Carbohydrate Polymers 86 (2011) 1651–1656

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Production, preliminary characterization and antitumor activity (SKOV-3 cell lines) in vitro of glycans from green tea

Fan DongMei[a], He Tao[a,*], Wang Yan[a], Kong GuoQiang[b], Jiang Tao[b], Zhou Dan[b]

[a] Department of Gynecology, The First Affiliated Hospital of Henan Science and Technologic University, LuoYang 471003, PR China
[b] Department of Oncology, The First Affiliated Hospital of Henan Science and Technologic University, LuoYang 471003, PR China

ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form 21 June 2011
Accepted 30 June 2011
Available online 7 July 2011

Keywords:
RSM
Antitumor
Tea
Polyphenols
SKOV-3

ABSTRACT

Glycan from green tea was extracted with boiling water. The extraction temperature, extraction time and ratio of liquid to solid were optimized by using response surface methodology (RSM). The maximum yield of tea glycan extracts (2.93%) was obtained when glycans in green tea were extracted. The optimal extraction conditions for the glycans from green teas were: extracting temperature 98 °C, extracting time 50 min and rate of liquid to solid 6:1. Tea glycan, with an average molecular weight of 8.3×10^5 Da, contained rhamnose (Rha), arabian (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc) in molar ratios of 1.06:2.31:5.17:0.91:3.06:4.24. Ethanol extract of tea showed significantly higher antitumor activity against the SKOV-3 cells than tea glycan. These results indicate that the higher antitumor activity of ethanol extracts from tea may be related to their polyphenol contents.

1. Introduction

Tea, from the plant *Camellia sinensis* L., is one of the most popular beverages consumed worldwide in its green, black, or oolong form. Tea polyphenols and tea polysaccharides are the main components in tea extracts. Tea polysaccharides are also reported to have the ability to inhibit pathogenic bacterial adhesion (Lee et al., 2006). Wang, Wang, Li, and Zhao (2001) reported that tea polysaccharides enhanced immunization of rats. It is evident that different tea polyphenols like epigallocatechin gallate (EGCG), epicatechin gallate (ECG) could modulate the tumorigenesis pathway by affecting the cellular proliferation, differentiation and apoptosis (Yang, Maliakal, & Meng, 2002). EGCG has been shown to induce differentiation in human epidermal keratinocytes (Balasubramanian, Sturniolo, Dubyak, & Eckert, 2005; Eckert, Crish, Efimova, & Balasubramanian, 2004; Hsu et al., 2006), eosinophilic leukemia EoL-1 cell line (Lung et al., 2002), and apoptosis in NNK induced lung tumorigenesis in female A/J mice (Xu, Ho, Amin, Han, & Chung, 1992), and also to inhibit angiogenic differentiation of human endothelial cells (Singh et al., 2002). Similarly, ECG can induce apoptosis and growth inhibition of colon cancer cells through NAG-1 gene expression (Baek et al., 2004). In general, it is known that food polysaccharides in mushrooms and plants activate macrophage immune responses and lead to immunomodulation, antitumor activity, and so on (Schepetkin & Quinn, 2006); however, there are still only few reports on the immunostimulating effect of tea polysaccharides.

Ovarian cancer is the most lethal gynecologic cancer in the Western world, and the fourth most common cause of cancer death in women, after breast, lung, and colorectal cancer. It has an incidence of 22,430 new cancer cases and accounts for 15,280 of death for 2007 in USA (Abuharbeid, Apel, Zugmaier, Knabbe, & Sander, 2005). The incidence of ovarian cancer increases with age and 70% of patients present with advanced disease, resulting in a 5-year survival rate of 20–40% for patients with stage III or IV (Zamboni, Strychor, Joseph, Parise, & Egorin, 2008). Low cure rates for ovarian cancer can be attributed to the wide variety of histologic cell types, grade and stage at diagnosis that exhibit different treatment responses (Teicher, Menon, Alvarez, Shih, & Faul, 2002).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the relative significance of several independent variables, and determining the optimum conditions for desirable responses. The central composite design (CCD) is one of the most common and well-known designs of experiments (DOE) in the optimization process. CCD is a multivariate optimization process that can evaluate the influence of several variables of an experimental procedure simultaneously, with a fewer number of experiments (Brum, Cassella, & Pereira Netto, 2008). The experimental design is more efficient for simplifying experiments and understanding mutual relationships among experimental parameters.

These previous studies suggested that the plant tea, especially its polyphenol fraction, has enormous potential as an antitumor

* Corresponding author. Tel.: +86 0379 64810716; fax: +86 0379 64810716.
E-mail address: het3915@yahoo.com.cn (T. He).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.06.079

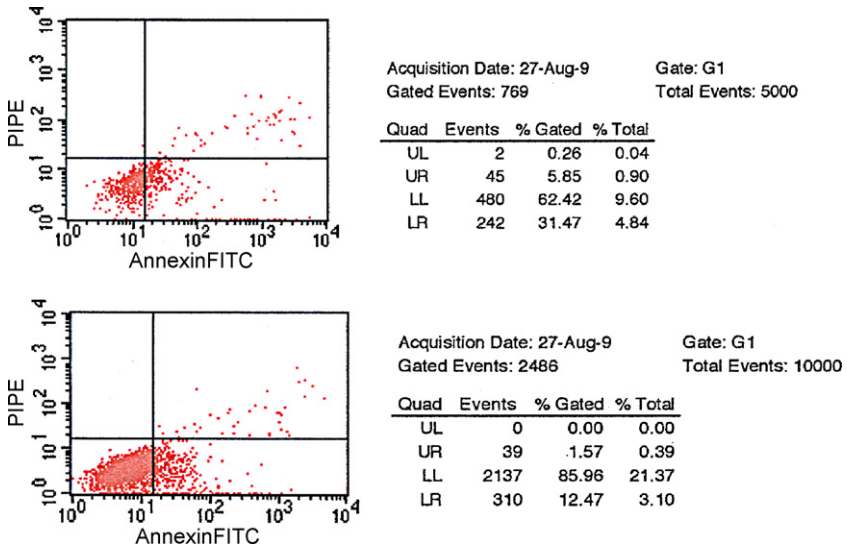

Fig. 1. Apoptosis of SKOV-3 before and after transfection.

agent. In this study, to achieve success in extraction yield improvements, the extraction temperature, extraction time and ratio of liquid to solid were evaluated for tea glycan production and statistically optimized to enhance productivity. Each variable was tested based on our prior experience over a range and fixed in central composite rotatable design of RSM. The role of each variable, their interactions and statistical analysis to obtain predicted yields of tea glycan was explained by applying second-order polynomial model. In addition, to ascertain the potential pharmacological activities of tea extracts and develop a new Chinese medicine for tumor treatment, the present work was carried out to evaluate the in vitro antitumor activity against SKOV-3 on the basis of our previous study (Fig. 1).

2. Materials and methods

2.1. Response surface methodology (RSM)

The basis of RSM is form of an experimental design and the most frequently used is the central composite rotatable design (CCRD) for the prediction and verification of model equation as well as the optimization of the response as the function of the independent parameters. CCRD experimental design is preferred and widely used for fitting a second order models represented as follows:

$$Y = \beta_0 + \sum_{J=1}^{K} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{1<j} \beta_{ij} X_i X_j \quad (1)$$

where β_0, β_i, β_{jj} and β_{ij} are regression coefficients for the intercept, linear, quadratic and interaction coefficients, respectively and X_i and X_j are the coded independent variables. Coded values are used since experimental parameters have different units and ranges in the experimental domain making regression analysis impossible to be preformed. Coded variables are forced to range from −1 to +1 so that they will response more evenly and the units of the parameters are irrelevant. The equation commonly used for coding is as:

$$X = \frac{(x - (x_{max} + x_{min}))/2}{(x_{max} - x_{min})/2} \quad (2)$$

where x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable. The 'Design Expert' software (version 6.0.10, Stat-Ease, Inc., Minneapolis, USA) was used for regression and graphical analysis of the experimental data. The statistical analysis of the model was

Table 1
The central composite experimental design and experiment data for hot water extraction of tea glycan.

Run	Extraction temperature ($X1$)	Extraction time ($X2$)	Ratio of liquid to solid ($X3$)	Response value [extraction yield ($R1$)]
1	−1.00	−1.00	−1.00	1.89
2	1.00	−1.00	−1.00	2.21
3	−1.00	1.00	−1.00	1.96
4	1.00	1.00	−1.00	2.45
5	−1.00	−1.00	1.00	2.33
6	1.00	−1.00	1.00	2.51
7	−1.00	1.00	1.00	2.43
8	1.00	1.00	1.00	2.57
9	−1.68	0.00	0.00	1.89
10	1.68	0.00	0.00	2.62
11	0.00	−1.68	0.00	2.61
12	0.00	1.68	0.00	2.67
13	0.00	0.00	−1.68	2.18
14	0.00	0.00	1.68	2.68
15	0.00	0.00	0.00	2.91
16	0.00	0.00	0.00	2.87
17	0.00	0.00	0.00	2.89
18	0.00	0.00	0.00	2.93
19	0.00	0.00	0.00	2.93
20	0.00	0.00	0.00	2.9

performed in the form of analysis of variance (ANOVA). The determination coefficient R^2 measures the goodness of fit of regression model. It also includes the *t*-value for the estimated coefficients and associated probabilities, $P(t)$. Response surface methodology (RSM) with central composite design (CCD) was employed to investigate the effect of extraction parameters on glycan yield from green tea. Three independent parameters namely, extracting temperature, extracting time and ratio of liquid to solid at three different levels each, were employed. The parameters chosen and their levels were based on preliminary experiments carried out in our laboratory. The level and ranges chosen for the factors are shown in Table 1.

2.2. Extraction process of tea polyphenolic compounds

The dried green tea (40 g dry weight) was extracted in a laboratory extractor of 6 cm inner diameter and 15 cm height (immersion operation), which was kept at constant temperature by an external water bath. A condenser was attached to the top of the extractor to avoid losses of solvent. A peristaltic pump was connected to the bottom of extractor, feeding continuously either ethanol or water. The samples were stored at 4 °C in darkness until the analysis of polyphenolic compounds was performed.

2.3. Monosaccharide composition analysis

The monosaccharide composition of tea glycan was determined according to a method described earlier (Kim, Laskowich, Michon, Kaiser, & Arumugham, 2006). Briefly, sample (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h in a sealed tube. After that, the removal of the excess amount of TFA was accomplished by co-evaporation at reduced pressure with ethyl alcohol added after reaction. The subsequent treatment of the resultant dry hydrolysate with acetic anhydride and pyridine afforded the corresponding alditol acetate, which was analyzed by an Agilent 6890N gas chromatography (GC) (Agilent, USA) fitted with a flame ionization detector (FID) and an AJW&HP-88 Capillary column (100 m × 250 μm, 0.25 μm). The analytical condition was 3 min at 180 °C, from 180 °C to 230 °C at 10 °C/min and held for 20 min at 230 °C, from 230 °C to 240 °C at 5 °C/min and held for 20 min at 240 °C, and from 240 °C to 250 °C at 5 °C/min and held for 5 min at 250 °C. Quantitation was carried out from the peak area.

2.4. Cell suspension culture

About 5 g of cancer 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 2500 lx as previously described (Pan, Wang, & Zhong, 2000). 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.

2.5. Evaluation of anticancer potential using MTT test

The cytotoxic potential of the ethanol extract from green tea against SKOV-3 cells was tested using MTT assay. About 1×10^6 cancer (SKOV-3) were seeded in 96-well plates, treated with varying content of extract/mL medium and incubated at 37 °C in an atmosphere of 5% CO_2 for 24, 48 and 72 h. Cells incubated in complete medium without extract served as control. At the end of incubation, medium was removed and 50 μL MTT (5 mg/mL) was added and the cells were further incubated for 4 h. After the incubation, the MTT solution covering the cells was removed. 100 μL of dimethylsulfoxide was added to the wells and the cell viability determined by measuring the absorbance in a microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. The experiment was repeated thrice. Cell viability was calculated using the following formula and from that the percentage of cytotoxicity and GI50 values of leaf extracts were calculated for the different time points studied.

$$\%\ \text{of cell viability} = \left[\frac{\text{mean OD of experimental wells}}{\text{mean OD of control wells}}\right] \times 100$$

2.6. Evaluation of Bcl-2 protein level

Bcl-2 protein levels were determined according to the method described by Zhang, Gong, Li, Zhang, and Wang (2011).

Table 2
Regression coefficients, and *P* or probability values for four dependent variables for hot water extraction of tea glycan.

Source	Sum of squares	df	Mean square	*F* value	*P* value Prob > *F*
Model	2.25	9	0.25	31.35	<0.0001
A–A	0.41	1	0.41	51.06	<0.0001
B–B	0.024	1	0.024	2.99	0.1142
C–C	0.35	1	0.35	43.29	<0.0001
AB	2.112E−003	1	2.112E−003	0.27	0.6179
AC	0.030	1	0.030	3.77	0.0810
BC	2.813E−003	1	2.813E−003	0.35	0.5657
A^2	0.94	1	0.94	117.65	<0.0001
B^2	0.20	1	0.20	25.59	0.0005
C^2	0.54	1	0.54	67.50	<0.0001
Residual	0.080	10	7.971E−003		
Lack of fit	0.077	5	0.015	27.99	0.0012
Pure error	2.750E−003	5	5.500E−004		
Cor total	2.33	19			
Std Dev	0.089		*R*-squared		0.9658
Mean	2.52		Adj *R*-squared		0.9350
C.V.%	3.54		Pred *R*-squared		0.7445
PRESS	0.59		Adeq precision		16.028

3. Results and discussion

3.1. Factor affecting tea glycan yield

The results from the experimental study are tabulated in Tables 1 and 2 at different level of factors which were studied. The estimates of main effects of the factors together with the interactions were plotted on a half normal probability graph as shown in Figs. 1–3.

The response surface demonstrated there must be the maximum in the stable range (Figs. 1–3). In light of multi-regressive-analysis of the central composite experiment shown in Table 1, the second-order polynomial prediction model was obtained as Eq. (3):

$$\begin{aligned} R1 = {} & +2.90887 + 0.17264 * A + 0.041804 * B + 0.15896 * C \\ & + 0.016250 * A * B - 0.061250 * A * C - 0.018750 * B * C \\ & - 0.25509 * A^2 - 0.11897 * B^2 - 0.19322 * C^2 \end{aligned} \quad (3)$$

The statistical significance of the second-order model was checked by an *F*-test (ANOVA) and data shown in Table 2. Response surface analysis (RSA) of the data in Table 2 demonstrates that the relationship between the extraction yield and extraction parameters is quadratic with good regression coefficient ($R^2 = 0.9658$).

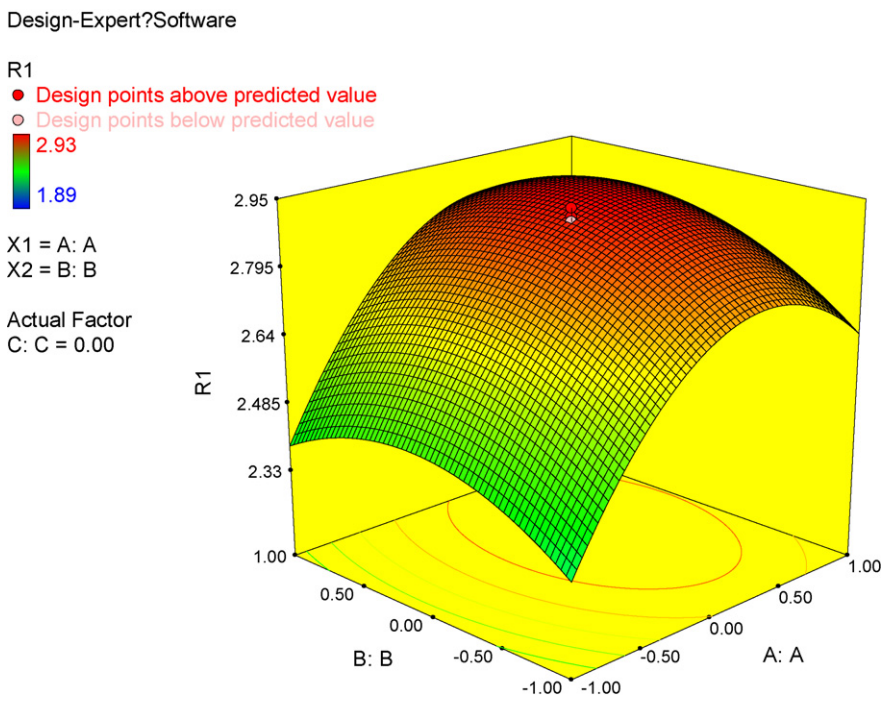

Fig. 2. Response surface showing the effect of extraction temperature ($X1$) and extraction time ($X2$) on tea glycan production.

The coefficient of determination (R^2) for production of tea glycan is 0.9350 (Table 2). This observed variation in production of tea glycan (2.98%) can be explained by the fitted model Eq. (3). This value shows a good agreement between experimental observations and predicted values.

Linear term extraction temperature ($X1$), linear term of ratio of liquid to solid ($X3$), and quadratic term of extraction temperature ($X1X1$), extraction time ($X2X2$) and ratio of liquid to solid ($X3X3$) showed the largest effect ($P < 0.0001$) on extraction yield (Table 2). These results again showed significant ($P < 0.005$) effects of these parameters (extraction temperature and ratio of liquid to solid) on extraction yield.

The three-dimensional graph and contour plot obtained from the calculated response surface are indicated in Figs. 2 and 4. It is evident from the plot that extraction yield of tea glycan reached the maximum at a combination of coded level 1.68 ($x1$), 0 ($x2$) and 1.68 ($x3$). This was a reconfirmation that the fitted surface had a maximum point, which was 98 °C, 50 min and 6:1 ratio of solvent to solid. The model predicted the maximum extraction yield of tea glycan at 2.95%.

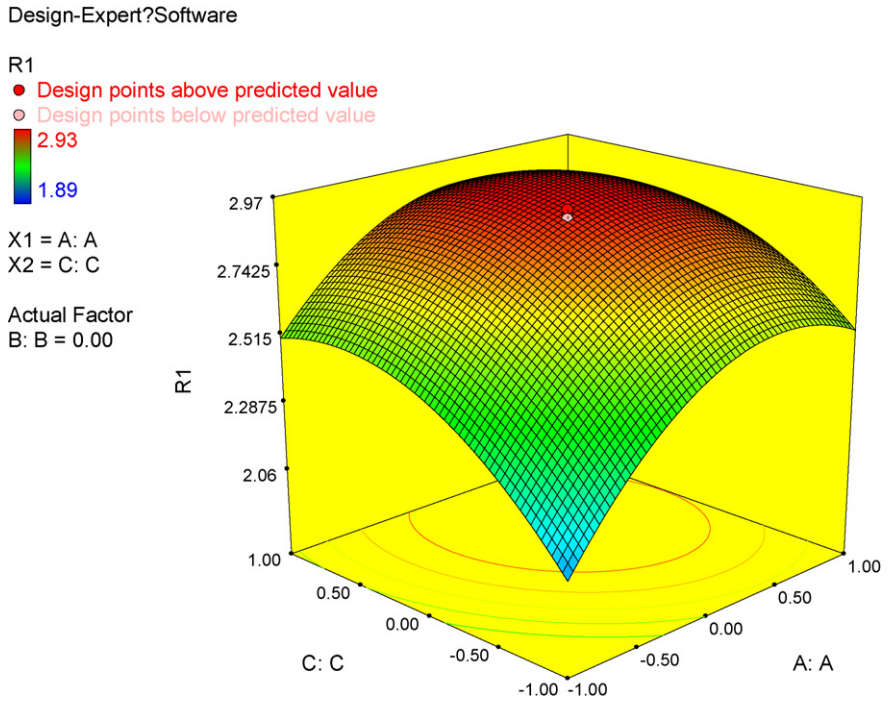

Fig. 3. Response surface showing the effect of extraction temperature ($X1$) and ratio of liquid to solid ($X3$) on tea glycan production.

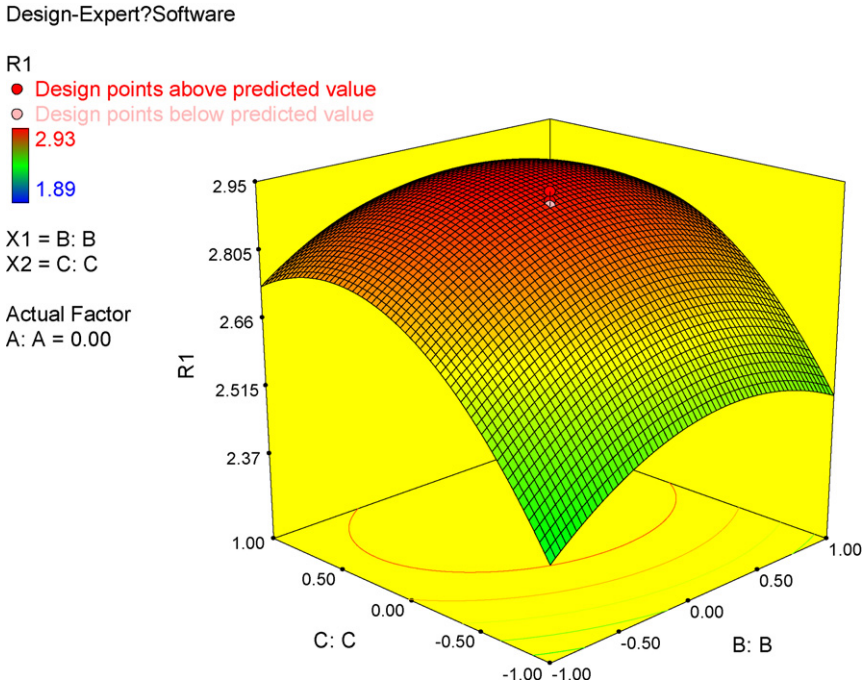

Fig. 4. Response surface showing the effect of extraction time (*X*2) and ratio of liquid to solid (*X*3) on tea glycan production.

3.2. Chemical composition

Average molecular weight of tea glycan was 8.3×10^5 Da. Chemical colorimetry analysis showed that the glycan contained 93.11% of carbohydrate polymers, 3.02% of protein, and 1.06% of nucleic acids. Monosaccharide composition analysis indicated that tea glycan was composed of rhamnose (Rha), arabian (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc) in molar ratios of 1.06:2.31:5.17:0.91:3.06:4.24.

3.3. Antitumor activity in vitro

Aqueous alcohol extract of tea plant root was reported to have a significant antitumor effect on ascites (Sur & Ganguly, 1994) and solid tumors (Chaudhuri et al., 1998). In addition, seed extracts of *C. sinensis* are known to have strong growth inhibition activity toward tumor cell lines at a relatively low concentration (Yoon, Choi, Lee, & Kim, 2005). Consumption of tea extract also inhibited tumor cell growth and metastasis in mice (Das, Sur, Gomes, Vedasiromoni, & Ganguly, 2002; Gupta, Hastak, Ahmad, Lewin, & Mukhtar, 2001). Many mechanisms have been proposed for the inhibition of carcinogenesis by tea (Kuo & Lin, 2003; Yang et al., 2002). 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.

Cytotoxic effects of ethanol extracts and glycan of tea were studied using the MTT assay. Ethanol extracts showed a dose dependent (Fig. 5) and time dependent (Fig. 6) inhibitory effect on the growth of SKOV-3 cells. Based on the OD values determined, the antitumor values of tea extract in human tumor cells were calculated and the results are shown in Figs. 5 and 6. Ethanol extract of tea showed strong antitumor activity against the SKOV-3 cells tested. However, tea glycan displayed weak antitumor activity against the SKOV-3 cells tested. Because polyphenol was most active antitumor constituent and was found at highest concentration in the ethanol

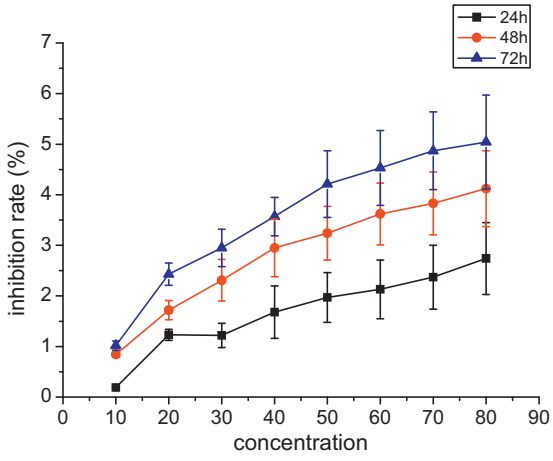

Fig. 5. Antitumor activity of glycan of tea.

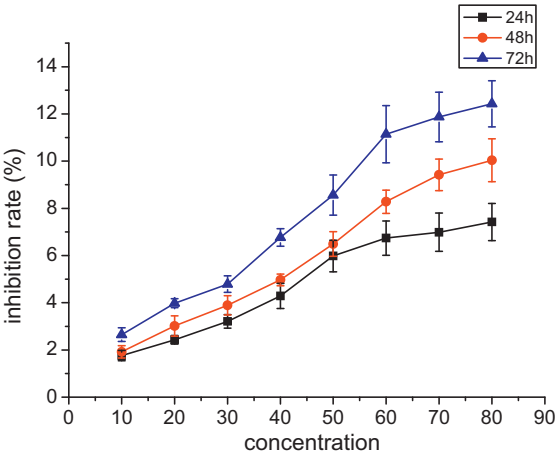

Fig. 6. Antitumor activity of ethanol extract of tea.

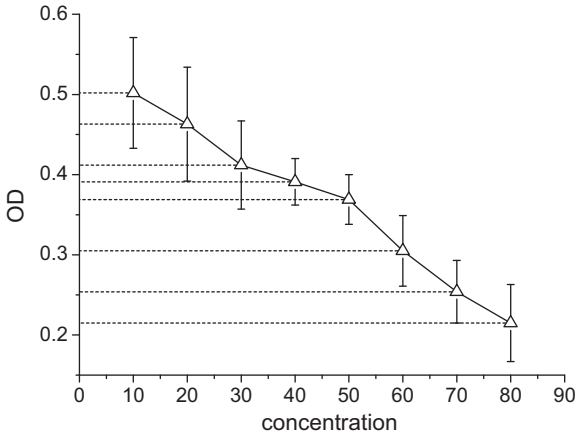

Fig. 7. Effect of ethanol extract of tea on the Bcl-2 level in SKOV-3 cells.

extract of tea, the total polyphenol fraction from green tea might be the most useful fraction from the perspective of the development of antitumor medicine. Ethanol extracts showed a dose dependent (Fig. 7) inhibitory effect on the Bcl-2 level in SKOV-3 cells. Treatment with ethanol extract for 72 h resulted in significant decrease in the Bcl-2 level in SKOV-3 cells in comparison to the control (Fig. 7).

In conclusion, the results of this research indicated that ethanol extract of tea could inhibit proliferation of human cancer cells and show strong cytotoxicity against ovarian cancer SKOV-3 cells. Ethanol extract of tea provoked different effects on SKOV-3 cells including down-regulation of cyclin A, E, D1 and CDK2 with cell-cycle arrest in S phase and triggered apoptosis via up-regulation of Bax, down-regulation of Bcl-2 and Bax translocation with mitochondrial release of cytochrome c into the cytosol and activation of effector caspase-3. However, further studies on antitumor activity in vivo of the total tea polyphenol fraction need to be performed to confirm this hypothesis.

References

Abuharbeid, S., Apel, J., Zugmaier, G., Knabbe, C., & Sander, M. (2005). Inhibition of HER-2 by three independent targeting strategies increases paclitaxel resistance of SKOV-3 ovarian carcinoma cells. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *371*, 141–151.

Baek, S. J., Kim, J. S., Jackson, F. R., Eling, T. E., McEntee, M. F., & Lee, S. H. (2004). Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis*, *25*(12), 2425–2432.

Balasubramanian, S., Sturniolo, M. T., Dubyak, G. R., & Eckert, R. L. (2005). Human epidermal keratinocytes undergo (−)-epigallocatechin-3-gallate-dependent differentiation but not apoptosis. *Carcinogenesis*, *26*(6), 1100–1108.

Brum, D. M., Cassella, R. J., & Pereira Netto, A. D. (2008). Multivariate optimization of a liquid–liquid extraction of the EPA-PAHs from natural contaminated waters prior to determination by liquid chromatography with fluorescence detection. *Talanta*, *74*, 1392–1399.

Chaudhuri, T., Sur, P., Gomes, A., Das, S. K., Das, M., & Ganguly, D. K. (1998). Effect of tea root extract (TRE) on solid tumours induced by 3-methylcholanthrene in mice. *Phytotherapy Research*, *12*, 62–64.

Das, M., Sur, P., Gomes, A., Vedasiromoni, J. R., & Ganguly, D. K. (2002). Inhibition of tumor growth and inflammation by consumption of tea. *Phytotherapy Research*, *16*, 40–44.

Eckert, R. L., Crish, J. F., Efimova, T., & Balasubramanian, S. (2004). Antioxidants regulate normal human keratinocyte differentiation. *Biochemical Pharmacology*, *68*(6), 1125–1131.

Gupta, S., Hastak, K., Ahmad, N., Lewin, J. S., & Mukhtar, H. (2001). Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 10350–10355.

Hsu, S., Yamamoto, T., Borke, J., Walsh, D. S., Singh, B., Rao, S., et al. (2006). Improved Taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biology International*, *30*, 262–269.

Kim, J. S., Laskowich, E. R., Michon, F., Kaiser, R. E., & Arumugham, R. G. (2006). Monitoring activation sites on polysaccharides by GC–MS. *Analytical Biochemistry*, *358*, 136–142.

Kuo, P. L., & Lin, C. C. (2003). Green tea constituent (−)-epigallocatechin-3-gallate inhibits Hep G2 proliferation and induces apoptosis through p53-dependent and fas-mediated pathways. *Journal of Biomedical Science*, *10*, 219–227.

Lee, J. H., Shim, J. S., Lee, J. S., Kim, J. K., Yang, I. S., Chung, M. S., et al. (2006). Inhibition of pathogenic bacterial adhesion by acidic polysaccharide from green tea (*Camellia sinensis*). *Journal of Agricultural and Food Chemistry*, *54*(23), 8717–8723.

Lung, H. L., Ip, W. K., Wong, C. K., Mak, N. K., Chen, Z. Y., & Leung, K. N. (2002). Antiproliferative and differentiation-inducing activities of the green tea catechin epigallocatechin-3-gallate (EGCG) on the human eosinophilic leukemia EoL-1 cell line. *Life Science*, *72*(3), 257–268.

Pan, Z.-W., Wang, H.-Q., & Zhong, J.-J. (2000). Scale-up study on suspension cultures of *Taxus chinensis* cells for production of taxane diterpene. *Enzyme and Microbial Technology*, *27*, 714–723.

Schepetkin, I. A, & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*(3), 317–333.

Singh, A. K., Seth, P., Anthony, P., Husain, M. M., Madhavan, S., Mukhtar, H., et al. (2002). Green tea constituent epigallocatechin-3-gallate inhibits angiogenic differentiation of human endothelial cells. *Archives of Biochemistry and Biophysics*, *401*(1), 29–37.

Sur, P., & Ganguly, D. K. (1994). Tea plant root extract (TRE) as an antineoplastic agent. *Planta Medica*, *60*, 106–109.

Teicher, B. A., Menon, K., Alvarez, E., Shih, C., & Faul, M. M. (2002). Antiangiogenic and antitumor effects of a protein kinase Cβ inhibitor in human breast cancer and ovarian cancer xenografts. *Investigational New Drugs*, *20*, 241–251.

Wang, D. W., Wang, C. W., Li, J. L., & Zhao, G. Z. (2001). Components and activity of polysaccharides from coarse tea. *Journal of Agricultural and Food Chemistry*, *49*(1), 507–510.

Xu, Y., Ho, C. T., Amin, S. G., Han, C., & Chung, F. L. (1992). Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis in A/J mice by green tea and its major polyphenol as antioxidants. *Cancer Research*, *52*, 3875–3879.

Yang, C. S., Maliakal, P., & Meng, X. (2002). Inhibition of carcinogenesis by tea. *Annual Review of Pharmacology and Toxicology*, *42*, 25–54.

Yoon, W. H., Choi, J. H., Lee, K. H., & Kim, C. H. (2005). Antimicrobial and antitumor activities of seed extracts of *Camellia sinensis* L. Kor. *Journal of Food Science and Technology*, *37*, 108–112.

Zamboni, W. C., Strychor, S., Joseph, E., Parise, R. A., & Egorin, M. J. (2008). Tumor, tissue, and plasma pharmacokinetic studies and antitumor response studies of docetaxel in combination with 9-nitrocamptothecin in mice bearing SKOV-3 human ovarian xenografts. *Cancer Chemotherapy and Pharmacology*, *62*, 417–426.

Zhang, L., Gong, M.-Y., Li, Y.-X., Zhang, L.-G., & Wang, X.-Y. (2011). Apoptosis of hepatoma cell line HepG2 induced by the combination of radiotherapy and thermotherapy and its relationship with Bcl-2/Bax protein expressions. *Chinese General Practice*, *14*(2), 627–630.