Sterols and Triterpenes in Cell Culture of *Hyssopus officinalis* L.

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Cell suspension cultures from hypocotyl-derived callus of *Hyssopus officinalis* were found to produce two sterols *i.e.* β-sitosterol (**1**) and stigmasterol (**2**), as well as several known pentacyclic triterpenes with an oleanene and ursene skeleton. The triterpenes were identified as oleanolic acid (**3**), ursolic acid (**4**), 2α, 3β-dihydroxyolean-12-en-28-oic acid (**5**), 2α, 3β-dihydroxyurs-12-en-28-oic acid (**6**), 2α, 3β, 24-trihydroxyolean-12-en-28-oic acid (**7**), and 2α,3β,24-trihydroxyurs-12-en-28-oic acid (**8**). Compounds **5**–**8** were isolated as their acetates (**6**, **8**) or bromolactone acetates(**5**, **7**)

Key words: Hyssopus officinalis, Cell Culture, Triterpenes, Sterols

Introduction

Hyssopus officinalis is an important medicinal plant of the family Lamiaceae originating from the region of South Europe and Asia Minor. As in other members of the family, the plant produces essential oil in its aerial parts (Tsankova and Konaktchiev, 1993). Only limited information is available on investigation of other bioactive constituents in the plant (Gollapudi *et al.*, 1995; Kochan *et al.*, 1998). We have established cell cultures of *H. officinalis* in order to study their ability to biosynthesize secondary metabolites. Analysis of dichloromethane extracts of cultured cells by TLC revealed the presence of sterols and triterpenes. We report here on the isolation and characterization of these compounds. Triterpenoids are one of the most abundant class of compounds in plants. It has frequently been suggested that triterpenoids play a defensive role against pathogens and herbivores. They also have several interesting pharmacological activities that include antiinflammatory (Recio *et al.*, 1995), antimycobacterial (Cantrell *et al.*, 2001), antiviral (Ohigashi *et al.*, 1986) and cytotoxic (Rios *et al.*, 2001) properties. For example, ursolic acid showed cytotoxic activity against lymphatic leukemia cells P-388 and L-1210 as well as human lung carcinoma cells A-549 (Macato and Sucharita, 1997). Ursolic acid is also referred to as being a strong inhibitor of tumor promotion in mouse skin (Tokuda *et al.*, 1986). Liu (1995) showed that both ursolic acid and oleanolic acid have antihyperlipidemic properties and were shown to be effective in protecting against chemically induced liver injury in laboratory animals.

Experimental

Plant material

Cell culture of *Hyssopus officinalis* was initiated from hypocotyl-derived callus (Grabias *et al.*, 1997). The culture was grown in a 300 cm^3 Erlenmeyer flask with 80 cm^3 B-5 medium (Gamborg *et al.*, 1968), supplemented with 1 mg/dm^3 α-naphthyl-1-acetic acid (NAA) and 0.2 mg/dm^3 6-benzylaminopurine (BAP) on the rotary shaker (100 rpm/min), at 26 ± 2 °C under continuous fluorescent light (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Subcultures were made every 21 days. The cell culture was maintained for two years before starting the experiments.

Instruments

Mass spectrometer: Finnigan MAT; NMR: Bruker AM 300; IR: ACT MATSAN. Chemical shift is given relative to tetramethylsilane (TMS) in δ-ppm, J-given in Hz. Column chromatography: Silica gel 60 F_{254} (70–230 mesh, Merck). Thin layer chromatography (TLC): Silica gel 60 F_{254} (Merck).

Detection: vanillin – H_2SO_4 reagent (1 g vanillin, 75 ml methanol, 15 ml acetic acid, 5 ml sulfuric acid). Spots were detected after heating to 120 °C for 2–3 min.

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GC-MS analysis condition: the sample (*ca.* 10 mg) was silylic with 1 ml *N,O*-bis – (trimethylsilyl) – trifluoroacetamide (BSTFA, Fluka). The solution was left to react at 90 °C for 20 min and then 2 ml of hexane was added. The analysis of TMS-derivatives was performed on HP-5890II (Hewlett-Packard) gas chromatograph equipped with a mass-selective detector HP-MBS 5972 (70 eV) and glass column HP-5MS (length 30 m, I. D. 0.25 mm, 0.25 μm film thickness). Helium was used as a carrier gas. Initial oven temperature was 100 °C and kept for 4 min, then a temperature programme of 10 °C/min was employed to 270 °C and held at 270 °C for 49 min; volume injected: 1 μl.

Extraction and isolation procedure

Dried at room temperature cells (76 g) were successively extracted with dichloromethane, dichloromethane-methanol 1:1 v/v and methanol using a Soxhlet apparatus. Each extraction was done with 700 ml of solvent for 8h. The resultant extracts were combined and concentrated *in vacuo* to yield 12.5 g of yellow-brown oil. The oil was dissolved in methanol (100 ml) and water (100 ml). The methanolic-aqueous solution was repeatedly extracted with chloroform (5 × 300 ml). The organic solvent was evaporated *in vacuo* to afford 3.8 g of light-yellow oil which was subjected to column chromatography (80 cm × 2.5 cm) on silica gel (250 g) by gradient elution with toluene-ethyl acetate (9:1 → 1:4 v/v). Fractions were analyzed by TLC (solvent system: toluene-ethyl acetate 3:7 v/v) and these gave rise to coloured spots (violet, blue) appearing after spraying with vanillin-H_2SO_4 reagent were collected and concentrated *in vacuo.* Thus, five fractions (F I–F V) containing sterols and triterpenes were obtained: F I (500 mg) eluted with toluene-ethyl acetate (4:1 v/v); F II (300 mg) eluted with toluene-ethyl acetate (7:3 v/v); F III (200 mg) toluene-ethyl acetate (3:2 v/v); F IV (500 mg) toluene-ethyl acetate (1:1 v/v); F V (410 mg) toluene-ethyl acetate (3:7 v/v).

Identification of compounds **1**–**8**

Three fractions (I–III) were subjected to GC and GC-MS analysis. In these fractions, by comparing the GC retention times and mass spectra with those of TMS-derivatives of respective standards, two sterols β-sitosterol (**1**), stigmasterol (**2**) and two triterpenes, oleanolic acid (**3**), ursolic acid (**4**) were found. The retention times (min) for β-sitosterol-TMS, stigmasterol-TMS, oleanolic acid-TMS, ursolic acid-TMS were: 37.00, 39.11, 51.87, 54.62, respectively. Fractions IV and V were mixtures of pentacyclic triterpenes as shown by ^{1}H-NMR spectrum. To separate them each of the fractions (IV and V) were successively bromined (with bromine in acetic acid) and acetylated (with anhydride acetic acid in pyridine) (Lewis and Tucker, 1983) and 200 mg of product *A* from fraction IV and 250 mg of product *B* from fraction V was obtained. Product *A* (200 mg) was further fractionated on silica gel (120 g) column (80 cm × 2.5 cm) using step gradient elution (toluene-ethyl acetate 9:1 → 4:1 v/v) to give: **5a** (13 mg) and **6a** (28 mg). Separation and purification of product *B* (250 mg) using the same column and conditions yielded: **7a** (11.6 mg) and **8a** (5.4 mg).

Olean 2α,3β-diacetyl-12α-bromo-13,28-lactone (**5a**) ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, s, 30-H); 0.90 (6H, s, 29-H and 26-H), 1.00 (3H, s, 23-H), 1.03 (3H, s, 24-H), 1.21 (3H, s, 25-H), 1.44 (3H, s, 27-H), 2.32 (1H, d, J = 10.8, 18-H), 4.28 (1H, t, J = 3.5, 12-H), 4.78 (1H, d, J = 10.4, 3-H), 5.11 (1H, ddd, J = 4.8, 10.5, 11.3, 2-H), 2.06 and 2.00 (2 × 3H, s, 2 × CH_3CO)

CI-MS *m/z* (rel. int.) 637.3 (18.9) $[M+2]^+$, 636.3 (7.1) $[M+1]^+$, 635.3 (18.26) $[M^+]$, 575.0 (24.8) $[M-CH_3COOH]^+$, 515.2 (97.6) $[M-2 \times CH_3COOH]^+$, 435.3 (58.3) $[M-2\times(CH_3COOH)-Br]^+$, 247.2 (10.2), 203 (12.3). IR ν_{max}^{KBr} cm^{-1}: 2960.7; 1773.2, 1745.4, 1370.8, 1233.9, 1046.4.

2α,3β-diacetyl-urs-12-en-28-oic acid (**6a**) ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, s, 26-H), 0.86 (3H, d, J = 6.1, 29-H), 0.89 (3H, s, 23-H), 0.90 (3H, s, 24-H), 0.94 (3H, d, J = 6.1, 30-H), 1.07 (6H, s, 25-H and 27-H), 2.18 (1H, d, J = 10.7, 18-H), 4.75 (1H, d, J = 10.3, 3-H), 5.10 (1H, ddd, J = 4.5, 10.5, 11.3, 2-H), 5.23 (1H like t, 12-H) CI-MS *m/z* (rel. int.) 555.8 (38.2) $[M]^+$, 495.8 (92) $[M-CH_3COOH]^+$, 435.8 (40.2) $[M-2 \times CH_3COOH]^+$, 248.2 (10.1), 203 (11.3).

Olean 2α,3β,24-triacetyl-12α-bromo-13,28-lactone (**7a**) ^{1}H-MNR($CDCl_3$) δ: 0.91 (3H, s, 30-H), 1.00 (3H, s, 29-H), 1,04 (3H, s, 26-H), 1.05 (3H, s, 23-H), 1.22 (3H, s, 25-H), 1.44 (3H, s, 27-H), 2.31 (1H, d, J = 9.8, 18-H), 4.20 (3H, s, 24-H), 4.30 (1H, t, J = 3.6, 12-H), 4.84 (1H, d, J = 10.7, 3-H), 5.18 (1H, ddd, J = 4.8, 10.7, 11.3, 2-H), 1.09, 2.06 and 2.07 (3 × 3H, s, 3 × CH_3CO). CI-MS *m/z* (rel. int.) 695.8 (34.06)

$[M+2]^+$, 693.8 (34.06) $[M]^+$, 633.7 (65.7) $[M-CH_3COOH]^+$, 573.6 (26.8) $[M-2 \times CH_3COOH]^+$, 513.6 (39.12) $[M-3 \times CH_3COOH]^+$, 433.6 (48.69) $[M-3 \times (CH_3COOH)\text{-}Br]^+$, 247.4 (12.69), 203.4 (16.0). IR ν_{max}^{KBr} cm^{-1}: 2960.7, 1773.2, 1745.4, 1465.8, 1373.8, 1233.9, 1132.8, 1046.4.

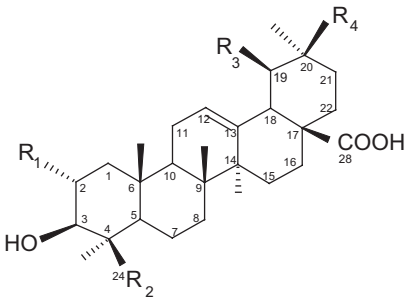
2α,3β,24-triacetyl-urs-12-en-28-oic acid (**8a**) ^{1}H-NMR($CDCl_3$) δ: 0.70 (3H, s, 26-H), 0.85 (3H, d, *J* = 6.2, 29-H), 0.94 (3H, d, *J* = 6.4, 30-H), 1.03 (3H, s, 27-H); 1.08 (3H, s, 25-H), 1.23 (3H, s, 23-H), 4.20 (3H, s, 24-H), 2.18 (1H, d, *J* = 10.7, H-18), 4.84 (1H, d, *J* = 10.7, H-3), 5.16 (1H, ddd, *J* = 3.40, 10.9, 11.9, 2-H), 5.25 (1H, like t, 12-H). CI-MS *m/z* (rel. int.) 615.4 (41.1) $[M]^+$, 555.4 (100) $[M-CH_3COOH]^+$, 495.3 (46.5) $[M-2 \times CH_3COOH]^+$, 435.3 (46.5) $[M-3 \times CH_3COOH]^+$, 248.2 (10.1), 203.0 (11.8).

Results and Discussion

The chloroform-soluble part of the dichloromethane-ethyl acetate extract of *Hyssopus officinalis* cells was separated by a column chromatography on silica gel by gradient elution with toluene-ethyl acetate mixture (8:2 → 3:7 v/v). This afforded five main fractions (I–V). The GC-MS examination of fractions I–III showed the presence of β-sitosterol (**1**), stigmasterol (**2**), oleanolic acid (**3**) and ursolic acid (**4**). The fractions IV and V showed a single spot on TLC plates with R_f value 0.31 and 0.20, respectively, in a solvent system of toluene-ethyl acetate (3:7 v/v). However, a more detailed examination (^{1}H-NMR analysis) of fractions IV and V suggested that they were mixtures and contained isomeric pairs of hydroxysubstituted olean-12-en-28-oic and urs-12-en-28-oic acids (compounds **5**–**8**). Because of the difficulty involved in separating these triterpenes by chromatographic methods, both fractions (IV and V) were treated with bromine in acetic acid. According to Lewis and Tucker (1983), in this way from each fraction mixture of unchanged derivatives of ursolic acid and bromo-lactone of oleanolic acid derivatives were obtained. The mixture was then acetylated and subjected to silica gel column chromatography. As a consequence, from fraction IV, two compounds **5a** and **6a** were isolated. Fraction V afforded compounds **7a** and **8a**. Identification of the compounds was performed by IR, CI-MS and ^{1}H-NMR spectroscopy.

The ^{1}H-NMR spectrum of **5a** showed the presence of signals of seven tertiary methyl groups (each as singlet between δ 0.85–1.44 ppm) indicating that **5a** belongs to the oleanene series of triterpenes. Two singlets of acetyl groups at 2.00 ppm and 2.06 ppm and two signals at 5.11 ppm (ddd *J* = 4.8, 10.5, 11.3) and 4.78 (d, *J* = 10.4) were attributed to protons for C-2 and C-3, respectively. The acetyl groups were assigned as being α- and β-oriented judging from the distance $\Delta\delta_{2-3}$ = 0.33 ppm between the chemical shifts of H-2 and H-3 protons and the values of the coupling constants *J* = 10.4 Hz for H-3 proton (Kojima and Ogura, 1989). The upfield shifts about 1 ppm of the proton signals attached to C-12 atom (δ 4.28 ppm) compared with olefin proton of oleanene and ursene-type triterpenes (δ 5.23 ppm) suggest the presence of bromine atom at this position. The intensity of peak M + 2 (*m/e* 637.3, 18.90%) in comparison with intensity of molecular peak M^+ (*m/e* 635.3, 18.26%) confirms the occurrence of one bromine atom in the molecule of **5a**. The IR of the **5a** spectrum indicates the intensity band of carbonyl group at the region 1745.4 cm^{-1} and 1773.2 cm^{-1}. It leads to the conclusion that the γ-lactone ring between C-13 and C-28 atoms is present in the structure of **5a**. The data shows that compound **5a** obtained from **5** by treatment with bromine in acetic acid and then the anhydride acid in pyridine is olean-2α,2β-diacetyl-12α-bromo-13,28-lactone, while compound **6a** is identified as a derivative of ursolic acid namely, 2α,3β-diacetylo-urs-12-en-28-oic acid.

Fraction V, after bromine treatment and acetylation reaction, affords compounds **7a** and **8a**. The ^{1}H-NMR spectrum of **7a** was very similar to that of **5a** and **8a** to that of **6a**. The only difference was the presence of an additional acetyl group linked to C-24 atom in both compounds **7a** and **8a**. The location of the acetyl group at the C-24 atom was deduced from the fact that the signal of the methylene protons at C-24 was downfield to δ 4.20 ppm region. The difference between chemical shifts of H-2 and H-3 protons gave the value Δδ 0.32 ppm. The spectral data showed further evidence that the compounds **7a** and **8a** have the same configurations as compounds **5a** and **6**a, *i. e.* α-oriented acetyl group at position C-2 and β-oriented acetyl group at position C-3. From the above, **7a** is identified as olean-2α,3β,24-diacetylo-12α-bromo-13,28-lactone, and **8a** as 2α,3β,24-triacetylo-urs-12-en-28-oic acid.



	R_1	R_2	R_3	R_4
3	H	CH_3	H	CH_3
4	H	CH_3	CH_3	H
5	OH	CH_3	H	CH_3
6	OH	CH_3	CH_3	H
7	OH	CH_2OH	H	CH_3
8	OH	CH_2OH	CH_3	H

Fig. 1. Triterpenes isolated from cell culture of *Hyssops officinalis* L.

Finally, it can be concluded that cells of *H. officinalis* cultured in B-5 medium, synthesized two sterols *i. e.* a steroidal drug product β-sitosterol (**1**) and stigmasterol (**2**) as well as six pentacyclic triterpenes (**3**–**8**) (Fig. 1.). These triterpenes were identified as oleanolic acid (**3**), ursolic acid (**4**) and two pairs of their hydroxyderivatives having hydroxy groups at C-2, C-3 and C-24 *i. e.* 2α,3β-dihydroxyolean-12-en-28-oic acid (**5**), 2α,3β-dihydroxy-urs-12-en-28-oic acid (**6**), 2α,3β,24-trihydroxy-olean-12-en-28-oic acid (**7**), 2α,3β,24-trihydroxy-urs-12-en-28-oic acid (**8**). Triterpenes (**5**–**8**) are reported for the first time from *H. officinalis*, although they have been found in plants of different species of several families including Lamiaceae. For example compound **6** named maslinic acid was previously detected in *Prunella vulgaris* L. (Lamiaceae) (Kojima and Ogura, 1986; Kojima *et al.*, 1997), and together with compound **5** in callus tissue of *Tecona grandis* L. (Verbenaceae) (Marwanii *et al.*, 1997). The latter authors also found that the fractions from *T. grandis* callus tissue which contained these compounds (**5** and **6**), showed a strong antibacterial action against *Escherichia coli* and *Bacillus subtilis*. Compound **5** was also isolated from *Paeonia suffruticosa* callus tissue (Ikuta, 1996) while compounds **6** and **8** were reported as secondary metabolites of *Actinidia polygama* callus inducted from the fruit galls (Sashida *et al.*, 1992). Our preliminary studies show that the intact plant of *Hyssopus officinalis* biosynthesized the same triterpenes as cell suspension culture. On the other hand, sterols β-sitosterol (**1**) and stigmasterol (**2**) were not detected in the aerial parts of the plants (data not shown), although they were present in the cultured cells.

The results of our work indicate that hydroxylation at the C-2, C-3 and C-24 positions is a common feature of the ursene and oleanene triterpenes isolated from *H. officinalis* cells. It is possible that ursolic acid and oleanolic acid may be transformed into more oxygenated compounds after biosynthesis (Pras, 1994). However, many steps in biosynthetic pathways of the triterpenes remain to be explained. Future research with cell cultures of *H. officinalis* could be carried out in two directions. First, the cell culture could become a good model system for the study of biosynthesis of triterpenoids as well as their roles in plants. Second, these cells have a strong capability for hydroxylation and can also be available as a system for the hydroxylation of some useful chemicals.

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