Biomass Accumulation of *Gynostemma Pentaphyllum* (Thunb.) Makino in Cell Suspension Cultures inhibiting Human Cancer Cell Growth

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Abstract

Gynostemma pentaphyllum (Thunb.) Makino *(GpM) is a medicinal plant in traditional medicine throughout Asia for the treatment of several diseases including cancer. GpM plant cell suspension cultures provide a time and cost effective well-controlled means promising a high-yielding biomass production of pharmaceutical compounds. The purpose of the current work is to investigate the effect of GpM cell suspension cultures on human cancer cell lines growth. The biomass was produced by cell suspension culture of GpM callus into 250 mL Erlenmeyer flask containing 50 mL of liquid medium culture. Gypenosides in GpM were confirmed by HPLC. Pharmacological activities of GpM extract were tested on human cancer cell line (HepG2, Huh7, A549 and HL-60) using Sulforhodamine B (SRB) cytotoxicity assay and Tetrazolium (MTT) assay.*

We successfully produced 5.485 ± 0.223 g GpM fresh biomass per 250 mL Erlenmeyer flask after 18 days culture. Total gypenosides and Rb1 in dry cell suspension were 48.844 ± 3.933 mg/g and 0.041 ± 0.004 mg/g. The crude extract from GpM cell suspension cultures exhibited significant cancerous cell growth inhibition in a dose dependent manner. From the MTT assay and SRB cytotoxicity assay, it is obvious that GpM cell suspension culture extract at 200 µg/mL significantly inhibited the growth of multiple human cancer cells including hepatoma cell lines (HepG2, Huh7), lung carcinoma cell line (A549) and leukemia cell line (HL-60). Anti-cancer cell proliferation properties of GpM cell suspension culture were significantly higher than those of natural plants' extracts. In this framework, GpM in cell suspension cultures was found to inhibit the proliferation of several human cancer cells. Biomass accumulation of GpM in cell suspension cultures may contribute to the development of efficient strategies for plant-derived anticancer compound production.

Keywords: Plant biomass production, cell suspension cultures, Gynostemma pentaphyllum, cancer cell, proliferation, inhibition.

Introduction

Gynostemma pentaphyllum (Thunb.) Makino (GpM) has been widely used in traditional medicine throughout Asia (China, Japan, Korea, Vietnam etc.) for the treatment of several diseases including hepatitis, diabetes and cardiovascular disease and cancer[13]. Recent reports have shown that GpM exhibits a variety of biological effects such as anti-inflammatory, antioxidative, lipid metabolism regulatory, antiproliferative, immunopotentiating, neuroprotective and anxiolytic activities[8]. In Vietnam, GpM has been used as a folk medicine to treat cough and chronic bronchitis and is distributed from the plains to mountainous areas at altitudes up to 2000 m[11].

The most pharmacologically active components in GpM is gypenosides, molecular formula $C_{55}H_{92}O_{24}$, which are well-known pharmacologically active components in *Panax ginseng*[3]. Gypenosides primarily exist as dammarane type-triterpene saponins or glycosides, responsible for the herbal pharmacological activities[8]. Gypenosides from GpM are same as ginsenoside Rb1 (Gypenoside III), Rc, Rb3, Rd (Gypenoside VIII), F2, Rg3, malonyl-Rb1 and malonyl-Rd from ginseng (*Panax* spp.)[19].

Cancer is among the leading cause of death globally. In 2018, the estimated number of new cases was 18.1 million and 9.6 million deaths were linked to cancer. In Vietnam, there were 164671 new cancer cases and 114 871 deaths in 2018[6,18]. Currently, herbal medicine-based therapies have unraveled anticancer effects without significant side effects[22].

GpM has recently been shown as a strong anticancer potential. Gypenosides in GpM played an important role in lung cancer cell cytotoxicity and could be used as potential cancer-preventive chemopreventive agents[23,29]. GpM water extraction and ethanol precipitation are effective supplementary agents for the treatment of hepatocellular carcinoma via improving immune responses of host organism[15]. GpM also has potential for oral cancer therapy. Gypenosides isolated from GpM induce oral cancer cell death, cell cycle arrest and apoptosis[16]. In colorectal cancer cells, GpM induced the changes of intracellular reactive oxygen species leading to apoptosis[31]. Anti-cancer activities of GpM have been proposed regarding multiple mechanisms including immunomodulating activities, inhibition of metastasis or glycolysis, cell cycle arrest and apoptosis[12].

Medicinal plant cell suspension cultures offer promise for high-yielding production of valuable secondary metabolites and could be a promising alternative "chemical factory"[32]. Plant cell culture in bioreactors provides a time and cost effective well-controlled means for large quantities of biomass production of pharmaceutical agents of industrial importance[25]. Up to date, numerous anticancer compounds in medicinal plant are produced through application of plant cell and tissue culture such as *Catharanthus roseus*, *Dioscorea doryphora*, *Ginkgo biloba*, *Arnebia euchroma* and *Coleus blumei*[2].

GpM suspension cells cultures are potential sources of biomass production of anti-cancer and other pharmacological agents. Biological activities of GpM suspension cells culture are not well studied. The purpose of this study is to investigate the human cancer cell lines growth inhibition of GpM cell suspension cultures.

Material and Methods

Plant biomass production: Cells suspension culture was conducted by transfer of 3 g of 30-day-old callus into 250 mL Erlenmeyer flask containing 50 mL of liquid medium culture. The medium consists of basic MS components[17] supplemented with 3% sucrose, 2.0 mg/L KIN and 0.5 mg/L IBA. Culture flasks were then incubated at 25±2ºC, shaking speed of 120 rpm[1]. The cell biomass was harvested after 18 days of culture for determination of the fresh and dry weights and gypenosides content.

Extraction preparation: Fresh cells biomass was filtered, washed with distilled water and weighed. The dry weight was determined by drying the fresh biomass at 50ºC until a constant weight was obtained. 10 g dried samples (cells biomass or natural leaf and stem of GpM) were extracted with 10 mL of 80% methanol assisted with ultrasonication for 30 mins, repeated for 3 times and evaporated at room temperature[28]. The residues were used for human cancer cytotoxicity assay.

Total gypenosides quantification: Total gypenosides concentration was estimated by colorimetric methods[7,9]. Ten milligram of gypenosides residue were dissolved in 5 mL of 80% methanol. Fifty ml of solution was transferred to another test tube and 0.25 mL of vanillin reagent (8% w/v) was added. The reaction solution was placed on ice and 2.5 mL of 72% (v/v) sulphuric acid was added, mixed and left for 3 min. The test tube was warmed to 60ºC for 10 min and then cooled in ice. Absorbance was measured at 544 nm using UV-Vis scanning spectrophotometer (Spectro UV-2650, Labomed, Inc.). Gypenosides content was calculated based on the ginsennoside Rb1 (Y0001347, CRS) standard curves.

Rb1 quantification: The residues were re-suspended in methanol to final concentration of apromaxitely 5 mg/mL and filtered (0.22 µm). Rb1 content was qualified by HPLC with Alliance E2695 system with C18 column (4.6 mm × 250 mm × 5 mm). The mobile phase consists of acetonitrile (34%) and distiller water (56%). HPLC procedure was conducted for 20 minutes with flow rate at 0.8 mL/min, injected volume of 20 µL and column temperature of 30°C. The signal was measured by PDA2998 detector at a wavelength of 203 nm[14]. Rb1 content was calculated based on the peak area of HPLC dendrogram compared with the standard Rb1.

Human cancer cell culture: Human cell line was a kind gift of Prof. Pezzuto J. M. (Long-Island University, New York, USA) and Prof. Maier J. (Milan University, Milan, Italy). The hepatocarcinoma cell lines (HepG2, Huh7) and lung carcinoma cell line (A549) were adherent cultured on T75 culture flasks (Nunc, Denmark) while human leukemia cell line (HL-60) was cultured in suspension. Cells were incubated in DMEM (Dulbecco s Modified Eagle Medium) or MEME (Minimum Essential Medium with Eagle salt) supplemented with 10% FBS (Fetal Bovine Serum), 100 units/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen Gibco) at 37^0C under 5% CO_2. The cells were sub-cultured upon reaching 80% confluence.

The cell viability was determined by trypan blue exclusion using a hemocytometer. Six thousand cells were seeded in 200 µL of medium per well of 96-well flat-bottom culture plates (Nunc, Denmark) and incubated for 24 h. Then, old medium was removed before treating cells with GpM extract at the concentration of 4, 20, 100 and 200 µg/mL for 72 hours. Another plate without sample treatment will be incubated for 30 minutes and served as day 0.

Sulforhodamine B (SRB) cytotoxicity assay: The sulforhodamine B (SRB) assay was performed as previously described[24]. After an incubation with GpM extract, cell monolayers of HepG2, Huh7 and A549 were fixed with trichloroacetic acid (10% wt/vol). The fixed cells were stained with sulforhodamine B for 30 min, then wash the cells repeatedly with acetic acid (1% vol/vol) to remove the unbound dye. The cell growth was assessed by dissolving the protein-bound dye in 10 mM Tris base solution and optical density specifically determination at 540 nm wavelength.

Tetrazolium (MTT) assay: Human leukemia HL-60 cells cytotoxicity and proliferation were assessed using the MTT chromophore[20]. Briefly, HL-60 cells suspension were seeded at a density of 4×10^4 cells/mL in 96 well tissue culture plates. Cells were treated with various concentrations of GpM for 24 h in culture medium. The tetrazolium salt MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37^0C in humidified 5% CO_2 incubator. Cell culture plates were centrifuged and carefully aspirated in supernatant culture medium.

The formazan crystals produced by cell metabolism were dissolved in DMSO and the absorbance (OD) values were determined at 570 nm using a microplate reader.

$$\% \text{ Inhibition } = 100\% - \frac{OD(treated\ cells) - OD(Day\ 0)}{OD(DMSO) - OD(Day\ 0)}$$

Statistical analysis: All experiments were repeated three times. Data were reported as mean ± standard deviation. To evaluate significant differences, the means were compared using a one-way analysis of variance (ANOVA) followed by Duncan's test. Differences were considered to be statistically significant if $P < 0.05$.

Results

Plant biomass production by cell suspension culture: GpM biomass was produced by cell suspension culture. 3 g of callus were cultured in 50 mL of liquid medium at shaking speed of 120 rpm for 18 days [1]. Biomass accumulation in the same condition was shown in table 1. In the suspension cells culture, the cells biomass reached 5.485 ± 0.223 g/flask corresponding of 0.155 ± 0.034 g dry biomass (Table 1 and fig. 1).

Bioactive compounds in cell suspension culture: In 50 mL GpM cells suspension culture, the accumulation of total gypenosides and Rb1 was 48.844 ± 3.933 mg/g and 0.041 ± 0.004 mg/g dry cells weight respectively. In methanol extraction, total gypenosides was 472.159 ± 59.098 mg/g dry weight, Rb1 content was 0.394 ± 0.021 mg/g dry weight and gypenosides occupied approximate 47% of methanol extraction.

In natural product of GpM including 50% of dry leaf and 50% of dry stem, the accumulation of total gypenosides was 37.270 ± 4.710 mg/g dry weight while Rb1 content was 0.072 ± 0.021 mg Rb1/g dry weight.

In methanol extraction, total gypenosides and Rb1 were 369.489 ± 25.317 mg/g and 0.697 ± 0.081 mg/g dry weight respectively. Gypenosides occupied approximate 37% of methanol extraction.

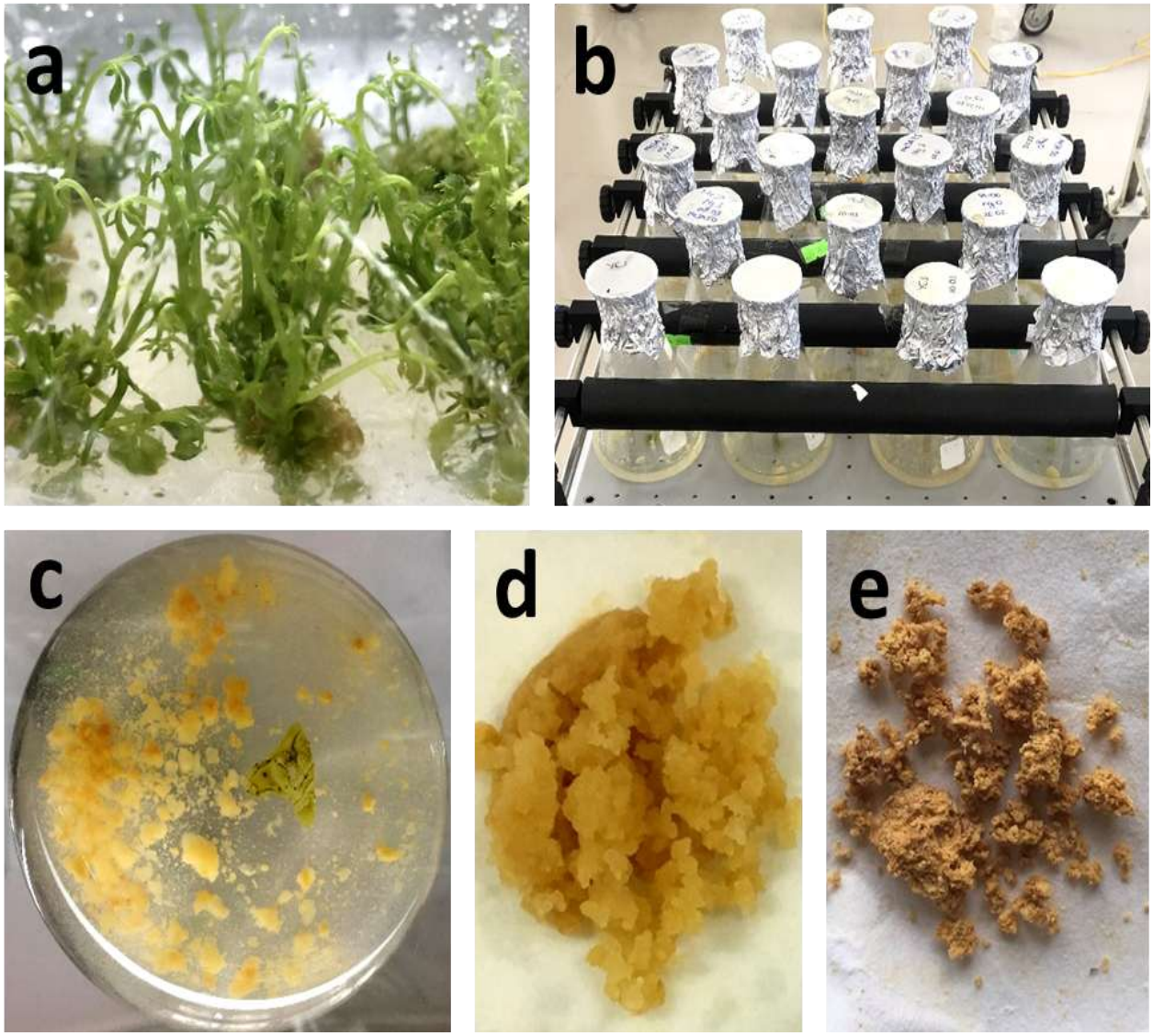

Figure 1: GpM biomass production by cell suspension culture.
a. *In vitro* propagation of GpM, b. cell suspension culture in shake flasks c. suspension cells, d. fresh cell suspension culture biomass, e. dry cell suspension culture biomass.

Total gypenosides in dry biomass in extract of cell suspension culture were significantly higher than those in natural plant biomass ($P < 0.05$) (Figure 2c and e). Meanwhile, RB1 in dry biomass in extract of cell suspension culture was significantly lower than those in natural plant biomass ($P < 0.005$) (Figure 2d and f). HPLC analysis showed that more compounds were detected in natural sample than in suspension cells such as retention times were 3.58 min, 6.11 min (Fig. 2). However, some compounds in cell had higher content than in natural sample, such as the retention time was 2.41 min.

The effect of GpM Extract on Cell Proliferation of Human lung carcinoma cell line (A549): To examine the cancer cell inhibitory effect of the GpM extract, we did SRB assay using human lung carcinoma cell line (A549). The GpM extracts presented concentration-dependent inhibition of A549 cell proliferation (Figure 3a). The inhibition effect of the highest concentration of extracts (200 µg/mL) was 25.15±2.03 and 37.68±1.26% for the natural GpM and plant cell suspension culture extracts. There was a significant difference of the inhibition of cell proliferation in the GpM extracts between natural and plant cell suspension culture for A549 cells.

The effect of GpM Extract on Cell Proliferation of Human hepatoma cell lines (HepG2 and Huh7): In human hepatoma cell lines, GpM extracts from natural and plant cell suspension culture inhibited proliferation of both HepG2 and Huh7 cells (Figure 3b and c). Growth inhibition of HepG2 and Huh7 cells was increased dramatically at higher concentrations (100-200 µg/mL). The extract in concentrations of 200 µg/ml showed the highest inhibiting activity in natural extract (34.36±1.12 in HepG2 and 36.07±1.89 in Huh7) as well as in cell suspension culture (41.16 ±2.28 in HepG2 and 44.43±1.65 in Huh7).

The Effect of GpM Extract on Cell Proliferation of Human leukemia cell line (HL-60): The extracts from natural and plant cell suspension culture inhibited human leukemia cell proliferation at the two highest concentrations 100 and 200 µg/mL.

Table 1
Total gypenosides accumulation in GpM samples (M ± SD)

Samples	Natural plant	Cell suspension culture
Fresh weight (g)	-	5.485 ± 0.223
Dry weight (g)	-	0.155 ± 0.034
Total gypenosides in dry cells (mg/g)	37.270 ± 4.710	48.844 ± 3.933
RB1 in dry cells (mg/g dry weight)	0.072 ± 0.021	0.041 ± 0.004
Total gypenosides in extraction (mg/g)	369.489 ± 25.317	472.159 ± 59.098
RB1 in extraction (mg/g dry weight)	0.697 ± 0.081	0.394 ± 0.021

Table 2
Comparison the cancer cell growth inhibitory potential of natural GpM and plant cell suspension culture

Concentration (µg/mL)	Inhibition (%)		
	Natural plant	Cell suspension culture	Significant
Human lung carcinoma cell line (A549)			
4	0.72 ± 0.79	0.9 ± 0.39	NS
20	6.98 ± 1.18	6.03 ± 0.62	NS
100	23.56 ± 1.52	13.88 ± 0.28	$P < 0.05$
200	25.15 ± 2.03	37.68 ± 1.26	$P < 0.05$
Human hepatoma cell line (HepG2)			
4	0.63 ± 0.38	0.69 ± 0.13	NS
20	8.93 ± 0.6	8.81 ± 0.93	NS
100	22.51 ± 0.58	32.89 ± 1.43	$P < 0.05$
200	34.36 ± 1.12	41.16 ± 2.18	$P < 0.05$
Human hepatoma cell line (Huh7)			
4	2.44 ± 0.13	1.72 ± 0.47	NS
20	7.24 ± 1.06	7.42 ± 0.57	NS
100	34.84 ± 1.72	15.19 ± 0.63	$P < 0.05$
200	36.07 ± 1.89	44.43 ± 1.65	$P < 0.05$
Human leukemia cell line (HL-60)			
4	3.57 ± 0.62	5.88 ± 0.93	NS
20	6.42 ± 0.93	11.23 ± 1.41	NS
100	33.51 ± 2.02	39.04 ± 1.07	NS
200	42.17 ± 2.48	48.76 ± 1.94	$P < 0.05$

In principle, there was a statistically significant difference in the inhibition of the proliferation between the extracts from natural and plant cell suspension culture. Human leukemia cell proliferation was significantly inhibited by the GpM extract in concentrations of 200 µg/ml in both GpM from natural (42.17±2.48) and cell biomass (48.76±1.94)

Discussion

Medicinal plants have been used worldwide since ancient time. The pharmacological properties of medicinal plants are based on their phytochemical components especially the secondary metabolites which are outstanding sources of value added bioactive compounds. Extended use of these metabolites in pharmaceutical industry has initiated a need to focus research on increasing the production by employing plant tissue culture techniques. Plant tissue culture techniques being independent of climatic and geographical conditions will provide an incessant, sustainable, economical and viable production of secondary metabolites[2].

In this study, gypenosides had good accumulation in the suspension cells, total gypenosides were 48.844 ± 3.933 mg/g dry weight, higher than that of natural product (37.270 ± 4.710 mg/g dry weight). However, biosynthesis of Rb1 in cells was lower than natural plant (0.041 ± 0.004 mg Rb1/g dry cells weight against 0.072 ± 0.021 mg/g dry weight).

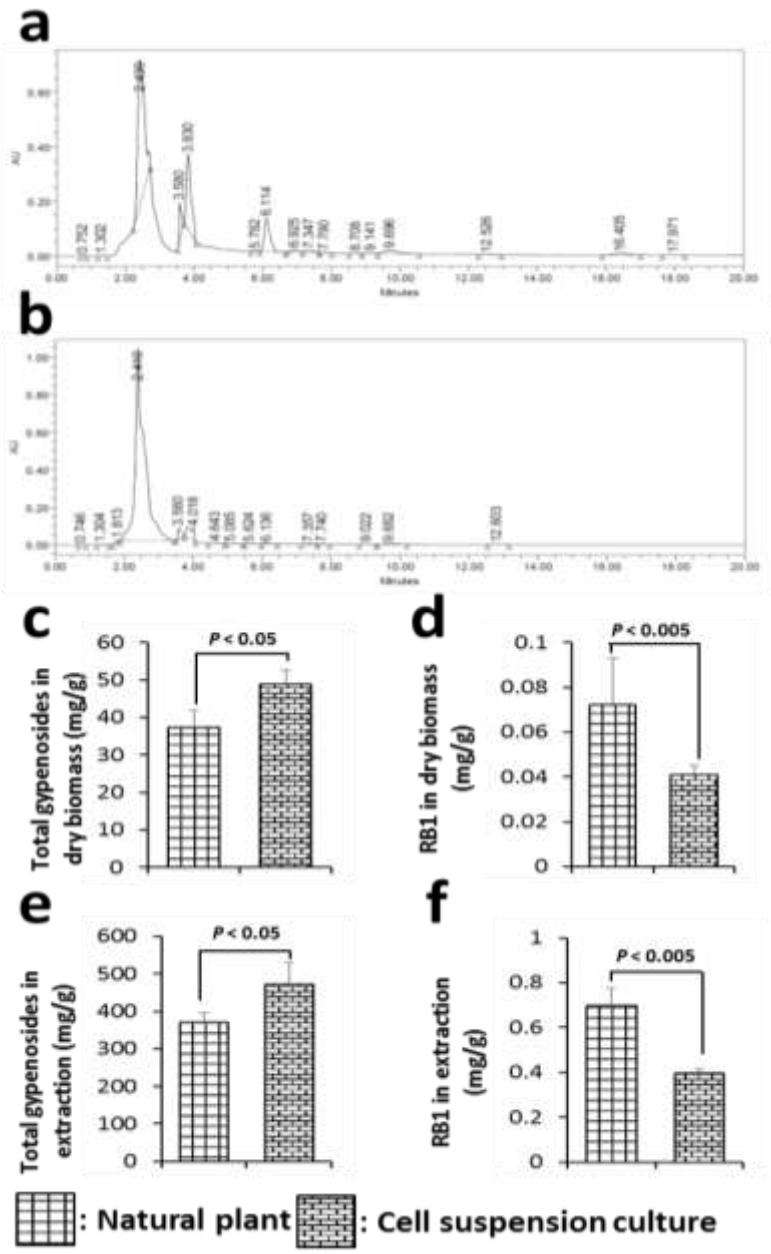

Figure 2: Bioactive compounds analysis of GpM biomass.
a. HPLC profile of GpM extract from natural plant biomas, b. HPLC profile of GpM extract from cells suspension culture, c. Comparison of total gypenosides in dry biomass of natural and plant cell culture, d. Comparison of RB1 in dry biomass of natural and plant cell culture, e. Comparison of total gypenosides in extract of natural and plant cell culture, f. Comparison of RB1 in extract of natural and plant cell culture

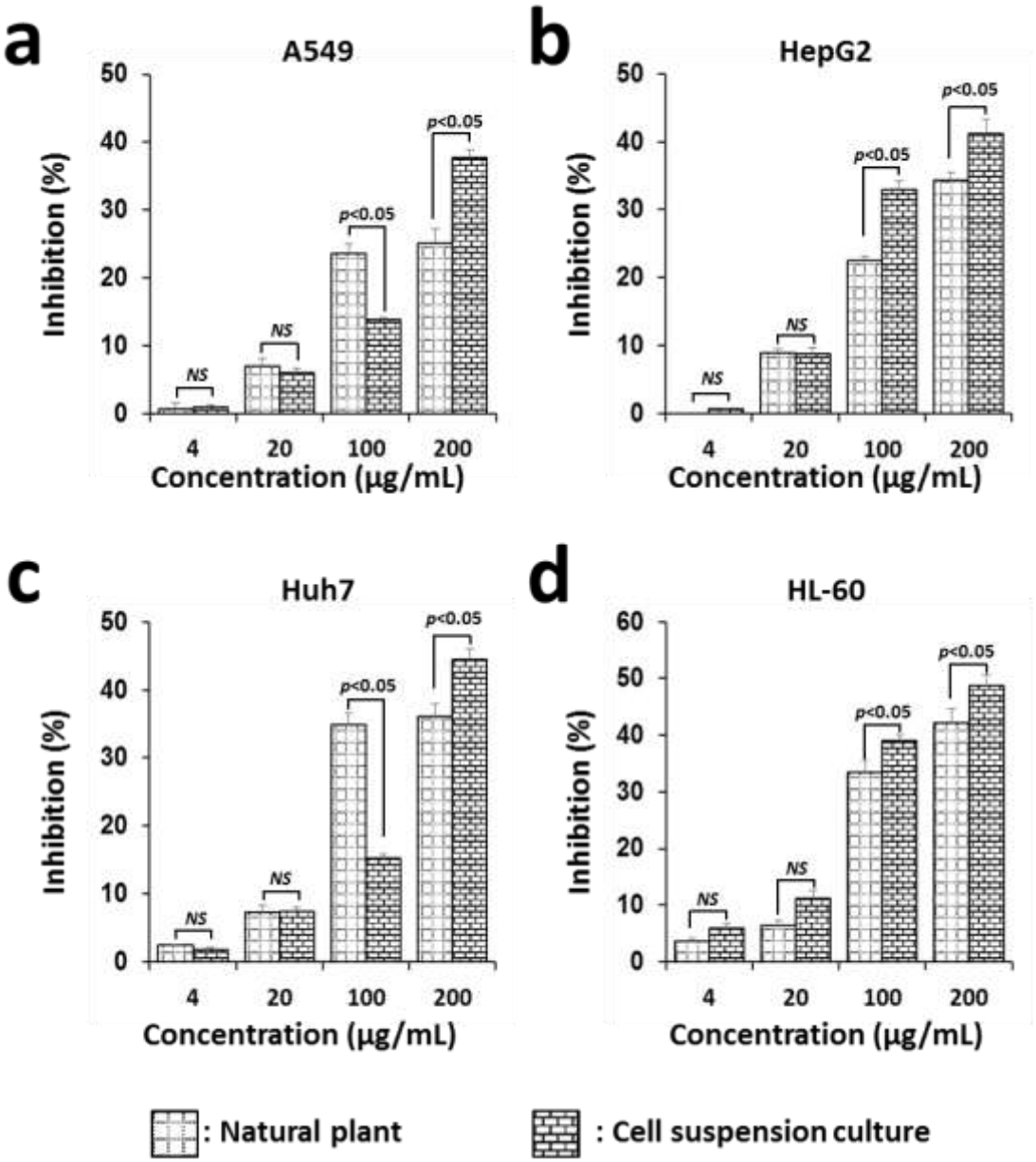

Figure 3: The effects of natural GpM and plant cell suspension culture on proliferation of human cancer cell lines. a. Human lung carcinoma cell line (A549). b. Human hepatoma cell line (HepG2). c. Human hepatoma cell line (Huh7). d. Human leukemia cell line (HL-60). *p*<0.05: Significant; NS: Non-significant

Biosynthesis of valuable secondary compounds in suspension cells higher than that of natural plants was previous reported for example production of rosmarinic acid from *Lavandula officinalis*[27], phenylethylamines dopamine and tyramine in *Piper cernuum*[5] cell culture. Secondary metabolites have complex chemical composition and are produced in response to various forms of stress to perform different physiological tasks in plants[2]. The diffirent stress in cell culture system and natural condition may led to the diffirent acumulation of gypenosides in GpM cell and natural biomass.

Natural extract of GpM has been investigated as a potent anti-cancer agent against several types of cancers cell *in vitro* proliferation including breast cancer, lung cancer, colorectal cancer cells, prostate cancer and liver cancer cell[4,10,13,26,30,31].

Li et al[12] isolated a nonpolar fraction EA1.3A from GpM (including alkaloids, organic esters, terpenes and catechol substance) and showed potent cancer suppression activities against four cancer cell lines (Human breast adenocarcinoma cell lines MDA-MB-453 and MCF7, human prostatic adenocarcinoma cell line LNCaP and human colorectal carcinoma cell line HCT116). The nonpolar fraction EA1.3A from GpM suppresses cancer cell proliferation through signaling pathways mediating apoptosis and cell cycle arrest.

Wang et al[26] showed that four compounds (including 3,4-dihydroxy phenyl-O-β-D-glucoside, gypenoside XLVI, gypenoside L and ginsenoside Rd) were isolated from the ethyl acetate and n-butanol fractions of GpM. Gypenoside L and ginsenoside Rd displayed a strong interference effect on the proliferation of breast cancer (MCF-7) and lung cancer (A549) cell line.

Cheng et al[4] isolated both flavonoid and saponin fractions from GpM, both fractions induced cell cycle arrest of prostate cancer cell PC-3 at both S and G2/M phases. GpM induced an increase both early and late apoptotic cell populations in dose-dependent. Xing et al[30] reported that Damulin B, a dammarane-type saponin isolated from GpM, showed the strong potent growth inhibitory activities against human lung carcinoma A549 cells. Damulin B exhibited an anti-cancer effect by inducing apoptosis, suppressing migration and cell cycle arest at early G0/G1 phase[30].

Mechanisms of effect of gypenosides from GpM on human colorectal cancer SW-480 cells were investigated by Yan et al.[31] Gypenosides from GpM may imply mitochondria damage, ROS generation and induce apoptosis on human colorectal cancer cells.

In 2020, Hussain et al[10] reported the anticancer activity potential of GpM extract on liver cancer cell (HepG2) cycle arrest and apoptotic induction. GpM extract inhibited the growth of HepG2 cells by arrested G0/G1 phase cell cycle via regulating the gene expression. In addition, GpM extract induced apoptosis of HepG2 cells via generation of intracellular reactive oxygen species, mitochondrial pathway and death receptor.

Conclusion

First evidence of GpM saponins as an effective anti-cancer agent for the treatment the $Apc^{Min/+}$ colorectal cancer mouse model was reported by Tai et al[21]. Results show that GpM saponins treatment could significantly inhibit polyp formation in colorectal cancer mice. The anti-cancer effect of GpM saponins was mediated through modulation of JNK/p38 MAPK signaling, upregulation of Prdx1 and Prdx2 and suppression of Ras, RAF/MEK/ERK/STAT, PI3K/AKT/mTOR signaling. Moreover, GpM saponins could enhance the anti-cancer efficacy of 5-FU as an adjuvant in the treatment of colorectal cancer[21].

Although many report show the anti-cancer effect of natural GpM extract on both *in vitro* and *in vivo*, there is no evidence to explain whether GpM cell suspension can inhibit cancer. Our results provide the evidence that biomass accumulation of GpM in cell suspension cultures inhibits human cancer cell growth *in vitro*.

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