Antitumor Activity of Cell Suspension Culture of Green Tea Seed (*Camellia sinensis* L.)

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Abstract The objective of this study was to investigate the antitumor activity of suspension cultures of tea callus cells grown in the presence of different concentrations of the growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D) with or without light irradiation. The methanol and ethanol extracts of precipitated cells (MEP, EEP) exhibited stronger inhibitory effects on the growth of tumor cell lines than the water extract of precipitated cells (WEP) or the supernatant. Compared to culture under dark conditions, exposure to light irradiation led to significantly higher antitumor activity. The MEP from light irradiated cells at 250 μg/mL with 2.0 mg/L 2,4-D displayed more than 64% growth inhibition of HEP-2 cells, whereas normal cells showed less than 25% growth inhibition. The some fractions of MEP obtained from Diaion HP-20 column chromatography displayed the majority of inhibitory activity against the HEP-2 cell line. These results show that 2,4-D, and light stimulated the synthesis of antitumor compounds.

Keywords: *Camellia sinensis* L., green tea seed, cell suspension culture, secondary metabolite, antitumor activity, MTT assay

INTRODUCTION

Green tea (*Camellia sinensis*) is an important commercial crop that is grown in over 30 countries and is consumed world wide primarily as a beverage made from the processed leaf [1]. It produces a variety of secondary metabolites that are valuable for human health. The main constituents of green tea are catechins that comprise up to 30% of the dry weight [2]. In green tea, they belong to the flavanol group of phenols and remain unoxidized in green tea. Catechins also form polymerized products, theaflavins and thearubigins, in black tea [3]. The catechins found in green tea are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), and catechin. These polyphenolic compounds in green tea are well known for their broad spectrum of biological activities such as antioxidant [4], antibacterial [5,6], antifungal [7], antigiardial [8], and antitumor functions [9,10].

Green tea also contains volatile oils, vitamins, minerals, and many other useful secondary metabolites such as caffeine [1,11], theanine [12-14], and saponin [15]. Large-scale production of these compounds has been attempted by means of *in vitro* tea cell cultures.

Aqueous alcohol extract of tea plant root was reported to have a significant antitumor effect on ascites [16] and solid tumors [17]. In addition, seed extracts of *C. sinensis* are known to have strong growth inhibition activity toward tumor cell lines at a relatively low concentration [15]. Consumption of tea extract also inhibited tumor cell growth and metastasis in mice [18,19]. Many mechanisms have been proposed for the inhibition of carcinogenesis by tea [20,21]. These include the modulation of signal transduction pathways that lead to the inhibition of cell cycle progression and transformation, induction of apoptosis, as well as inhibition of metastasis and angiogenesis. However, these mechanisms need to be verified in humans in order to gain more public attention on the effectiveness of tea against human tumors.

Plant cell culture is a promising biotechnological approach, and has been adopted for production of various categories of secondary metabolites [22]. Although the lower yield of these compounds in cell culture has restricted the commercial utilization of the approach, several strategies have been proposed to enhance product yield and productivity in cell culture system. In this study, the suspension cultures of green tea seed (*C. sinensis* L.) were used as an alternative source of secondary metabolites independent from seasonal variation and disease in order to obtain antitumor compounds. We prepared sus-

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pension cultures of tea callus cells grown in the presence of different concentrations of the growth regulator (2,4-D) with or without light irradiation, and investigated their antagonizing effects on the growth of various tumor cell lines. Here we present the first report that tea suspension cells produce antitumor compounds whose production is dependent on light, and increased by 2,4-D.

MATERIALS AND METHODS

Plant Materials

The seeds of green tea plant, *C. sinensis* L., were kindly provided by Hankooktea Co., Gwangju, Korea in October 2003.

Callus Induction

Seeds were washed with 70% alcohol for 1 min, 0.25% sodium hypochlorite for 10 min, and rinsed with sterile water 4~5 times. Seeds were sowed onto Murashige and Skoog (MS) medium (pH 5.7) containing 3% sucrose and 1.8% agar. The callus was induced about one month after incubation at 26°C in the dark. The highest rate of callus growth was observed in MS medium (Sigma, USA) containing 2.0 mg/L 2,4-D (Sigma).

Cell Suspension Culture

About 5 g of induced callus cells were suspended in liquid MS culture media containing the same ingredients as the solid MS culture media except for agar. Liquid cell cultures were maintained at 100 rpm in a 250 mL Erlenmeyer flask at 26°C in the presence or absence of light with an intensity of 2,500 Lux as previously described [13,23,24]. The cell suspension cultures were subcultured every 15 days by inoculating 25 mL of suspension cells into 100 mL of fresh medium in a 250 mL Erlenmeyer flask. One-month-old cultures were used for our initial experiments.

Sample Preparation

The suspended cells and the culture medium were separated by centrifugation at 3,000 rpm, 4°C for 20 min. Immediately after the cells were harvested by vacuum filtration, they were washed four times with cold distilled water. Cells (10 g) were ground in absolute methanol, absolute ethanol, or distilled water (100 mL) with a mortar and pestle to produce each extract. Absolute methanol and absolute ethanol were purchased from Samchun Chemical Co. (Seoul, Korea). The extracts were centrifuged as above and filtered using Whatman paper No. 41. The supernatant and filtrate were evaporated under vacuum and then used for assays measuring cytotoxic activity. Among the filtrates, MEP displayed the strongest activity and thus was fractionated further. MEP was dissolved in distilled water, then the aqueous solution was injected to a column (size; $\phi 100 \times 400$ mm) packed with Diaion HP-20 resin (Samchun Chemical Co.) at a ratio of 1:2 (volume of Diaion HP-20 resin:filtrate). Elution was carried out in steps with 0, 20, 40, 60, 80, and 100% methanol in water (1 L each in total) at a flow rate of 10 mL/min. Each fraction (F-1, F-2, F-3, F-4, F-5, and F-6) obtained by Diaion HP-20 column chromatography was concentrated using a rotary vacuum evaporator (N-1; EYELA Co., Japan). After drying, samples were dissolved in phosphate buffered saline (PBS; pH 7.2), aseptically filtered with a 0.45 μm pore size filter, and used for assays.

Determination of Total Polyphenol Content

Total polyphenol content was measured by the Folin-Denis method [25]. One mL of the diluted sample solution (5 mg/mL) was mixed with 9 mL of distilled water for 3 min. One mL of commercial Folin reagent was added to 10 mL of the diluted samples and set for 5 min before adding 10 mL of 7% sodium carbonate aqueous solution. The final volume was adjusted to 45 mL with distilled water. After holding the mixed solution for 1 h, absorbance at 760 nm was measured at room temperature using a UV-VIS spectrophotometer (Optizen 2120; Mecasys Co., Seoul, Korea). Total polyphenol contents were determined from a standard curve obtained using tannic acid (Sigma) and were expressed as tannic acid equivalents (mg TAE/g sample).

Tumor and Normal Cell Lines

Five human carcinoma cell lines from lung (SK-MES-1), colon (DLD-1), larynx (HEP-2), endometrial adenocarcinoma (HEC-1B), and melanoma (Farrow) tissue, and along with the mouse embryo cell line (NIH/3T3) were obtained from the Korean Cell Bank. All except for the HEP-2 cell line were grown in RPMI 1640 medium (2.0 g/L, sodium bicarbonate, Gibco BRL, MD, USA). The HEP-2 cell line was grown in MEM medium (2.2 g/L, sodium bicarbonate, BRL). Each medium was supplemented with 10% fetal bovine serum (BRL), penicillin (10,000 unit/mL), and streptomycin (10 mg/mL). All cell lines were incubated at 37°C in a 5% CO_2 and 95% air chamber.

Assay for Cytotoxic Activity

Cytotoxicity was examined using the MTT assay [26,27]. Cells undergoing exponential growth were suspended in each medium at a concentration of 2×10^5 cells/mL, dispensed in a 96-well flat plate in a volume of 100 μL per well, and stabilized by incubation at 37°C in 5% CO_2 for 24 h. Then, 100 μL of each sample dissolved in PBS was added to the wells. One hundred μL of PBS was used as the control. After incubation at 37°C for 44 h, 50 μL of MTT (0.5 mg/mL PBS, Sigma) was added to each well, and the resulting 250 μL mixture was further incubated at 37°C for 4 h. The formazan crystals formed in each well were dissolved in 100 μL of dimethylsulfoxide (DMSO) solution by gentle mixing on a plate shaker.

Table 1. Growth inhibitory effects of cell suspension culture against tumor cell lines

Cell lines	Cell suspension culture[1]			
	Supernatant[2,3]	Precipitate[2]		
		Methanol extract[3]	Ethanol extract[3]	Water extract[3]
Farrow	18.7	98.4	97.1	17.2
HEP-2	23.0	95.8	92.5	18.6
SK-MES-1	3.70	97.6	97.0	10.3

[1] Growth in MS medium plus 2,4-D (2.0 mg/L).
[2] The number indicates % growth inhibition of tumor cell lines.
[3] Sample was added at a final concentration of 10 mg/mL in PBS buffer (pH 7.2). The data indicate average percentages of three independent cultures.

The optical densities of the colored reaction samples were measured at 570 nm on a multi-well scanning spectrophotometer (Microplate Autoreader, Bio-Tek Instrument, USA).

Data Analysis

Data were expressed as means ± standard deviation (SD). The statistical significance of differences between groups was determined by applying the Student t-test. Values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Establishment of Cell Suspension Culture

A *C. sinensis* callus was induced from an axenic seed on MS agar medium containing 3% sucrose and 2.0 mg/L 2,4-D. The callus appeared pale yellow and tended to aggregate and form clusters. This compact callus was subcultured on the same solid medium every month at 26°C in the dark and stably maintained for more than one year. To achieve homogeneous synchronization of cell cultures, the callus cells were cultivated in the same liquid media, and the suspension cultures were used to extract secondary metabolites. Cultivating 100 mL of suspension cells for two weeks typically resulted in approximately 1.84 g of cells (fresh weight). The cell concentration continued to increase until 14 days after inoculation and then remained relatively unchanged. The doubling time of the cell suspension cultures was approximately 14 days, which has remained consistent thus far, indicating no physiological changes during subculture. Thus, we established a stable cell suspension line that produces a constant yield of secondary metabolites.

Cytotoxic Activities of the Metabolites

Inhibitory Effects of Various Extracts

At day 15, suspension cells grown in MS medium plus 2.0 mg/L 2,4-D were harvested and extracted with methanol, ethanol, or water. We then examined the growth inhibitory effects of methanol extract from precipitated green tea callus cells (MEP), ethanol extract (EEP), water extract (WEP), and supernatant, each at a final concentration of 10 mg/mL, on the HEP-2, SK-MES-1, and Farrow cell lines. As shown in Table 1, the methanol and ethanol extracts displayed major inhibitory activity toward human tumor cell lines, whereas no significant activity was detected in the water extract and the supernatant. This contrasts with previous results involving the water extract of green tea seeds which showed a high anti-proliferative effect on human tumor cell lines [15]. These results suggest that the antitumor activity of methanolic and ethanolic extracts of tea seed suspension cells is higher than the water extract.

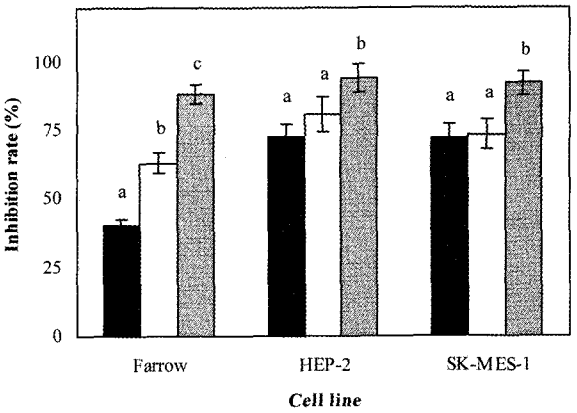

Fig. 1. Inhibitory effects of MEP on tumor cell lines. Cells were grown in MS medium containing: ■ 2,4-D (0.5 mg/L); □ 2,4-D (1 mg/L); ▨ 2,4-D (2 mg/L). Methanol extract of precipitate was added at a final concentration of 5 mg/mL in PBS buffer (pH 7.2). The data indicate averages of triplicate cultures ± SD. Data were analyzed by student t-test (two-tailed). [a-c]Values with different superscripts are significantly different ($p<0.05$).

Enhancement of Inhibitory Effects by 2,4-D

We also compared antitumor activities of cell suspension cultures grown in MS medium containing 0.5, 1.0, and 2.0 mg/L 2,4-D. The MEP and EEP of each suspension culture were tested at a final concentration of 5 mg/mL on human tumor cell lines (Figs. 1 and 2). As shown in Fig. 1, MEP with 0.5, 1.0, and 2.0 mg/L 2,4-D inhibited cell growth by 40, 63, and 88% in the Farrow cell line, 73, 81, and 94% in the HEP-2 cell line, and 72, 74, and 92% in the SK-MES-1 cell line, respectively ($p<0.05$). In a similar fashion, growth inhibition by EEP with 0.5, 1.0, and 2.0 mg/L 2,4-D was 39, 51, and 67% in the Farrow cell line, 51, 57, and 74% in the HEP-2 cell line, and 49, 50, and 58% in the SK-MES-1 cell line, respectively (Fig. 2). Therefore, 2,4-D appears to stimulate *de novo* synthesis of antitumor compounds. Overall, the antitumor activity of MEP was stronger than that of EEP at 5 mg/mL. However, both MEP and EEP showed the highest antitumor activity when produced from cells treated with 2.0 mg/L 2,4-D ($p<0.05$). Notably, growth

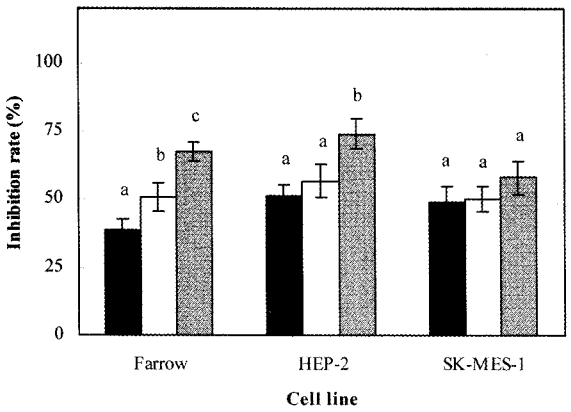

Fig. 2. Inhibitory effects of EEP on tumor cell lines. Cells were grown in MS medium containing: ■ 2,4-D (0.5 mg/L); □ 2,4-D (1 mg/L); ▨ 2,4-D (2 mg/L). Ethanol extract of precipitate was added at a final concentration of 5 mg/mL in PBS buffer (pH 7.2). The data indicate averages of triplicate cultures ± SD. Data were analyzed by student *t*-test (two-tailed). [a-c]Values with different superscripts are significantly different ($p<0.05$).

of the human larynx carcinoma cell line HEP-2 was strongly inhibited. Taken together, these results indicate that the growth of cell suspension culture in MS medium plus 2.0 mg/L 2,4-D combined with subsequent methanol extraction leads to the strongest antineoplastic effects.

Enhancement of Inhibitory Effects by Light Irradiation

We investigated whether light irradiation of the cell suspension cultures affects the quality of extracts with respect to tumor cell growth inhibition. The cell precipitates were extracted with methanol, and the extracts were tested at three different concentrations for growth inhibition of five human tumor cell lines (Fig. 3). At 250, 500, and 1,000 μg/mL, the extracts inhibited tumor cell growth by 38, 69, and 75% for the DLD-1 cell line, 20, 33, and 56% for the Farrow cell line, 37, 56, and 75% for the HEC-1B cell line, and 51, 71, and 78% for the SK-MES-1 cell line, respectively. The 250 μg/mL extracts exhibited less than 50% growth inhibition activity against all tumor cell lines mentioned above except for the SK-MES-1 cell line ($p<0.05$).

In contrast to the other cell lines, growth of the HEP-2 cell line was inhibited by over 64% irrespective of the three different concentrations of extract. As compared with the non-irradiated control (Fig. 3), light irradiation caused a significant increase in growth inhibition of all tumor cell lines. In addition, there is a significant increase in the yields of extract precipitates. The light-irradiated extract yielded about 29 mg of precipitate per 1 g of suspension cells, compared to 11 mg obtained from the same amount of non-irradiated cells. Light irradiation also did not cause suspension cells to grow faster than the non-irradiated cells as determined by the fresh weights of precipitated cells grown for 15 days. All these results together suggest that light irradiation increases the biosynthesis of antitumor compounds.

It has been reported that under light irradiation, total

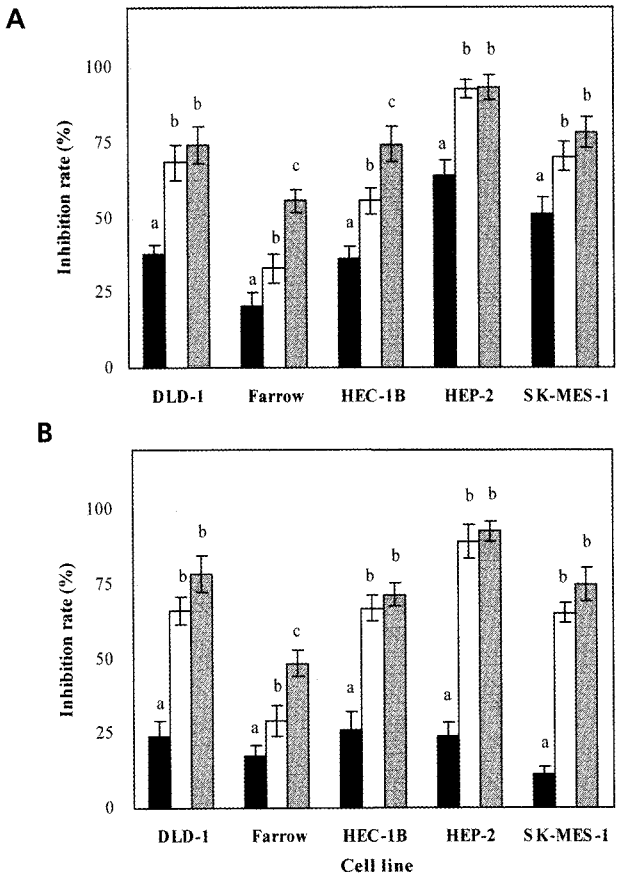

Fig. 3. Inhibitory effects of MEP of cell suspension culture in the presence (A) and absence (B) of light. Tumor cell lines were treated with MEP: ■ 250 μg/mL; □ 500 μg/mL; ▨ 1,000 μg/mL in PBS buffer (pH 7.2). The data indicate averages of triplicate cultures ± SD. Data were analyzed by student *t*-test (two-tailed). [a-c]Values with different superscripts are significantly different ($p<0.05$).

flavonoid content in tea suspension cells was significantly increased without inhibiting cell growth of the suspension culture [28], and that chloroplasts are involved in the biosynthesis of flavonoids [24]. In this study, the total polyphenol content of light-irradiated extract was 34.2 mg TAE/g whereas the total polyphenol content of the non-irradiated extract was 26.6 mg TAE/g ($p<0.05$). Also, tea flavonoids were shown to have inhibitory effects toward both tumor cells and angiogenesis [9,10]. Accordingly, light-dependent polyphenolic compounds could be responsible for the antitumor activity. Although such bioactive substances remain to be identified, the catechins, the main components of green tea, are likely candidates since they are well known as chemopreventive agents for tumors [9,21].

Inhibitory Effects on a Normal Cell Line

One of the most desirable characteristics of antitumor compounds is their selective cytotoxicity toward tumor cells. Therefore, we compared the effects of methanol extracts from the light-irradiated suspension cells on the

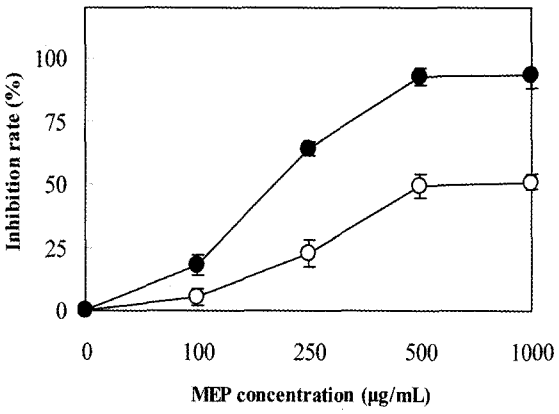

Fig. 4. Inhibitory effects of various concentrations of light-irradiated cell suspension MEP on tumor cell line HEP-2 (●) and normal cell line NIH/3T3 (○). The data indicate averages of triplicate cultures ± SD.

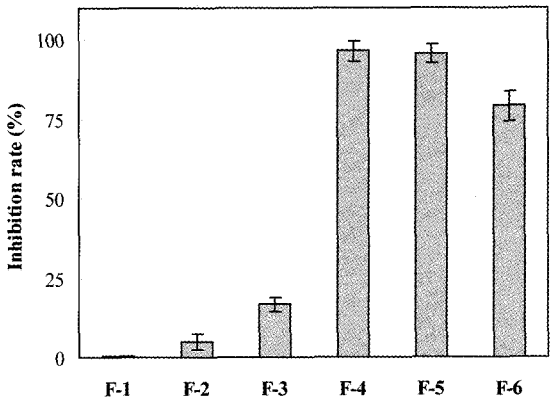

Fig. 5. Inhibitory effects of fractions of MEP separated by a Diaion HP-20 column chromatography on HEP-2 cell line at 100 μg/mL. F-1: non-absorption fraction, F-2, F-3, F-4, F-5, and F-6: fraction eluted with 20, 40, 60, 80, and 100% methanol, respectively. The values are mean ± SD (n = 3).

normal cell line NIH/3T3 and the human larynx carcinoma cell line HEP-2 (Fig. 4). When NIH/3T3 cells were exposed to 100 to 1,000 μg/mL methanol extracts, cell growth inhibition continued to increase up to 1,000 μg/mL. On the other hand, the HEP-2 cell line showed 64% inhibition with 250 μg/mL methanol extracts. In comparison, the NIH/3T3 cell line was inhibited less than 25% by the 250 μg/mL methanol extract. These results suggest that the methanol extract of suspension cells is selectively more cytotoxic toward HEP-2 cells than NIH/3T3 cells.

Inhibitory Effects of Partially Purified Substance(s) on HEP-2 Cells

Fig. 5 shows the growth inhibitory effect of MEP fractions obtained using Diaion HP-20 column chromatography. As shown in Fig. 5, the F-4, F-5, and F-6 fractions obtained by Diaion HP-20 column chromatography displayed significant inhibitory activity toward the HEP-2 cell line, whereas no significant activity was detected in the F-1, F-2, and F-3 fractions. The F-4, F-5, and F-6 fractions inhibited HEP-2 cell growth by 96, 96, and 79%, respectively, at a concentration of 100 μg/mL. At a concentration of 250 μg/mL, all fractions except for the F-1 fraction showed growth inhibition of the HEP-2 cell line (data not shown). These results suggest that the adsorbed fractions from Diaion HP-20 columns contain antitumor substances. Overall, the F-4 and F-5 fractions showed the highest antitumor activity at 100 μg/mL. Notably, growth of the human larynx carcinoma cell line HEP-2 was the most strongly inhibited among the cell lines used in this study.

CONCLUSION

We investigated the inhibitory effect of *C. sinensis* L. cell suspension extract under many conditions on the growth of various human tumor cell lines. The cell precipitate from *C. sinensis* L. suspension culture shows stronger inhibitory activity toward various tumor cell lines than does the culture supernatant. MEP is more effective than EEP in terms of growth inhibition. Also, the MEP of suspension cultures grown in MS medium containing 2.0 mg/L 2,4-D showed the highest degree of antitumor activity. In addition, light irradiation results in higher yields of extractable compounds than non-irradiation, and a significant difference in growth inhibition activity toward tumor cells was noted between the light-irradiated and non-irradiated cells. Thus, light appears to be necessary for the production of elevated levels of antitumor compounds. In response to 250 μg/mL MEP produced with light irradiation, HEP-2 cells displayed more than 64% growth inhibition, whereas the normal cell line NIH/3T3 showed less than 25% growth inhibition. These results indicate that 2,4-D and light stimulate the synthesis of antitumor compounds in tea cell suspension culture. Among the fractions of MEP obtained using Diaion HP-20 column chromatography, the F-4, F-5, and F-6 fractions inhibited HEP-2 cell growth by 96, 96, and 79%, respectively, at a concentration of 100 μg/mL. Theses results suggest that the adsorbed fractions from Diaion HP-20 columns contain antitumor substances. Therefore, it is presumed that the antitumor substances of tea cell suspension culture are hydrophobic. In the future, follow-up studies should be done to purify and define the bio-active substances in the F-4, F-5, and F-6 fractions regarding antitumor activity.

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