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Sterol and Triterpene Profiles of the Callus Culture of *Solanum mammosum*

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Abstract

This study aimed to compare the sterol and triterpene profiles of two types of *Solanum mammosum* callus cultures, i.e., compact globular structure (CGS) and normal fine (F) calluses. The CGS callus resulted from the differentiation of the F callus culture after many years of subculturing. The growth rate, microscopic characteristics, and morphologies of the two callus types were determined and compared. Sterols and triterpenes were identified through thin-layer chromatography, gas chromatography–flame ionization detection, and gas chromatography–mass spectrometry analyses. The growth rate of the CGS callus was lower than that of the F callus. Microscopic identification revealed that thick, lignin-containing cell walls formed in the CGS callus but not in the F callus. The chromatographic analysis suggested that the CGS and F callus cultures had different sterol and triterpenoid profiles. The sterols and triterpenes produced by the CGC culture were more diverse than those produced by the F callus culture.

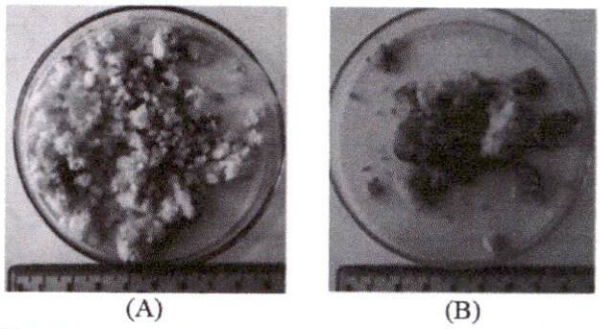
Keywords: Solanum mammosum, callus, tissue culture, sterol, triterpene

Introduction

Sterols can be found in all eukaryotic organisms as membrane components that regulate the fluidity and the permeability of phospholipid bilayers. Most plant cells can produce sterols, such as cholesterol, campesterol, and β-sitosterol, via the cycloartenol pathway [1]. Sterols in plants originate from cycloartenol, whereas those in fungi or animals are derived from lanosterol [2]. Sterols are members of the terpenoid family. Specifically, sterols are triterpenoids. Terpenoids are produced by plants and have various basic functions in growth and development but mainly participate in chemical interactions and protection against abiotic and biotic environmental stressors [3]. Several sterols have important biological activities, including anti-inflammatory, antidiabetic, anticancer, and lipid-lowering activities [4]. Triterpenes, such as betullinic acid, are natural products that exert activities against a variety of cancer types by directly influencing mitochondrial membrane permeabilization [5].

Some sterols and triterpenes have been identified in several cell cultures of *Solanum* spp. Cholesterol, stigmasterol, β-sitosterol, isofucosterol, lanosterols, lupeol, betulin, betulin aldehyde, and betulinic acid have been identified in callus cultures of *S. laciniatum* [6,7]. Cholesterol, campesterol, stigmasterol, β-sitosterol, 28-isofucosterol, and 24-methylene cycloartanol have also been found in callus cultures of *S. malacoxylon* (current name: *S. glaucophyllum*) [8]. The callus cultures of *S. mammosum* and *S. wrightii* produce cholesterol, campesterol, stigmasterol, and β-sitosterol but not solasodine [7,9]. The application of cell cultures of *S. mammosum* for the production of phytosteroids and the biotransformation of some chemical compounds have been reviewed [10].

Some plant cell cultures can self-develop compact globular structure (CGSs). In general, the secondary metabolite profiles of CGS cultures are different from those of normal callus cultures [11-13]. In addition, CGS cultures exhibit cell wall thickening. Two forms of *S. mammosum* callus cultures, CGS and normal (F), are available in our laboratory (Figure 1). CGS callus cultures were obtained after more than 26 years of subculturing since 1984. To the best of our knowledge, reports on the



(A) (B)

Figure 1. *Solanum Mammosum* Callus Cultures: F Callus (A) and CGS Callus (B)

CGS culture of *S. mammosum* have not yet been published. The present work describes the sterol and triterpene profiles of the CGS and F callus cultures of *S. mammosum*.

Materials and Methods

Preparation of callus cultures. The CGS and F callus cultures of *S. mammosum* were grown in a 300 mL Erlenmeyer flask containing 50 mL of modified Murashige-Skoog medium supplemented with 7 g/L agar, 30 g/L sucrose, 2 mg/L kinetin, and 0.5 mg/L 1-Naphtaleneacetic acid at 25 °C ± 2 °C under continuous light (ca. 2000 lux). The cultures were subcultured every 4 weeks of incubation as described in our previous work (Indrayanto et al., 1998). Growth rate was determined by measuring the weight of four callus cultures every week by calculating the ratio of the callus weight at certain time (n^{th} week) to the initial inoculation weight (n = 0). Data were reported as average rate ± standard deviation (SD).

Microscopic characterization. Thin sections of fresh F and CGS callus cultures were observed under light microscopy with 400× magnification. A few drops of phloroglucinol–HCl were added to the CGS callus cultures to identify the presence of lignin.

Sample preparation. Extraction was performed in accordance with a previously published method [7]. Oven-dried (40 °C; moisture content 10%) powdered callus (6 g) was extracted with 50 mL of n-hexane by ultrasonification (3 × 15 min). The extract and residue were separated through filtration. The residue was further extracted with acetone (50 mL) using the same procedure. All collected extracts were evaporated to dryness under a nitrogen stream. The dried extracts of n-hexane (200 mg) and acetone (200 mg) were dissolved in 2 mL of chloroform. A total of 5 mg of different standards (cholesterol, campesterol, stigmasterol, β-sitosterol, lupeol, betulin, and betullinic acid) (Sigma) was dissolved in 2 mL of chloroform.

Thin-layer chromatography. Standards (β-sitosterol, lupeol, and betullinic acid) and 2 µL aliquots of n-hexane and acetone extracts from the CGS and F callus cultures in chloroform were applied to thin-layer chromatography (TLC) plates (Kieselgel 60 F 254). Two different solvents were used in the TLC analysis: 1) n-hexane : ethyl acetate (4:1) for n-hexane extraction, and 2) chloroform : methanol (6:1) for acetone extraction. Plates were sprayed with anisaldehyde–H_2SO_4 for visualization.

Gas chromatography–flame ionization detection. Samples (2 µL) and standards (2 µL) containing cholesterol, campesterol, stigmasterol, β-sitosterol, lupeol, betulin, or betullinic acid were injected into the gas chromatography (GC) instrument. Acetate derivatization was performed by mixing ca. 1 mg of acetone extract with 2 mL of pyridine and 2 mL of an acetic acid anhydride. The mixture was incubated for 24 h in dark, evaporated into dryness under nitrogen, and then dissolved with 2 mL of ethyl acetate. The solution (2 µL) was then injected into the GC instrument. Gas chromatography–flame ionization detection (GC–FID) was performed with a capillary column 5% phenyl methyl siloxane (30.0 m × 320 µm × 0.25 µm) (Agilent Technologies 6890N) at a flow rate 40 mL/min (He) and FID 300 °C. The column temperature was started at 220 °C and then increased to 270 °C at a rate of 10 °C /min.

Gas chromatography–mass spectrometry analyses. Gas chromatography–mass spectrometry analyses (GC–MS) analysis was performed using Agilent GC 6890N and Mass Selective Detector (MSD) 5973 equipped with HP-5 column (30 m × 0.250 mm × 0.25 µm i.d.) and completed with Wiley 7n.1 database (2004). Inlet temperature was set at 250 °C. Analysis was performed in splitless injection mode at a flow rate of 1 mL/min (He). The oven temperature was programmed as 220 °C–270 °C at 10 °C/min. Electron impact ionization (EI–MS) source temperature was set at 230 °C. The transfer line temperature from oven to the detector was 280 °C. The energy of ionization was set at 70 eV.

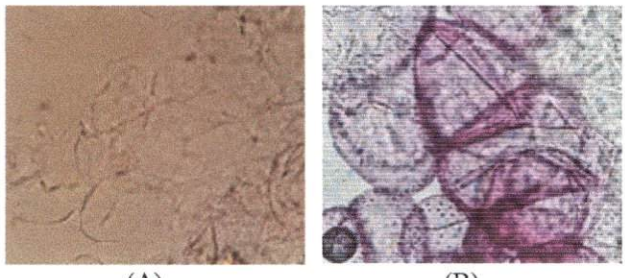
Identification method. The EI–MS spectra of peaks 1–15 (Figures 5 and 6) were compared with the EI–MS spectra of standards and EI–MS spectra from the Wiley database, the online databases of National Institute of Standard and Technology [14], the Spectral Database for Organic Compounds [15], MassBank [16], and published reports [17-24]. Peaks 1–15 were identified in accordance with the method of Commission Decision 2002/657/EC [25].

Results and Discussion

Microscopic identification and growth rate of CGS and F calluses. The CGS culture could be easily differentiated macroscopically from the F callus culture. The CGS culture showed a cohesive callus aggregate with diameters of 0.5–2 cm. By contrast, the normal callus culture was highly dispersed. Microscopic examination revealed that the formation of a thick cell wall limited the differentiation of the CGS culture. The thick cell walls of the CGC culture contained lignin as indicated by the development of a red color after the addition of phloroglucinol–HCl reagent. By contrast, F callus cultures lacked cell wall thickening and lignin (Figure 2). The growth rate of CGS cultures was slower than that of the F callus culture (Figure 3).

Identification of sterol and triterpenes in CGS and F calluses. Sterols and other triterpenes in the callus cultures were identified through TLC, GC–FID, and GC–MS analyses. The n-hexane and acetone extracts of CGS and F callus cultures were subjected to TLC analysis,

visualized with anisaldehyde sulfuric acid spray reagent, and compared with standards. The results indicate that all n-hexane and acetone extracts contained sterol (β-sitosterol) and triterpene (lupeol). However, betullinic acid was only detected in the acetone extract of the CGS callus culture (Figure 4). Further analysis was performed through GC–FID (Tables 1 and 2). The peaks of the cholesterol, campesterol, stigmasterol, and β-sitosterol peaks at 31.678, 32.692, and 45.096 min were further identified through GC–MS as isofucosterol, cycloartenol, and 24-methylene cycloartanol, respectively. GC–FID analysis indicates that β-sitosterol was the major compound in the n-hexane extract of the F and CGS calluses, whereas β-sitosterol and stigmasterol were the major compounds in acetone extracts of the F and CGS calluses, respectively.



(A) (B)

Figure 2. Microscopy Identification of *S. Mammosum* Callus Cultures: F Callus (A) and CGS Callus (B) After the Addition of Phloroglucinol–HCl

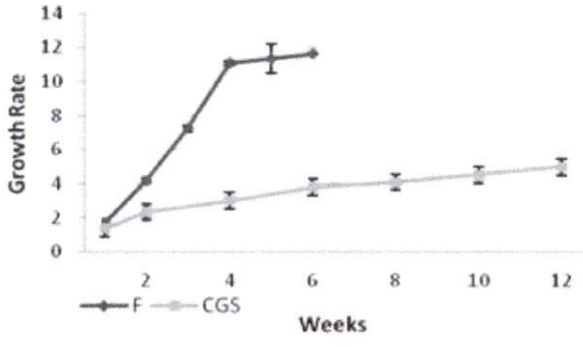
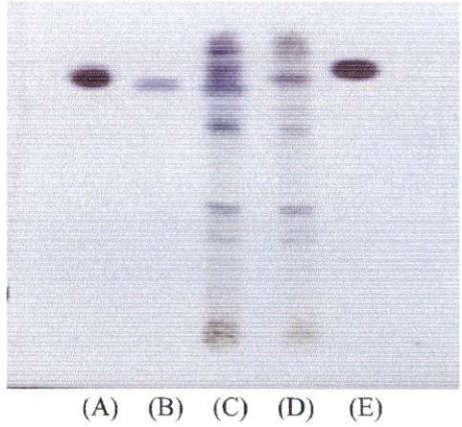


Figure 3. Growth Rate of the F and CGS Callus Cultures of *S. Mammosum*



(A) (B) (C) (D) (E)

Figure 4. TLC Profiles of The Acetone Extracts of *S. Mammosum* Callus Cultures Visualized with Anisaldehyde–H_2SO_4 Reagent. (A) Sitosterol Standard, (B) Betullinic Acid Standard, (C) Acetone Extract of The CGS Callus, (D) Acetone Extract of the F Callus, and (E) Lupeol Standard. Solvent:Chloroform:Methanol (6:1)

Table 1. GC–FID Results for the N-Hexane Extracts of F and CGS Callus Cultures

Standard	Rt std (min)	RRT standard[a]	Rt callus F (min)	RRT callus F[a]	Rt callus CGS (min)	RRT callus CGS[a]
Cholesterol	23.383	1.00	23.126	1.00	23.272	1.00
Campesterol	27.042	1.16	27.119	1.17	27.060	1.16
Stigmasterol	28.256	1.21	28.395	1.23	28.294	1.22
β-Sitosterol	30.782	1.32	31.043	1.34	30.939	1.33
-	-	-	-	-	31.678[b]	1.36
-	-	-	-	-	32.692[b]	1.40
Lupeol	35.145	1.50	34.608	1.50	35.138	1.51
-	-	-	-	-	45.096[b]	1.94

[a] RRT was calculated on the basis of the Rt of the cholesterol standard;
[b] later identified by GC-MS as isofucosterol, cycloartenol, and 24-methylene cycloartanol. - not detected.

Table 2. GC-FID Result for the Acetone Extracts of F and CGS Callus Cultures

Standards	Rt std (min)	RRT standard[a]	Rt Callus F (min)	RR Callus F[a]	Rt callus CGS (min)	RRT callus CGS[a]
Cholesterol	23.383	1.00	23.205	1.00	23.260	1.00
Campesterol	27.042	1.16	27.070	1.17	27.041	1.16
Stigmasterol	28.256	1.21	28.283	1.22	28.713	1.23
β-Sitosterol	30.782	1.32	30.855	1.33	30.841	1.32
Lupeol	35.145	1.50	35.577	1.53	35.032	1.51

[a] RRT was calculated on the basis of the Rt of the cholesterol standard

The acetone extract of the CGS callus was subjected to acetate derivatization to confirm the presence of betullinic acid further. n-Hexane and acetone-derivatized extracts were further subjected to GC–MS analysis. The total ion chromatograms (TICs) of the n-hexane and acetone extracts of the CGS callus exhibited 8 and 7 peaks, respectively (peak 1–15) (Figures 5 and 6). The EI–MS spectra of the extracts were compared with those from the Wiley database available in the GC–MS instrument, as well as those from other online databases, i.e., NIST, SDBS, and MassBank [14-16], to determine the identity of the peaks. The EI–MS spectra of compounds **1, 2, 3, 4,** and **7** were identical to those of cholesterol, campesterol, stigmasterol, β-sitosterol, and lupeol, standards, respectively. The EI–MS spectra of their acetate derivatives (**9–13**) were identical to published EI–MS spectra and EI–MS spectra from three online databases [14-24]. The important EI–MS fragments of compounds **1–15** and their molecular ions $[M]^+$ are presented in Tables 3 and 4. The chemical structures of sterols and triterpenes that were identified in this work are illustrated in Figure 7. Although compounds **5**, **6**, **8**, **14**, and **15** lacked standards, their EI–MS spectra were identical to those from the three databases and published data [14-24]. The Commission Decision 2002/657/E states that the identity of a compound can be confirmed if its MS spectrum showed at least four fragments that are identical to those in the MS spectrum of the standard [25]. Compounds **1–15** showed numerous fragments (>4 fragments) that were identical to the fragments of standards, online databases, and published data [14-24]. Thus, the identities of peaks **1–15** were unambiguously confirmed.

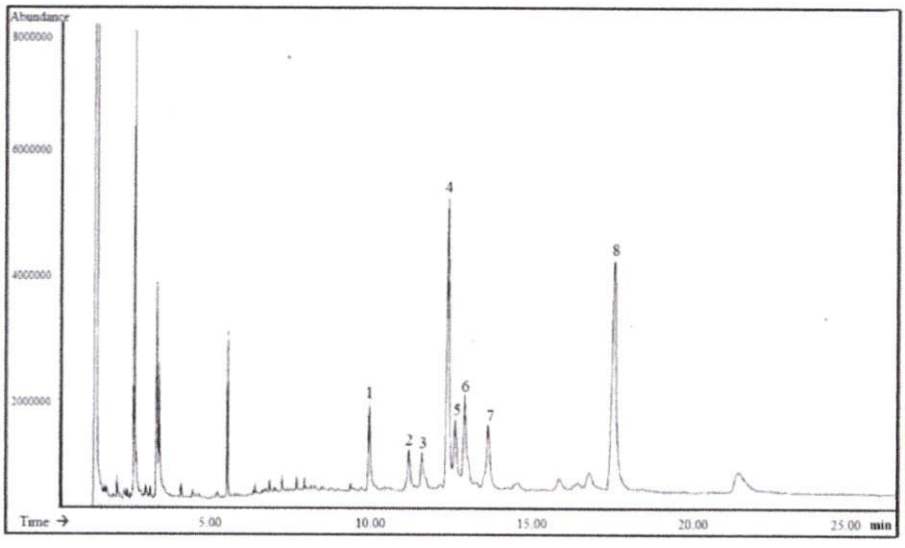


Figure 5. Total Ion Chromatogram of the N-Hexane Extract of The CGS Callus

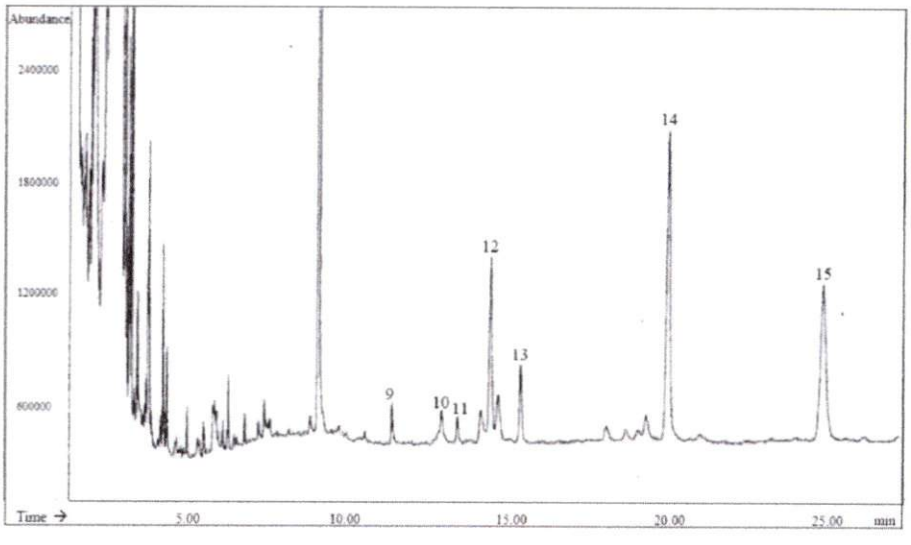


Figure 6. Total Ion Chromatogram of the Acetone Extract of the CGS Callus After Derivatization

Table 3. Molecular Ions and Fragments of the Identified Sterols in CGS Callus

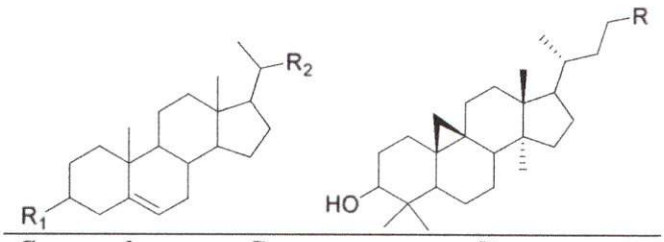
Molecular ion $[M]^+$ and fragment	Compound (*m/z*)								
	R = OH				R = CH_3COO				
	1	**2**	**3**	**4**	**5**	**9**	**10**	**11**	**12**
$[M]^+$	386	400	412	414	412	-	-	-	-
$[M\text{-}CH_3]^+$	371	385	397	399	-	-	-	-	-
$[M\text{-}R]^+$	368	382	394	396	-	368	382	394	396
$[M\text{-}CH_3\text{-}R]^+$	353	367	379	381	379	353	367	379	381
$[M\text{-}C_3H_5]^+$	-	-	369	-	-	-	-	-	-
$[M\text{-}C_3H_5\text{-}R]^+$	-	-	351	-	-	-	-	351	-
$[M\text{-}C_5H_7\text{-}R]^+$	301	315	327	329	-	-	-	-	-
$[M\text{-}C_7H_{14}]^+$	-	-	314	-	-	-	-	-	-
$[M\text{-}C_7H_9\text{-}R]^+$	275	289	301	303	-	275	-	-	-
$[M\text{-}C_8H_{12}\text{-}R]^+$	260	274	286	288	-	260	274	-	288
$[M\text{-}C_9H_{13}\text{-}R]^+$	247	261	273	-	-	-	-	-	275
$[M\text{-}C_6H_{11}\text{-}2CH_3\text{-}R]^+$	-	-	-	-	281	-	-	-	-
[M-Side chain]$^+$	273	273	273	273	273	-	-	-	-
[M-Side chain-2H]$^+$	-	-	271	-	271	-	-	-	-
[M-Side chain-R]$^+$	255	255	255	255	-	255	255	255	255
[M-Side chain-H_2O]$^+$	-	-	-	-	255	-	-	-	-
[M-Side chain-42]$^+$	231	231	231	231	231	-	-	-	-
[M-Side chain-42-R]$^+$	213	213	213	213	-	-	213	213	213
[M-Side chain-44]$^+$	-	-	-	-	229	-	-	-	-
[M-Side chain-42-H_2O]$^+$	-	-	-	-	213	-	-	-	-

1: Cholesterol; **2**: Campesterol; **3**: Strigmasterol; **4**: β-Sitosterol; **5**: Isofucosterol;
9: Cholesterol acetate; **10**: Campesterol acetate; **11**: Stigmasterol acetate; **12**: Sitosterol acetate.

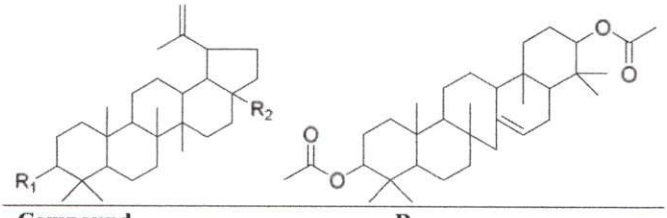
Table 4. Molecular Ions and Fragments of the Identified Triterpenes in CGS Callus

Molecular ion $[M]^+$ and fragment	Compound (m/z)					
	R = OH			R = CH_3COO^-		
	6	7	8	13	14	15
$[M+H]^+$	427	-	441	-	-	-
$[M]^+$	426	426	440	468	-	526
$[M\text{-}CH_3]^+$	411	411	425	453	483	-
$[M\text{-}R]^+$	-	-	422	408	438	466
$[M\text{-}CH_3\text{-}R]^+$	393	393	407	393	423	451
$[M\text{-}3CH_3\text{-}R]^+$	-	-	-	-	-	423
$[M\text{-}2R]^+$	-	-	-	-	-	406
$[M\text{-}2R\text{-}CH_3]^+$	-	-	-	-	-	391
$[M\text{-}C_3H_7]^+$	-	-	397	-	-	-
$[M\text{-}C_3H_7\text{-}R]^+$	-	-	379	-	-	-
$[M\text{-}COOH]^+$	-	-	-	-	453	-
$[M\text{-}HCOOH]^+$	-	-	-	-	452	-
$[M\text{-}HCOOH\text{-}CH_3]^+$	-	-	-	-	395	-
$[M\text{-ring C,D,E}^1]^+$	-	-	-	-	262	-
$[M\text{-ring C,D,E}^2]^+$	-	-	-	-	249	249
[M-ring A,B,C]$^+$	-	-	-	-	248	-
[M-ring A,B,C-COOH]$^+$	-	-	-	-	203	-
[M-ring C,D,E-CH_3COO^-]$^+$	-	-	-	-	189	-
[M-Side chain-CH3]	-	370	-	-	-	-
[M-Cyclopropane ring cleavage]$^+$	286	-	300	-	-	-
[M-Cyclopropane ring cleavage-CH_3]$^+$	271	-	285	-	-	-
[M-Cyclopropane ring cleavage-C_7H_{14}]$^+$	-	-	203	-	-	-
[M-Side chain-ring E-CH_3]$^+$	-	329	-	-	-	371
[M-ring C,D,E]$^+$	-	207	-	-	-	249

6: Cycloartenol; **7**: Lupeol; **8**: 24-methylene cycloartanol; **13**: Lupeol acetate;
14: Acetyl betulinic acid; **15**: Serratenediol diacetate.



Compound	R_1	R_2
1	OH	
9	CH_3COO-	
2	OH	
10	CH_3COO-	
3	OH	
11	CH_3COO-	
4	OH	
12	CH_3COO-	
5	OH	



Compound	R
6	
8	

Compound	R_1	R_2
7	OH	CH_3
13	CH_3COO–	CH_3
14	CH_3COO–	COOH

Figure 7. Structures of Identified Sterols and Triterpenes

Conclusion

The sterol profile of the CGS culture was more diverse than that of the normal callus culture. The production of several triterpenes, including betullinic acid, by the CGS culture but not by the normal callus culture could be attributed to differentiation by the CGS culture. This is the first report of betullinic acid from the in vitro callus culture of *S. mammosum*.

Acknowledgments

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