นิพนธ์ต้นฉบับ

สารกลุ่มเทอร์ปินอยด์จากใบและเซลล์เพาะเลี้ยงโกฐจุฬาลำพา และการประยุกต์ใช้เทคโนโลยีชีวภาพเพื่อเพิ่มระดับดาวาโนน

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บทคัดย่อ

การเพาะเลี้ยงเซลล์ของโกฐจุฬาลำพาเริ่มต้นจากการชักนำให้เกิดเป็นแคลลัส โดยนำชิ้นส่วนของใบที่ปลอดเชื้อแล้ว มาวางบนอาหารเพาะเลี้ยงกึ่งแข็งชนิด MS ที่ประกอบด้วย 2,4-dichlorophenoxyacetic acid 1 มิลลิกรัมต่อลิตร และ kinetin 0.1 มิลลิกรัมต่อลิตร หลังจากนั้นจึงเปลี่ยนถ่ายแคลลัสที่สมบูรณ์ลงในอาหารเหลวชนิดเดียวกันแต่ปราศจากวุ้น เพื่อชักนำให้เกิดเป็นเซลล์เพาะเลี้ยงแขวนลอย เมื่อจำแนกสารกลุ่มเทอร์ปินอยด์ที่ได้จากเซลล์เพาะเลี้ยงของโกฐจุฬาลำพาโดยใช้วิธี Gas Chromatography/Mass Spectrometry และนำไปเปรียบเทียบกับสารเทอร์ปินอยด์ที่ได้จากต้นจริง พบว่าเซลล์เพาะเลี้ยงของโกฐจุฬาลำพาสามารถสร้างดาวาโนนเป็นสารเทอร์ปินอยด์หลักได้เหมือนกับต้นจริง แต่มีปริมาณน้อยมาก ดังนั้นจึงนำวิธีทางเทคโนโลยีชีวภาพมาใช้เพื่อเพิ่มความสามารถของเซลล์ในการสร้างสารดาวาโนน ได้แก่ การใช้ตาข่ายไนลอนเพื่อตรึงเซลล์ ร่วมไปกับการเติม geranyl acetate ความเข้มข้นต่างๆ เพื่อเป็นสารต้นของกระบวนการเปลี่ยนแปลงทางชีวภาพ รวมทั้งใช้หลอด Porapak Q เพื่อดูดซับดาวาโนนที่ถูกขับออกมาจากเซลล์ด้วย ระดับของดาวาโนนจึงสามารถวัดได้สูงขึ้นหลังจากใช้วิธีการเหล่านี้

กุญแจคำ

โกฐจุฬาลำพา, สารกลุ่มเทอร์ปินอยด์, ดาวาโนน, เทคโนโลยีชีวภาพ

Original Article

Terpenoid Constituents from Leaves and Cell Cultures of *Artemisia vulgaris* var. *indica* and Application of Biotechnological Techniques to Increase Davanone Level

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Abstract

Sterilised leaf explants of *Artemisia vulgaris* var. *indica* were placed on semisolid basal MS media, containing 1 mg/l of 2,4-dichlorophenoxyacetic acid and 0.1 mg/l of kinetin in order to initiate callus cultures. Cell suspension cultures were then initiated by subculturing healthy callus cultures in the same media (except without agar). Terpenoid constituents of the cell cultures were identified by Gas Chromatography/Mass Spectrometry and compared to leaves of intact plant. It was found that cell cultures could produce the same major terpenoid constituent as intact plants, named davanone, but its level recovered from cultures was very low. Some biotechnological techniques were used for improving cell capacity to produce davanone, these were nylon meshes for immobilising cells, along with various concentrations of geranyl acetate fed for the precursor of biotransformation process. Porapak Q tubing was also used to adsorb davanone excreted from cells. Increasing davanone level can be detected after using these techniques.

Key words

Artemisia vulgaris var. *indica*, Terpenoid constituents, Davanone, Biotechnological techniques

Introduction

Artemisia vulgaris L. var. *indica* Maxim. (syn. *A. dubia* Wall ex. Bess.) belongs to a fairly large genus within the family Asteraceae. It is an ethnomedicinal plant native to Europe and continental Asia which has been used for anthelmintic, asthma, scabies, skin rashes, head-ache and stomachache, homeostatic for nose bleeding and bleeding wound, antiseptic, and antipyretic (1). Essential oils from genus *Artemisia* are rich source of terpenoid constituents that are useful in perfumery and pharmaceutical industries, such as anthelmintic, antispasmodic, anti-inflammatory and antibacterial (2). Essential oil obtained from leaves of *A. vulgaris* var. *indica* contained davanone (Fig. 1) as major constituent. The compound is a sesquiterpene ketone compound that had been first characterized in 1968 by Sipma and Van der Wal (3), and total synthesis of (±)-davanone had been described in 1999 (4). It had revealed a strong dose-dependent antispasmodic action (5).

Many *Artemisia* spp. have been studied on cell cultures since 1990 (6-13). However, cell cultures of *A. vulgaris* var. *indica* has never been described. The purposes of this study were also to identify terpenoid constituents from *A. vulgaris* var. *indica* intact plant and cell cultures, and use some biotechnological techniques to increase the level of its major constituent, davanone.

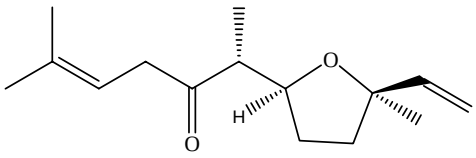

Figure 1. Davanone, the major constituent in *A. vulgaris* var. *indica*

Materials and Methods

Plant materials

Leaves of *A. vulgaris* var. *indica* were collected from the garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. These explants were identified by Dr. Nijsiri Ruangrungsi of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Hydrodistillation of leaves of **Artemisia vulgaris *var.* indica**

Fresh leaves of *A. vulgaris* var. *indica* were hydrodistilled by a Clevenger-type apparatus. The essential oil was collected and stored at 4 °C until being analysed for its chemical constituents by Gas Chromatography/Mass Spectrometry (GC/MS).

Plant cell cultures

Leaf explants of *A. vulgaris* var. *indica* were surface sterilized with surface sterilizing agent (sodium hypochlorite, streptomycin, tetracycline, and cis- N -[(trichloromethyl)thio]-4-cyclohexane-1,2-di-carboximide), followed by H_2O_2 7% (w/v) and 5% (w/v), containing Tween 80 as wetting agent, for 15 and 7 minutes, respectively. Callus cultures were initiated and maintained in MS basal media (14) containing sucrose (3% w/v), ascorbic acid (0.5% w/v), 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.1 mg/l), and (1%). The medium was adjusted to pH 5.75 before autoclaving. Callus cultures were subcultured every 4-6 weeks. Suspension cultures initiated from the fourth generation of callus were maintained in the same media as callus (except without the agar) 24 hr with continuous light at 25 ± 2 °C on an orbital shaker (120 rpm), and subcultured to new fresh liquid media with a 1:2 dilution every 15-21 days.

Extraction

Leaves of *A. vulgaris* var. *indica*, callus cultures, cell suspension cultures and immobilised cells, along with Porapak Q glass tube used for improving davanone content, were extracted with dichloromethane. The organic layer was evaporated under nitrogen to 100 μl and subjected to analysis by GC and GC/MS.

GC analysis

Shimadzu gas chromatograph with FID detector was used for gas chromatography analysis. The column was OV-1, high performance fused silica linked methyl silicon capillary column (30 m × 0.22 mm; 0.25 (m). Oven temperature programming was 50-200 (C at 5 (C/min. Injector and detector temperatures were 250 (C. Volume of injector was 1 (l, and carrier gas was nitrogen. 2-Hydroxy-4-methoxy-benzaldehyde, with the purity of 98%, was used as internal standard and the concentrations of samples were calculated with respect to internal standard. For quantitative analysis of davanone content, the peak area of davanone under GC chromatogram was calculated by using the internal normalisation methods.

GC/MS analysis

Varian Saturn III instrument was used for gas chromatography/mass spectrometry analysis. The column was DB-5 fused silica capillary column (30 m (0.25 mm i.d., 0.25 (m). Oven temperature programming was 60-240 (C at 3 (C/min. Injector and detector temperature were 240 (C. Volume of injector was 1 (l; split ratio was 100:1, and carrier gas was helium.

Identification was based on sample retention time data and electron impact mass spectral data compared to NIST library (15) and literature (16).

Methods for improving davanone content in cell suspension cultures

Healthy cell suspension cultures were selected for improving davanone content by these following techniques:

1. Cell immobilisation

Nylon meshes ($1 \times 1 \times 1$ cm^3), pore size 10 ppi, were transferred into new media, and cell suspension cultures were then added to the same media to enable formation of immobilisation. The immobilisation was successful after 21 days. The immobilised cells were then transferred to new media prior to use in biotransformation methods.

2. Precursor feeding and biotransformation

Four concentrations of geranyl acetate (5, 10, 50 and 100 ppm), with the purity being over 98%, were selected as precursor in this experiment. They were mixed with 70% (v/v) ethanol to get clear solution and feed to new liquid media by passing through pre-autoclaved Mobile Phase Filter (0.45 μm pore size, Whatman).

The immobilised cells, which were used in biotrans-formation method, were transferred to these liquid media containing different amount of geranyl acetate.

3. Use of adsorbent

Porapak Q (ethyl vinyl benzene-divinyl benzene copolymer) was selected as adsorbent in this experiment. It was packed into glass tube and washed with dichloromethane prior to use. This tube was fixed on top of the flask containing immobilised cells with various concentrations of geranyl acetate. Air was passed through these cells during cell growth via pre-autoclaved Mobile Phase Filter (0.45 μm pore size, Whatman).

Results

Terpenoid constituents obtained from hydrodistillation of* A. vulgaris *var.* indica *leaves

The yield of essential oil obtained from the leaves by hydrodistillation was 0.25% (v/w) of fresh weight. Its chemical constituents, identified by GC/MS as shown in Table 1, were mostly terpenoids. They were shown as six monoterpenes, seven oxygenated monoterpenes, four sesquiterpenes, and six oxygenated sesquiterpenes. Amongst these compounds, davanone appeared to be the major constituent (71.56%), followed by chrysanthenone (6.65%) and 9-*epi*-β-caryophyllene (3.80%).

Analyses

Table 2 showed davanone content obtained from dichloromethane extracts of intact plant compared with cultures before and after using biotechnological techniques for improving essential oil constituents, calculated by using the internal normalisation methods.

(1) Before using biotechnological techniques for improving davanone

Callus and cell suspension cultures were extracted and analysed by GC and GC/MS. Davanone was found to be the major constituent in both cultures. The davanone level found in cell suspension cultures was higher than in callus cultures (15.09 and 13.90 μg/g fresh weight, respectively). However, these levels were still lower than that of the intact plant.

(2) After using biotechnological techniques for improving davanone

After using biotechnological techniques, including cell immobilisation, feeding precursors and biotransformation, and use of adsorbent, davanone level has been improved successfully. It was improved from 13.90 μg/g fresh weight (FW) in callus cultures and 15.09 μg/g FW in cell suspension cultures to 18.02, 20.06, 56.73 and 52.17 μg/g FW in immobilised cell after feeding geranyl acetate 5, 10, 50 and 100 ppm, respectively. Porapak-Q tubing was used in all these experiments to adsorb davanone content.

Discussion

Plant cell cultures offer an alternative method for natural product production because they process the full set of genes necessary for all functions of a plant. However, having no special storage sites for them compared with their intact plants, the product accumulation in cell cultures is low. Some

Table 1. Essential constituents obtained from *A. vulgaris* var. *indica* leaves by hydrodistillation

Compound	% Yield
Monoterpenes	
santolina triene	0.15
sabinene	0.21
α-phellandrene	0.29
2-δ-carene	0.26
o-cymene	1.94
γ-terpinene	0.14
Oxygenated monoterpenes	
1,8-cineole	2.07
cis-chrysanthenol	0.46
chrysanthenone	6.65
4-terpineol	0.51
α-terpineol	0.31
cis-chrysanthenyl acetate	0.37
bornyl acetate	0.09
Sesquiterpenes	
α-copaene	t
9-*epi*-β-caryophyllene	3.80
α-humulene	0.98
germacrene	1.92
Oxygenated sesquiterpenes	
nordavanone	0.16
cis-threo-davanafuran	0.12
artedouglasia oxide	0.24
davanone	71.56
juniper camphor	1.63
α-bisabolol acetate	0.55
Others	
(*Z*)-3-hexenol	0.43
unidentified 1	1.00
3-octanone	0.21
3-octanol	0.27
unidentified 2	2.86
unidentified 3	0.43
unidentified 4	0.22

t = trace amount

Table 2. Davanone contents (μg/g fresh weight) obtained from dichloromethane extracts of intact *A. vulgaris* var. *indica* compared with cultures before and after using biotechnological techniques for improving essential oil constituents, calculated by using the internal normalization methods.

Dichloromethane extracts	Davanone (μg/g FW)
Intact plant	70.82
Callus cultures	13.90
Cell suspension cultures	15.09
Immobilised cells + 5 ppm geranyl acetate	18.02
Immobilised cells + 10 ppm geranyl acetate	20.26
Immobilised cells + 50 ppm geranyl acetate	56.73
Immobilised cells + 100 ppm geranyl acetate	52.17

biotechnological techniques e.g. cell immobilization, feeding of precursor and biotransformation have been used for improving the productivity of plant cell cultures. Cells are immobilised in matrix such as nylon mesh in order to increase cell-to-cell communication and degree of cell organisation, while precursor feeding aims to increase the level of its precursor in biosynthetic pathway. Moreover, Porapak Q is used to adsorb volatile constituents released from the cell cultures.

After using these biotechnological techniques described above with cell suspension cultures of *A. vulgaris* var. *indica*, the level of davanone, the major terpenoid constituent, has been improved successfully.

Since monoterpenes are known to be toxic to plant cells due to their hydrophobicity (17-19), high concentration of geranyl acetate may damage cells and cause cell death. Therefore, feeding geranyl acetate at the highest concentration (100 ppm) produced lower davanone content than when 50 ppm of the precursor was fed.

According to the data from this experiment, the use of these techniques successfully improved the production of essential oil from plant cell cultures. The prospect of producing useful secondary metabolites based on undifferentiated cell cultures is feasible, with possible large-scale production of volatile compounds.

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