HUE UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY
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INVESTIGATION OF THE PRODUCTION AND ACTIVITY DETERMINATION OF GYPENOSIDES FROM CELL CULTURES OF *Gynostemma pentaphyllum* (Thunb.) Makino

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1. National Library of Vietnam.
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LIST OF PUBLISHED WORKS

[1]. Hoang Tan Quang, Pham Thi Diem Thi, Dang Ngoc Sang, Tran Thi Ngoc Tram, Nguyen Duc Huy, Tran Quoc Dung, Quach Thi Thu The (2022) Effects of plant elicitors on growth and gypenosides biosynthesis in cell culture of *Giao co lam* (*Gynostemma pentaphyllum*). *Molecules* 27(9): 2972.

[2]. Nguyen Thanh Tung, Pham Thi Diem Thi, Dang Ngoc Sang, Do Thi Thao, Hoang Tan Quang (2022) Biomass accumulation of *Gynostemma pentaphyllum* (Thunb.) Makino in cell suspension cultures inhibiting human cancer cell growth. Research Journal of Biotechnology 17(3): 61-68.

[3]. Tung Nguyen-Thanh, Sang Dang-Ngoc, Dung Tran-Quoc, Quang Hoang-Tan (2023) Gypenosides production and spermatogenesis recovery potentials of extracts from cell suspension cultures of *Gynostemma pentaphyllum*. *Avicenna Journal of Medical Biotechnology* 15(4): 216-222. https://doi.org/10.18502/ajmb.v15i4.13491

[4]. Dang Ngoc Sang, Nguyen Quang Hoang Vu, Nguyen Thi Nguyen Man, Nguyen Huu Thuan Anh, Tran Quoc Dung, & Hoang Tan Quang. (2025). Isolation, characterization, and transcriptional analysis of the Cytochrome P450 (CYP89A2) gene from *Gynostemma pentaphyllum* (Thunb.) Makino. *Proceedings of the 3rd National Conference on Plant Physiology – 2025* (pp. 79–86).

[5]. Dang Ngoc Sang, Pham Thi Diem Thi, Tran Thi Thoa, Tran Quoc Dung & Hoang Tan Quang (2025). Biomass and gypenoside production from cell cultures of *Gynostemma pentaphyllum*. *Hue University Journal of Science: Agriculture and Rural Development*, 134(3A), 119–129. https://doi.org/10.26459/hueunijard.v134i3A.7717

INTRODUCTION

1. RATIONALE OF THE STUDY

Gynostemma pentaphyllum (Thunb.) Makino is a medicinal plant of the Cucurbitaceae family, widely distributed in East and Southeast Asia, and known for diverse pharmacological activities such as hepatoprotective, antitumor, and anti-inflammatory effects, as well as regulation of glucose and lipid metabolism. Its major bioactive constituents, gypenosides, are dammarane-type triterpenoid saponins derived from the isoprenoid pathway. Compared with *Panax ginseng*, *G. pentaphyllum* accumulates substantially higher levels of triterpenoid saponins and exhibits a faster growth rate, making it a promising and cost-effective alternative source of ginsenoside-like compounds.

To reduce dependence on wild resources, the development of in vitro production systems for *G. pentaphyllum* is necessary. Suspension cell culture, typically established from callus, is an effective platform for producing plant secondary metabolites. Moreover, elicitors are widely used to stimulate secondary metabolite biosynthesis and represent one of the most efficient strategies to enhance gypenoside accumulation. Therefore, elicitor-treated suspension cultures of *G. pentaphyllum* provide a sustainable and efficient approach for gypenoside production.

Based on the aforementioned practical considerations, we conducted the study entitled: ***"Investigation of the production and activity determination of gypenosides from cell cultures of Gynostemma pentaphyllum* (Thunb.) Makino".**

2. RESEARCH OBJECTIVES

Establish a procedure for the production and isolation of gypenosides from suspension cell cultures of *Gynostemma pentaphyllum* (Thunb.) Makino.

3. OBJECTS AND SCOPE OF THE STUDY

Research Subject: *Gynostemma pentaphyllum* (Thunb.) Makino.

Scope of the Study

Time frame: from March 2021 to September 2025.

Research subjects: suspension cell cultures producing gypenosides from *G. pentaphyllum*, cancer cell lines, and mouse testes.

Research locations: Institute of Biotechnology, Hue University; Hue University of Medicine and Pharmacy; Institute of Biotechnology, Vietnam Academy of Science and Technology.

4. RESEARCH CONTENTS

Content 1: Investigation of the effects of culture conditions on gypenoside accumulation in suspension cells.

Content 2: Investigation of the effects of elicitors on enhancing gypenoside production from suspension cultures.

Content 3: Investigation of the isolation process and bioactivity testing of gypenosides derived from suspension cells.

5. SCIENTIFIC AND PRACTICAL SIGNIFICANCE OF THE STUDY

Scientific significance:

The results of this study will provide new scientific evidence on the potential for gypenoside production through suspension cell cultures of *Gynostemma pentaphyllum* under elicitor treatment. It will also generate data on the transcriptional levels of genes involved in the gypenoside biosynthetic pathway in suspension cells, as well as the anticancer potential of gypenosides.

Practical significance:

The findings will serve as a valuable source of information and reference for the application of suspension cell culture techniques, gene expression analysis, and the evaluation of gypenoside bioactivity against cancer cell lines.

6. NOVEL CONTRIBUTIONS OF THE DISSERTATION

6.1. For the first time, a protocol for establishing suspension cell cultures of *Gynostemma pentaphyllum* has been developed. The culture conditions for *G. pentaphyllum* suspension cells in Erlenmeyer flasks consisted of liquid basal MS medium supplemented with 2.0 mg/L kinetin (KIN) and 0.5 mg/L indole-3-butyric acid (IBA), with an inoculation ratio of 3 g per 50 mL medium, maintained under shaking at 120 rpm.

6.2. For the first time, a protocol for gypenoside production through suspension cell culture with elicitor treatment has been established. The highest gypenoside content was recorded at 79.721 mg/g dry weight when treated with 100 µM salicylic acid (SA) after six days of culture, approximately twice the level found in natural samples. When combining the optimal elicitor concentrations of MeJA (50 µM), SA (100 µM), and YE (3 g/L), the maximum gypenoside content reached 103.810 mg/g dry weight.

6.3. The transcriptional levels of 12 genes involved in the gypenoside biosynthetic pathway in *Gynostemma pentaphyllum* suspension cells were evaluated under the influence of different elicitors. Among them, CYP86A8 increased 8.25-fold, CYP94A1 increased 10.01-fold, and CYP89A2 increased 11.14-fold compared with the control when treated with MeJA. The UGT73B4 gene exhibited the strongest transcriptional activation under SYM treatment, with a 15.35-fold increase relative to the control.

6.4. For the first time, the protective effects of *Gynostemma pentaphyllum* cell extract on mouse testicular cells have been reported. The extract derived from suspension cell cultures of *G. pentaphyllum* demonstrated the ability to protect testicular cells, enhance spermatogenesis, and increase testosterone levels in heat-stressed mice.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

Research subject: *Gynostemma pentaphyllum* (Thunb.) Makino.

Experimental materials:

- Suspension cells of *G. pentaphyllum*.
- Gypenosides derived from suspension cells.
- Cancer cell lines including hepatocellular carcinoma(HepG2, Huh7), lung carcinoma (A549), and leukemia (HL-60) cells.

2.2. METHODS

2.2.1. Callus culture

Callus was maintained on basal MS medium supplemented with 3% sucrose, 0.8% agar, 2.0 mg/L kinetin (KIN), and 0.5 mg/L indole-3-butyric acid (IBA), adjusted to pH 5.8.

2.2.2. Suspension cell culture

2.2.2.1. Establishment of growth curve and gypenoside accumulation

Suspension cells were cultured in 250 mL Erlenmeyer flasks containing 50 mL of liquid medium with the same composition as the optimized callus culture medium (without agar). Cell biomass was harvested every two days of culture to determine fresh weight and dry weight.

2.2.2.2. Determination of gypenoside content

Extraction of gypenosides: To determine the gypenoside content in samples, cells were dried at 50 °C to constant weight and extracted with 80% methanol using ultrasonic treatment at 40 °C.

Determination of gypenoside content: The total gypenoside content was quantified by the vanillin colorimetric method. The colorimetric reaction was measured using a spectrophotometer at 544 nm.

Determination of Rb1 content: HPLC analysis was carried out on an Alliance E2695 system equipped with a Symmetry® C18 column (4.6 mm × 250 mm × 5 μm). The mobile phase consisted of water and acetonitrile (34%) over a 20-minute run, with a column temperature of 30 °C, a flow rate of 0.8 mL/min, and an injection volume of 20 μL.

2.2.2.3. Effects of medium composition and culture conditions on cell growth

- Plant growth regulators: combinations of cytokinins (BAP and KIN) and auxin (IBA).
- Carbon sources: sucrose, glucose, fructose, galactose, lactose, and glycerol.
- Inoculum size: different initial inoculum sizes ranging from 4% to 8%.
- Shaking speed: various agitation rates (100, 120, and 150 rpm).

2.2.3.4. Bioreactor culture experiment

The LiFlux GX system (Hanil, Korea) was used with a working culture volume of 6 liters. The inoculation ratio was 10%

(v/v), and agitation was maintained at 150 rpm. All other parameters were set at default conditions.

2.2.3. Elicitor treatment

2.2.3.1. Preparation of elicitors

The elicitors used in this study included MeJA, SA, YE, and *Fusarium* (Fox) biomass.

2.2.3.2. Evaluation of elicitor concentration effects

The effects of elicitor concentrations were assessed by supplementing each elicitor individually into the medium at different concentrations at the beginning of the culture. Specifically, MeJA and SA were applied at concentrations ranging from 25 to 200 µM, while YE and Fox biomass were applied at concentrations from 1 to 5 g/L. The control treatment consisted of cultures without elicitor supplementation.

2.2.3.3. Evaluation of elicitor application timing

Elicitors were added to the medium at different culture times ranging from day 3 to day 15. The control treatment consisted of elicitor supplementation at the initial time of culture.

2.2.4. Characterization of selected CYP and UGT genes

2.2.4.1. Total DNA extraction and gene amplification

Total genomic DNA was extracted from in vitro leaf samples using the TopPURE Plant DNA Extraction Kit (ABT, Vietnam). PCR reactions were carried out using total DNA as the template and 12 primer pairs previously reported by Zhang et al. (2021).

2.2.4.2. Gene sequencing and phylogenetic tree construction

The nucleotide sequences of PCR products representing two groups of CYP and UGT genes were directly analyzed using the Sanger sequencing method.

2.2.4.3. Prediction of enzyme characteristics and functions

The structural characteristics and potential functions of representative CYP and UGT enzymes were predicted using bioinformatics tools.

2.2.5. Determination of transcriptional levels of CYP and UGT genes

2.2.5.1. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from cultured cells using the GeneJET Plant RNA Purification Kit (Thermo Scientific, USA). First-strand cDNA was synthesized using the First Strand cDNA Synthesis Kit (#K1612, Fermentas) according to the manufacturer's instructions.

2.2.5.2. Real-time PCR analysis

To evaluate the transcriptional levels of CYP and UGT genes, real-time PCR reactions were performed with gene-specific primers, using 18S rRNA as the internal reference (housekeeping gene).

2.2.6. Preparation of gypenoside extracts

2.2.6.1. Preparation of Gynostemma pentaphyllum crude extract

After extraction, the crude extract was evaporated to dryness and subsequently re-dissolved to the desired concentrations.

2.2.6.2. Purification of gypenosides

The extraction and purification procedure was performed according to Liang et al. (2014), with modifications adapted to laboratory conditions.

2.2.7. Evaluation of anticancer activity

2.2.7.1. Sulforhodamine B (SRB) assay

Cytotoxicity was analyzed using the sulforhodamine B staining method, as described by Vajrabhaya et al. (2018).

2.2.7.2. Tetrazolium (MTT) assay

The effect of *G. pentaphyllum* extracts on the growth and proliferation of HL-60 leukemia cells was evaluated using the MTT assay.

2.2.8. Evaluation of the protective effects on mouse testes

Mice were randomly assigned to experimental groups: a control group (10 mice) maintained at 25°C, a heat-stress group exposed to 40°C (H40, 10 mice), a heat-stress group administered natural *Gynostemma pentaphyllum* extract at 200 mg/kg (H40+N), and a heat-stress group administered *G. pentaphyllum* suspension cell extract at 200 mg/kg (H40+CS).

Heat stress treatment protocol

Heat stress was applied at 40 °C for 10 minutes, twice per day with a 10-minute interval, six days per week (with rest on Sundays) for a total of five consecutive weeks.

Determination of testosterone concentration

Plasma testosterone levels were measured using ELISA on an automated immunoassay analyzer (Cobas e411, ROCHE).

Hematoxylin and Eosin (H&E) staining

Histological evaluation was performed using the Hematoxylin and Eosin staining method, following the technical guidelines for histopathology and cytology issued by the Ministry of Health.

2.2.9. Statistical analysis

The culture experiments were arranged in a completely randomized design, with each experiment repeated three times. Experimental results were expressed as mean values and analyzed using ANOVA followed by Duncan's test at $p < 0.05$, performed with SPSS software version 20.0./.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. EFFECTS OF CULTURE CONDITIONS ON GYPENOSIDE ACCUMULATION IN SUSPENSION CELLS

3.1.1. Establishment of the growth curve and gypenoside accumulation

From the beginning of culture until day 10, cell growth was minimal, with biomass increasing only from 2 g to 2.280 g. Subsequently, cell biomass increased continuously from day 10 to day 20, reaching a maximum of 3.780 g fresh weight per flask, corresponding to 0.087 g dry weight per flask, with a growth index of 1.89 after 20 days of culture (Figure 3.1). After reaching this peak, the biomass declined steadily, and by day 24 only 3.010 g fresh weight (0.059 g dry weight) was obtained, with a growth index of 1.51.

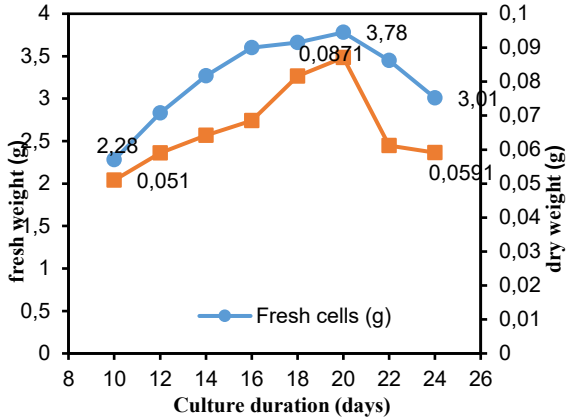

Figure 3.1. Growth curve of suspension cells

Compared with cell growth, gypenoside accumulation in *Gynostemma pentaphyllum* suspension cells reached its maximum 2–4 days earlier. The accumulation capacity gradually increased from day 10 to day 16, remained stable between days 16–18, and then declined from day 20 onwards. The highest gypenoside content obtained was 42.122 mg/g on day 18 (not significantly different from day 16) (Table 3.1). In contrast, the maximum Rb1 content was observed between days 18–20 (approximately 0.05 mg/g dry weight), which was about two days later than the peak of total gypenoside accumulation.

Table 3.1. Effects of culture duration on cell growth and gypenoside accumulation

Culture duration (days)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
10	28,291^{cd}	0,010^{d}
12	30,343^{c}	0,011^{d}
14	36,210^{b}	0,012^{d}
16	41,321^{a}	0,022^{c}
18	42,122^{a}	0,049^{a}
20	34,100^{c}	0,050^{a}
22	25,510^{d}	0,041^{a}
24	24,081^{d}	0,024^{c}

Based on the accumulation of fresh biomass, dry biomass, total gypenoside content, and Rb1 levels, day 18 was selected as the optimal harvesting time point, as it ensured both high biomass yield and elevated accumulation of the target compounds.

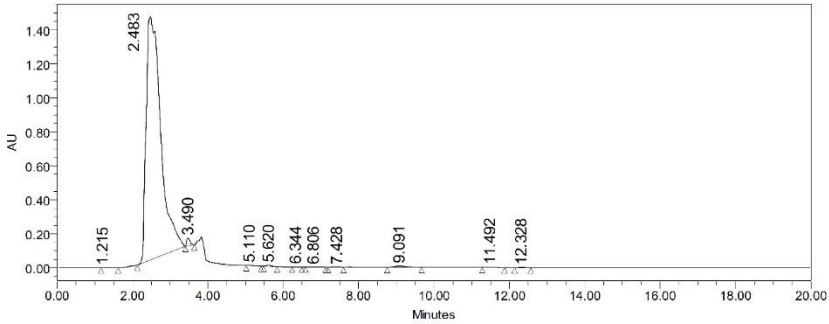

Figure 3.2. HPLC chromatogram of cell samples

Thus, the biosynthesis of gypenosides in general, and Rb1 in particular, occurred in the suspension cells of *Gynostemma pentaphyllum*. The levels of both total gypenosides and Rb1 in suspension cells were higher than those in callus, comparable to stem samples, but still lower than those in natural leaves.

3.1.2. Effects of culture medium on gypenoside accumulation in suspension cells

3.1.2.1. Effects of combinations of plant growth regulators

In terms of biomass production, two media yielded the highest biomass after 18 days of culture: the medium supplemented with 2.5 mg/L BAP combined with 0.5 mg/L IBA (3.501 g fresh weight per flask), and the medium containing 2 mg/L KIN combined with 0.5 mg/L IBA (3.693 g fresh weight per flask). Other media resulted in lower biomass yields.

Regarding gypenoside accumulation, media containing KIN and IBA were more effective than those containing BAP and IBA. Among them, KIN concentrations ranging from 1.5 to 2 mg/L resulted in the highest gypenoside contents (40–41 mg/g dry weight). The highest Rb1 content was obtained in the medium supplemented with 2 mg/L KIN and 0.5 mg/L IBA (0.041 mg/g dry weight).

Table 3.2. Effects of BAP or KIN in combination with 0.5 mg/L IBA on cell growth and gypenoside accumulation

BAP (mg/L)	KIN (mg/L)	Fresh weight (g/flask)	Dry weight (g/flask)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
1	-	3,203^{b}	0,149^{ab}	26,360^{d}	0,025^{d}
1,5	-	2,822^{c}	0,120^{c}	34,601^{b}	0,043^{b}
2	-	2,997^{bc}	0,138^{b}	34,932^{b}	0,049^{a}
2,5	-	3,501^{a}	0,161^{a}	35,850^{b}	0,048^{a}
3	-	2,732^{c}	0,113	25,053^{d}	0,019^{e}
-	1	2,573^{cd}	0,121^{c}	23,287^{d}	0,025^{d}
-	1,5	2,860^{c}	0,118^{c}	40,900^{a}	0,034^{c}
-	2	3,693^{a}	0,157^{a}	40,240^{a}	0,041^{b}
-	2,5	3,274^{b}	0,144^{ab}	31,733^{c}	0,032^{c}
-	3	2,369^{d}	0,095^{d}	24,190^{d}	0,013^{f}

3.1.2.2. Effects of carbon sources

The results presented in Table 3.3 show that sucrose remained the most effective carbon source for both cell growth (3.641 g fresh weight per flask) and gypenoside accumulation (39.845 mg gypenoside/g dry weight), followed by glucose. For all other tested carbon sources, both cell growth and gypenoside accumulation were less efficient.

Table 3.3. Effects of carbon sources on cell growth and gypenoside accumulation

Carbon source	Fresh weight (g/flask)	Dry weight (g/flask)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
Sucrose	3,641^{a}	0,157^{a}	39,845^{a}	0,037^{a}
Glucose	3,247^{b}	0,106^{b}	22,349^{d}	0,031^{b}
Fructose	2,383^{c}	0,095^{b}	27,862^{c}	0,030^{b}
Galactose	2,795^{c}	0,114^{b}	15,033^{e}	0,034^{b}
Lactose	2,311^{c}	0,091^{b}	31,397^{b}	0,034^{b}
Glycerol	2,471^{c}	0,094^{b}	29,905^{bc}	0,031^{b}

3.1.3.1. Effects of inoculation ratio

With an initial inoculum of 3 g, the culture process yielded the best results, with good cell growth (5.392 g fresh weight per flask) and

high gypenoside accumulation (46.498 mg/g dry weight).

Table 3.4. Effects of inoculum size on cell growth and gypenoside accumulation

Inoculum size (g)	Fresh weight (g/flask)	Dry weight (g/flask)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
2	3,661[b]	0,082[b]	42,876[b]	0,049[a]
3	5,392[a]	0,128[a]	46,498[a]	0,038[b]
4	5,430[a]	0,130[a]	34,520[c]	0,021[c]

3.1.3.2. Effects of shaking speed

In our experiments, the shaking speed of 120 rpm yielded the best results, while both higher and lower shaking speeds were less effective.

Table 3.5. Effects of shaking speed on cell growth and gypenoside accumulation

Shaking speed (rpm)	Fresh weight (g/flask)	Dry weight (g/flash)	Rb1 (mg/g dry)	Gypenoside (mg/g dry)
100	3,462[c]	0,066[b]	-	13,542[c]
120	5,430[a]	0,131[a]	0,039[a]	46,870[a]
150	4,521[b]	0,049[b]	0,022[b]	38,041[b]

3.1.4. Suspension cell culture in a bioreactor

During the first 10 days of culture, the fresh biomass of cells increased slowly, reaching only 38.860 g compared with the initial inoculum of approximately 37.221 g. After this initial adaptation phase, cell biomass increased rapidly in the following days, from 38.860 g to 45.611 g (corresponding to 1.341–1.792 g dry biomass), with the maximum biomass observed on day 18.

3.2. EFFECTS OF ELICITORS ON ENHANCING GYPENOSIDE PRODUCTION IN SUSPENSION CULTURES

3.2.1. Effects of elicitor concentrations on cell growth and gypenoside accumulation

The results presented in Table 3.7 show that all concentrations of MeJA inhibited cell growth, with dry biomass ranging from 0.063 to 0.118 g per flask (compared with 0.190 g per flask in the control). Relative to the control, gypenoside content increased when 25–100 μM MeJA was added, with the highest level observed at 50 μM (58.116 mg/g dry weight). In cells treated with SA, biomass accumulation decreased, but the highest gypenoside content was recorded at 100 μM SA (76.809 mg/g dry weight), approximately 1.52-fold higher than the untreated control.

The results presented in Table 3.8 demonstrate that YE was the most effective elicitor when added at the initial stage of culture. The highest gypenoside content was achieved when 3 g/L YE was supplemented into the medium (73.767 mg/g dry weight), approximately 1.46-fold higher than the control.

Table 3.6. Effects of MeJA and SA on cell growth and gypenoside accumulation

Concentration (mM)	Elicitor	Fresh weight (g)	Dry weight (g)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
0	-	5,732^{a}	0,190^{a}	50,545^{d}	0,048^{cde}
25	MeJA	5,043^{bcd}	0,165^{bc}	54,546^{cd}	0,052^{bcd}
	SA	5,763^{a}	0,204^{a}	58,475^{bc}	0,060^{bc}
50	MeJA	5,003^{bcd}	0,163^{bc}	58,116^{bcd}	0,053^{bcd}
	SA	5,456^{ab}	0,163^{bc}	63,980^{b}	0,062^{b}
100	MeJA	4,876^{bcde}	0,094^{de}	53,617^{cd}	0,041^{de}
	SA	5,187^{abc}	0,141^{c}	76,809^{a}	0,075^{a}
150	MeJA	4,854^{bcde}	0,088^{de}	32,552^{e}	0,037^{f}
	SA	4,659^{cde}	0,107^{d}	32,100^{e}	0,061^{b}
200	MeJA	4,472^{de}	0,063^{e}	29,514^{e}	0,037^{f}
	SA	4,331^{e}	0,026^{f}	9,724^{f}	0,056^{bc}

Table 3.7. Effects of YE and Fox on cell growth and gypenoside accumulation

Concentration (g/L)	Elicitor	Fresh weight (g)	Dry weight (g)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
0	-	5,433^{a}	0,171^{abc}	50,478^{de}	0,052^{b}
1	YE	5,499^{a}	0,170^{abc}	54,974^{d}	0,061^{a}
	Fox	4,597^{bc}	0,181^{a}	36,790^{f}	0,049^{bc}
2	YE	5,411^{a}	0,131^{de}	67,135^{b}	0,063^{a}
	Fox	4,885^{ab}	0,174^{ab}	34,924^{f}	0,050^{bc}
3	YE	5,410^{a}	0,137^{de}	73,767^{a}	0,066^{a}
	Fox	4,521^{bc}	0,170^{abc}	31,115^{fg}	0,043^{cd}
4	YE	5,424^{a}	0,123^{ef}	60,915^{c}	0,063^{a}
	Fox	4,076^{cd}	0,165^{abcd}	27,411^{gh}	0,040^{d}
5	YE	5,387^{a}	0,105^{f}	46,338^{e}	0,062^{a}
	Fox	3,931^{d}	0,142^{bcde}	23,066^{h}	0,039^{d}

3.2.2. Effects of elicitor application timing on cell growth and gypenoside accumulation

SA treatment was most effective when added to the medium on day 6, yielding a gypenoside content of 79.721 mg/g dry weight. This value was 1.58-fold higher than the untreated control, 1.4-fold higher than stem samples, and twice as high as natural leaf samples.

Table 3.8 . Effects of elicitor application timing on cell growth and gypenoside accumulation

Treatment timing	Elicitor	Fresh weight (g)	Dry weight (g)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
0	MeJA	4,968^{cde}	0,207^{fg}	57,852^{hi}	0,052^{g}
	SA	4,908^{cde}	0,148^{l}	76,480^{ab}	0,075^{c}
	YE	4,855^{e}	0,186^{i}	74,579^{bc}	0,061^{f}
3	MeJA	5,110^{bcde}	0,212^{ef}	65,633^{ef}	0,055^{ghi}
	SA	4,906^{de}	0,149^{l}	76,661^{ab}	0,088^{b}
	YE	4,980^{cde}	0,192^{h}	61,202^{gh}	0,056^{gh}
6	MeJA	5,168^{bcde}	0,216^{de}	73,461^{bc}	0,055^{ghi}
	SA	4,907^{bde}	0,157^{k}	79,721^{a}	0,093^{a}

	YE	5,041^{bcde}	0,204^{g}	57,543^{hi}	0,035^{k}
9	MeJA	5,302^{bc}	0,244^{b}	73,901^{bc}	0,066^{de}
	SA	5,025^{cde}	0,178^{j}	73,558^{bc}	0,063^{ef}
	YE	5,137^{bcde}	0,209^{f}	54,163^{ij}	0,031^{ij}
12	MeJA	5,254^{bcd}	0,229^{c}	68,622^{de}	0,067^{d}
	SA	5,259^{bcd}	0,211^{f}	70,547^{cd}	0,059^{fg}
	YE	5,424^{b}	0,221^{d}	51,321^{j}	0,027^{jk}
15	MeJA	5,018^{cde}	0,191^{hi}	60,814^{gh}	0,045^{j}
	SA	5,327^{bc}	0,221^{d}	62,628^{fg}	0,054^{ghi}
	YE	6,048^{a}	0,252^{a}	49,989^{j}	0,024^{k}

3.2.3. Effects of combined elicitors on cell growth and gypenoside accumulation

The optimal combination (including all three elicitors) resulted in a gypenoside content of 103.810 mg/g dry weight, which was approximately 2.22-fold higher than untreated cells and about 2.78-fold higher than natural samples.

Table 3.10. Effects of combined elicitors on cell growth and gypenoside accumulation

Elicitor	Fresh weight (g/flask)	Dry weight (g/flask)	Gypenoside (mg/g dry)	Rb1 (mg/g dry)
MeJA	4,958^{a}	0,172^{a}	73,114^{b}	0,055^{c}
SA	3,887^{b}	0,122^{bc}	79,830^{b}	0,087^{b}
YE	4,031^{b}	0,152^{b}	73,767^{b}	0,067^{bc}
MeJA + SA	3,475^{c}	0,106^{c}	79,487^{b}	0,075^{b}
MeJA + YE	4,623^{a}	0,197^{a}	77,554^{b}	0,079^{b}
SA + YE	4,275^{ab}	0,105^{c}	98,044^{a}	0,108^{a}
MeJA + SA + YE	4,633^{a}	0,187^{a}	103,810^{a}	0,127^{a}

HPLC overlay analysis of the obtained samples revealed that under SA treatment (green line), several peaks exhibited increased intensities, while other peaks remained comparable to those observed in different treatment conditions (Figure 3.12). The analysis also indicated that a total of 24 peaks were clearly detected across all samples, with MeJA treatment yielding the highest number of peaks

(21/24). Certain peaks, with retention times of 7.73; 8.49; 15.78; 17.03; 17.81; 38.45, and 40 minutes, were absent in the control but appeared or were enhanced in elicitor-treated samples. These compounds were either undetectable or below the detection limit in untreated cells and were induced upon elicitor application (Table 3.11).

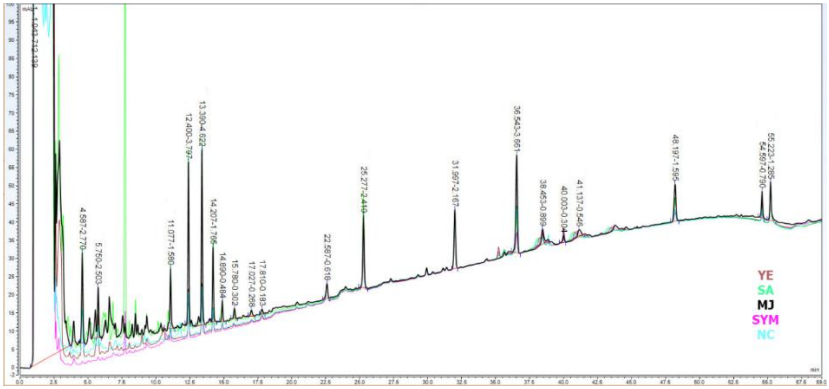

Figure 3.12. HPLC chromatogram of *Gynostemma pentaphyllum* suspension cells

3.2.4. Determination of *CYP* and *UGT* gene expression levels in suspension cells

3.2.4.1. Isolation and characterization of CYP and UGT genes

Screening with gene-specific primers for 12 genes described by Zhang et al. (2021) showed that 4 out of 7 CYP genes and 1 out of 5 UGT genes produced specific amplification products. Among them, CYP89A2 and UGT91A1 exhibited the strongest amplification. The PCR product of the CYP89A2 gene (CpQN) showed 99.17% sequence similarity to the CYP89A2 mRNA sequence of *Gynostemma pentaphyllum* (accession no. MT416753), whereas the UGT91A1-QN gene from *G. pentaphyllum* (Quang Nam) showed only 92.55% similarity compared with the reference sequence (accession no. MN128698).

3.2.4.2. Prediction of three-dimensional structure and physicochemical properties of enzymes

The prediction results indicated that the *CYP89A2* protein shared 26% structural similarity with sugiol synthase (*CYP76AH3*) from *Salvia miltiorrhiza*. The *UGT91A1-QN* enzyme showed 40% structural similarity with glycosyltransferase *OsUGT91C1* (apo form) from *Oryza sativa* subsp. *japonica* (Table 3.12).

Table 3.12. Predicted three-dimensional structure and physicochemical properties of enzymes

Characteristic	CYP89A2	UGT91A1-QN
Full-length gene (bp)	-	1.460
cDNA	1.521	1.383
Polypeptide chain	506 aa	460 aa
Molecular weight (kDa)	58,44	52,786
Isoelectric point (pI)	7,06	5,66
Tm	55°C~65°C	55°C~65°C
Acid/base	base	base
Domain	PRM, CBM_X, PX, PLCXc, HSF, GLUCA, BRICHOS, BACK, HPT, KNOX2, TPK_B1_binding, ClpB_D2_small và MoCF_biosynth	UDPGT (224-443)
α helix	15	16
β strain	8	15

3.2.4.3. Determination of transcriptional levels of CYP and UGT genes

For CYP genes in suspension cells, CYP71B19, CYP86A8, CYP89A2, and CYP94A1 were strongly transcribed, whereas the remaining genes were either not transcribed or expressed at very low levels. Among them, CYP71B19 exhibited the highest transcriptional activation under SYM treatment, showing a 6.14-fold increase compared with the untreated control. In contrast, other genes displayed substantial transcriptional induction in response to MeJA treatment: CYP86A8 increased 8.25-fold, CYP94A1 increased 10.01-fold, and CYP89A2 increased 11.14-fold (Table 3.13).

Regarding UGT genes in suspension cells, only UGT73B4 showed strong transcription, while the others were not transcribed (undetectable). UGT73B4 exhibited the highest transcriptional activation under SYM treatment, with a 15.35-fold increase compared with the untreated control (Table 3.14).

3.3. ISOLATION AND BIOACTIVITY ASSAY OF GYPENOSIDES FROM SUSPENSION CELLS

3.3.1. Preparation of extracts and gypenoside preparations

3.3.1.1. Preparation of extracts

The extracts were analyzed for gypenoside content using spectrophotometric methods and for Rb1 content using HPLC. The gypenoside content presented in Table 3.15 shows that the suspension cell extract contained 472.159 mg/g dry weight, which was higher than that of the natural extract at 369.489 mg/g dry weight. These extracts were subsequently used in further experiments to evaluate bioactivity.

3.3.1.2. Preparation of gypenoside preparations

To produce gypenoside preparations, *Gynostemma pentaphyllum* cells were cultured in 250 mL Erlenmeyer flasks containing 50 mL of culture medium (294 flasks, divided into 7 culture batches). A combined elicitor treatment consisting of MeJA (50 μM), SA (100 μM), and YE (3 g/L) was added on the 6th day of culture. After extraction of the dried biomass with 80% methanol, 11.13 g of crude extract was obtained. This extract was further purified to yield approximately 5.2 g of gypenosides, which were subsequently used to evaluate cytotoxic activity.

3.3.2. Cytotoxic activity assays against cancer cells

3.3.2.1. Cytotoxic activity of crude extracts

Effect of extracts on A549 cells

The *Gynostemma pentaphyllum* extracts exhibited dose-dependent inhibition of A549 cell proliferation. However, the inhibitory effect on A549 cells was not strong.

Effect of extracts on HepG2 and Huh7 cells

In human liver cancer cell lines, both suspension cell extracts and natural extracts inhibited the proliferation of HepG2 and Huh7

cells.

Effect of extracts on HL-60 cells

Both suspension cell extracts and natural extracts inhibited the proliferation of human leukemia HL-60 cells at the two highest concentrations, 100 and 200 µg/mL.

3.3.2.2. Cytotoxic activity of gypenosides

Effect of gypenosides on A549 cells

At the highest concentration tested (200 µg/mL), the inhibitory effect reached 83.37 ± 5.04% for the suspension cell sample and 33.76 ± 2.70% for the natural extract.

Effect of gypenosides on HepG2 and Huh7 cells

At 200 µg/mL, the natural extract showed maximal inhibitory activity (42.30 ± 3.37% for HepG2 and 42.17 ± 2.03% for Huh7), whereas the suspension cell extract exhibited significantly stronger inhibition (88.21 ± 4.87% for HepG2 and 63.11 ± 3.12% for Huh7).

Effect of gypenosides on HL-60 cells

Both the natural and suspension cell extracts inhibited the proliferation of human leukemia HL-60 cells at the highest tested concentration of 200 µg/mL, with a statistically significant difference between the two samples. The proliferation of HL-60 cells was markedly suppressed by *G. pentaphyllum* gypenosides at 200 µg/mL, reaching 37.80 ± 3.81% inhibition for the natural extract and 96.35 ± 3.48% for the suspension cell extract.

3.3.3. Evaluation of the protective effects of extracts on testicular cells

3.3.3.1. Effects of the extract on the improvement of spermatogenesis

The extract derived from suspension-cultured *Gynostemma pentaphyllum* cells significantly improved spermatogenesis occurring within the seminiferous tubules. The number of germ cells present in the lumen of the seminiferous tubules markedly increased (Figure 3.21d).

In the heat-treated mouse group, those administered the suspension-cultured *G. pentaphyllum* extract exhibited a serum testosterone concentration of 3.19 ± 2.65 ng/mL, which was higher

than that observed in the heat-treated group administered the natural plant extract (1.57 ± 0.89 ng/mL).

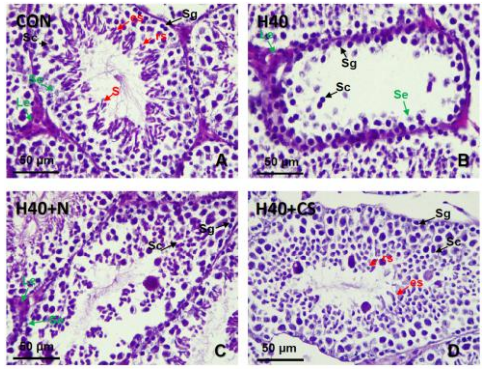

Figure 3.21. Effects of *Gynostemma pentaphyllum* extract on the microscopic structure of seminiferous tubules in mouse testicular tissue.

3.3.3.2. Effects of the extract on the improvement of blood testosterone levels

The extract of Gynostemma pentaphyllum restored the testosterone production capacity of heat-stressed mice. The extract derived from suspension cells demonstrated an improvement in testosterone levels in heat-stressed mice comparable to that of the natural plant extract.

CONCLUSION AND RECOMMENDATIONS

CONCLUSION

1. The dissertation successfully established a *Gynostemma pentaphyllum* cell suspension culture system at the shake-flask scale and conducted an initial scale-up to a bioreactor system. Under optimized culture conditions, the suspension cells exhibited a high capacity for gypenoside accumulation (approximately 46.9 mg/g dry weight in shake flasks and approximately 40.1 mg/g dry weight in the bioreactor), demonstrating that cell suspension culture represents a feasible approach for generating a sustainable and controllable biomass source for gypenoside production.

2. Both individual and combined elicitors were able to induce enhanced gypenoside accumulation in suspension-cultured cells. In particular, single SA treatment increased gypenoside content to approximately 80 mg/g dry weight, whereas the combined application of MeJA, SA, and YE resulted in the highest gypenoside level, exceeding 100 mg/g dry weight. These results indicate the presence of synergistic effects among elicitors in regulating gypenoside biosynthesis.

3. Elicitors have the capacity to regulate the expression of numerous genes belonging to the *CYP* and *UGT* families in cell suspensions. Several *CYP* and *UGT* genes exhibited significant transcriptional upregulation, exceeding a 10-fold increase compared to the control, notably *CYP89A2* (11.14-fold), *CYP94A1* (10.01-fold), and *UGT73B4* (15.35-fold). These results demonstrate that the triterpenoid saponin biosynthetic pathway has been activated at the molecular level. The concurrent upregulation of genes involved in oxidation and glycosylation steps contributes to elucidating the mechanism underlying the observed increase in gypenoside accumulation.

4. The thesis established an optimized protocol for processing crude extracts through the removal of non-polar and hydrophobic components, yielding a fraction with high solubility in methanol and

DMSO, which is well-suited for HPLC analysis and subsequent bioactivity assays.

5. Gypenosides and crude extracts derived from *Gynostemma pentaphyllum* cell suspensions exhibited significant biological activities. Notably, gypenosides demonstrated inhibitory effects on the HepG2 liver cancer cell line ($IC_{50} \approx 86.7$ μg/mL). Furthermore, the crude extract exhibited protective effects on testicular cells, facilitated spermatogenesis, and increased testosterone levels in a heat-stressed mouse model.

RECOMMENDATIONS

Future research should focus on scaling up the cultivation process and optimizing gypenoside production within pilot-scale systems. This is essential to evaluate growth stability, active compound accumulation efficiency, and the overall feasibility of utilizing plant cell suspension culture as a viable platform for the industrial production of pharmaceutical raw materials.

The study aims to characterize the functional roles of *CYP* and *UGT* genes involved in the gypenoside biosynthetic pathway, integrating elicitation strategies with large-scale cultivation to enhance gypenoside production efficiency in *Gynostemma pentaphyllum* cell suspension cultures.