SH_ML	1441,5	1	1441,5	49,91	***0,0021***
RESIDUOS	115,525	4	28,8813		
TOTAL (CORREGIDO)	1557,03	5			

Todas las razones-F se basan en el cuadrado medio del error residual

B2-.Pruebas de Múltiple Rangos para valores germinacion para SH_SH_ML

Método: 95,0 porcentaje Tukey HSD

SH_SH_ML	Casos	Media LS	Sigma LS	Grupos Homogéneos
SH	3	64,0	3,10276	X
SH-MI	3	95,0	3,10276	X

Anexo de análisis estadísticos

Anexo tabla 2-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas entre los medios de inducción por tallo. En la tabla "a" demuestra que hay diferencias entre los tratamientos puesto que el P-valor<0,05, mientras que en la tabla "b" permite determinar cuales medias de los medios probados son diferentes estadísticamente.

a-. Análisis de Varianza para valores medio tallo - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Medios inducción tallo	6619,34	2	3309,67	168,36	***0,0000***
RESIDUOS	117,949	6	19,6582		
TOTAL (CORREGIDO)	6737,29	8			

b-. Pruebas de Múltiple Rangos para medios inducción de tallo

Método: 95,0 porcentaje Tukey HSD

medios inducción tallo	Casos	Media LS	Sigma LS	Grupos Homogéneos
IBA (0,1 mg/l) + BA (2 mg/l)	3	10,5	2,55983	X
AIA (2 mg/l) + KIN (0,2 mg/l)	3	61,8	2,55983	X
NAA (2 mg/l) + BA (2 mg/l)	3	72,7	2,55983	X

Anexo tabla 3-.Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas entre los medios de inducción por hoja. En la tabla "a" demuestra que hay diferencias entre los tratamientos puesto que el P-valor<0,05, mientras que en la tabla "b" permite determinar cuales medias de los medios probados son diferentes estadísticamente.

a-. Análisis de Varianza para valores medio inducción en hoja - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Medios inducción hoja	2022,74	3	674,248	16,90	***0,0008***
RESIDUOS	319,134	8	39,8917		
TOTAL (CORREGIDO)	2341,88	11			

b-. Pruebas de Múltiple Rangos para valores de medios de inducción hoja

Método: 95,0 porcentajes Tukey HSD

Medios inducción hoja	Casos	Media LS	Sigma LS	Grupos Homogéneos
2,4-D (1mg/l) + KIN (0,1 mg/l)	3	56,9	3,64654	X
IBA (0,1 mg/l) + BA (2 mg/l)	3	80,7	3,64654	X
AIA (2 mg/l) + KIN (0,2 mg/l)	3	81,4	3,64654	X
NAA (2 mg/l) + BA (2 mg/l)	3	92,5	3,64654	X

Anexo tabla 4-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas entre los medios de crecimiento. En la tabla "a" demuestra que hay diferencias entre los medios utilizados puesto que el P-valor<0,05, mientras que en la tabla "b" permitió determinar cual de los medios es estadísticamente diferentes al resto.

a-.Análisis de Varianza para valores_medio_crecimiento - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	0,131748	3	0,043916	306,24	***0,0000***
B:Medio crecimiento 4-PU-30	0,00248581	2	0,00124291	8,67	***0,0015***
INTERACCIONES					
AB	0,0119558	6	0,00199263	13,90	***0,0000***
RESIDUOS	0,0034417	24	0,000143404		
TOTAL (CORREGIDO)	0,149631	35			

b-.Método: 95,0 porcentaje Tukey HSD

Medio crecimiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
MS+PU 2 mg/l	12	0,128538	0,00345692	X
MS+PU 1mg/l	12	0,136	0,00345692	X
MS+PU 3 mg/l	12	0,148669	0,00345692	X

Anexo tabla 5-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en la producción de los centellósidos entre los medios de crecimiento. En la tabla "a" demuestra que hay diferencias entre los medios utilizados puesto que el P-valor<0,05, mientras que en la tabla "b" permitió determinar cual de los medios es estadísticamente diferente al resto.

a-. Análisis de Varianza para producción total - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	0,0468667	2	0,0234333	17,90	***0,0001***
B:Crecimiento 4-PU30	0,0164667	2	0,00823333	6,29	***0,0085***
INTERACCIONES					
AB	0,0185333	4	0,00463333	3,54	***0,0268***
RESIDUOS	0,0235622	18	0,00130901		
TOTAL (CORREGIDO)	0,105429	26			

Anexo de análisis estadísticos

b-. Pruebas de Múltiple Rangos para crecimiento producción totales por crecimiento 4-PU30

Método: 95,0 porcentaje Tukey HSD

Crecimiento 4-PU30	Casos	Media LS	Sigma LS	Grupos Homogéneos
2 mg/l	9	0,426667	0,0120601	X
1 mg/l	9	0,436667	0,0120601	X
3 mg/l	9	0,483333	0,0120601	X

Anexo tabla 6-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en la citoquinina. No hay diferencias entre las dos citoquininas utilizados puesto que el P-valor>0,05.

Análisis de Varianza - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Citoquinina	0,0	1	0,0	0,00	1,0000
B:Principio activo	0,19155	3	0,06385	386,98	***0,0000***
RESIDUOS	0,00313488	19	0,000164994		
TOTAL (CORREGIDO)	0,194685	23			

Anexo tabla 7-.Análisis estadístico mediante ANOVA, para determinar si existen diferencias entre las plantas elicitadas a la control. Hay diferencias entre las plantas tratadas y las no tratadas, el P-valor<0,05.

Análisis de Varianza para peso seco - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tratamiento	0,386334	1	0,386334	184,65	***0,0000***
B:Parte planta	0,521265	1	0,521265	249,15	***0,0000***
C:Tiempo	0,141834	1	0,141834	67,79	***0,0000***
INTERACCIONES					
AB	0,0271354	1	0,0271354	12,97	***0,0024***
AC	0,000975375	1	0,000975375	0,47	0,5045
BC	0,164838	1	0,164838	78,79	***0,0000***
ABC	0,00413438	1	0,00413438	1,98	0,1789
RESIDUOS	0,0334753	16	0,00209221		
TOTAL (CORREGIDO)	1,27999	23			

Anexo tabla 8-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en la producción de planta elicitada. Existen diferencias entre los tratamientos y la parte de la planta analizada, el P-valor<0,05.

Análisis de Varianza - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tratamiento	2,42348	1	2,42348	17,94	***0,0004***
B:Parte planta	10,8502	1	10,8502	80,30	***0,0000***
C:Tiempo	0,921399	1	0,921399	6,82	***0,0167***
RESIDUOS	2,70234	20	0,135117		
TOTAL (CORREGIDO)	16,8975	23			

Anexo tabla 9-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en la producción de esteroles en plantas elicitadas. Existen diferencias en los niveles de fitosteroles entre las plantas elicitadas y las control, P-valor<0,05.

A-.Análisis de Varianza para plantas elicitada esteroles total - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: tiempo	0,00346492	1	0,00346492	30,45	***0,0000***
B: parte	0,00755809	1	0,00755809	66,42	***0,0000***
C: tratamiento	0,03609	1	0,03609	317,14	***0,0000***
INTERACCIONES					
AB	0,0105624	1	0,0105624	92,82	***0,0000***
AC	0,0138695	1	0,0138695	121,88	***0,0000***
BC	0,0191051	1	0,0191051	167,89	***0,0000***
ABC	0,00784206	1	0,00784206	68,91	***0,0000***
RESIDUOS	0,00182076	16	0,000113797		
TOTAL (CORREGIDO)	0,104145	23			

B1-.Pruebas de Múltiple Rangos para tiempo

Método: 95,0 porcentaje Tukey HSD

Tiempo	Casos	Media LS	Sigma LS	Grupos Homogéneos
2ª semana	12	0,162942	0,00307947	X
4ª semana	12	0,187345	0,00317424	X

B2-.Pruebas de Múltiple Rangos para parte

Método: 95,0 porcentaje Tukey HSD

Parte	Casos	Media LS	Sigma LS	Grupos Homogéneos
Raíz	11	0,157122	0,00326627	X
Hoja	13	0,193165	0,00298168	X

B3-.Pruebas de Múltiple Rangos para tratamiento

Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	12	0,135764	0,00307947	X
control	12	0,214523	0,00317424	X

Anexo tabla 10-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en el control interno utilizado. No hay diferencias entre las diferentes concentraciones, P-valor>0,05.

Análisis de Varianza para control interno - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:0,5 µl	171,6	4	42,9	1,47	0,2818
A: 1 µl	956,4	4	239,1	3,32	0,0564
A:1,5 µl	873,6	4	218,4	2,42	0,1176

Anexo tabla 11-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en la producción de centellósidos en el estudio de crecimiento de suspensiones celulares. Existen diferencias, P-valor<0,05.

Análisis de Varianza para producción total - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	0,0324044	6	0,00540073	88,86	***0,0000***
RESIDUOS	0,000850903	14	0,0000607788		
TOTAL (CORREGIDO)	0,0332553	20			

Anexo tabla 12-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas entre los medios MS + 2,4D2 + BA0,1 y MS + ANA1 + BA1. No existen diferencias entre los dos medios, P-valor>0,05.

Análisis de Varianza para valores totales - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:ANA_24D	0,00387269	1	0,00387269	2,27	0,1412
B:Tiempo	0,469321	6	0,0782202	45,83	***0,0000***
RESIDUOS	0,058027	34	0,00170668		
TOTAL (CORREGIDO)	0,531221	41			

Anexo tabla 13-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en el crecimiento celular de la línea control y tratadas con jasmonato de metilo, 0,1 mM. Hay diferencias entre las tratamientos, P-valor<0,05.

Análisis de Varianza para valores_100 - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	0,2229	3	0,0743	2,13	0,1361
B:Control / MeJa	0,1536	1	0,1536	4,41	0,0519
INTERACCIONES					
AB	0,4611	3	0,1537	4,41	***0,0192***
RESIDUOS	0,557249	16	0,034828		
TOTAL (CORREGIDO)	1,39485	23			

Anexo tabla 14-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,1 mM provoca diferencias significativas en la producción de centellósidos. Hay diferencias entre tratamientos, P-valor<0,05.

a-. Análisis de Varianza para valores totales - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	5,73304	1	5,73304	927,39	***0,0000***
B: Tiempo	3,94119	3	1,31373	212,51	***0,0000***
INTERACCIONES					
AB	1,67755	3	0,559184	90,45	***0,0000***
RESIDUOS	0,0989107	16	0,00618192		
TOTAL (CORREGIDO)	11,4507	23			

b-. Pruebas de Múltiple Rangos para diferenciar entre compuestos en Control.

Método: 95,0 porcentaje Tukey HSD

compuesto	Casos	Media LS	Sigma LS	Grupos Homogéneos
Asiaticósido	12	0,0537993	0,0037388	X
Madecasósido	12	0,160487	0,0037388	X

b-.Pruebas de Múltiple Rangos para diferencias entre compuestos en las elicitadas

Método: 95,0 porcentaje Tukey HSD

compuesto	Casos	Media LS	Sigma LS	Grupos Homogéneos
Madecasósido	12	0,471777	0,0165318	X
Asiaticósido	12	0,719745	0,0165318	X

Anexo de análisis estadísticos

Anexo tabla 15-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,1 mM provoca diferencias significativas en la producción de fitosteroles individuales. Hay diferencias entre tratamientos, P-valor<0,05.

A1-.Análisis de Varianza para sitosterol - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	13282,8	1	13282,8	298,88	***0,0000***
B: Tiempo	13135,8	3	4378,59	98,52	***0,0000***
INTERACCIONES					
AB	7393,99	3	2464,66	55,46	***0,0000***
RESIDUOS	711,062	16	44,4414		
TOTAL (CORREGIDO)	34523,6	23			

A2-.Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	12	50,8728	1,92443	X
Control	12	97,9238	1,92443	X

B1-.Análisis de Varianza para estigmasterol + campesterol - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Tiempo	25511,1	3	8503,71	78,61	***0,0000***
B: Control / MeJa	2038,05	1	2038,05	18,84	***0,0005***
INTERACCIONES					
AB	2824,43	3	941,478	8,70	***0,0012***
RESIDUOS	1730,86	16	108,179		
TOTAL (CORREGIDO)	32104,5	23			

B2-.Pruebas de Múltiple Rangos para estigmas estigmasterol + campesterol

Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	12	115,834	3,00248	X
MeJa	12	134,265	3,00248	X

Anexo tabla 16-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,1 mM provoca diferencias en la producción de fitosteroles totales. Hay diferencias entre tratamientos, P-valor<0,05.

Análisis de Varianza para totales fitosteroles - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	4635,86	1	4635,86	57,01	***0,0000***
B: Tiempo	11522,9	3	3840,97	47,23	***0,0000***
INTERACCIONES					
AB	14804,7	3	4934,89	60,68	***0,0000***
RESIDUOS	1301,12	16	81,3202		
TOTAL (CORREGIDO)	32264,6	23			

Anexo tabla 17-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la escualeno sintasa a una concentración de 0.1 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para la escualeno sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	3,12769E9	1	3,12769E9	58,46	***0,0000***
B: Tiempo	1,26873E11	9	1,4097E10	263,51	***0,0000***
INTERACCIONES					
AB	3,55784E10	9	3,95316E9	73,89	***0,0000***
RESIDUOS	2,13991E9	40	5,34977E7		
TOTAL (CORREGIDO)	1,67719E11	59			

b-.Pruebas de Múltiple Rangos para la escualeno sintasa

Método: 95,0 porcentaje Tukey HSD

temps100	Casos	Media LS	Sigma LS	Grupos Homogéneos
2h	6	33616,3	11326,7	X
20h	6	34240,1	11326,7	X
4h	6	38882,3	11326,7	X
1h	6	41712,5	11326,7	X
8h	6	44629,4	11326,7	X
30d	6	50794,9	11326,7	X
25d	6	76543,4	11326,7	X
48h	6	134343,	11326,7	X
15d	6	143090,	11326,7	X
20d	6	150437,	11326,7	X

Anexo tabla 18-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la α-amirina sintasa a una concentración de 0,1 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para la α-amirina sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	2,86897E9	1	2,86897E9	151,82	***0,0000***
B: Tiempo	1,92134E10	9	2,13482E9	112,97	***0,0000***
INTERACCIONES					
AB	1,62605E10	9	1,80672E9	95,61	***0,0000***
RESIDUOS	7,55863E8	40	1,88966E7		
TOTAL (CORREGIDO)	3,90987E10	59			

b-.Pruebas de Múltiple Rangos para α-amirina sintasa

Método: 95,0 porcentaje Tukey HSD

Expresión α-amirina sintasa	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	30	41184,4	793,653	X
MeJa	30	55014,2	793,653	X

Anexo tabla 19-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la cicloartenol sintasa a una concentración de 0,1 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para la cicloartenol sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	3,734E9	9	4,14888E8	27,48	***0,0000***
B:Tratamiento	3,9898E9	1	3,9898E9	264,27	***0,0000***
INTERACCIONES					
AB	3,56117E9	9	3,95686E8	26,21	***0,0000***
RESIDUOS	6,03894E8	40	1,50974E7		
TOTAL (CORREGIDO)	1,18889E10	59			

b-.Pruebas de Múltiple Rangos para cicloartenol sintasa

Método: 95,0 porcentaje Tukey HSD

Expresión cicloartenol sintasa	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	30	38745,0	709,398	X
Control	30	55054,1	709,398	X

Anexo tabla 20-. Análisis estadístico mediante ANOVA, para determinar si existen diferencias significativas en el crecimiento celular de la línea control y tratadas con jasmonato de metilo, 0,2 mM. Hay diferencias entre las tratamientos, P-valor<0,05.

Análisis de Varianza para crecimiento - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Tiempo	2,92177	3	0,973924	18,85	***0,0000***
B: Control / MeJa	1,01846	1	1,01846	19,71	***0,0003***
RESIDUOS	0,981815	19	0,0516745		
TOTAL (CORREGIDO)	4,92205	23			

Anexo de análisis estadísticos

Anexo tabla 21-. Análisis estadístico mediante ANOVA, para determinar si la viabilidad celular presenta diferencias significativas entre 0,1 mM, 0,2 mM y el control. Existen diferencias entre los tratamientos, P-valor<0,05.

Análisis de Varianza - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Tiempo	12558,2	7	1794,03	45,53	***0,0000***
B: Control / MeJa	761,79	2	380,895	9,67	***0,0003***
INTERACCIONES					
AB	1413,81	14	100,986	2,56	***0,0079***
RESIDUOS	1891,36	48	39,4033		
TOTAL (CORREGIDO)	16625,1	71			

Anexo tabla 22-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,2 mM provoca diferencias significativas en la producción de centellósidos. Hay diferencias entre tratamientos, P-valor<0,05.

a-.Análisis de Varianza para producción - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	0,00178113	1	0,00178113	95,07	***0,0000***
B: Tiempo	0,00528568	3	0,00176189	94,04	***0,0000***
INTERACCIONES					
AB	0,0057719	3	0,00192397	102,69	***0,0000***
RESIDUOS	0,000299758	16	0,0000187349		
TOTAL (CORREGIDO)	0,0131385	23			

Anexo tabla 23-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,2 mM provoca diferencias significativas en la producción de esteroles totales. Hay diferencias entre tratamientos.

Análisis de Varianza para fitosteroles totales - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	11997,5	1	11997,5	36,73	***0,0000***
B: Tiempo	27856,9	3	9285,64	28,43	***0,0000***
INTERACCIONES					
AB	13998,6	3	4666,2	14,29	***0,0001***
RESIDUOS	5226,06	16	326,629		
TOTAL (CORREGIDO)	59079,0	23			

Anexo tabla 24-. Análisis estadístico mediante ANOVA, para determinar si el MeJa a 0,2 mM provoca diferencias significativas en la producción de esteroles individuales. Hay diferencias entre tratamientos, P-valor<0,05.

A1-.Análisis de Varianza para sitosterol- Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	25157,8	1	25157,8	371,99	***0,0000***
B: Tiempo	19538,4	3	6512,79	96,30	***0,0000***
INTERACCIONES					
AB	6786,02	3	2262,01	33,45	***0,0000***
RESIDUOS	1082,09	16	67,6306		
TOTAL (CORREGIDO)	52564,3	23			

A2-.Pruebas de Múltiple Rangos para sitosterol

Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	12	59,3959	2,374	X
Control	12	124,149	2,374	X

B1-. Análisis de Varianza para estigmasterol + campestesterol - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Control / MeJa	2408,96	1	2408,96	21,24	***0,0003***
B: Tiempo	21122,7	3	7040,9	62,08	***0,0000***
INTERACCIONES					
AB	1690,77	3	563,591	4,97	***0,0126***
RESIDUOS	1814,73	16	113,421		
TOTAL (CORREGIDO)	27037,2	23			

B2-.Pruebas de Múltiple Rangos para estigmasterol + campestesterol

Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	12	119,098	3,07436	X
MeJa	12	139,135	3,07436	X

Anexo de análisis estadísticos

Anexo tabla 25-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la escualeno sintasa a una concentración de 0,2 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

Análisis de Varianza la escualeno sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	2,05706E10	8	2,57132E9	74,43	***0,0000***
B:Control / MeJa	1,45117E9	1	1,45117E9	42,01	***0,0000***
RESIDUOS	1,51997E9	44	3,45448E7		
TOTAL (CORREGIDO)	2,35417E10	53			

Anexo tabla 26-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la α-amirina sintasa a una concentración de 0,2 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para α-amirina sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Tiempo	2,37289E10	8	2,96611E9	195,14	***0,0000***
B:Control / MeJa	2,30662E8	1	2,30662E8	15,17	***0,0004***
INTERACCIONES					
AB	6,20577E9	8	7,75721E8	51,03	***0,0000***
RESIDUOS	5,47205E8	36	1,52001E7		
TOTAL (CORREGIDO)	3,07125E10	53			

b-.Pruebas de Múltiple Rangos para α-amirina sintasa

Método: 95,0 porcentaje Tukey HSD

Expresión α-amirina sintasa	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	27	40552,5	750,312	X
Control	27	44686,1	750,312	X

Anexo tabla 27-. Análisis estadístico mediante ANOVA, para determinar si presenta diferencia significativa la expresión de la cicloartenol sintasa a una concentración de 0,2 mM. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para cicloartenol sintasa - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	6,67434E10	8	8,34293E9	115,44	***0,0000***
B:Control / MeJa	7,00325E10	1	7,00325E10	969,05	***0,0000***
INTERACCIONES					
AB	3,05788E10	8	3,82235E9	52,89	***0,0000***
RESIDUOS	2,60169E9	36	7,2269E7		
TOTAL (CORREGIDO)	1,69956E11	53			

b-.Pruebas de Múltiple Rangos para cicloartenol sintasa

Método: 95,0 porcentaje Tukey HSD

Expresión cicloartenol sintasa	Casos	Media LS	Sigma LS	Grupos Homogéneos
MeJa	27	54502,0	1636,04	X
Control	27	126527,	1636,04	X

Anexo tabla 28-. Análisis estadístico mediante ANOVA, para determinar si existen diferencia significativa entre las concentraciones de DMSO. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para DMSO_valores - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	908,755	5	181,751	5,97	***0,0002***
B:Concentraciones DMSO	3179,23	3	1059,74	34,83	***0,0000***
INTERACCIONES					
AB	980,255	15	65,3503	2,15	***0,0231***
RESIDUOS	1460,28	48	30,4225		
TOTAL (CORREGIDO)	6528,52	71			

b-.Pruebas de Múltiple Rangos para DMSO_valores por porcentaje

Método: 95,0 porcentaje Tukey HSD

porcentaje	Casos	Media LS	Sigma LS	Grupos Homogéneos
2,50%	**18**	59,3833	1,30005	X
1,25%	**18**	64,7667	1,30005	X
0,63%	**18**	74,3333	1,30005	X
C	**18**	75,25	1,30005	X

c-.Análisis de Varianza para control / 0.65% - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tiempo	852,703	5	170,541	4,74	***0,0037***
B:Control / 0.65%	7,5625	1	7,5625	0,21	0,6508
INTERACCIONES					
AB	165,463	5	33,0925	0,92	0,4852
RESIDUOS	863,583	24	35,9826		
TOTAL (CORREGIDO)	1889,31	35			

Anexo tabla 29-. Análisis estadístico mediante ANOVA, para determinar si existen diferencia significativa entre las diferentes biotransformaciones. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para biotransformaciones - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A: Compuesto	1,58346	3	0,527818	60,85	***0,0000***
B: Tratamiento	21,2579	3	7,08597	816,95	***0,0000***
INTERACCIONES					
AB	2,97634	9	0,330704	38,13	***0,0000***
RESIDUOS	0,277556	32	0,00867364		
TOTAL (CORREGIDO)	26,0952	47			

b-. Pruebas de Múltiple Rangos para biotransformaciones

Método: 95,0 porcentaje Tukey HSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	12	0,330434	0,026885	X
Acetona	12	0,433291	0,026885	X
Acetona + DMSO	12	0,845251	0,026885	X
Amirina + DMSO	12	2,0074	0,026885	X

Anexo tabla 30-. Análisis estadístico mediante ANOVA, para determinar si existen diferencia significativa entre las diferentes biotransformaciones mediadas por la β-ciclodextrina. Existen diferencias entre los tratamientos, P-valor<0,05.

a-.Análisis de Varianza para total - Suma de Cuadrados Tipo III

Fuente	Suma de Cuadrados	Gl	Cuadrado Medio	Razón-F	Valor-P
EFECTOS PRINCIPALES					
A:Tratamiento	1,0352	3	0,345068	18,75	***0,0006***
RESIDUOS	0,147191	8	0,0183989		
TOTAL (CORREGIDO)	1,18239	11			

b-. Pruebas de Múltiple Rangos para total por tratamiento

Método: 95,0 porcentaje LSD

Tratamiento	Casos	Media LS	Sigma LS	Grupos Homogéneos
Control	3	1,32174	0,0783132	X
Ciclodextrina + acet	3	1,501	0,0783132	XX
Ciclodextrina	3	1,75285	0,0783132	X
Amirina + ciclodextr	3	2,104	0,0783132	X

Anexo de publicaciones

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SHORT COMMUNICATION

Identification of triterpenoid compounds of *Centella asiatica* by thin-layer chromatography and mass spectrometry

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ABSTRACT: The identification of the four principal triterpenoid components of *Centella asiatica* has been achieved by TLC on silica gel plates and mass spectrometry, as a modification of the method described in the *European Pharmacopoeia* (5th edn). A combination of ethyl acetate and methanol as the mobile phase was found to be successful in separating these compounds from the rest of the main components of the extract. The spots were detected with anisaldehyde solution. The separated compounds were confirmed by MALDI -TOF mass spectrometry. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: *Centella asiatica*; triterpenes; normal-phase TLC; mass spectrometry

INTRODUCTION

Asiatic acid, madecassic acid, asiaticoside and madecassoside are the principle terpenoids with an ursane skeleton found in *Centella asiatica* (L) Urb. (Apiaceae), a plant which has traditionally been used as an antipyretic, diuretic and treatment for skin inflammations.

Several studies have identified the triterpenoid compounds of *Centella asiatica* using TLC coupled to high-speed counter-courrent chromatography (Diallo *et al*., 1991; Du *et al*., 2004).

A preliminary approach to the identification of these compounds by TLC can be found in the *European Pharmacopoeia* (2005) using drug powder as the starting material. However, this method was limited to pharmaceutical preparations, as other types of extracts (plant extracts, calli extracts) contain substances that interfere with the resolution of these compounds.

In order to improve the resolution of centellosides from different kinds of extracts using normal-phase TLC on silica gel plates, we have developed a specific TLC protocol with a modified elution solution. The compounds can be identified using anisaldehyde solution to locate the bands, but if they are recovered on the plate without using this solution, it is possible to carry out an identification using mass spectrometry. Moreover, this protocol can be used as a method to prepare samples for separation by HPLC.

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EXPERIMENTAL

Materials. Asiatic acid, madecassic acid, asiaticoside and madecassoside were obtained from ChromaDex Inc. The plant material was obtained from Plameca, S. A. (Hospitalet de Llobregat, Barcelona, Spain) and calli came from our *Centella in vitro* cultures. *Centella* powder (Blastoestimulina) was a pharmaceutical preparation from Almirall Prodesfarma (Barcelona, Spain) containing *Centella* extract. All other chemicals and solvents were of analytical or chromatographic grade.

Sample preparation. The stock standard solutions of the four compounds were prepared (10 mg/mL of each) in methanol. These stock solutions were diluted to obtain solutions of 2 mg/mL.

For extraction, 1 g of pharmaceutical powder was sonicated in methanol–water (9:1, v/v; 2 × 25 mL) for 2 h at ambient temperature and filtered through a 0.45 µm filter (Waters Millipore). The filtrate was evaporated to dryness. The dried extract was disolved in 1 mL of methanol and filtered through a 0.45 µm filter (Waters Millipore) and the clear filtrate was used for TLC analysis (Inamdar *et al*., 1996).

For plant and calli extraction, 1 g of fine powder was first sonicated in 10 mL ethyl acetate for 2 h at ambient temperature and filtered through a 0.22 µm filter (Waters Millipore). The filtrate was evaporated to dryness and redissoled in 1 mL of ethyl acetate for TLC analysis. The plant and callus powder was collected, dried and sonicated in methanol–water (9:1) followed by the procedure described above for pharmaceutical powder.

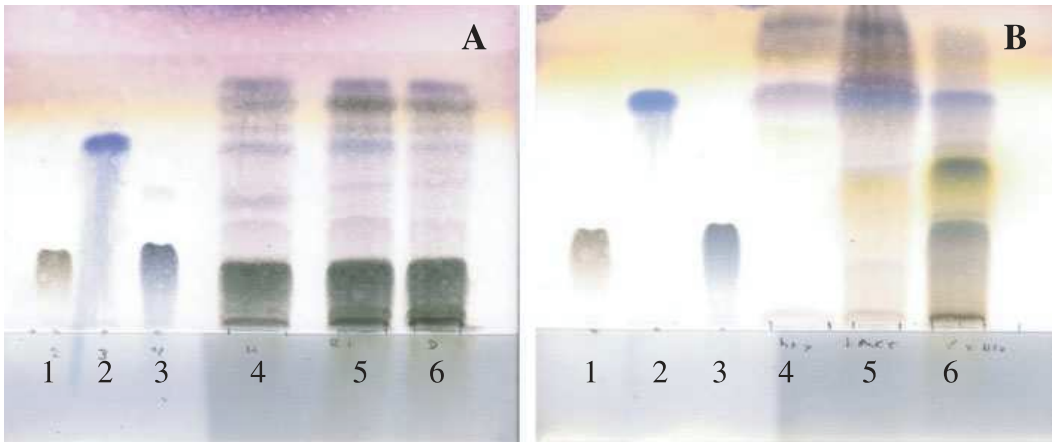

Figure 1. Photograph of chromatograms showing resolution of centellosides, (A) from callus extracts, (B) from plant extracts. Lane 1: madecassoside; lane 2: asiatic acid; lane 3: asiaticoside; lane 4: hexane extract; lane 5: ethyl acetate extract; lane 6: methanol extract. Development time, 40 min; temperature, 19 ± 2°C; detection, anisaldehyde solution.

Chromatographic studies. The separation was carried out at room temperature. The four centellosides were dissolved in methanol at a concentration of 2 mg/mL and 4 µL of this solution were applied to the plate with adapted Pasteur pipettes and dried. Samples of 100 µL from plant extract, callus extract and pharmaceutical preparation were applied to the plate as above. TLC separations were performed on 10 × 10 cm silica gel 60 F_{254}-covered plates with concentrating zone of 10 × 2.5 cm (Merck, Darmstadt, Germany) without a fluorescent indicator. The solvent used was ethyl acetate–methanol (60:40, v/v). Chromatograms were run in small glass tanks lined with chromatography paper equilibrated with the running solvent. The developed plates were dried at room temperature and centellosides were identified on TLC plates sprayed with anisaldehyde solution, heated at 100–105°C and visualized under daylight.

MS identification. Some developed TLC bands were only revealed by spraying the part corresponding to the standards but not the part corresponding to the samples. The bands corresponding to the samples were scraped off and the powder was diluted in 1 mL methanol, filtered through a 0.45 µm filter (Waters Millipore) and the filtrate was taken to dryness and used for MALDI-TOF identification. MALDI-TOF MS was done in positive ionization mode using Applied Biosystems Voyager-DE RP apparatus. The absorbing matrix compound used was α-cyano-4-hydroxycinnamic acid.

RESULTS

Using the TLC identification method from the *European Pharmacopoeia* (2005), the separation of the centellosides from our plant and callus extracts was not achieved due to a large amount of compounds that interfered particularly with asiaticoside and madecassoside in the lower half of the plate, near the origin. This problem was resolved by testing different solvents that permit separation of these two trisacharides from the interfering compounds, at the same time maintaining good resolution of the acids (asiatic acid, madecassic acid). It was also necessary to clean the samples by washing with ethyl acetate before the extraction with methanol–water (9:1, v/v). We also tried cleaning the samples with hexane, which removed a high amount of asiatic acid and madecassic acid; however, we decided to use only ethyl acetate. In relation to the pharmaceutical preparation, it was not necessary to pre-clean the sample with ethyl acetate.

The solvent chosen for the plate development was ethyl acetate–methanol (60:40, v/v). Figure 1(A) shows the photograph of a chromatogram with three standards and the three types of callus extracts (hexane, ethyl acetate and methanol) and Fig. 1(B) shows the same with the plant extracts. The R_f values for the identified centellosides are shown in Table 1. The results are averages of at least five identical runs.

To further confirm the identification of the four centellosides obtained by TLC, MALDI-TOF mass spectrometry was carried out, and gave mass ions $[M + Na]^+$ at *m/z* 997.6, 981.6, 527.4 511.3 for

Table 1. The R_f values of centellosides on plates developed with 60% ethyl acetate–40% methanol

Compound	R_f from mixture
Madecassoside	0.35
Asiaticoside	0.37
Madecassic acid	0.88
Asiatic acid	0.90

madecassoside, asiaticoside, madecassic acid and asiatic acid, respectively.

Moreover, this method can be used to prepare samples for HPLC analysis. For each HPLC analysis, the fringes on the TLC plate corresponding to the four compounds were cut and eluted with methanol. The solutions were filtered and concentrated. In the case of the plant extract, the ethyl acetate solution (see Experimental section) contained significant amounts of asiatic acid and madecassic acid, and for this reason the zone corresponding to these compounds on the plate was also recovered for a later analysis by HPLC (data not shown). Each of the solutions contained the four centellosides without the interfering compounds.

DISCUSSION

The aim of this work is to offer an alternative TLC method for the identification of asiatic acid, madecassic acid, asiaticoside and madecassoside, which can be particularly useful in the case of extracts that can give problems in the resolution of glycosidic saponines.

In addition to the separation of the standards, we have assayed three different kinds of extracts and the resolution of the four centellosides in each one was good enough to be used in a later HPLC analysis.

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The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* cultured plants

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Abstract

Considering that exogenously applied methyl jasmonate can enhance secondary metabolite production in a variety of plant species and that 2,3-oxidosqualene is a common precursor of triterpenes and sterols in plants, we have studied *Centella asiatica* and *Galphimia glauca* (both synthesizing triterpenoid secondary compounds) and *Ruscus aculeatus* (which synthesizes steroidal secondary compounds) for their growth rate and content of free sterols and respective secondary compounds, after culturing with or without 100 μM methyl jasmonate. Our results show that elicited plantlets of *G. glauca* and to a higher degree *C. asiatica* (up to 152-times more) increased their content of triterpenoids directly synthesized from 2,3-oxidosqualene (ursane saponins and nor-seco-friedelane galphimines, respectively) at the same time as growth decreased. In contrast, the free sterol content of *C. asiatica* decreased notably, and remained practically unaltered in *G. glauca*. However, in the case of *R. aculeatus*, which synthesizes steroidal saponins (mainly spirostane type) indirectly from 2,3-oxidosqualene after the latter is converted to the plant phytosterol-precursor cycloartenol, while the growth rate and free sterol content clearly decreased, the spirostane saponine content was virtually unchanged (aerial part) or somewhat lower (roots) in presence of the same elicitor concentration. Our results suggest that while methyl jasmonate may be used as an inducer of enzymes involved in the triterpenoid synthesis downstream from 2,3-oxidosqualene in both *C. asiatica* and *G. glauca* plantlets, in those of *C. asiatica* and *R. aculeatus* it inhibited the enzymes involved in sterol synthesis downstream from cycloartenol.

Keywords: *Centella asiatica*; *Ruscus aculeatus*; *Galphimia glauca*; Elicitation; Triterpenes; Ursane saponins; Spirostane saponins; Galphimine-B; Phytosterols

1. Introduction

Perhaps one of the most diverse groups of plant secondary metabolites are terpenoids, which are also found in microorganisms and animals. Included in this group are the triterpenes (C_{30}) and sterols (C_{18}–C_{29}), whose structurally diverse molecules proceed from a common precursor, the squalene. Metabolic pathways originating from squalene form an extensive net of compounds with defined branching points that diversify the end products, including compounds with primary roles in membrane architecture (sterols such as sitosterol, stigmasterol and campesterol) as well as a variety of secondary metabolites specific to each plant species (Grunwald, 1980; Seigler, 1998).

Centella asiatica (L.) Urban is a herbaceous plant with great medicinal value belonging to the Apiaceae family. Notable bioactive compounds of *C. asiatica* are the triterpene saponins madecassoside and asiaticoside, with their respective ursane type sapogenins madecassic and asiatic acid. As shown in Fig. 1, these compounds, referred to as centellosides, proceed from the cyclisation of 2,3-oxidosqualene by a specific oxidosqualene cyclase (OSC), β-amyrin synthase.

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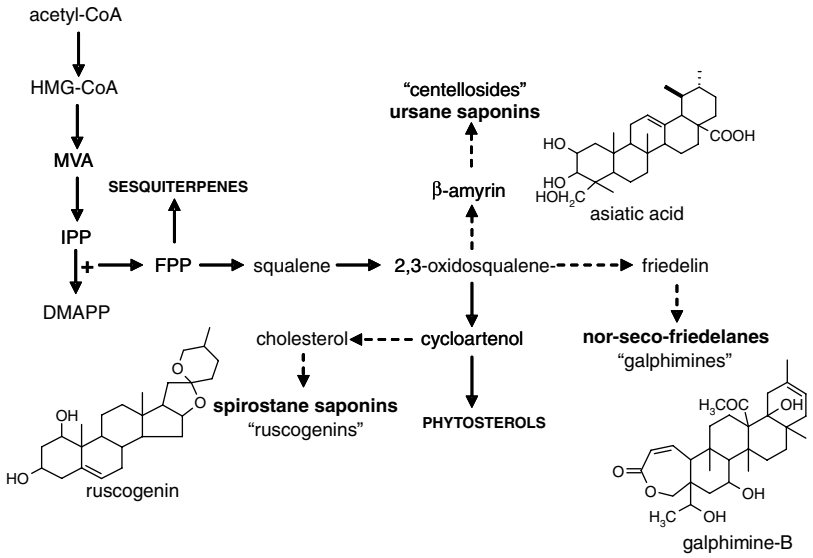

Fig. 1. Relationships between sterol and triterpene biosynthesis in *C. asiatica*, *R. aculeatus* and *G. glauca*.

Ruscus aculeatus L. (butcher's broom) of the Liliaceae family is a small evergreen shrub whose active components are steroidal saponins. Most of these are of the spirostane type, including the aglycones neoruscogenin and ruscogenin. Like triterpene saponins in *C. asiatica*, these compounds proceed from the cyclisation of 2,3-oxidosqualene, but their pathways differ in that steroidal saponins are formed via cycloartenol, a precursor they share with phytosterols (Fig. 1). Despite the interest of this medicinal plant, no studies on the biosynthesis of its active principles have been carried out until now.

Galphimia glauca Kav (Malpighiaceae) is a small evergreen tropical shrub extending from Mexico to Guatemala in Central America. Its main active component is galphimine-B, a nor-seco-triterpene of the friedelane type, with sedative and spasmolytic activities. Although the complete galphimine-B biosynthesis is not known, Corsino et al. (2000) have reported that the key branching point in the pathway to friedelane terpenoids is the cyclisation of 2,3-oxidosqualene by OSC. Similar to the biosynthesis of *C. asiatica* triterpenoids (Fig. 1), and differing from that of *R. aculeatus* steroidal saponins, the route to friedelanes is not through cycloartenol. Corsino et al. (2000) have observed that the conversion of 2,3-oxidosqualene to friedelin takes place in the leaves of *Maytenus aquifolium* and *Salacia campestris*, in the same way that galphimine-B is biosynthesised in the aerial parts of *G. glauca* (Lara-Ochoa et al., 2005).

Generally, when plant cells perceive environmental changes via specific receptors or perception mechanisms, they generate biological responses through specific signal transduction. Jasmonic acid and its methyl ester (methyl jasmonate, MeJA) have been reported to play an important role in a signal transduction process that regulates defense genes in plants (Farmer and Ryan, 1990). However, although exogenously applied MeJA is widely used in plant cell cultures to activate secondary metabolism, there are surprisingly few studies about its impact on plant growth, considering that jasmonates have a variety of biological activities, including inhibition of seed and pollen germination (Feys et al., 1994; McConn and Browse, 1996) or inhibition of root growth and photosynthetic apparatus (Staswick et al., 1992; Reinbothe et al., 1993a,b; Rossato et al., 2002).

Recently, Kim et al. (2004) have studied the accumulation of asiaticoside in whole plant cultures of *C. asiatica*, reporting an enhancement of its production by MeJA treatment. However, no elicitation studies with MeJA have been done with whole *R. aculeatus* and *G. glauca* plants, although it is of interest that Osuna et al. (1999) observed a higher production of galphimine-B in *G. glauca* calli after increasing the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium.

With respect to sterol metabolism it is known that the amount of sterols is generally constant among plant species (Benveniste, 2004). Triterpene and sterol biosynthesis begins with the conversion of farnesyl diphosphate into squalene (2,3-oxidosqualene), which determines the channelling of the isoprenoid pathway into the branches that produce phytosterols (Fig. 1). The sterol pathway involves a sequence of more than 30 enzyme-catalyzed reactions, all of which are membrane linked. Nothing is known about the catabolism of plant sterols. Upregulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in transgenic tobacco, corn and tomatoes results in the accumulation of only cycloartenol, rather than sterols. Potential strategies for sterol production are, however, likely to focus on overexpression of terminal enzymes in the pathway (Hartmann, 1998).

In this study we show that the production of potentially downstream 2,3-oxidosqualene compounds, such as sterols with a primary role and pharmacologically active triterpenoids and steroids, can be affected in the aerial part and roots of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets by the addition of 100 μM methyl jasmonate to the culture medium.

2. Results and discussion

2.1. Characterization of cultured plants

As shown in Table 1, in all cases the growth capacity of the treated plantlets declined in comparison with the controls. In the case of *Ruscus*, the reduction of growth mainly affected the roots. After 2 weeks of MeJA treatment, the fresh weight of elicited roots was 20% lower than those of the control, and after 4 weeks the reduction of root growth was more than 50%, due to a total inhibition of root growth during weeks 2–4. Furthermore, these roots also showed abnormal morphology traits, such as necrosis of the root apex (Fig. 2).

C. asiatica plantlets treated with MeJA also showed a considerable reduction of growth (Table 1), with the fresh weight of aerial parts reduced by over 50% and a decline in root growth of more than 70%. As in the *Ruscus* cultures, root growth was completely blocked during weeks 2–4. In addition, the elicited *Centella* plantlets showed necrosis symptoms in leaves and roots at the end of the culture period (Fig. 2).

The inhibitory effect of MeJA on the development of cultured *G. glauca* plantlets (compared to controls) was even more evident than in *Centella* and *Ruscus* cultures. After 2 weeks of culture, treated *Galphimia* plantlets not only showed a drastic reduction of growth (Table 1), but also accelerated symptoms of senescence and necrosis (Fig. 2), mainly in the roots, which led to the plants' death before the end of the culture period (4 weeks).

The effect of MeJA or of any elicitor (biotic or abiotic) is dependent on a number of factors which may interact. These include the elicitor's specificity and concentration, the duration of treatment and the growth stage of the culture (Holden et al., 1988). Researchers have frequently used MeJA at a concentration of 100 μM to increase secondary metabolism in in vitro cultures (Ketchum et al., 1999; Cusidó et al., 2002; Palazón et al., 2003; Kim et al., 2004), with the plant cells or organs coming into direct contact with the elicitor. In contrast, in our plant cultures only the roots were directly exposed to MeJA, which was probably why its effects on root development were more pronounced. In this context, it is worth noting that jasmonates inhibit the growth of seedlings, roots and cell division. As Koda et al. (1996) have reported, growth inhibition by MeJA appears to be caused mainly by the disruption of cortical microtubules, a phenomenon ubiquitous in plants.

2.2. Effects of the elicitor on specific secondary compound content

In *C. asiatica* the levels of the main active compounds, the triterpene saponins madecassoside and asiaticoside

Table 1
Effect of methyl jasmonate on plant growth measured as fresh (FW) and dry weight (DW) after 2 or 4 weeks of treatment

Growth	Week	Control		Elicited	
		AP	Roots	AP	Roots
Ruscus aculeatus					
FW	2	1.257 ± 0.093	4.818 ± 0.136	1.032 ± 0.098	4.231 ± 0.237
	4	1.915 ± 0.108	6.702 ± 0.288	1.448 ± 0.124	3.271 ± 0.338
DW	2	0.347 ± 0.013	0.735 ± 0.088	0.248 ± 0.023	0.595 ± 0.011
	4	0.435 ± 0.049	0.953 ± 0.078	0.357 ± 0.021	0.516 ± 0.057
Centella asiatica					
FW	2	4.506 ± 0.109	5.695 ± 0.366	3.391 ± 0.428	2.514 ± 0.398
	4	8.083 ± 0.437	7.116 ± 0.387	5.198 ± 0.511	2.381 ± 0.255
DW	2	0.612 ± 0.067	0.524 ± 0.044	0.412 ± 0.038	0.242 ± 0.022
	4	0.918 ± 0.088	0.551 ± 0.029	0.745 ± 0.066	0.191 ± 0.041

Growth	Week	Control	Elicited
		WP	WP
Galphimia glauca			
FW	2	0.277 ± 0.022	0.167 ± 0.024
	4	nd	nd
DW	2	0.057 ± 0.010	0.029 ± 0.011
	4	nd	nd

Each value is the mean of 8–10 determinations ± SE.
AP, aerial part; WP, whole plant.

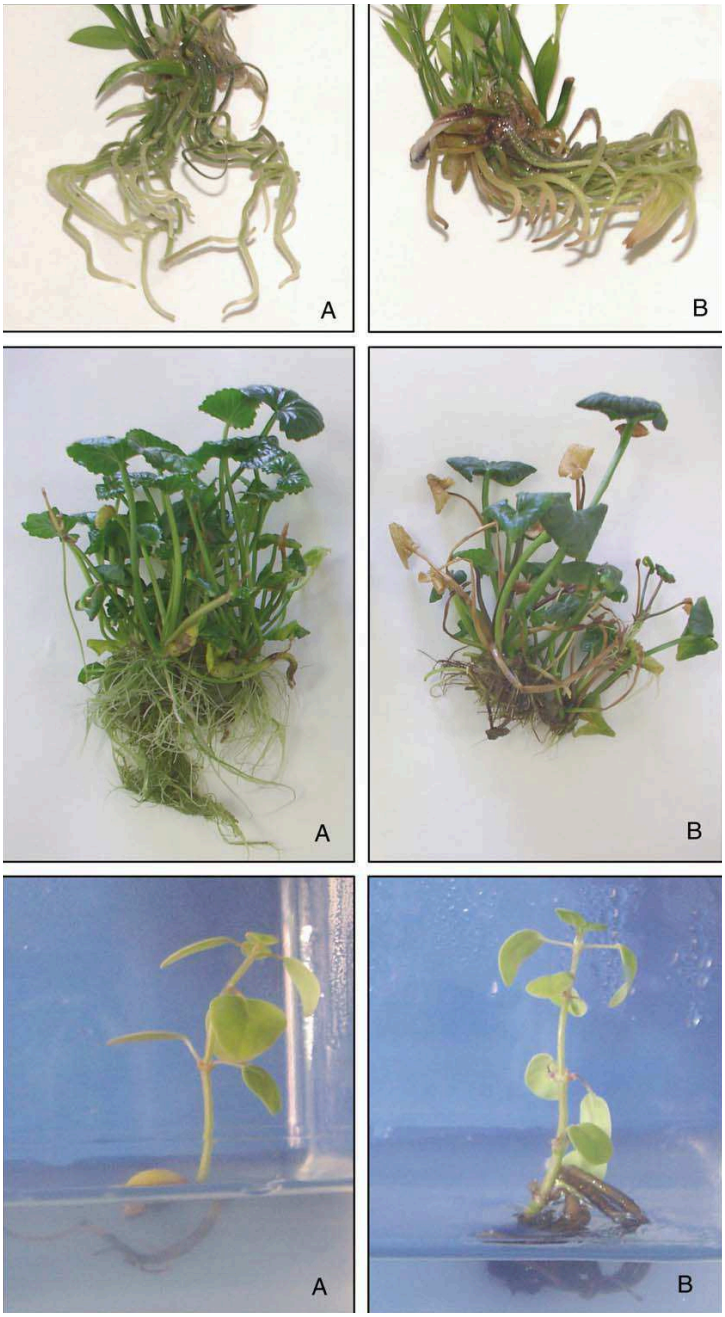

Fig. 2. Effects of methyl jasmonate on developing plantlets of *R. aculeatus* (up) and *C. asiatica* (center) and *G. glauca* (down). (A) Control plant, (B) elicited plant.

and their respective ursane sapogenins madecassic and asiatic acid, were determined in the aerial part and roots of both control and elicited plantlets after two and four weeks of culture. As shown in Fig. 3, in the aerial part of untreated plantlets the main compound was found to be asiaticoside followed by madecassoside and then asiatic acid and madecassic acid, while in the roots the asiaticoside content was very similar to or lower than that of madecassoside. The triterpene saponin content was always significantly ($p < 0.001$; *t*-test) higher in the aerial part than in the roots of plantlets, especially at 2 weeks of culture. The triterpenoid pattern in *Centella* differs according to

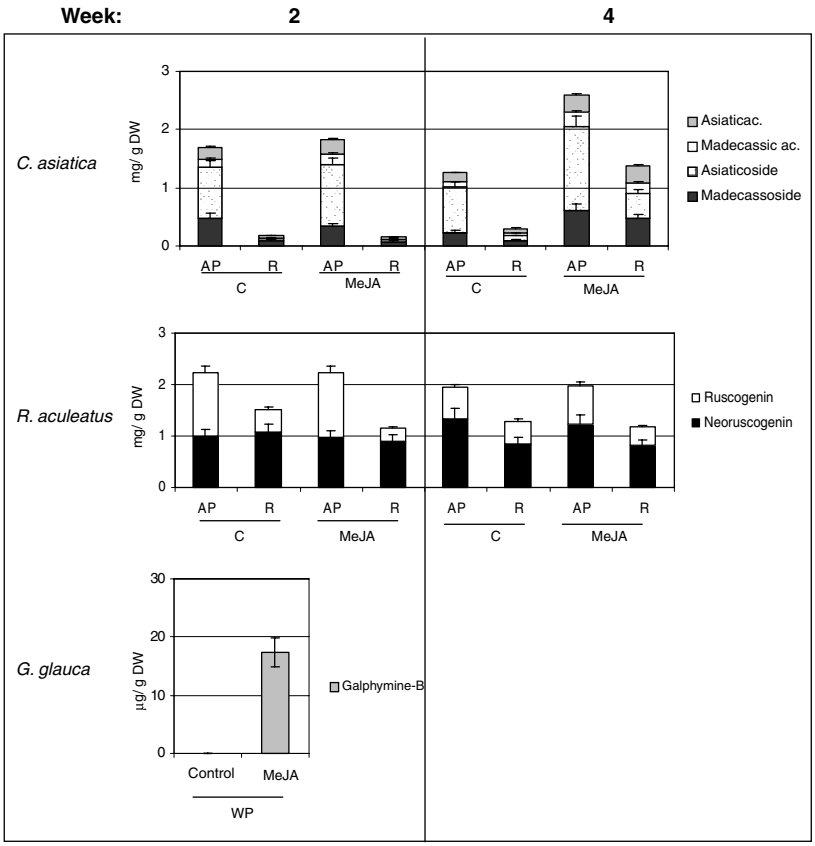

Fig. 3. Effects of methyl jasmonate on terpenoid secondary compound production of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–6 determinations. Bars represent ±SE. AP, aerial part; R, roots; WP, whole plant; C, control; MeJA, methyl jasmonate.

the species and culture region (Rouillard-Guellec et al., 1997), but a survey of the bibliography shows both asiaticoside and madecassoside as the predominant compounds in whole plants, although very little is known about their content in roots. As shown in Fig. 4, in our *Centella* cultures a similar centelloside pattern was found in the aerial part and roots of plants. This pattern was not changed by elicitor treatment and in all samples the ratios of the different centellosides were asiaticoside > madecassoside > asiatic acid > madecassic acid.

Compared to the controls, the content of triterpene saponins and ursane sapogenins in elicited *C. asiatica* plantlets increased only slightly in the aerial part at week 2 and increased significantly ($p < 0.001$) in both the aerial part and roots at week 4. At the end of the culture period, as can be deduced from the values in Fig. 3, the level of

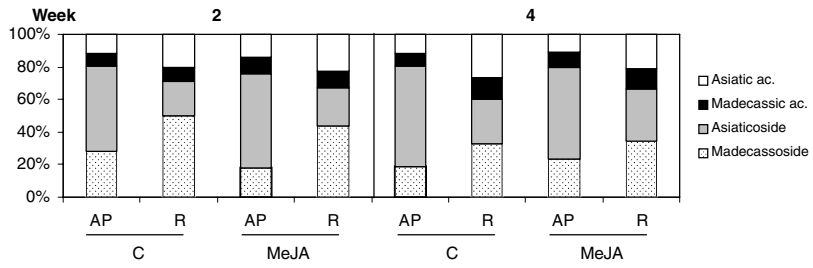

Fig. 4. Effects of methyl jasmonate on centelloside patterns (expressed as percentage of total centellosides) of *C. asiatica* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–6 determinations. Bars represent ±SE. AP, aerial part; R, roots; C, control; MeJA, methyl jasmonate.

madecassoside, asiaticoside, madecassic acid and asiatic acid in the aerial part was 2–3-fold higher, and 4–6-fold higher in the roots. These data suggest that the longer the elicitor was in contact with the roots, the greater the increase in all these triterpenoid compounds in both the aerial part and roots of cultured plantlets. The relatively high asiaticoside content in our elicited roots differs from previous observations that have suggested its production in *C. asiatica* is tissue-specific, with synthesis occurring mainly in the leaves (9.56 mg/g DW) and very little in the roots (0.17 mg/g DW) of whole plants cultured in presence of MeJA 100 μM (Kim et al., 2004). This difference is probably due to the fact that in our study only the roots were in contact with the elicitor.

In contrast, the production of steroidal saponins ruscogenin and neoruscogenin in the aerial part and roots of elicited *R. aculeatus* plantlets (Fig. 3) after 2 and 4 weeks was similar have any specific effect on the different metabolic steps of the biosynthesis of both these steroidal saponins in our *Ruscus* cultures. MeJA concentrations higher than 100 μM produced several symptoms of necrosation in the roots and the cultured plantlets died before the end of the experiment (data not shown). Considering the lack of previous studies on the biosynthesis of these active compounds at the level of the whole plant, it was interesting to find that neoruscogenin was the main steroidal saponin to accumulate in both aerial parts and roots of the *R. aculeatus* plantlets. The only exception to this general observation was at week 2, when the aerial parts showed similar amounts of both steroidal saponins. Throughout the culture period, the content of steroidal saponins was higher in the aerial parts than in the roots. As reported by Palazón et al. (2006), *Ruscus* callus cultures show a limited capacity to biosynthesize steroidal saponins, but when the calli regenerate aerial shoots it increases drastically. This observation, together with our results, suggests that the synthesis of steroidal saponins ruscogenin and neoruscogenin occurs mainly in the leaves of *R. aculeatus*.

As previously mentioned, the inhibitory effect of MeJA on the development of cultured *G. glauca* plantlets was more evident than in both *Centella* and *Ruscus* cultures, and consequently, studies on the biosynthesis of their main active compound, the nor-seco-friedelane galphimine-B, had to be done using the whole plant. The lower MeJA concentration (50 μM) did not elicit the biosynthesis of the secondary compound of interest, since the nor-seco-friedelane galphimine-B content was not detectable, as in the control cultures (data not shown). In contrast, the presence of 100 μM MeJA in the culture medium of *G. glauca* plantlets caused a relatively considerable increase in their galphimine-B content (Fig. 3).

From the values depicted in Fig. 3, it can be deduced that the elicitor action specifically affected some metabolic steps of the ursane saponin (asiaticoside and madecassoside) and nor-seco-friedelane (galphimine-B) biosynthesis in the respective *C. asiatica* and *G. glauca* cultures, but not the steroidal saponins ruscogenin and neoruscogenin of *R. aculeatus*, which are not directly formed from 2,3-oxidosqualene but via its previous conversion to cycloartenol (Gross et al., 1985; Combarieu et al., 2002).

2.3. Effects of the elicitor on the content of free sterols

From the data shown in Fig. 5, it may be inferred that under the conditions of this work the level of free sterols with a primary role (sitosterol, stigmasterol and campesterol) and that of cholesterol was affected mainly by differences in biosynthetic activity among the cultured plantlets. Cholesterol, which appears not to have any primary role in plants, is found in small amounts in many plant species, where it serves as a precursor for other steroid derivatives (Heftman, 1984). When comparing sterol content in *C. asiatica* plantlets cultured for 2 and 4 weeks with and without MeJA, it can be deduced that the free sterol pattern in both aerial parts and roots was stigmasterol + campestrol > sitosterol while very small quantities of cholesterol were detected only in the roots of untreated plantlets (≈10 μg/g DW). Additionally, when considering the total content of free sterols (the sum of the sterols measured), our results show that in both control and elicited cultures it was higher in the aerial part than in the roots, and this was especially evident after 2 weeks of culture in plantlets grown without MeJA. It is also clear that the presence of MeJA in the culture medium reduced the free sterol levels, which were lower in the aerial parts and roots of all elicited plantlets than in the controls after 2 and 4 weeks of culture (2.4- and 1.3-fold at week 2, respectively, and 1.8- and 1.3-fold at week 4, respectively). The decrease in free sterols could be the result of elicited plantlets having a high capacity to synthesize triterpene saponins, and also might be explained by MeJA acting differently on the two metabolic pathways considered. This is supported by previous results reported by Kim et al. (2005a,b) which show an activation of β-amyrin synthase (*Cab*AS), a key enzyme in the biosynthesis of triterpene saponins, in *C. asiatica* plants elicited by MeJA but an inhibition of the expression of cycloartenol synthase (*Ca*CYS), the enzyme responsible for the first step in sterol biosynthesis. This also concurs with the fact that both sterols and triterpene saponins are synthesised from a common precursor, 2,3-oxidosqualene, via two different pathways (Fig. 1).

In the case of *R. aculeatus* (Fig. 5), the total free sterol content of plantlets grown without MeJA was always clearly higher in the roots than in the aerial part, and as observed in *C. asiatica* plantlets, the addition of the elicitor decreased this content in both the aerial part and roots throughout the experiment. This decrease could also be due to the aforementioned inhibition of the CYS enzyme expression by MeJA, although the elicitor did not affect the capacity of *Ruscus* to produce both ruscogenin and neoruscogenin in the aerial part and only slightly in the roots (Fig. 3). Moreover, considering the proposal that

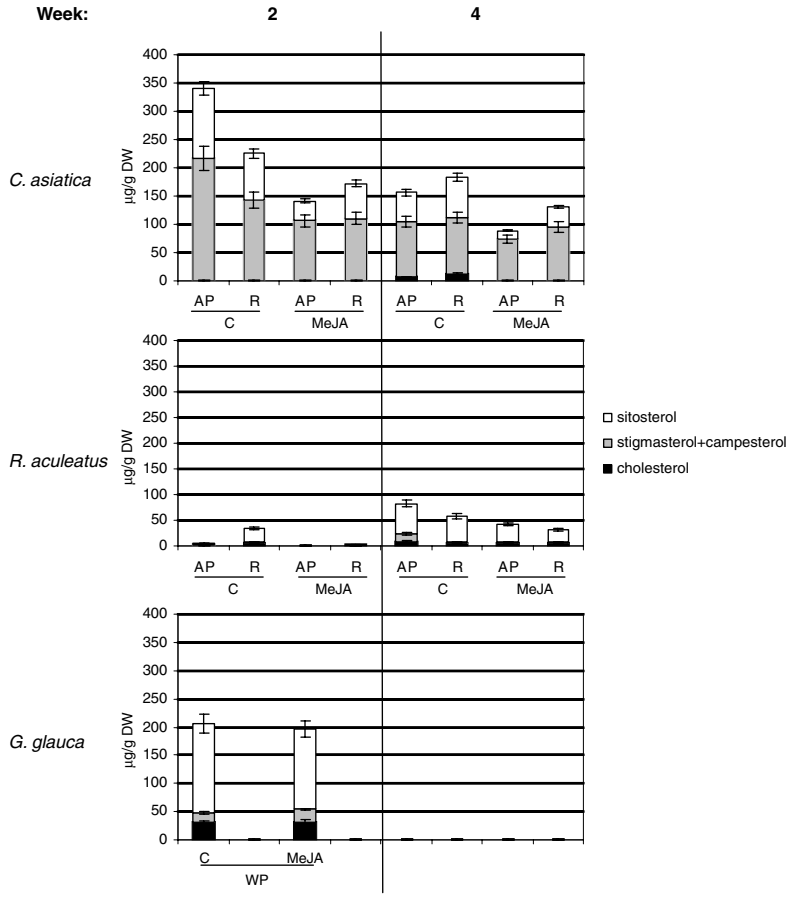

Fig. 5. Effects of methyl jasmonate on free sterol production of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–4 determinations. Bars represent ±SE. AP, aerial part; R, roots; WP, whole plant; C, control; MeJA, methyl jasmonate.

Ruscus spirostane saponins (ruscogenins) are synthesized from the key precursor in plant sterol synthesis, cycloartenol, in a biosynthetic route including cholesterol (Gross et al., 1985; Combarieu et al., 2002), it was of interest that cholesterol, usually a minor sterol in most plant species, was present at relatively high levels in our *Ruscus* plantlets, comprising approximately 7–10% of total free sterol content.

The total free sterol content in *G. glauca* plants after 2 weeks of culture (Fig. 5) was very similar in plantlets grown with and without MeJA, always in the pattern of sitosterol > cholesterol > stigmasterol+campesterol. The level of cholesterol in our *Galphimia* plantlets was considerable, comprising approximately 15% of total free sterol content, which places *G. glauca* among the small number of plant species in which cholesterol is more than a minor sterol. In contrast with *R. aculeatus* plantlets, *G. glauca* cultures appeared to show an activation of the enzymes involved in the biosynthesis of galphimine-B, a nor-seco friedelane triterpenoid directly synthesized from 2,3-oxidosqualene, probably due to the presence of MeJA, since it was only detected in plantlets grown in presence of the elicitor (≈17 µg/g DW).

Our results demonstrate for the first time how in *R. aculeatus* and *G. glauca* cultured plants the production of both free sterols and specific secondary compounds (the steroidal saponins ruscogenin and neoruscogenin and the triterpenoid galphimine-B, respectively) can be affected by exogenous application of MeJA to the culture medium. We have also shown that the production of centellosides (the triterpene saponins madecassoside and asiaticoside, and their respective ursane type sapogenins madecassic and asiatic acids) clearly increased in both the aerial part and roots of *C. asiatica* plantlets in response to the presence of MeJA, although the triterpenoid pattern was not affected. Finally, considering that 2,3-oxidosqualene is a precursor of all the compounds studied in this experiment, another explanation for their contents in our *Centella*, *Ruscus* and *Galphimia* cultures could be that the reduced growth induced by MeJA feeds the precursors

for secondary metabolite production if they were endogenously limiting.

3. Experimental

3.1. General experimental procedures

Madecassoside, asiaticoside, asiatic acid, madecassic acid, ruscogenin, neoruscogenin and campesterol were obtained from CromaDex Inc. (USA). β-Sitosterol, stigmasterol and cholesterol were obtained from Sigma–Aldrich (USA). The standard compound galphimine-B was isolated from aerial parts of *G. glauca* as described in Osuna et al. (1999). Acetonitrile and MeOH (HPLC grade) and other chemicals used (analytical grade) were obtained from commercial sources without further purification. Methyl jasmonate was purchased from Sigma–Aldrich (USA).

The HPLC system consisted of a Pharmacia LKB-HPLC 2150 pump (Pharmacia-LKB, Uppsala, Sweden), an LC 2152 Controller (Pharmacia-LKB), an HPLC autosampler 465 (Kontron Instruments), a 2141 Variable Wavelength Monitor (Pharmacia-LKB) and a Biodacs integrator (Pharmacia-LKB).

3.2. Plant material

C. asiatica plants were obtained from seeds provided by the School of Pharmacy, Second Military Medical University (Shangai, China). *R. aculeatus* plants were grown in the greenhouse of the Faculty of Pharmacy of the University of Barcelona. A voucher specimen is kept in the Botanical Section of the Faculty of Health and Life Sciences of the University of Pompeu Fabra (Barcelona, Spain). Mature seeds of *G. glauca* were collected from wild plants in Guanajuato, Mexico, and adapted to the culture conditions of the Plant Physiology Laboratory of the University of Barcelona. Voucher specimens were deposited at the Instituto Mexicano del Seguro Social Herbarium (IMSSM) under the code numbers 8645 and 8646.

Nodes from plants of *C. asiatica*, embryos of *R. aculeatus* (Moyano et al., 2006) and seeds of *G. glauca* were cultured on MS medium (Murashige and Skoog, 1962) solidified with 0.27% of phytagel. When the plants were 4–7 cm high they were used as experimental material for controls and elicitor treatment.

3.3. Treatment with methyl jasmonate

Plantlets of *C. asiatica*, *R. aculeatus* and *G. glauca* obtained as described above were cultured for 4 weeks in MS solid medium (Murashige and Skoog, 1962) with and without methyl jasmonate (MeJA). Different concentrations of MeJA (50, 100 and 200 μM) were preliminarily assayed. Since 50 μM was not enough to produce elicitation in the three species, and 200 μM produced several symptoms of necrosation in the roots after 2 weeks of treatment, we chose to carry out our studies with 100 μM of MeJA. Control plants (grown without MeJA) and treated plants were sampled after 2 and 4 weeks of treatment. At each harvesting, 8–10 plants were separated into roots and aerial parts, except in the case of *G. glauca* cultures whose limited root development forced us to collect the whole plant. Each plant fraction was washed, weighed for fresh weight (FW) and lyophilised to obtain dry weight (DW) and analysed the triterpenes and free sterols.

3.4. Extraction and analysis of specific secondary compounds

To determine the quantity of madecassoside, asiaticoside, madecassic acid and asiatic acid, aerial parts and root, were taken separately from control and elicited *C. asiatica* plants at 2 and 4 weeks of culture. The samples were lyophilized and powdered, and 1 g was extracted as reported by Bonfill et al. (2006). The chromatographic analysis was performed at room temperature with a Spherisorb 5μ ODS2 (250 × 4 mm) column (Waters) using gradient elution, the eluents being acetonitrile (A) and water with ammonium dihydrogenphosphate 10 mM (pH 2.5 with ortophosphoric acid) (B) according to the following profile: 0–15 min, 80% A; 15–30 min, 62% A; 30–37 min, 30% A; 37–45 min, 80% A. The flow rate was 1 ml/min and the detector was set at 214 nm.

To determine the quantity of ruscogenin and neoruscogenin, aerial parts and roots were taken separately from control and elicited *R. aculeatus* plants at 2 and 4 weeks of culture. Lyophylized powdered samples (500 mg) were extracted as reported by Palazón et al. (2006) for HPLC analysis.

To determine the quantity of galphimine-B, whole plants were taken from control and elicited *G. glauca* plants at 4 weeks of culture. Lyophilized samples were extracted with MeOH (75 ml) during 72 h shaking and sonicated for 2 min. After filtration the solvent was evaporated to dryness and the residue was dissolved in 3 ml of MeOH and applied to HPLC. Quantification of galphimine-B was performed at room temperature using a Chromolit Performance RP_{18} column (10 cm length). The mobile phase, consisting of acetonitrile/water (35:65), was eluted isocratically at a constant flow rate of 1.7 ml/min. The detector was set at 232 nm. The identity and purity of the galphimine-B standard were confirmed by comparison with published spectral data.

3.5. Extraction and analysis of free sterols

The extraction method used was the same as that used for the extraction of ursane saponins from *C. asiatica*, as described above. The chromatographic analysis is based on the method of Manzi et al. (1996) but with some modifications. The separation was carried out isocratically at room temperature using a HYPERSIL 5μ ODS (250 × 4.6 mm) column (Thermo electron corporation)

with MeOH as the mobile phase. The flow rate was 0.9 ml/min and the detector was set at 210 nm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.06.025.

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ORIGINAL RESEARCH PAPER

Triterpenoid saponin content and the expression level of some related genes in calli of *Centella asiatica*

Susana Mangas · Elisabeth Moyano · Lidia Osuna · Rosa M. Cusido · Mercedes Bonfill · Javier Palazón

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Abstract *Centella asiatica* has been extensively studied but there has been no report to date that relates gene expression and centelloside production in non-differentiated tissues. We have determined the content of the four principal triterpenoid bioactive compounds of *C. asiatica* (asiaticoside, madecassoside, asiatic acid and madecassic acid) in calli grown in different media and checked the expression level of some of the genes in the centelloside biosynthetic pathway. The results when compared with data from in vitro plant cultures showed a significantly lower expression of the gene encoding β-amyrin synthase in calli, which is consistent with the observed lower production of centellosides (less than 900 μg/g DW), while in the plants the production was around 1.5–2 mg/g DW. Moreover, we find an efficient housekeeping gene for this plant. The biosynthesis of phytosterols is also discussed.

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Keywords Asiaticoside · Callus cultures · *Centella asiatica* · Centellosides · Housekeeping · Madecassoside · Triterpenoid saponins

Abbreviation
4PU-30 *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea

Introduction

Centella asiatica (L) Urban of the Apiaceae family is a herbaceous plant with great medicinal value. Notable bioactive compounds of *C. asiatica* are triterpenoid saponins and sapogenins, among which the saponines madecassoside and asiaticoside are of particular interest, as well as their respective sapogenins madecassic and asiatic acid. Asiaticoside stimulates wound healing and is useful in the treatment of leprosy (Chakrabarty and Deshmukh 1976) and tuberculosis, while madecassoside has antiinflamatory properties and is able to increase collagen III secretion significantly (Solet et al. 1986). As shown in Fig. 1, two molecules of farnesyl diphosphate are converted by squalene synthase (SQS) into squalene, a common precursor of sterol and triterpenoid biosynthesis. Epoxidation of squalene at the second and third carbon positions results in the formation of 2,3-oxidosqualene. Oxidosqualene cyclases, which are situated at the branching step for biosynthesis of phytosterols and triterpene saponins, catalyse the

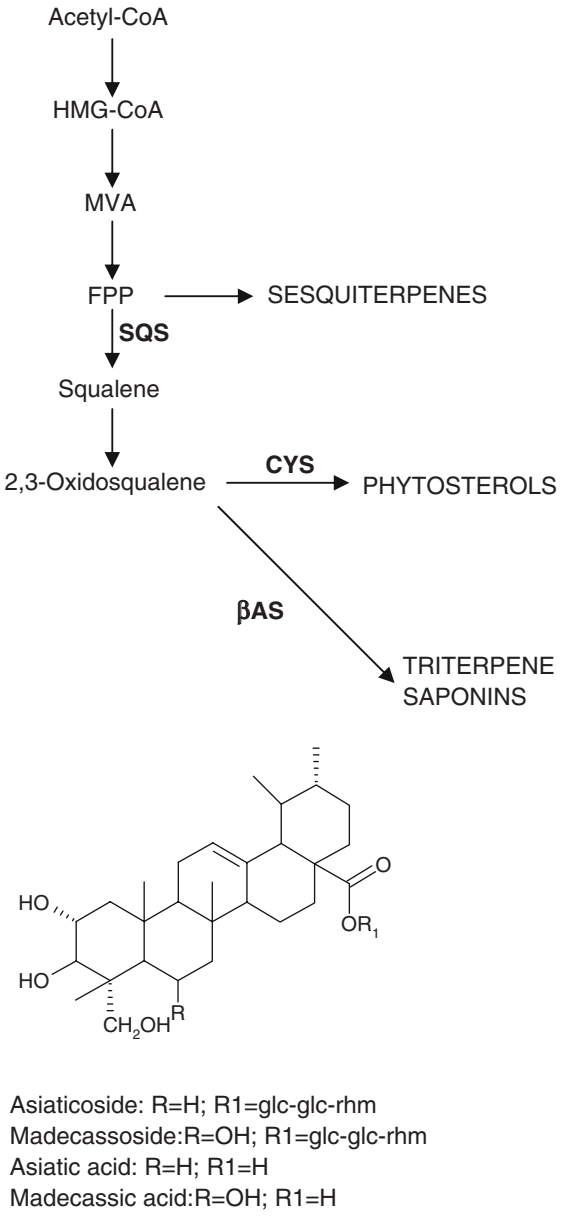

Fig. 1 Relationships between sterol and triterpene saponins in *C. asiatica*. Abbreviations: SQS, squalene synthase; CYS, cycloartenol synthse; βAS, β-amyrin synthase

cyclization of 2,3-oxidosqualene. Phytosterols arise from the cyclisation of 2,3-oxidosqualene by cycloartenol synthase (CYS), while the triterpenoid saponins of *C. asiatica* (centellosides) are formed via the cyclisation of 2,3-oxidosqualene by a specific oxidosqualene cyclase, β-amyrin synthase (βAS).

Due to its medicinal properties, the interest in *C. asiatica* has increased in recent years and many researchers have attempted to overproduce centellosides through in vitro cultures (Matsuda et al. 2001; Nath and Buragohain 2005). Recently, Kim et al. (2005a, b, c) have cloned some of the genes involved in the biosynthetic pathway of triterpenoid saponins in *C.asiatica*, such as β-amyrin synthase (*Cab*AS) (Kim et al. 2005a), cycloartenol synthase (*Ca*CYS) (Kim et al. 2005b) and squalene synthase (*Ca*SQS) (Kim et al. 2005c).

In this work, we have compared the production of centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) in *C. asiatica* plantlets and callus tissue with the expression level of the genes encoding the following enzymes: βAS, the specific oxidosqualene cyclase for centelloside production, CYS, which directs the pathway toward phytosterols, and SQS, which directs the pathway toward both phytosterols and centellosides. Additionally, we have checked a housekeeping gene for the gene expression studies.

Methods and materials

Whole-plant and callus cultures

Plants of *C. asiatica* obtained from seeds provided by the School of Pharmacy, Second Military Medical University (Shanghai, China) were cultured on MS medium (Murashige and Skoog 1962) supplemented with 2.7% (w/v). Gelrite and maintained in a controlled growth chamber at approximately 23°C, under continuous light. When the plants were 4–7 cm high they were subcultured in the same conditions and sampled after 4 weeks. At each harvesting, 8–10 plants were separated into roots and leaves. For centelloside and free sterol content analysis, both plant organs were lyophilised, and for molecular work they were frozen with liquid nitrogen and stored at −80°C.

To obtain calli, leaves from the aforementioned plants were dissected into 2–3 equal parts and inoculated in MS medium supplemented with 2 mg 6-benzylaminopurine (BA)/l + 0.1 mg indole-3-butyric acid (IBA)/l; 2 mg (BA)/l + 2 mg α-naphthaleneacetic acid (NAA)/l; 0.2 mg/l kinetin (Kn)/l + 2 mg indole-3-acetic acid (IAA)/l; and 0.1 mg Kn/l + 1 mg 2,4-D/l. Explants were incubated in the dark at 25°C in Petri dishes, and subcultures after the callus

induction were done every 25 days. The growth of calli was studied in 200-ml Magenta flasks, containing 30 ml of the solid MS medium supplemented with 0.1 mg IBA/l and 1, 2 and 3 mg 4PU-30/l for 6 weeks. The inoculum mass of calli was 0.5 ± 0.05 g, the results being the mean of 3–4 determinations ± SD.

Extraction and analysis of centellosides and free sterols and HPLC analysis

In callus tissue, the quantification of the four triterpenoid saponins (madecassoside, asiaticoside, madecassic acid and asiatic acid) was carried out during 6 weeks in the solid MS medium supplemented with 0.1 mg IBA/l and 1, 2 and 3 mg 4PU-30/l.

To determine the quantity of triterpene saponins and free sterols, lyophilized samples of roots, leaves and calli were powdered and 1 g of each was extracted as reported in Bonfill et al. (2006). HPLC analysis was performed following the method of Inamdar et al. (1996) modified as described in our previous paper (Mangas et al. 2006). Purity of standards was determined by MALDI-TOF mass spectrometry.

Total RNA extraction and cDNA preparation

Duplicates of root, leaf and callus samples frozen with liquid nitrogen and stored at −80°C were used to check the expression level of squalene synthase, cycloartenol synthase, β-amyrin synthase and and the housekeeping gene (5.8S rRNA).

Total RNA was extracted using the TRIZOL reagent (InVitroGen, USA) according to the manufacturer's instructions. Concentration of each RNA sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Only the RNA samples with a 260:280 ratio between 1.9 and 2.1 were used for the analysis. The integrity of RNA samples was also assessed by agarose gel electrophoresis. One microgram of total RNA from each sample was reverse-transcribed by First-Strand cDNA Synthesis using M-MLV RT (InVitroGen, USA), according to the manufacturer's instructions.

Primer designing and semiquantitative RT-PCR analysis

The primer sequences corresponding to the three genes under study (*Caβ*AS, *Ca*CYS and *Ca*SQS) cloned and sequenced by Kim et al. (2005a, b, c), respectively, and to the housekeeping gene (5.8S rRNA) were chosen using the BTI software Gene Tool Lite (version 1.0.0.1) and are given in Table 1. Oligonucleotides for the three genes were synthesized by TIB Molbiol Inc (Berlin, Germany) and for the housekeeping gene by Metabion International AG (Germany). Primers were designed to amplify the bands that are listed in Table 1, which were amplified. Sequencing of the 5.8S rRNA gene band confirmed its identity. PCR was performed with 2 µl of cDNA template using puReTag Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) and was carried out on a programmable thermocycler (MiniCycler MJ Research, Watertown, MA, USA). All the PCRs were performed under the following conditions: initial denaturation at 94°C for 5 min, followed by multiple cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 45 s and then a final extension at 72°C for 7 min. The number of cycles was standardized for each gene separately, being 28 cycles for 5.8S rRNA and *Cab*AS, and 25 cycles for *Ca*SQS and *Ca*CYS. Three replicates for each biological sample were used for reverse transcriptase (RT-PCR) analysis.

Table 1 Sequences of the primers used to amplify the housekeeping gene (5.8S rRNA) and the genes coding for squalene synthase (*Ca*SQS), cycloartenol synthase (*Ca*CYS), and β-amyrin synthase (*Cab*AS)

Genes	Sequences	Amplicon size (bp)
*Ca*CYS	Forward primer 5′-GAATCCACGCCATGAAGTCT–3′	421
	Reverse primer 5′-ACCACCATGATCCAGAATCC-3′	
*Cab*AS	Forward primer 5′-TGGTTGGGGAGAAAGTCTTG-3′	302
	Reverse primer 5′-ACAAGCGTTTGCGGTACTCT-3′	
*Ca*SQS	Forward primer 5′-TGGGTTAGGGTTGTCAAAGC-3′	324
	Reverse primer 5′-CGGAAGATAGCAGGATCTCG-3′	
5.8S rRNA	Forward primer 5′-CGGCAACGGATATCTCGGCTCT-3′	201
	Reverse primer 5′-TCCGCCCCGACCCCTTTC-3′	

Results and discussion

Assay of different growth regulators in callus cultures

Based on the work of several authors, different growth regulators in MS medium were assayed to obtain calli from leaf explants (see Methods). Calli began to grow after 2 weeks and were developed at week four. In the case of BA (2 mg/l) + NAA (2 mg/l) and Kn (0.2 mg/l) + IAA (2 mg/l) media, calli were without significant growth, as described by Patra et al. (1998) when using media containing BA in combination with NAA; with BA (2 mg/l) + IBA (0.1 mg/l), the medium used by Banerjee et al. (1999) for in vitro multiplication of *C.asiatica*, calli were white and large.

As we observed that growth was greater with IBA than with other auxins (data not shown), in a second series of experiments to obtain more callus biomass, we maintained the same IBA concentration (0.1 mg/l), and tested the effect of the cytokinin 4PU-30 at 1, 2 and 3 mg/l. Cytokinins derived from phenylurea are the best supplement for the promotion of shoot growth in *C. asiatica* (Kim et al. 2004) and we have previously observed that 4PU-30 promotes the development of large calli in tobacco callus cultures (Piñol et al. 1987). The biomass production of calli in MS medium with IBA and 4PU-30 is shown in Table 2, the calli being non-organogenic in all cases and showing a considerable growth rate.

Relationship between triterpenoid saponin—phytosterol contents and gene expression level

In callus tissue, the main triterpenoid was found to be madecassoside, followed by madecassic acid, asiaticoside and asiatic acid. The total production in the calli, with all the media tested, was less than 900 μg/g DW (Fig. 2), while in the plants it was around 1.5–2 mg/g DW. In calli, the concentration of madecassoside was approximately 6–8 times greater than that of asiaticoside, and the concentration of madecassic acid was approximately 2–3 times higher than that of asiatic acid. Comparing the content of saponins and sapogenins, the amount of madecassoside was approximately

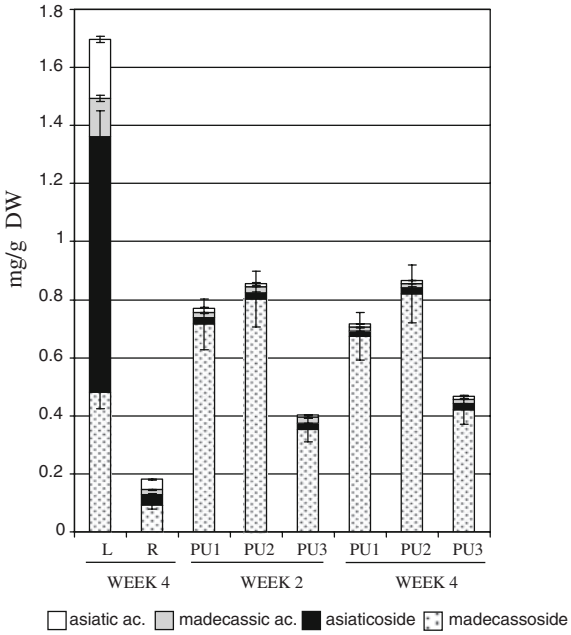

Fig. 2 Comparison of centelloside (asiaticoside, madecassoside, asiatic acid and madecassic acid) content in *C. asiatica* plants grown for 4 weeks in MS basal medium and in derived non-organogenic calli cultured in the same medium for 4 weeks supplemented with IBA (0.1 mg/l) and 4PU-30 at: 1 mg/l (PU1), 2 mg/l (PU2) and 3 mg/l (PU3). In all cases, the inoculum mass of calli was 0.5 ± 0.05 g. Each value is the mean of 3–4 determinations. Bars represent ± SD. L, leaves; R, roots

Table 2 Growth rate measured as fresh (FW) and dry weight (DW) of *C. asiatica* whole plant organs and derived non-organogenic calli grown in MS basal medium, the former without growth regulators and the latter with IBA (0.1 mg/l) and different concentrations of 4PU-30: 1 mg/l (PU1), 2 mg/l (PU2) and 3 mg/l (PU3)

Growth	Week	Whole plant organs		Non-organogenic calli		
		Leaves	Roots	PU1	PU2	PU3
FW (g)	2	–	–	0.79 ± 0.14	0.80 ± 0.16	0.88 ± 0.23
	4	8.08 ± 0.437	7.12 ± 0.39	1.0 ± 0.17	1.18 ± 0.22	1.55 ± 0.37
DW (g)	2	–	–	0.125 ± 0.002	0.12 ± 0.02	0.10 ± 0.02
	4	0.92 ± 0.09	0.55 ± 0.03	0.182 ± 0.013	0.18 ± 0.01	0.26 ± 0.02

In all cases, the inoculum mass of calli was 0.5 ± 0.05 g. Each value is the mean of 3–4 determinations ± SD

double that of the madecassic acid, and the amount of asiaticoside was similar to that of asiatic acid.

In vitro plants (see Methods) presented a different saponin production pattern compared to the calli, and it varied according to the part of the plant. Thus, the main triterpenoid found in the aerial part of the plant was asiaticoside followed by madecassoside, but in the roots it was madecassoside (Fig. 2).

Comparing the expression level of the three genes assayed (*Ca*SQS, *Ca*CYS and *Cab*AS) with the centelloside content, in roots and callus cells where the centelloside content was low, the expression of the *Ca*SQS and *Cab*AS genes was also low, while in leaves both parameters were high. Comparing the levels of *Cab*AS in the low producing roots and calli, the expression of downstream *Cab*AS was similar in roots and calli grown for 2 and 4 weeks with different concentrations of 4PU-30, although the total content of centellosides was significantly ($P < 0.001$) higher in calli than in roots. The observation that triterpene aglycones act as structural components of plant membranes (Nes and Heftmann 1981) could explain this higher centelloside content in calli under the conditions of our study (Fig. 3).

Another factor in the lower centelloside content of roots and calli could be the notable decrease in *Ca*SQS expression level (Fig. 2). Since this enzyme separates farnesyl diphosphate from the isoprenoid pathway and converts it to squalene, the first specific precursor of sterols and triterpene saponins, it would appear that SQS plays a regulatory role in centelloside formation.

Concerning the total content of free sterols (Fig. 4), a direct relationship was found between their level and the concentration of 4PU-30 in the culture medium, the total content of free sterols being highest in calli grown in the presence of 3 mg 4PU-30/l and lowest in those grown with 1 mg 4PU-30/l. Although secondary metabolite production does not usually depend on growth, the calli cultured at the highest 4PU-30 concentration showed the highest accumulation at one time.

When comparing free sterol content in leaves and roots of our plants with that of the calli grown with 1, 2 and 3 mg 4PU-30 l^{-1}, it can be seen that the free sterol pattern of plant leaves and roots was the same as in calli, being stigmasterol + campesterol > sitosterol.

Additionally, the total content of free sterols, was significantly ($P < 0.001$) higher in leaves and roots of donor plants than in calli, especially those grown in the presence of the lowest concentration of 4PU-30 (1 mg/l). This general decrease in free sterol content might well result from a lower expression of the gene coding for cycloartenol synthase (CYS), which catalyses the first committed step in sterol biosynthesis. This is

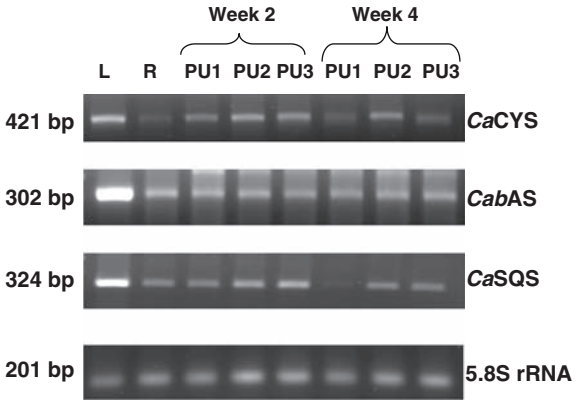

Fig. 3 Expression of squalene synthase (*Ca*SQS), cycloartenol synthase (*Ca*CYS), and β-amyrin synthase (*Cab*AS) genes in *C. asiatica* whole plant organs and derived non-organogenic calli grown in MS basal medium, the former without growth regulators and the latter with IBA (0.1 mg/l) and of 4PU-30 at: 1 mg/l (PU1), 2 mg/l (PU2) and 3 mg/l (PU3). L, leaves; R, roots

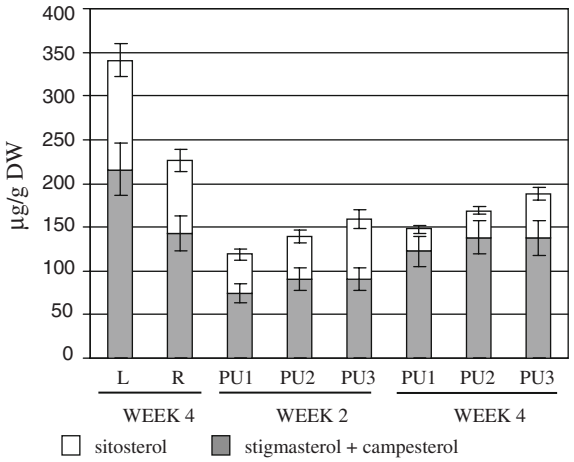

Fig. 4 Comparison of free sterol (sitosterol, stigmasterol and campesterol) content in *C. asiatica* plants grown for 4 weeks in MS basal medium and in derived non-organogenic calli cultured for 4 weeks in the same medium supplemented with IBA (0.1 mg/l) and 4PU-30 at: 1 mg/l (PU1), 2 mg/l (PU2) and 3 mg/l (PU3). In all cases, the inoculum mass of calli was 0.5 ± 0.05 g. Each value is the mean of 3–4 determinations. Bars represent ± SD. L, leaves; R, roots

supported by the higher transcript levels for CYS observed in plant leaves (Fig. 3).

Housekeeping gene validation

Although *C. asiatica* has been extensively studied, thus far there has been no report relating gene expression using reverse transcriptase (RT-PCR) analysis with centelloside production. It was thus necessary to find an adequate housekeeping gene.

The 5.8S rRNA gene from *C.asiatica* was assayed for its stability and also its level of expression. A high quality total RNA was isolated from different tissue samples and reverse transcribed. Expression studies using biological duplicates were done by RT-PCR analysis in a set of 8 tissue samples (see Methods). We compared the amplification of this gene in all the samples at various cycles to check when the amplicon was within the exponential phase, as evidenced by its intensity data, and in this manner 28 cycles were selected. Additionally, we checked the cDNA template for 0.5, 1 and 1.5 μl, and the amplification yield also increased accordingly (Fig. 5). This result supported the use of 28 cycles for quantitative PCR. Expression of the 5.8S rRNA gene was found to be the same in leaf, root and callus samples, and also in calli at the second and fourth week of culture (Fig. 3), showing that it was suitable for housekeeping use in *C. asiatica*.

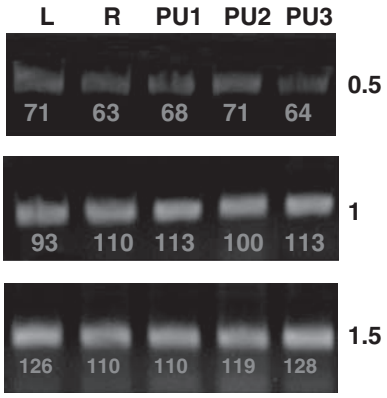

Fig. 5 Amplification of the 5.85 rRNA gene using 0.5, 1 and 1.5 μl of a cDNA template in all five studied samples. Numerals above each line represent microlitres of the cDNA template used in PCR. Values inside the panel represent the intensities of the amplicon as measured using Kodak Molecular Imaging Software. PCR was performed using 28 cycles. L, leaves; R, roots; 4PU-30: 1 mg/l (PU1), 2 mg/l (PU2) and 3 mg/l (PU3)

Conclusions

We have discussed how the level of accumulated centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) in non-organogenic callus cultures of *C. asiatica* appears to be significantly diminished by the low expression level of both β-amyrin synthase (*Cab*AS) and squalene synthase (*Ca*SQS) genes, seems to be linked with callus cell dedifferentiation. The observed tissue-specific biosynthesis of centellosides presents a limitation that could be partially overcome by genetic manipulation of the triterpenoid pathway to ensure the greatest possible flux into these active triterpene saponins. Considering the versatility and controlled experimental conditions offered by cell suspension systems, we believe that our findings open the way for the use of *C. asiatica* cell cultures overexpressing the genes encoding for βAS and/or SQS to increase centelloside production.

These results also suggest that the high asiaticoside content in *C.asiatica* leaves is due to its production in leaf cells rather than transport from roots.

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Valuable phytochemicals with medicinal and bioactive properties: Commercial medicinal plants and resins as a source of α-amyrin, β-amyrin and *epi*-lupeol

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Valuable phytochemicals with medicinal and bioactive properties: Commercial medicinal plants and resins as a source of α–amyrin, β-amyrin and *epi*-lupeol

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Running title: Isolation of α-amirin, β-amirin and *epi*-lupeol from medicinal plants and resins

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Abstract

A simple, fast and efficient method was developed for the isolation of α– and β-amyrin and 3-*epi*-lupeol from commonly available commercial resins and medicinal plants. The method involves rapid qualitative detection of triterpenes by thin-layer chromatography, efficient extraction with *n*-hexane, quantitative analysis by HPLC and preparative separation (preparative TLC and crystallization) to obtain high-purity α-amyrin (98.5%), 3-*epi*-lupeol (98%) and β-amyrin (82%). The identity of these compounds was confirmed by NMR. The presence of α- and β-amyrin, or a mixture of both, was detected by TLC on silica gel in all the sources investigated, while 3-*epi*-lupeol was only found in the Mexican Copal resins. The results demonstrated that HPLC is a selective method for the analysis of α- and β-amyrin and 3-*epi*-lupeol, and that the best source of α-amyrin was Mexican Copal Tepoztlan, that of β-amyrin was Nanche wastes and that of 3-*epi*-lupeol was Mexican Copal Sonora.

1. Introduction

Pentacyclic triterpenes are ubiquitously distributed throughout the plant kingdom, in a free form as aglycones or in combined forms, and have long been known to have a number of biological effects [1]. The compounds α-amyrin, β-amyrin and 3-*epi*-lupeol, commonly found in medicinal plants, have many bio-active properties. Widely distributed in the genera *Rhizophora* and *Betula*, α-amyrin is also found in several medicinal plants as well as the foliage of the corn plant [2]. In recent years ιτ has attracted interest because of its pharmacological effects. The analgesic, anti-inflammatory, anti-ulcerogenic, and anti-hyperglycemic activities of the herbal tea *S. taurica* as well as the anti-inflammatory and analgesic activities of *Himatanthus sucuuba*, are attributed to this compound. The anti-lipoxygenase and anti-arthritic activities of amyrin derivatives are also well documented [2]. In addition, there are abundant examples of *α*-amyrin-containing plants in folk medicine, for example, *Strobilanthes callosus,* which has anti-inflammatory and anti-microbial

activities [3], and *Decalepis hamiltonii,* which is used as an appetite stimulant, blood purifier and food preservative [4].

Both *in vitro* and in *vivo* studies have shown that β-amyrin also has important biological functions. This triterpenoid is found in the foliage of many species of *Rhizophora* [5], rhododendron leaves, the bark of birch species and the foliage of many corn plants [2]. It has many bioactive properties, being responsible for the sedative effect of *M. forsskaolianam*, the anti-spasmodic, anti-arrhythmic and anti-cholinergic properties of *S. persica*, anti-inflammatory, anti-pyretic, analgesic and estrogen-like activities of *D. sissoo*, the analgesic activity of *A.* the anti-inflammatory and analgesic of *C. amblyocarpa,*[2] the anti-bacterial activity of *H. ellipticum* [6], the significant anti-anflammatory effect of *Stevia rebaudiana* [7] and potent *in vitro* α-glucosidase inhibitory activities of *M. conraui.*(8) Other studies have demonstrated that the α/β amyrin triterpene mixture also has many biological functions, including analgesic, antimicrobial, anti-inflammatory activities [9].

Low concentrations of 3-*epi*-lupeol have been reported in some plants, including steam barks of *Commiphora dalzielii* [10], *Erythrina variegate* [11] and *Bursera simaruba* [12], roots of *Glochidion eriocarpum* Champ [13] and leaves of *Sebastiana adenophora* (Euphorbiaceae) [14]. This triterpenoid has also been found in high concentrations in resin from several species of *Bursera* [15]. Although 3-*epi*-lupeol has not been clearly associated with any specific medicinal properties, some studies link it with the allelopathic action of *S. adenophora* [14]. Additionally, 3-*epi*-lupeol isolated from *Glochidion eriocarpum* has show a growth inhibitory effect against tumor cell lines [13]. It has also shown moderate anti-tubercular activity [16].

One of the sources tested for the above mentioned triterpenes was Copal, an oleo-resin obtained by bark incision of several species of *Bursera or Protium* of the Burseraceae family. This resin is a complex mixture containing a series of mono-, sesqui-, di-, and triterpenoids, the most frequent constituents being by far the pentacyclic triterpenes α- and β-amyrin.

Another investigated source was Manila elemi resin obtained from the species *Canarium*

luzonicum and *Canarium commune* found in the Philippine Islands. The major constituents described from non-volatile fractions of this resin are diterpenes, tetra-, and pentacyclic triterpenes, including α-amyrine, β-amyrenone, taraxerol and β-sitosterol [15].

The objectives of the present study were two-fold. After using TLC to rapidly identify the valuable and bioactive phytochemicals α-amyrin, β-amyrin and 3-*epi*-lupeol, which are available in large quantities from common and economical sources (commercial resins, residues and commercial medicinal plants), the first aim was to develop a method combining soxhlet extraction and HPLC for their simultaneous determination. The second objective was to separate these bioactive analogous triterpenoids on a preparative scale using crystallization for β-amyrin and preparative TLC for α-amyrin and 3-*epi*-lupeol. The compounds would be identified using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra.

2. Materials and Methods

2.1 Resins and plant material

The Manila Elemi resin (*Canarium luzonicum*) (MER) was provided by Droguería Riesgo (Madrid, Spain) and the resins Mexican Copal Sonora (MCS), Mexican Copal Tepoztlan (MCT), Mexican Copal Negro (MCN) and Mexican Copal Piedra (MCP) were purchased from herbal stores in Sonora market (México City) in December, 2007. The commercial medicinal plants, bearberry leaves (*Arctotaphylos uva-ursi*), pot marigold leaves (*Calendula officinalis*), dandelion leaves (*Taraxacum officinale*), olive leaves (*Olea europaea*), and "cancerina" root bark (*Hippocratea excelsa HBC*), were provided by Soria Natural S.A. (Barcelona, Spain) and herbal stores of Sonora market (México City). The residues of Nanche steam barks (*Byrosonima crassifolia*) were obtained from a local company in the Veracruz province of México in December, 2007.

2.2 Reagents and standards

Commercially available hexane, ethyl acetate, acetone, methanol and dichloromethane (HPLC grade) were obtained from Tecnokroma and redistilled water was filtered through a 0.45 μm membrane before

use. All other chemical reagents used were of analytical grade.

Crystalline reference samples of α-amyrin and 3-*epi*-lupeol (Fig. 1) compounds were isolated from Mexican Copal Tepoztlan and Mexican Copal Sonora (see section **2.5**) and characterized in our laboratory. The purity (>98) of the isolated compounds was confirmed by chromatography (HPLC and RMN) and structures of α-amyrin and *epi*-lupeol were identified on the basis of chemical and spectral evidence including two-dimensional NMR (COSY, 1H-1H, HMQC and HMBC) and mass spectrometry. The certified standard of β-amyrin (Fig. 1) was purchased from Sigma (St. Louis, MO).

2.3 TLC analysis of pentacyclic triterpenes

Dried and pulverised commercial medicinal plants (about 5g) were extracted with 50 mL of *n*-hexane in a Soxhlet apparatus for 12 h and then concentrated in a rotary evaporator.

Standards of α-amyrin, β-amyrin and 3-*epi*-lupeol (2 mg/mL), crude resins (10 mg/mL) and hexane concentrated crude extracts of the medicinal plants (10 mg/mL) dissolved in dichloromethane were spotted onto 20X20 cm silica gel 60 F254 TLC plates (Merck, Darmstadt, Germany). The chromatrograms were developed in chromatogram tanks in a hexane-dichloromethane-methanol mobile phase (10:10:1). The triterpenoids were detected by spraying the plates with anisaldehyde-sulphuric acid (AS) reagent, and the chromatograms were heated at 80-100^{o}C for five min. Identification was carried out by comparing the colour and R_f values of spots of analyzed compounds (see Fig. 2). Photos of the developed TLC plates were taken with an HPScanjetG3010 scanner.

For the analysis of 3-*epi*-lupeol (2 mg/mL) the TLC was performed on silica plates that had earlier been coated with a 10% aqueous solution of silver nitrate and activated at 100 °C for one hour. The mobile phase was chloroform-diethyl ether (19:1).

2.4 Quantitative analysis of triterpenes by HPLC

120 **2.4.1 Sample preparation**

121 Dried and pulverised medicinal plants (10 g) were extracted with 50 mL of *n*-hexane in a Soxhlet
122 apparatus for 12 h. and then concentrated in a rotary evaporator at 40 ºC and dried overnight at 60
123 °C. The plant extract was dissolved in methanol (10 mL) and analyzed by HPLC. The resin extract
124 was obtained by dissolving 50 mg of crude resin in 10mL of methanol. All samples were filtered
125 through a 0.45-um syringe filter and injected for HPLC analysis to calculate the triterpene
126 concentration from the calibration graphs.

127 **2.4.2 HPLC analysis**

128 The analysis was based on the method described by Bonfill et al. [17] with minor modifications.
129 Chromatography was carried out on a Waters 600 Controller and Waters 717 plus Autosampler.
130 The three triterpenes were satisfactorily separated with a reversed phase Hypersil column (5µ ODS,
131 250 x 4.6mm, Thermo electron corporation). The separation was carried out isocratically using
132 methanol as the mobile phase (25min). The system was operated at room temperature and
133 monitored at 210 nm. The flow rate was 0.9 mL/min and the injection volume was 20 µL. The
134 compounds α-, β-amyrin and 3-*epi*-lupeol were identified and quantified by comparing their
135 retention time with the standards and calculating the concentration from the respective calibration
136 curves. The analysis was carried out in duplicate.

137

138 **2.5 Preparative scale isolation of α-amyrin, β-amyrin and 3-*epi*-lupeol**

139 **2.5.1 Preparative TLC separation of α-amyrin from Mexican copal Tepoztlan**

140 The crude Mexican Copal Tepoztlan resin was dissolved in hexane, and the insoluble fraction was
141 used for the extraction of α-amyrin, 200 mg being dissolved in 0.5 mL of dichloromethane and
142 applied to the preparative TLC plates (Merck Silica Gel 60, 20 x 20 cm, 0.25 mm thickness). To
143 correctly identify and locate the α-amyrin molecule, a reference sample containing α-amyrin was
144 also applied to the TLC plate. After developing the plate twice in hexane:dichloromethane:methanol
145 (10:3:0.5), the reference band was exposed to the developer (anisaldehyde-sulphuric acid), while

the sample area was covered with a glass plate. Two zones (α-amyrin and a mixture of 3-*epi*-lupeol and another unidentified compound) were scraped and each fraction was extracted three times with 20 mL ethyl acetate. The resulting solution was filtered and the solvent evaporated, yielding 160 mg (80%) of pure α-amyrin with 98% purity by NMR and HPLC, and ^{1}H and ^{13}C NMR identical to reported data [12]. This preparative TLC was repeated several times.

2.5.2 Preparative separation of β-amyrin from Nanche residues

The air-dried and powdered stem barks of Nanche (*Byrosonima crassifolia*) residues (1 kg) were extracted with hexane (2 L x 2) at room temperature for 48 h, and 20.6 g (2.06% dry weight) of a solid white extract was obtained after filtration and evaporation of the solvent under reduced pressure. Crystallization of β-amyrin was performed by dissolving 4 g of solid white extract in 20 mL of dichloromethane at room temperature. After dissolution, 15-20 mL of methanol was added, and the mixture was kept at room temperature for 12 h before being isolated by suction filtration from the mother liquor. NMR spectral data [18] revealed a mixture of β- and α-amyrin crystals (82% and 18%, respectively) although they had appeared as homogenous on TLC. Attempts to separate the mixture by $AgNO_3$-TLC failed.

2.5.3 Preparative TLC separation of 3-*epi*-lupeol from Mexican copal Sonora

Preparative TLC of 200 mg of Mexican Copal Sonora (spot with 3-*epi*-lupeol mixed with another unidentified compound) was carried out on 60 silica gel coated glass plates (Merck) immersed in a solution of 15% silver nitrate in deionized water/ethanol (1 + 1, v/v) for 30 min and dried at 100°C for 5 min. The residue was dissolved in dichloromethane and spotted onto the plate, which was developed by dichloromethane-diethyl ether (19:1) and sprayed (one portion) with anisaldehyde reagent for visualisation. The preparative TLC fraction containing 3-epi-lupeol was extracted with ethyl acetate and the solvent was evaporated. An amount of 160 mg (80%) pure *epi*-lupeol (with 99% purity by NMR and HPLC and ^{1}H and ^{13}C NMR identical to reported data was obtained [13]

3. Results and discussion

TLC provided an easy and rapid way to study plant extract profiles and partially identify compounds. The first step for the identification of α-amyrin, β-amyrin and 3-*epi*-lupeol was to compare R_F values of reference standards with those of sample extracts. TLC on silica gel revealed that α-amyrin on tracks 7 and 15, β-amyrin on track 14 and the α-, β-amyrin mixture on track 16, as well as two standards, all had the same R_F (Fig. 2). The α-amyrin band was observed as brown, while the β-amyrin band appeared violet, as did the band for the α-, β-amyrin mixture. TLC analysis revealed the presence of α-amyrin, β-amyrin and 3-*epi*-lupeol by a comparison of the position and color of the triterpene spots with those of the authentic compounds (Fig. 2). The bands of α- and β-amyrin or their mixture were observed in all commercial resin tracks 1-5 and medicinal plant tracks 8-13, while 3-*epi*-lupeol track 7 was detected only in the commercial Mexican Copal resin tracks 1-4. Attempts were made to separate the α-, β-amyrin mixture, which had appeared as homogenous on TLC (see HPLC results), but without success. These results are in concordance with those previously reported [19].

On the other hand, as shown in Figure 2, *epi*-lupeol was absent in Manila Elemi resin and the tested medicinal plants, but present in all the Mexican resins as a mixture with another minor constituent. This resolution was only possible using TLC silica gel plates impregnated with $AgNO_3$. These results showed that TLC can be used as a simple method for a preliminary analysis of these triterpenes in extracts of commercial resins and plants, but cannot be employed for the analysis of the α-, β-amyrin mixture. $AgNO^3$-TLC can be used for a rapid and easy qualitative analysis of 3-*epi*-lupeol in resins.

3.1 Quantitative analysis of triterpenes by HPLC

The triterpenes α-amyrin, β-amyrin and 3-*epi*-lupeol were identified and quantified by HPLC, their retention time compared with standards and previously reported data, and their concentration

198 calculated from the respective calibration curves. The order of elution in the HPLC was from more
199 polar compounds to less polar (i.e., 3-*epi*-lupeol, β-amyrin and α-amyrin). The chromatogram of
200 the standard solutions (1 mg/mL) is shown in Fig. 3A, in which the retention times are 16.6 min for
201 α-amyrin, 15.3 min for β-amyrin and 12.4 min for 3-*epi*-lupeol, with some additional impurities in
202 the solvent. The typical chromatograms of the resin solutions and the hexane extracts of medicinal
203 plants with complete resolution of all peaks are shown in Fig 3B, 3C and 3D. It can be observed
204 that in addition to the peaks of α-amyrin, β-amyrin and 3-*epi*-lupeol, there are at least 3 other
205 insignificant peaks, indicating the presence of some unidentified substances.

206 The other resins had a similar amount of α-amyrin. Figure 4 also shows that among the
207 commercial medicinal plants Nanche wastes had the highest content of α-amyrin, which was
208 similar to that of the resins. Bearberry also had significant amounts of this compound. The highest
209 amounts of β-amyrin were determined in the hexane extract of Nanche wastes. Regarding the other
210 plant extracts, β-amyrin levels were highest in dandelion and bearberry and lowest in pot marigold,
211 olive and "cancerina". As Figure 4 clearly shows, the main triterpene found in the hexane extract of
212 MCS was 3-*epi*-lupeol, with MCN and MCP showing similar quantities and MCT the lowest.

213 The HPLC method permitted an estimation of the triterpenoid concentration in resins and
214 medicinal plants. The extraction yields of the three compounds, shown in Table 1, indicate that the
215 best source of the three triterpenes was MCS resin (98.4%): α-amyrin (21%), β–amyrin (4.4%) and
216 3-*epi*-lupeol (73%). Among the medicinal plants, the highest overall content was in Nanche wastes:
217 α-, β-amyrin mixture (0.90%), α-amyrin (0.24%) and β-amyrin (0.66%).

218 The chromatogram in Fig. 3A shows that the purity of the α-amyrin and 3-*epi*-lupeol
219 isolated from resins was high (98.5% and 98.0%), indicating that the isolation method for these
220 triterpenoids was satisfactory. It was clear from HPLC analysis that the best sources were Mexican
221 Copal Tepoztlan for α-amyrin (α/β amyrin ratio 4/1), Mexican Copal Sonora for 3-*epi*-lupeol and
222 Nance wastes for β-amyrin (β/α amyrin ratio 3/1). The commercial Mexican Copals showed a

similar α- and β-amyrin content to the reported values for other Bursera resins (*Bursera delpechiana*, *Bursera galleotina* Eng and *Bursera simaruba*) and better than those reported for other commercial resins *(Protium heptaphyllum*) [15]. These results led to the selection of the Mexican Copal Tepoztlan and Mexican Copal Sonora resins and Nanche wastes for triterpenoid isolation on a preparative scale.

3.2 Preparative isolation of α-amyrin, β-amyrin and 3-*epi*-lupeol

In preparative TLC separation of α-amyrin from Mexican Copal Tepoztlan the developing solvent was hexane-dichloromethane (3:7). The separated color zone was cut off and quantitatively extracted by ethyl acetate, and the product was obtained after solvent evaporation. The purity (98.5%) of the α-amyrin was demonstrated by NMR, HPLC analysis and melting point determination. The purification yield was 60%. HPLC analysis of the isolated α-amyrin showed a single peak, as indicated in Fig. 3, with a retention time of 15.5 min. These values are similar to those previously reported [19]. Oleo-resins such as Copal obtained from several species of the genus *Bursera* are characterized by a high content of pentacyclic triterpenes and essential oils, giving high yields of triterpenoid fractions(9,13). Consistent with these earlier studies, in this work the preparative TLC isolation yield of α-amyrin from Mexican Copal Tepoztlan resin was high.

3.3 Preparative separation of β-amyrin.

The first crystallization from methanol of the α-, β-amyrin mixture (1 g) of the Nanche wastes extract resulted in crystals (0.01 g) containing 82% β-amyrin and 18% α-amyrin (by ^{1}H MNR and HPLC, see Fig. 3). Successive crystallizations did not improve the purity of the β-amyrin. Further purification of the crystals by preparative CCF-$AgNO_3$ was not possible.

The hexane extract from Nanche wastes (*Byrosonima crasssifolia*) showed a content of the α-, β-amyrin mixture similar to or better than the reported values for other plants of the genus

Byrosonima (*Byrsonima verbascifolia* L) [20].

3.4 Preparative TLC separation of 3-*epi*-lupeol of Copal Sonora resin.

Crude Mexican Copal Sonora resin on TLC showed the presence of two major compounds (Rf = 0.3 and 0.4) and was chromatographed over preparative TLC, eluting with a hexane-dichloromethane mixture (3:7). The separated colour zone was cut off and quantitatively extracted by ethyl acetate, and the product was obtained after solvent evaporation. The separated R_F 0.4 zone was re-chromatographed over preparative silica gel impregnated with $AgNO_3$ (15%), eluting with a hexane-dichloromethane mixture (3:7). The product was obtained after extraction with ethyl acetate and solvent evaporation, yielding a crystalline 3-*epi*-lupeol (60%) and α-amyrin (20%).

4. Concluding remarks

Considering the emerging importance of the pentacyclic triterpenes, α-amyrin, β-amyrin and 3-*epi*-lupeol, the results of the present work may prove valuable for further research on the use of the different phytochemicals present in resins and plants to sustain human health. The TLC procedure described here is a simple and rapid method for the determination of pentacyclic triterpenes in plants and resins. The HPLC method proved to be suitable for the analysis of the α-,β-amyrin mixture and 3-*epi*-lupeol in a complex matrix such as a copal resin. Classic preparative TLC, prep-TLC-AgNO3 and crystallization were used for the isolation and purification of α-amyrin, β-amyrin and 3-*epi*-lupeol from Mexican copal resins and Nanche wastes. The high yield of purified α-amyrin and 3-*epi*-lupeol suggests that these commercial resins have a potential economic use as a source of these triterpenoids.

Acknowledgments

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275 **5. References**

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Table 1. Percentage of triterpenes in the plants and resins studied.

	% α-amyrin*	% β-amyrin*	% *epi*-lupeol*	% Total triterpenes
MCT	11.2	2.6	27.0	40.4
MCS	21.0	4.4	73.0	98.4
MCN	11.0	3.5	37.4	51.9
MCP	7.7	1.8	34.1	43.6
Elemi Manila	24.0	10.1	0.0	34.1
Dandelion	0.02	0.05	0.0	3.90
Olive	<0.01	<0.01	0.0	0.12
"cancerina"	0.01	<0.01	0.0	0.05
Nanche wastes	0.24	0.72	0.0	0.96
Bearberry	0.6	0.14	0.0	0.74
Pot marigold	0.04	0.13	0.0	0.17

* Percent of 10 g in resins and dried plants

Legends of Figures:

Figure 1. Structures of the investigated molecules in commercial resins and medicinal plants.

Figure 2. TLC plate. Tracks: 1=MCT; 2=MCS; 3=MCN; 4=MCP; 5=MER; 6 and 15=α-amyrin; 7=3-*epi*-lupeol; 8=Dandelion; 9=Olive; 10=Cancerina; 11= Nanche wastes; 12=Bearberry; 13=Pot marigold; 14=β-amyrin; 15=α-amyrin and 16=mixture α-, β-amyrin.

Figure 3. A: Chromatogram of standard; B: Chromatogram of Mexican Copal Tepoztlan; C:Chromatogram of Mexican Copal Sonora; D: Chromatogram of Nanche wastes.

Figure 4. Contents of amyrins and *epi*-lupeol in the extracts analysed expressed as a percentage. Each value is the average of 3 determinations.

Fig. 1

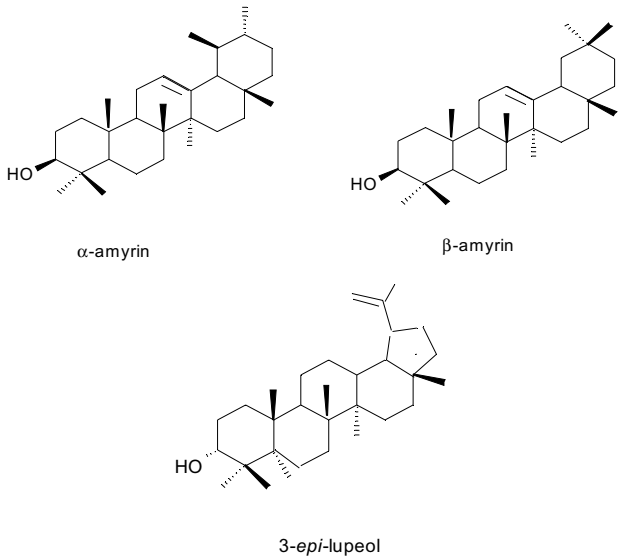


Fig. 2

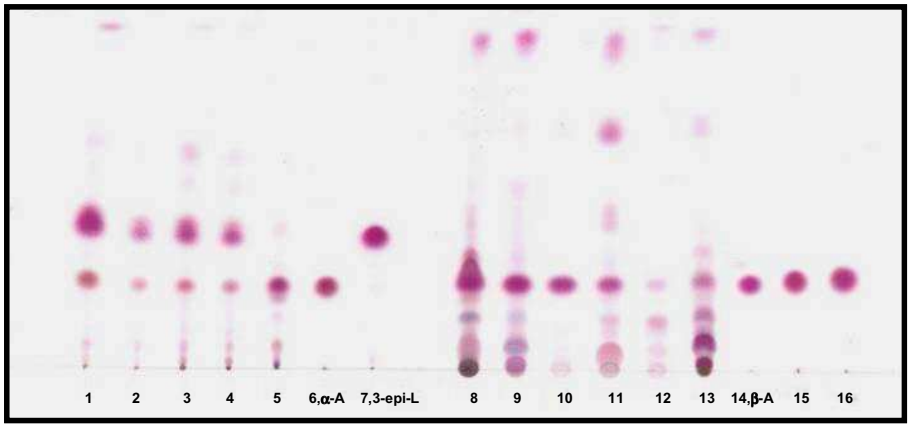
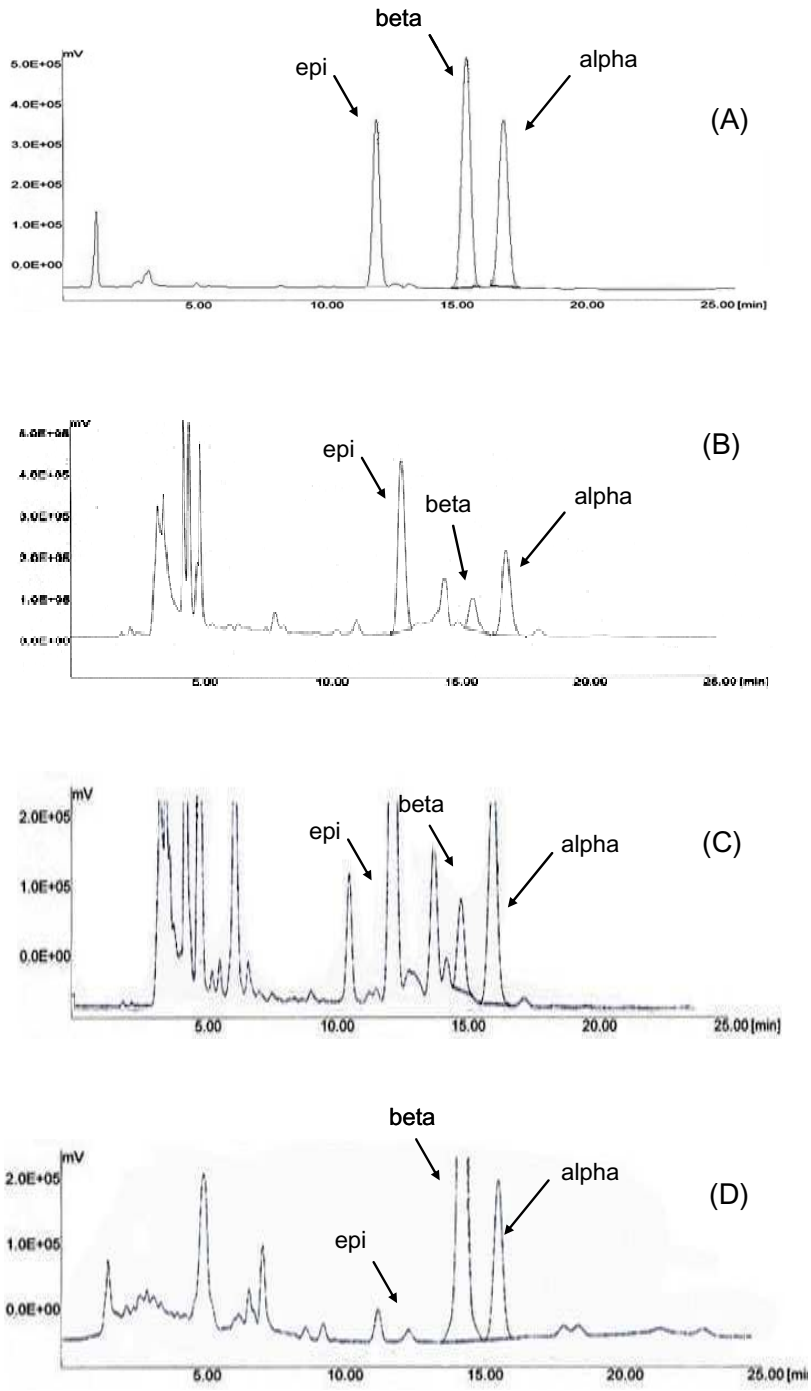


Fig. 3


beta
epi
alpha
(A)
mV
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
5.00
10.00
15.00
20.00
25.00 [min]
epi
beta
alpha
(B)
mV
5.00
10.00
15.00
20.00
25.00 [min]
beta
epi
alpha
(C)
mV
2.0E+05
1.0E+05
0.0E+00
5.00
10.00
15.00
20.00
25.00 [min]
beta
alpha
epi
(D)
mV
2.0E+05
1.0E+05
0.0E+00
5.00
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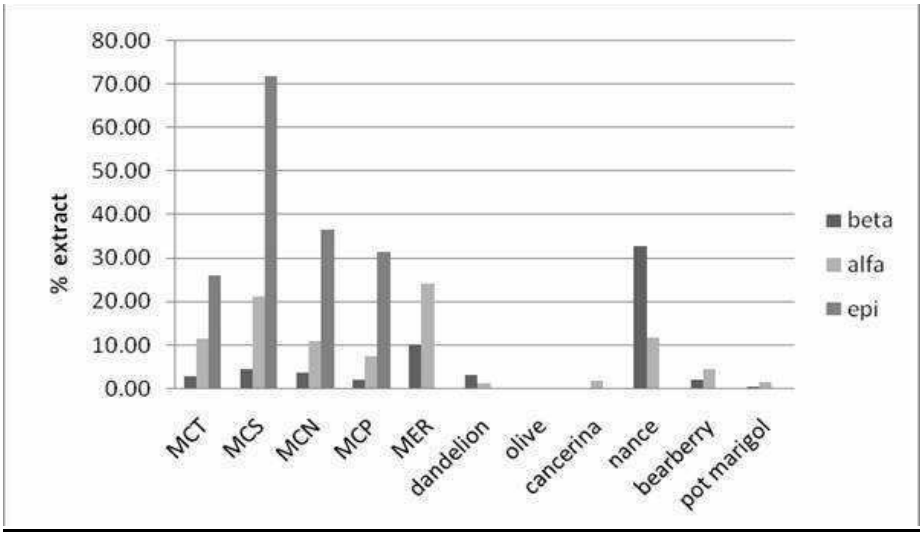

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4 *Centella asiatica* (L) Urban: An updated approach

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Abstract

Centella asiatica (L.) Urban is much used for its antiinflamatory and antiedematic properties, which are due to its bioactive compounds, the triterpene saponins. Several studies with in vitro cultures have been directed at developing a biotechnological process able to produce these interesting compounds. Some of them are summarized in this work, which also describes the conditions for the induction and establishment of a good biomass of friable calli as a prior step to achieving cell suspension cultures.

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Introduction

Centella asiatica (L.) Urban, previously also named *Hydrocotyle asiatica* L., and commonly referred to as Indian pennywort and gotu kola, is an herbaceous plant belonging to the *Apiaceae* family with great medicinal value (Fig 1). It has been used in traditional medicine as an antipyretic, diuretic and antibacterial drug and in the treatment of skin diseases, vein insufficiency and mental disorders [1, 2].

Figure 1. *Centella asiatica* (L) Urban.

It is native to Asia and mainly found in India, Pakistan and Madagascar, but the plant also grows in tropical and equatorial Africa, America, and the tropical regions of Oceania. It has been widely cultivated as a vegetable or spice in China, Southeast Asia, India, Sri Lanka, Africa, and Oceanic countries.

The bioactive compounds of *Centella asiatica* are triterpenoid saponins and sapogenins with an ursane skeleton (Fig. 3), among which the saponines madecassoside and asiaticoside are of particular interest, as well as their respective sapogenins madecassic and asiatic acid. Asiaticoside appears to stimulate the wound healing process and is useful in the treatment of leprosy [3] and tuberculosis, while madecassoside has antiinflamatory properties and is able to significantly increase collagen III secretion [4].

Due to its medicinal properties, the interest in this plant has increased in recent years and included in the literature are studies that describe the production of triterpenoid saponins in various parts of the plant [2, 5] the whole plant [5, 6, 7], and also in suspension cultures [8, 9]. Most of the authors only determine asiaticoside [5, 7, 9], or asiaticoside and madecassoside [2, 10, 11]. There are three studies, including ours, that determine the four compounds [6, 8].

Triterpenoids are one of the most abundant natural products commonly occuring in plants. They are often glycosylated and stored in underground parts as saponins [12]. According to a recent study [5] 98.5% of the total asiaticoside content in *Centella* is found in aerial parts, and only 1.5% accumulates in the roots. Among the three varieties described in relation to their geographic origin, the most studied is *C. asiatica* L. var. *typica*, found in Southern Asia and Madagascar [4]. Surveys of the chemical constituents of *C. asiatica* have shown that although triterpenoid saponins are the major bioactive compounds, their content can differ not only among the plant varieties but also within the same variety according to region. For example, *C.asiatica* from Madagascar produces more secondary compounds than the Indian variety [13]. As reported by Aziz et al. (2007), such variations of asiaticoside content in *C.asiatica* are associated with the origin of the plant. Thus, a population growing at high altitude contains more asiaticoside than plants from a lower altitude [14]. Plants collected from 609m above sea level contained 0.11% of asiaticoside per leaf dry mass, whereas those from ca.5 m above sea level contained less than half of this value. The same authors found that the content of asiaticoside and madecassoside was higher (0.36% and 0.68% dry mass respectively) in one of two phenotypes of *C.asiatica* (L) Urban of Malaysia. When trying to increase centelloside production by biotechnological processes, it is important to select a plant line with a naturally high content.

Biosynthesis of centellosides and cloned genes in the pathway

Centellosides are triterpenoid compounds that are biosynthesized through the mevalonate pathway in the cytoplasm, (see Fig. 2). Their biosynthesis can be divided in three stages:

1. The synthesis of the universal precursor of all terpenoids, isopentenyl diphosphate (IPP).
2. The synthesis of the first triterpene, squalene.
3. The synthesis of centellosides.

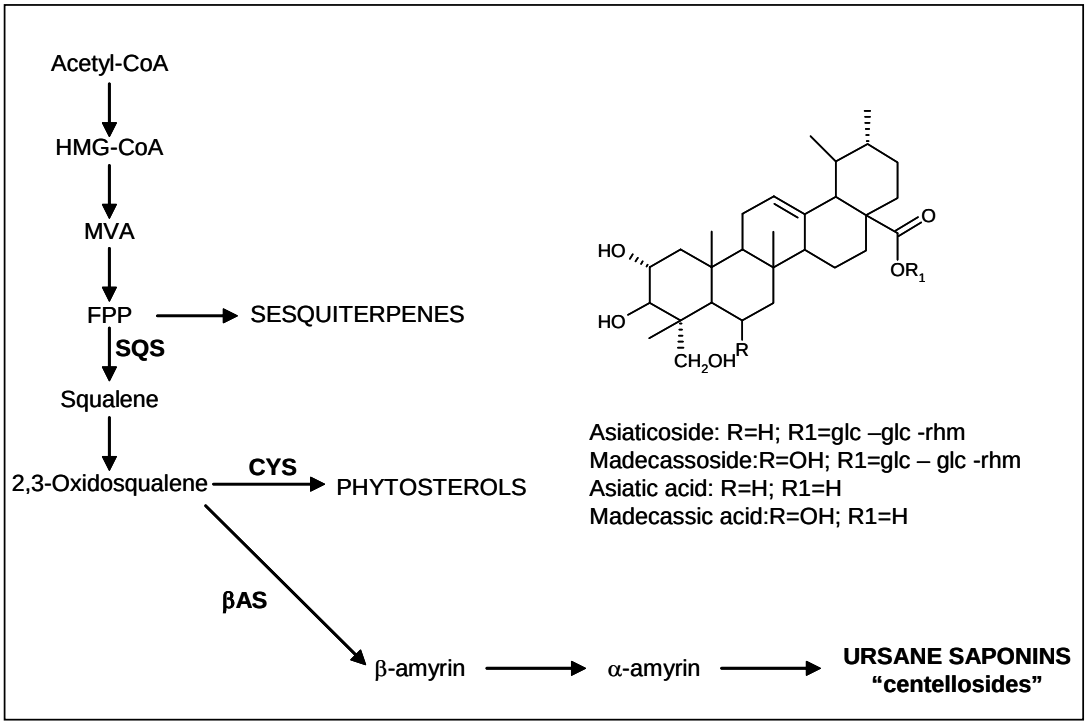

Figure 2. Ursane saponin biosynthesis.

1. The first precursors in the mevalonate pathway are mevalonate and hydroxymethylglutaryl-CoA, which lead to the formation of the universal precursor of all terpenoids: 5-carbon isopentenyl diphosphate. In this route, for which not only the participating enzymes but also their codifying genes are known, 6-carbon mevalonate is synthesised by condensation of 3 acetyl-CoA molecules in a reaction catalysed by the hydroxymethylglutaryl-CoA reductase (HMGR). This reaction is now considered to be the key regulatory step in the synthesis of cytosolic isoprenoids [15]. Mevalonate is then phosphorylated by two different soluble kinases, mevalonate and phosphomevalonate, to form 5 pyrophosphomevalonate. This compound is decarboxylated by the pyrophosphomevalonate decarboxylase into IPP [16], which is in equilibrium with its isomer, the dimethylallyl diphosphate (DMAPP), formed by the isopentenyl diphosphate isomerase.

2. Two molecules of IPP successively bond with the DMAPP to form the first sesqiterpene, the 15-carbon farnesyl diphosphate (FPP). This type of bonding is catalysed by specific prenyltransferases. The two FPP molecules are subsequently converted by squalene synthase (SQS) into squalene, which has 30C and is a common precursor of sterol and triterpenoid biosynthesis [17]. This reaction, catalysed by the squalene

synthase, is considered to be a key regulatory step in the synthesis of mevalonate-derived terpenes, since it controls the branching of the main isoprenoid pathway to the biosynthesis of triterpenes and phytosterols [18].

3. Epoxidation of squalene at the second and third carbon positions results in the formation of 2,3-oxidosqualene. Several oxidosqualene cyclases catalyze the cyclization of 2,3-oxidosqualene, which is situated at the branching step for the biosynthesis of phytosterols and triterpene saponins. Centellosides proceed from the cyclisation of 2, 3-oxidosqualene, via a chair-chair-chair-boat transition stage, by a specific oxidosqualene cyclase (OSC), the β-amyrin synthase. The last steps in this biosynthetic pathway are unknown, but centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) are ursane-type saponines.

Recently, Kim et al. [19, 20, 21, 22] have cloned some of the genes involved in the biosynthetic pathway of triterpenoid saponins in *C. asiatica*, such as β-amyrin synthase (*CabAS*) [19], cycloartenol synthase (*Ca*CYS) [20], squalene synthase (*Ca*SQS) [21] and farnesyl diphosphate synthase [22]. In this context, quantitative expression data of these genes can provide insight into the switched-on and switched-off genes as well as the up-regulated and down-regulated genes in the *C. asiatica* biosynthetic pathway.

These authors showed that the levels of *Cab*AS mRNA during leaf development peaked at 2-3 weeks and decreased after 4 weeks. However, despite such decreases in the level of *Cab*AS mRNA, the content of asiaticoside in leaves was found to increase with time. In order to explain this inverse relationship between the level of *Cab*AS mRNA and saponin content in tissue, it has been proposed that triterpene aglycones act as structural components of membranes during normal plant growth and development [23, 24]. Therefore, since the number of *Cab*AS transcripts increases in the early stages of *C. asiatica* leaf development, it is thought that *Cab*AS may play a role in synthesizing structural components of membranes [19]. The same authors have determined that 0.1mM methyl jasmonate is sufficient to up-regulate the levels of *Ca*SQS mRNA and *Cab*AS mRNA and thus enhance the triterpene saponin content.

Another cloned gene, *Ca*CYS, codifies for cycloartenol synthase, a key enzyme for the regulation of phytosterol biosynthesis. Cycloartenol is the major product of the condensation of squalene 2,3-epoxide in a chair-boat-chair-boat transition state, and is the precursor of sterols in plants. A series of reactions from cycloartenol, including modification of the side chain, lead to

the most abundant phytosterols, such as β-sitosterol, campesterol and stigmasterol. Cycloartenol plays a central role in the synthesis of other plant sterols, such as cholesterol, which in turn serves as a precursor for other steroid derivatives, such as progestagens, cardiac glycosides [25] and steroidic saponines. Phytosterols play a major structural role in membranes of plant cells, the greatest sterol content being in the plasma membrane [26, 27, 28]. Kim et al. [20] found the highest expression of CaCYS mRNA transcripts in leaves but they decreased significantly when MeJA was added to the medium. However, the down-regulation of *Ca*CYS mRNA transcripts following exposure to MeJA was overcome by adding a cytokinin to the medium. It seems that MeJA inhibits the formation of cycloartenol and promotes the triterpenoid route.

Centellosides content *in vitro* cultures vs. plants

A survey of published articles about the content of centellosides in *C.asiatica* reveals very diverse data according to the plant origin and the type

Table 1. Centelloside production (mg/g DW). Values from several articles.

Analyzed Centellosides	C.asiatica origin	Plant culture			*In vitro* culture					Authors
					Calli	Cell susp		Plant		
		W	AP	R		W	AP	R		
asiaticoside madecassoside	Sri Lanka		7 0.136							Matsuda et al. 2001
asiaticoside madecassoside asiatic acid madecassic ac	Sri Lanka	9.8% 15% 13% 28.8%								Schaneberg et al. 2003
asiaticoside	Korea	4.32								Kim et al. 2004a
asiaticoside madecassoside asiatic acid madecassic ac	Malasia					4.26 2.34 0.2-1.4 0.1-0.7				Omar et al. 2004
asiaticoside	India				190.48	494.62		125		Nath and Buragohain 2005
asiaticoside madecassoside	Botanical International Germantown MD	7.2 10.1								Schieffer 2005
asiaticoside madecassoside	Malasia				ND ND			1.15% 1.65%		Aziz et al. 2007

W: Whole plant. AP: Aerial part. R: Roots

of culture carried out. It is noteworthy that the highest values correspond to extracts from wild plants or those cultured traditionally, with the exception of one study that obtained very high values using an *in vitro* culture [9]. As mentioned above, centellosides are mainly concentrated in the aerial part.

According to Kim et al. [5], compared to field-grown plants the asiaticoside content in micropropagated shoots of *C.asiatica* is 50% lower and in transformed hairy roots is was as low as 8.3% of the wild plant content. Table 1 shows centelloside production values from several articles. In spite of such a low centelloside content, *in vitro* cultures offer the necessary controlled conditions for biotechnological manipulation aiming to increase the production of these bioactive compounds in both *in vitro* plants and cell suspension cultures.

Elicitation of *in vitro* plants with methyl jasmonate

Elicitors have been found to induce triterpene saponin accumulation in plants. Among them, it has been reported that exogenously applied methyl jasmonate (MeJA) induces the biosynthesis of many secondary metabolites [29], including terpenoids [30, 31, 32] and it plays an important role in a signal transduction process that regulates defense genes in plants [33]. Therefore, exogenously applied MeJA is widely used in plant cell cultures to activate secondary metabolism and it can enhance the secondary metabolite production in a variety of plant species. However, it has been observed that when MeJA is exogenously applied to plants, it produces effects such as growth inhibition, induction of leaf senescence [34] and promotion of ethylene production [35].

Kim et al. [5] have studied the accumulation of asiaticoside in whole plant cultures of *C.asiatica*, reporting an enhancement of its production by MeJA treatment. Taking into account the aforementioned effect of this elicitor on growth and senescence, they have observed that when a cytokinin, which acts as an antisenescence agent in several plants, is also added to the medium, the production of asiaticoside is greater. They explain that this is due to an increased growth of shoots, where asiaticoside is mainly synthesized, rather than the stimulation of secondary metabolites.

We have also treated *C.asiatica in vitro* plants with the elicitor MeJA and compared their ursane saponin content with that of non-treated plants [30]. Plants of *C.asiatica*, obtained from seeds provided by the School of Pharmacy of the Second Military Medical University (Shangai, China), were cultured for four weeks in Murashige and Skoog (MS) solid medium [36] with and without 100μM MeJA. Fig.3A shows the development of an untreated plant of *C.asiatica* during three four-week subcultures (A, B, C)

and Fig. 3B the appearance of a *C.asiatica* plant treated with the elicitor compared with an untreated plant. In the treated plants after four weeks of culture, we observed a general decrease in growth and necrotic symptoms in the roots. Researchers have frequently used MeJA at a concentration of 100μM to increase secondary metabolism in *in vitro* cultures [37, 38, 39], with the plant cells or organs coming into direct contact with the elicitor. In contrast, in our plant culture only the roots were directly exposed to MeJA, which is probably why it had a greater effect on root development.

In relation to triterpenoid saponin content in *C.asiatica* plants, treated and untreated with MeJA, the levels of the four bioactive compounds were determined in the aerial parts and roots. In all cases the saponin content of the roots was lower than in the aerial parts, and the elicitor treatment significantly increased the content of these four compounds at the week 4 (Fig. 4A).

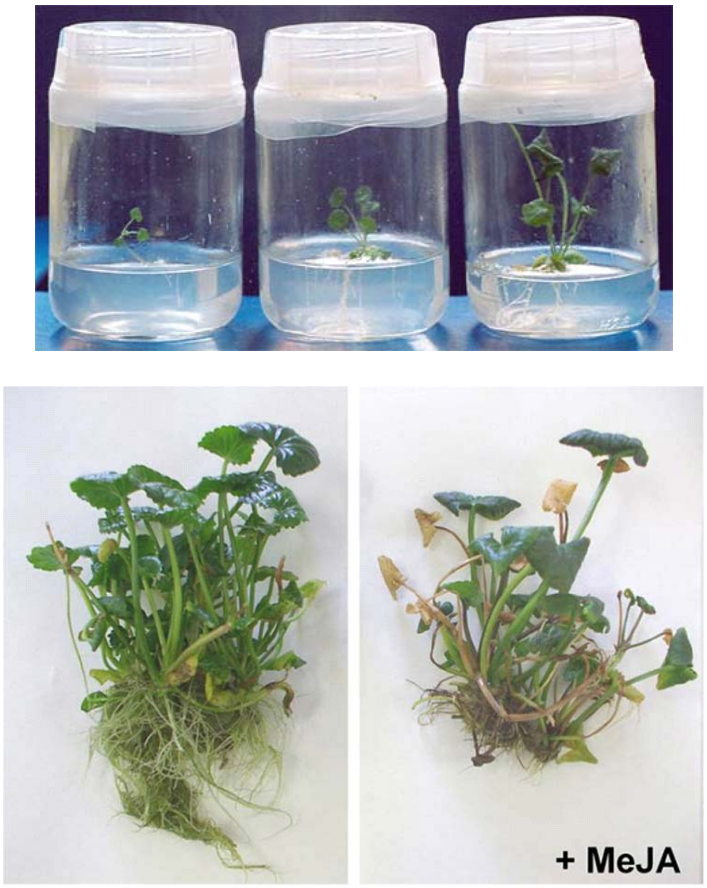

Figure 3. A: *In vitro* development of an untreated plant of *Centella asiatica. Centella asiatica* plant treated with MeJA compared with an untreated plant after 4 weeks of growth.

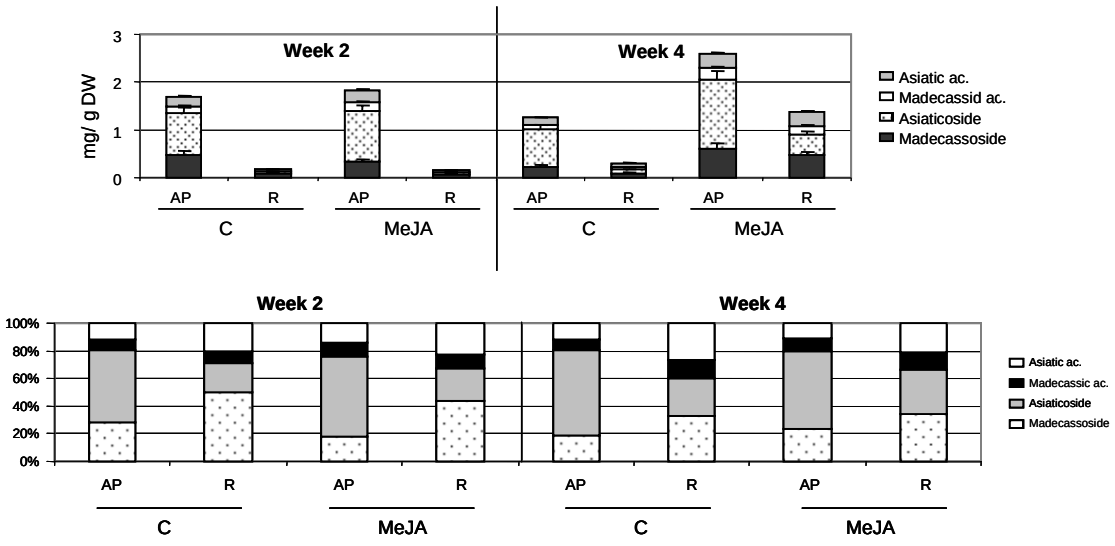

Figure 4. A: Centelloside content in aerial parts and roots of *C.asiatica* treated and untreated with MeJA **B**: Centelloside pattern in aerial parts and roots of *C.asiatica* treated and untreated with MeJA.

Interestingly, the centelloside pattern in the aerial parts and roots was not changed by the elicitor treatment (Fig. 4B). The most abundant centellosides were asiaticoside and madecassoside, which corresponds with other data in the literature [8].

Establishment of cell suspension cultures: Assaying different media to obtain large and friable calli

Before establishing cell suspension cultures, it is necessary to obtain a good biomass of friable callus culture. Based on the work of several authors, different growth regulators in MS medium were assayed to obtain calli from leaf and petiole explants.

Explants from young leaves and petioles were sterilized and cultured in MS medium containing the different concentrations and combinations of auxins and cytokinins that appear in Table2.

The assayed media for callus induction appear in italics in Table 2. Explants were incubated in the dark at 25ºC in Petri dishes. Calli began to grow after two weeks and had developed at the fourth week. The induction percentage of calli from leaf explants in the assayed media is shown in Table 2.

The best media for callus induction from leaves were: [MS + IAA (2 mg/L) + Kn (0.2 mg/L); MS + IBA (0.1 mg/L) + BA (2 mg/L) and MS + NAA (2 mg/L) + BA (2 mg/L)]. These three media were used to induce calli

Table 2. Induction and morphological traits of Calli from *C.asiatica* explants in MS medium.

PGR[a]				% Callus induction[b]		Morphological traits
Auxin: mg/L		Cytokinins: mg/L		Leaf	Petiole	
IAA	*2*	*KIN*	*0.2*	91.2	10.5	Good induction, small calli
2,4-D	*1*		*0.1*	67.8	-	Little induction, friable calli
			1		*1*	No induction
			2	**4PU-30**	**3**	**Large and friable calli**
IBA	*0.1*	*BA*	*2*	93	77.3	Good induction but not friable
		4PU-30	0.2			Small calli and not very friable
			1			Good growth but not friable
			2			Good growth but not friable
			3			Good growth but not friable
NAA	*2*	*BA*	*2*	92.4	72.7	Good induction but not friable

[a] PGR: plant growth regulator, IAA: indoleacetic acid; 2,4-D: 2,4-dichlorophenoxy acetic acid; IBA: indole-3-butyric acid; NAA: α-naphtaleneacetic acid; KIN: kinetin; BA: 6-benzyladenine; 4PU-30: N-(2-cloro-4-piridil)-N'-fenilurea.

[b] Data represent mean of 20 replicates/medium in two repeated experiments.

Italic: Media assayed for callus induction
Capital: The best medium to establish cell suspension cultures. See also Figure 5.

from petiole explants but this material proved to be less effective for induction than leaf explants. On [MS + IAA (2 mg/L) + Kn (0.2 mg/L)] medium, callus induction was good but growth was poor [40]. On [MS + IBA (0.1 mg/L) + BA (2 mg/L)] and [MS + NAA (2 mg/L) + BA (2 mg/L)] media, profuse callusing was observed (Paramageetham et al., 2004). Media with NAA, IAA and IBA produced hard and compact calli, which was not desirable. In contrast, on media containing 2,4-D (1 mg/L) in combination with Kn (0.1 mg/L) calli were friable, but significant growth was not induced, and when Kn was 1 mg/L there was no callus induction.

Once the callus tissue was induced, the aim was to find an optimum medium to obtain large, white and friable calli. The three aforementioned media chosen for induction were assayed: in the case of [MS + NAA (2 mg/L) + BA (2 mg/L)] and [MS + IAA (2 mg/L) + Kn (0.2 mg/L)] media, calli were compact and without significant growth, as described by Patra et al. [41] when using media containing BA in combination with NAA. On the medium with [MS + IBA (0.1 mg/L) + BA (2 mg/L)], as used by Banerjee et al. [42] for *in vitro* multiplication of *C. asiatica*, calli were white, large but not friable. We also used these three media with 1/2 MS salts [43], in which case calli were whiter but their growth remained the same and they were still not friable.

As we observed that growth was greater with IBA than with other auxins (IAA, NAA, 2,4-D), to obtain friable calli we maintained IBA and only changed the cytokinin BA for N-(2-cloro-4-piridil)-N'-phenilurea (4PU-30) at different concentrations. It has been reported that cytokinins derived from phenylurea are the best supplement for the promotion of shoot growth in C. asiatica [44], and in previous work with tobacco callus cultures we had observed that 4PU-30 developed large and friable calli [45]. With the exception of the [MS + IBA (0.1 mg/L) + 4PU-30 (0.2 mg/L)] medium, the other 4PU-30 concentrations tested (1, 2 and 3 mg/L) gave good growth, although in no case was friable callusing induced.

Since we had previously obtained friable calli with 2,4-D (2 mg/L) [46] we decided to use it instead of the auxin IBA, maintaining the kinetin 4PU-30 (3 mg/L). The resulting medium, MS + 2,4-D (2 mg/L) + 4PU-30 (3 mg/L), was optimal for obtaining large, white and friable calli (Fig. 5). Work is currently being developed in our laboratory with the aim of increasing the triterpene saponin production in cell suspension cultures of *C. asiatica*.

Figure 5. Aspect of friable calli obtained with 2,4-D (2 mg/L) + 4PU-30 (3 mg/L) prior to the establishment of cell suspension cultures.

Pattern of centellosides in calli and *in vitro* plants

The content of madecassoside, asiaticoside, madecassic acid and asiatic acid in our calli cultured in MS medium [36] with different growth regulators.is shown in Figure 6. This figure shows that the most abundant centellosides in our calli cultures were madecassoside and asiaticoside. When the medium combined NAA and BA the content of both centellosides was similar. The content in calli was about ten times lower than in *in vitro* plants, whose content ranged between 1.3 to 2.5 mg/g DW after elicitation (Fig. 7). This might seem low but, as mentioned before, the content of asiaticoside in *in vitro* plants is reported to be about 50% lower than in field-grown plants [5].

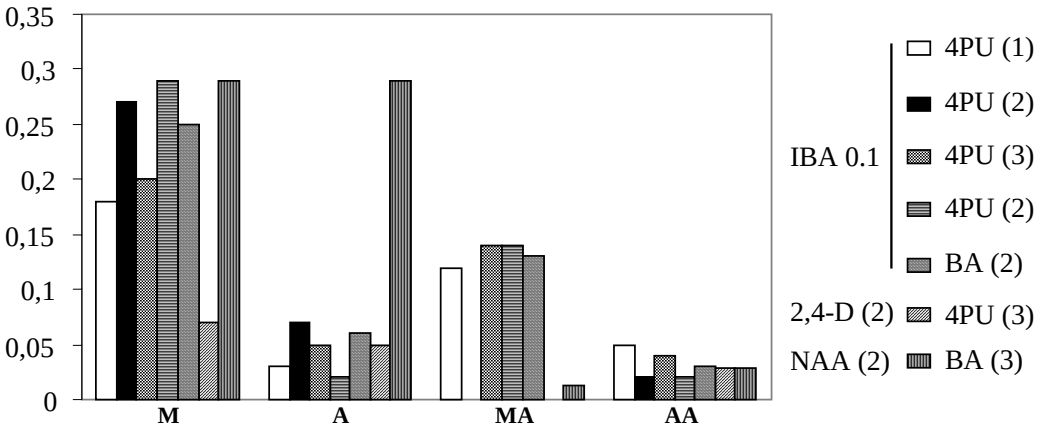

Figure 6. Triterpenoid saponin content in calli of *Centella asiatica* (mg/g DW) M: madecassoside, A: asiaticoside, MA: madecassic acid, AA: asiatic acid.

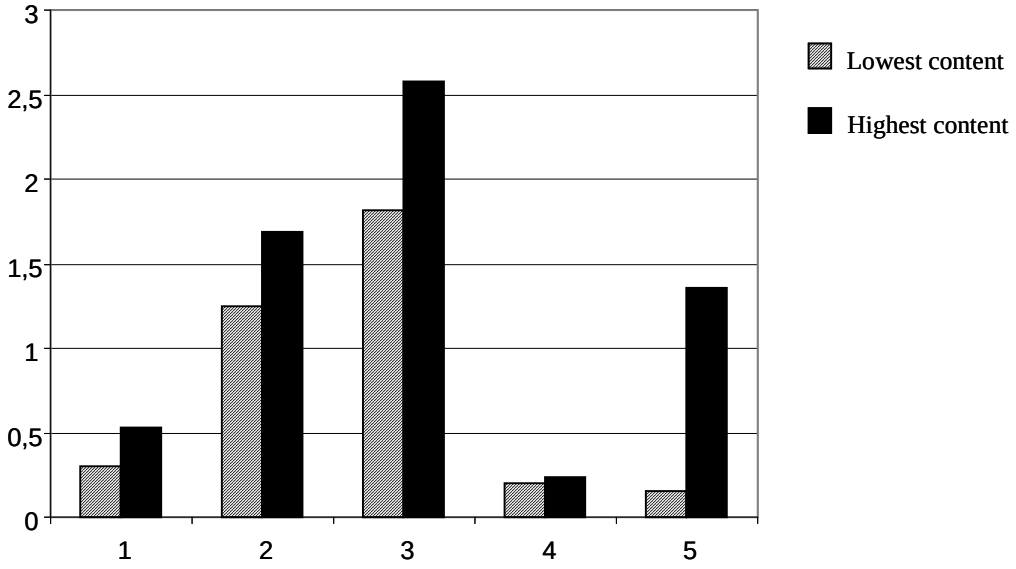

Figure 7. Highest and lowest level of centellosides in calli (C), aerial parts (AP) and roots (R) of *C.asiatica in vitro* plants. (mg/ g DW). MeJa = Methyl Jasmonate.

Analytical techniques

For the identification and quantifying of the four principal triterpenoid components of *C.asiatica* we carried out qualitative analysis using Thin-Layer Chromatography (TLC) and Matrix-Assisted Laser Desorption/ Ionisation - Time-Of-Flight (MALDI-TOF) mass spectrometry, and a quantitative analysis using High Performance Liquid Chromatography (HPLC). The identification of madecassoside, asiaticoside, madecassic and

asiatic acid was achieved by TLC on silica gel plates and mass spectrometry, as a modification of the method described in the European Pharmacopoeia (2005). A combination of ethyl acetate and methanol as the mobile phase was found to be successful in separating these compounds from the rest of the main components of the extract. The separated compounds were confirmed by MALDI-TOF mass spectrometry. HPLC analysis was done with a modifying procedure [47].

We started with one gram of dry weight, and we used methanol-water for the extraction. After repeating the process we obtained an extract, which was dissolved in MeOH (Figure 8).

The extract was used for a qualitative analysis using Thin-Layer Chromatography (TLC) and MALDI-TOF mass spectrometry, and for a quantitative analysis using High Performance Liquid Chromatography (HPLC).

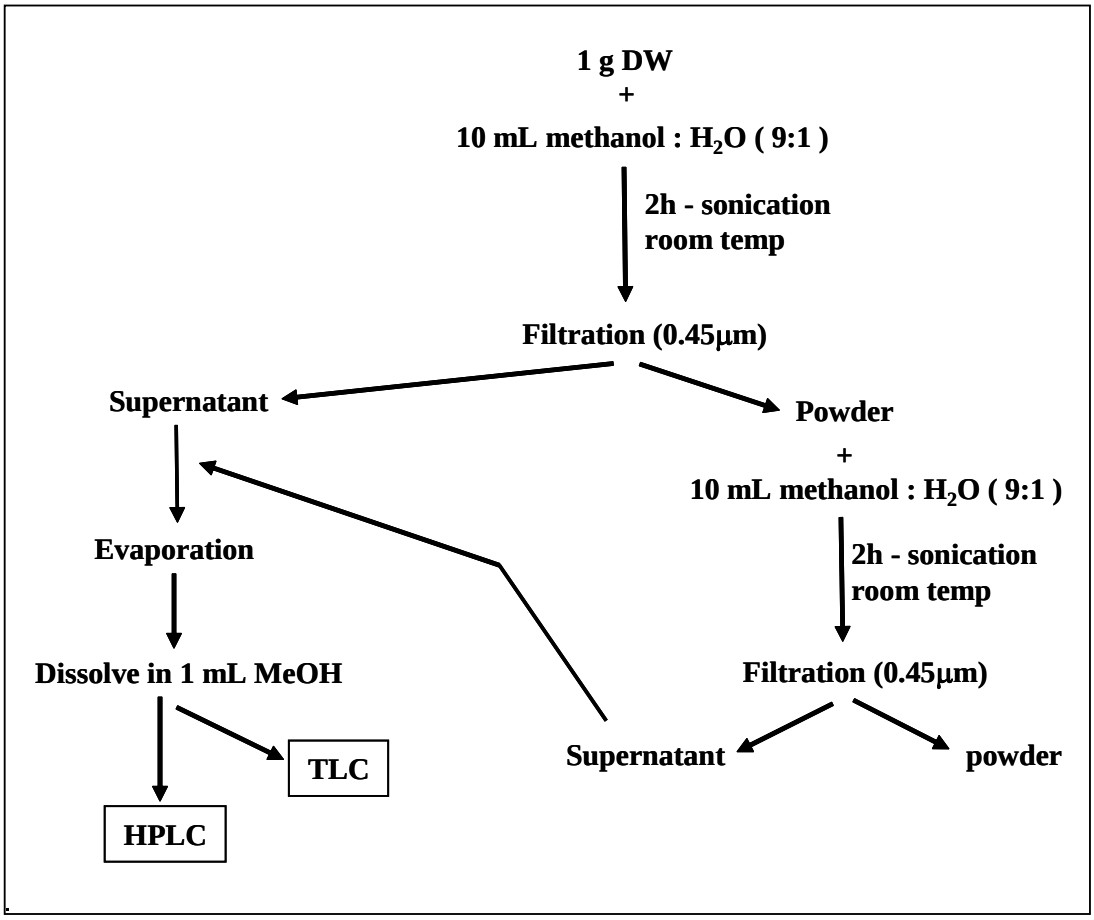

Figure 8. Centelloside extraction protocol.

TLC identification

As a preliminary approach to identify the four triterpenoid compounds of *C.asiatica* we followed a TLC protocol from the *European Pharmacopoeia* (2005). Using this protocol, we were unable to separate madecassoside and asiaticoside, especially from the callus extracts because a large amount of compounds interfered with them, particularly in the lower half of the plate. We had to change the solvent system, which permitted the separation of these two trisaccharides from the interfering compounds.

Figure 9 shows the plates with the plant and callus extracts: The plant extract appears more clearly than the callus extract. The dark band at the bottom hided madecassoside and asiaticoside. We tried to clean the samples with hexane and ethyl acetate before the extraction but we observed that these solvents removed a considerable amount of asiatic and madecassic acids, for this reason, we decided to use only the methanol extract [48].

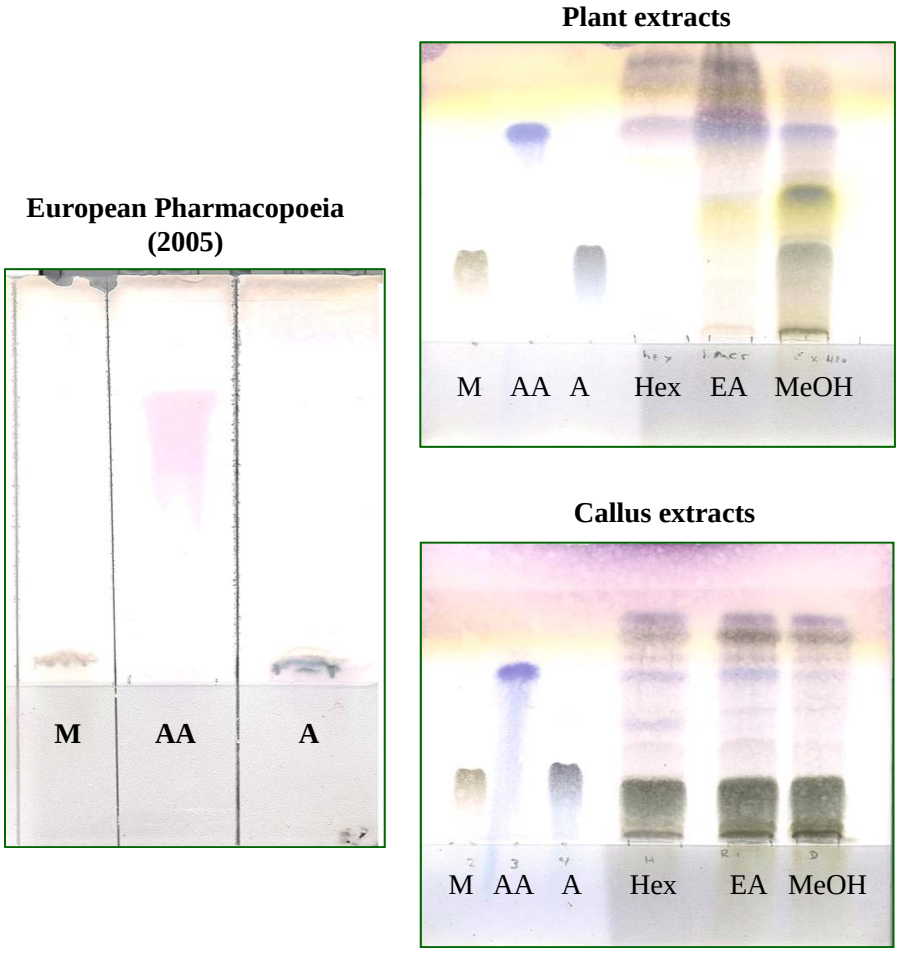

Figure 9. Centelloside identification by TLC. M: madecassoside, AA: asiatic acid, A: asiaticoside, Hex: hexane extract, EA: ethyl acetate extract, MeOH: methanol extract.

The TLC protocol used was: silica gel plates, ethyl acetate-methanol (60:40, v/v) as the solvent system and anisaldehyde solution as the spray detector. Figure 10 shows a plate with the 4 standards, a pharmaceutical preparation containing *Centella* extract and a plant extract.

This system allows the identification of triterpenoid compounds of *C. asiatica* and their separation into saponins and sapogenins, and also can be used to prepare samples for HPLC analysis.

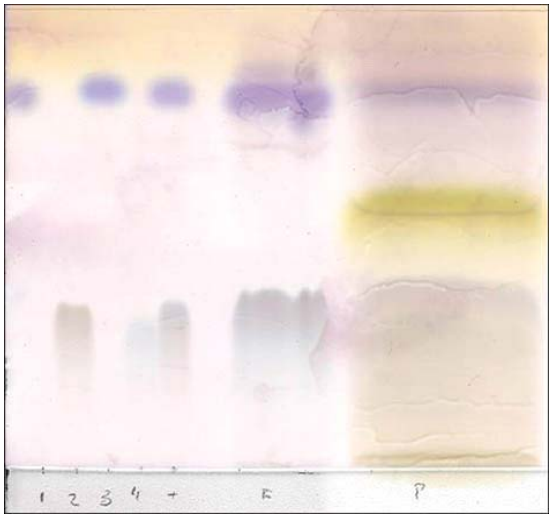

Figure 10. Centelloside identification by TLC. MA: madecassic acid, M: madecassoside, AA: asiatic acid, A: asiaticoside, 1 and 2: pharmaceutical preparation, P: plant.

MALDI-TOF identification

To further confirm the identification of the four centellosides obtained by TLC, we carried out a MALDI-TOF spectrometry in positive ionization mode. Figure 11 shows the MALDI-TOF spectra of standards: asiaticoside, madecassoside, asiatic acid and madecassic acid with their respectives mass ions $[M + Na]^+$ and in Figure 12 we can see a MALDI-TOF spectra corresponding to a plant extract. The arrows indicate the peaks belonging to madecassic acid and the two saponins.

HPLC analysis

The quantification of the four compounds was done by HPLC [30]. The chromatographic analysis was performed by a reversed phase using gradient elution. Figure 13A shows a chromatogram of the four separated standards and Figure 13B a chromatogram of a plant extract with the arrows indicating the centellosides. The purity of peaks was checked using a photodiode-array detector (Figure 14).

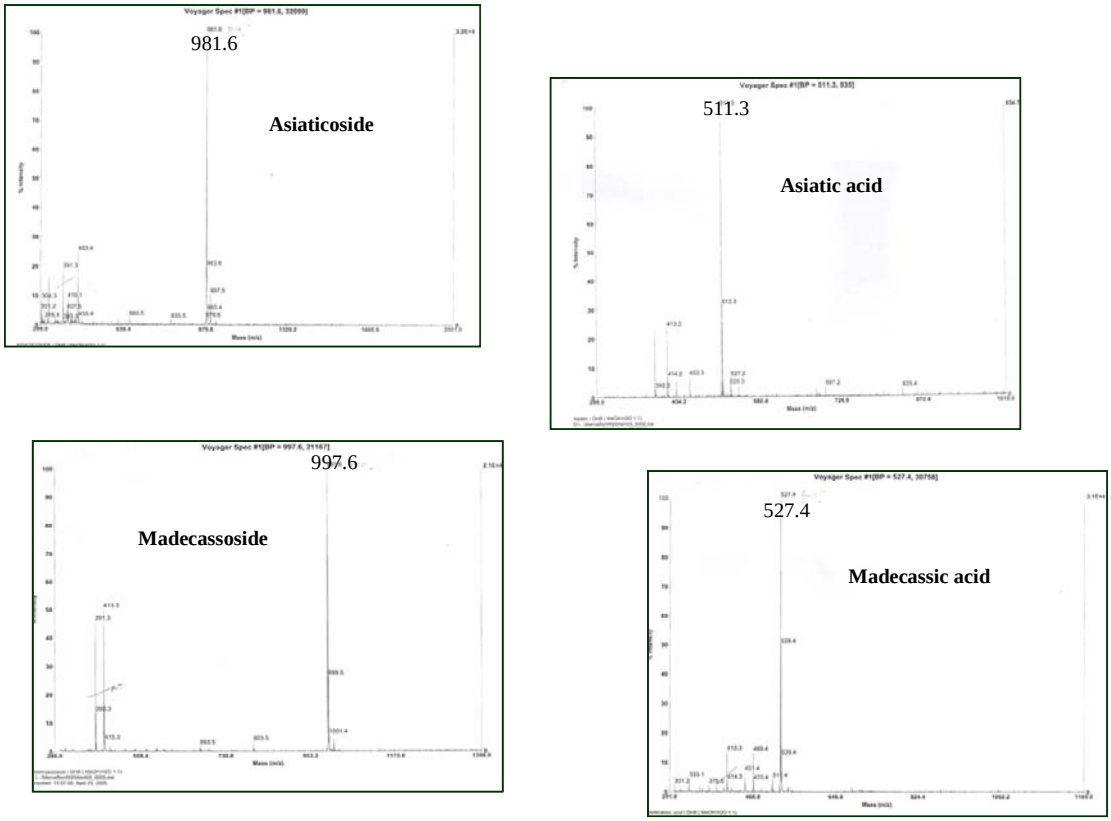

Figure 11. MALDI-TOF spectra of centellosides.

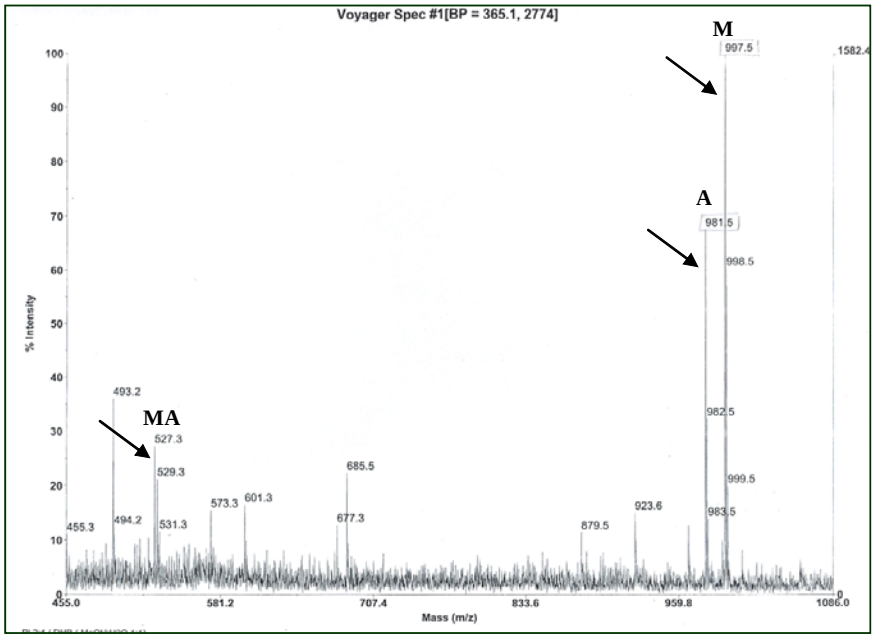

Figure 12. MALDI-TOF spectra of a plant extract. MA: madecassic acid, M: madecassoside, A: asiaticoside. Arrows indicate the peaks.

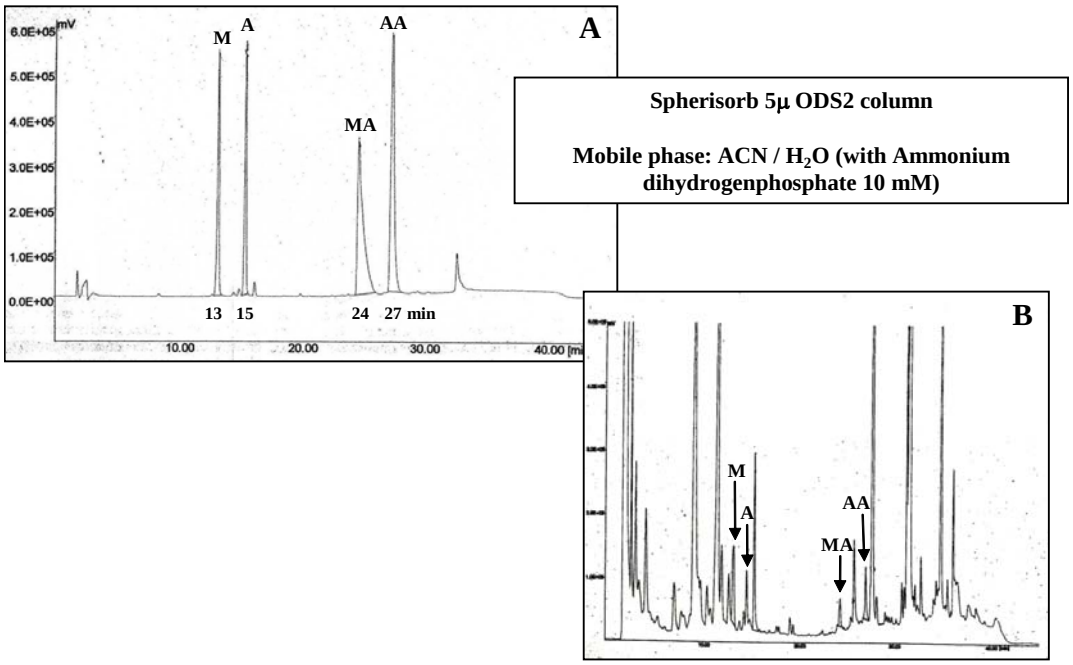

Figure 13. HPLC analysis of the four centellosides using gradient elution. A: Standard mixture B: Centella plant extract. (MA: madecassic acid, M: madecassoside, AA: asiatic acid, A: asiaticoside).

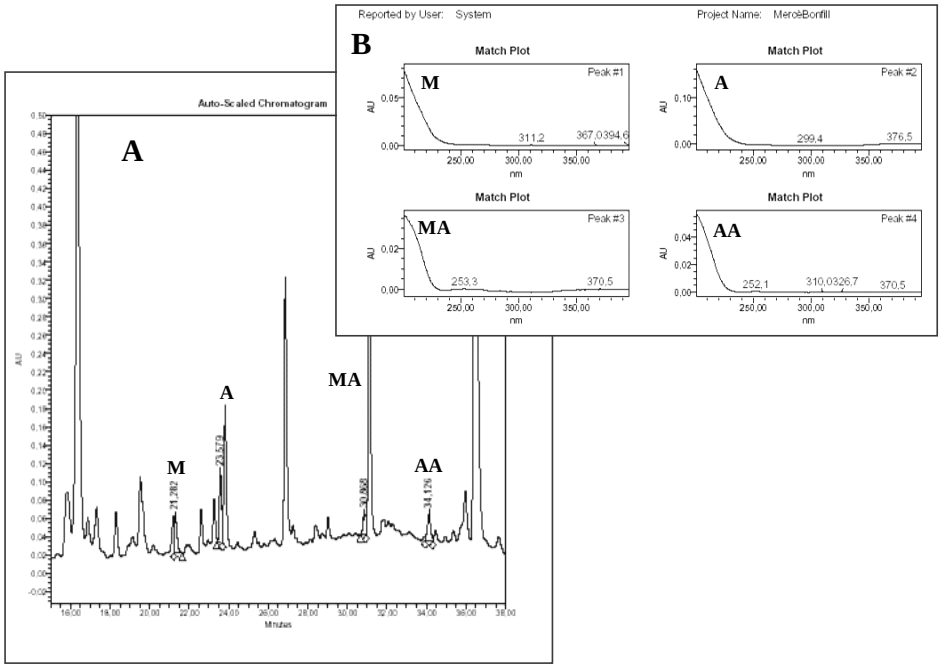

Figure 14. HPLC Analysis: Photodiode - Array Detection. A: plant extract, B: absorption spectra of standards. (MA: madecassic acid, M: madecassoside, AA: asiatic acid, A: asiaticoside).

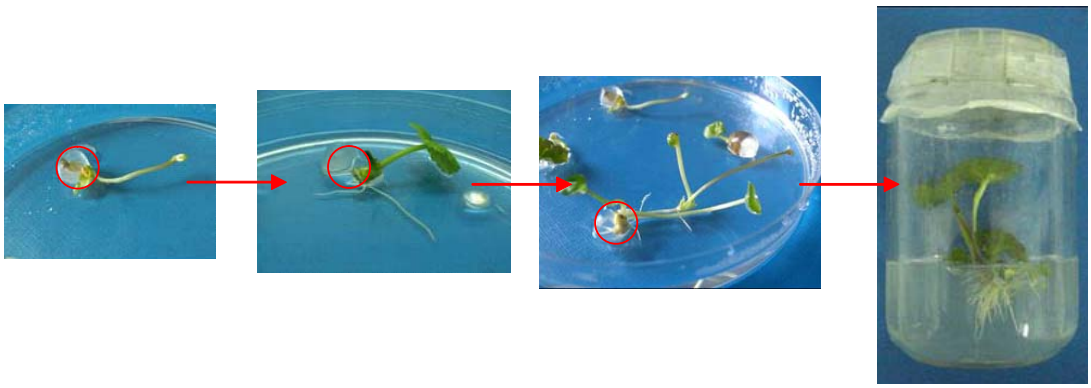

Figure 15. *Centella* micropropagation by somatic seeds.

Centella micropropagation by somatic seeds

A method that allows sterile clones of *C. asiatica* to be kept for the culture of *in vitro* plants is micropropagation by somatic seeds obtained from steril meristems. This method, succesfully implemented by the team of Dr. M.Furmanowa (Biology and Pharmaceutical Botany Department. The Medical University of Warsaw. Warsaw, Poland), has also been used by our group to maintain a stock of *C. asiatica* plants conserving interesting genotypes. The protocol is the following:

C. asiatica plants grown *in vitro* are transferred to a Petri dish with wet filter paper to keep the plant moist and the meristematic structures are selected (apical meristems of roots and stems and axilar meristems). Fragments of approximately 0.5 cm of these meristems are cut and submerged in 100 ml of 5% sodium alginate solution. The submerged meristems are picked up with a 10 ml wide-mouth pipette and released drop by drop into a 0.55% calcium chloride solution. The contact of the sodium alginate with the calcium chloride solution produces the alginate beads, which should remain in the calcium chloride solution for 30 minutes. The calcium-alginate beads are then cleaned with sterile distilled water and placed on filter paper to remove the excess water. The beads containing meristems are selected, placed on Petri dishes with MS medium and kept at 4ºC for up to 6 months. Figure 15 shows several steps in the germination of a somatic seed of *C. asiatica*.

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