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Structure and antitumor (LOVO) activity of Cortex moutan heteroglycan and Curcumin

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ABSTRACT

The Box–Behnken design combined with response surface methodology was used to optimize the extraction of curcumin. The optimized results showed that the highest extraction yield of curcumin could arrive 1.12%. The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. The predicted extraction yield of heteroglycan was 1.13%, which was consistent with the practical extraction yield of heteroglycan of 1.17%. Cortex moutan heteroglycan (500 mg) was applied to a column (3.2 cm × 32 cm) of DEAE Sepharose CL-6B (Cl^-) that was equilibrated with H_2O. The two adsorbed fractions (Fraction I and Fraction II) were obtained as lyophilisate after dialysis The antitumor activities of the Cortex moutan heteroglycan and curcumin were evaluated in vitro. The results indicated that the Cortex moutan heteroglycan and curcumin could inhibit colorectal carcinoma (LOVO) cells growth and stimulate apoptosis. Moreover, antitumor activities of curcumin was stronger than that of Cortex moutan heteroglycan. This indicated that curcumin was useful for therapy of colorectal carcinoma.

1. Introduction

Moutan Cortex ("Mok-Dan-Pi" in Korean), the root bark of Paeonia suffruticosa Andrew (Paeoniaceae), is an analgesic, sedative, and anti-inflammatory agent that has been used as a remedy for cardiovascular disorders, extravagated blood, and female genital diseases (Ding et al., 1999). The drug has also been used for gynaecological disease, and to relieve neuropathic pain. MCR reportedly inhibits IL-8 and MCP-1 secretions (Oh et al., 2003; Tatsumi et al., 2004), and reactive oxygen species production (Rho et al., 2005). Although Moutan Cortex is a well known herb found in anti-diabetic traditional Chinese medicine formulae (Shimizu, Zenko, Tanaka, Matsuzawa, & Morita, 1993), the extract ingredients of Moutan Cortex responsible for the hypoglycemic effect and the underlying molecular mechanisms of their actions have not been systematically investigated (Chan et al., 2007). Numerous monoterpenes and acetophenones have been isolated from this plan (Kaneda, Iitaka, & Shibata, 1972; Shimizu et al., 1983; Fukuhara & Yoshida, 1987; Kuwajima et al., 1996; Lemmich, 1996; Lin, Ding, Wu, & Wu, 1996; Ha et al., 2009).

Curcumin
, commonly called diferuloyl methane, is a hydrophobic polyphenol derived from the rhizome (turmeric) of the herb Curcuma longa. Turmeric has been used traditionally for many ailments because of its wide spectrum of pharmacological activities. Curcumin has been identified as the active principle of turmeric; chemically, it is a bis-α, β-unsaturated β-diketone that exhibits keto–enol tautomerism (Fig. 1). Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities. It also has hepatoprotective and nephroprotective activities, suppresses thrombosis, protects against myocardial infarction, and has hypoglycemic and antirheumatic properties. Moreover, curcumin has been shown in various animal models and human studies to be extremely safe even at very high doses (Aggarwal, Kumar, & Bharti, 2003; Goel, Jhurani, & Aggarwal, 2008a; Anand, Kunnumakkara, Newman, & Aggarwal, 2007; Aggarwal and Harikumar, 2009; Anand, Sundaram, Jhurani, Kunnumakkara, & Aggarwal, 2008; Goel, Kunnumakkara, & Aggarwal, 2008b).

The Box–Behnken is a second-order multivariate technique based on three-level incomplete factorial designs that received a wide application for assessment of critical experimental conditions, that is, maximum or minimum of response function. The number of experiments (N) needed for the development of Box–Behnken matrix is defined as $N = 2k\ (k-1) + C0$, where (k) is the factor number and (C0) is the replicate number of the central point (Montgomery, 1997; Box, Hunter, & Hunter, 1997; Santelli, Bezerra, SantAna, Cassella, & Ferreira, 2006).

Colorectal cancer accounts for >90% of malignant tumors of the large intestine and is the third most common cause of death from malignant disease in the Western world (Shen & Falzon, 2005). It

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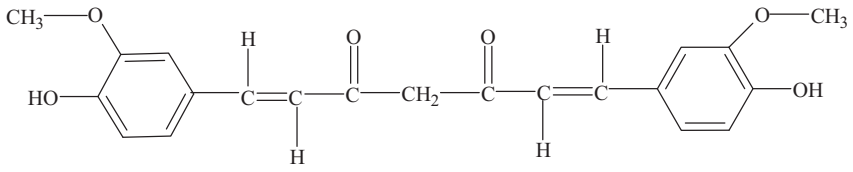

Fig. 1. Chemical structure of Curcumin.

was reported that extract of Moutan Cortex and Curcumin inhibited LoVo cells. The aim of the work is to analyze the influence of different parameters such as time, temperature, and ratio of liquid to solid and extraction number on the extraction yield. A Box–Behnken experimental design method was used to investigate the effects of various parameters on the extraction performance. In this study, we examined the direct effects of Moutan Cortex and Curcumin on human colorectal cancer cells (Lovo cells) growth.

2. Materials and methods

2.1. Extraction of Cortex moutan heteroglycan

The dry Cortex moutan (50 g) was firstly extracted with 500 mL distilled water at 100 °C for 2 h. The extracted slurry was centrifuged at 10,000 rpm/min for 20 min to collect the supernatant, and the insoluble residue was treated again for 2–3 times as mentioned above. The supernatant was incorporated and concentrated to one-fifth of initial volume using a rotary evaporator (SENCO Technology and Science Inc., Shanghai, China) at 55 °C under vacuum. The resulting solution was mixed with four volumes of dehydrated ethanol (ethanol final concentration, 80%) and kept overnight at 4 °C. Then the solution was centrifuged at 10,000 rpm/min for 20 min, washed six times with dehydrated ethanol and the precipitate was collected as Cortex moutan heteroglycan.

2.2. Isolation, fractionation, and purification of crude extracts

Cortex moutan heteroglycan (500 mg) was applied to a column (3.2 cm × 32 cm) of DEAE Sepharose CL-6B (Cl^-) that was equilibrated with H_2O. The column was first eluted with H_2O until sugar was no longer detected. The two adsorbed fractions (Fraction I and Fraction II) were obtained as lyophilisate after dialysis (Fig. 2).

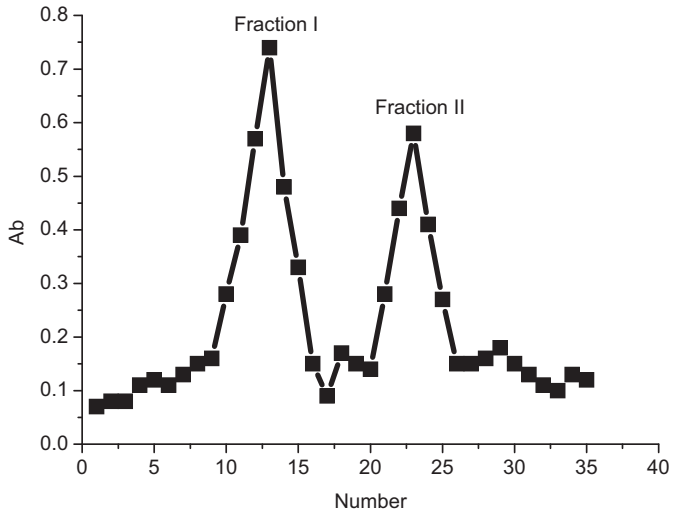

Fig. 2. Elution curve of Cortex moutan heteroglycan separated by DEAE Sepharose CL-6B column.

2.3. Capillary electrophoresis procedure

Before first use, a new capillary was conditioned by flushing with 1MNaOH, 0.1 M NaOH and water for 10 min each, and finally for 30 min with the running buffer. Between runs, the capillary was flushed with running buffer for 5 min. At the end of a working session, the capillary was rinsed with 0.1 M NaOH for 5 min, water for 5 min and N_2 for 1 min. The optimum running buffer was 20 mM acetic acid/sodium acetate, pH 5.0, with 1.2 mMI_2 and 7.2 m MKI. Standards or samples were injected hydrodynamically for 2 s at 3400 Pa (0.5 psi). Separations were performed at 25 °C, and the applied voltage was 20 kV. Detection of iodine complexes was at 560 nm, although the entire spectra in the range 300–600 nm were recorded.

2.4. Box–Behnken design

The Box–Behnken design is used in order to optimize the number of experiments to be carried out to ascertain the possible interactions between the studied parameters and their effects on the extraction yield of curcumin. The Box–Behnken design is a spherical, revolving design; it consists of a central point and the middle points of the edges of the cube circumscribed on the sphere (Evans, 2003). It is a three level fractional factorial design consisting of a full 22 factorial seeded into a balanced incomplete block design. It consists of three interlocking 22 factorial designs having points, all lying on the surface of a sphere surrounding the center of the design. It has been applied in the optimization of several chemical and physical processes, and the number of experiments are decided accordingly (Kumar, Prasad, & Mishra, 2007).

In the present study, the Box–Behnken experimental design is applied to investigate and validate the treatment process parameters affecting extraction yield of curcumin. Extraction temperature ($X1$), extraction time ($X2$), ratio of liquid to solid ($X3$) and particle size ($X4$) are input variable parameters. The interval of the allowed values for these factors was deduced from the preliminary tests carried out. The factor levels were coded as – 1 (low), 0 (central point or middle) and 1 (high).

The extraction yield of curcumin, Y was designed as a response of the studied system. For this response (Y), a polynomial model of the second degree is established to quantify the influence of the variables.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y is the process response or output (dependent variable), k is the number of the patterns, i and j are the index numbers for pattern, β_0 is the free or offset term called intercept term, $x_1, x_2, \ldots, x_k$ are the coded independent variables, β_i is the first-order (linear) main effect, β_{ii} is the quadratic (squared) effect, β_{ij} is the interaction effect, and ε is the random error or allows for discrepancies or uncertainties between predicted and measured values. In develop-

Table 1
Box–Behnken design for optimization of extraction yield.

Run	Extraction time (min) *X*1	Ratio of liquid to solid *X*2	Extraction temperature (C) *X*3	Extraction number *X*4	Extraction yield (g/kg) *Y*1
1	−1 (80)	−1 (8)	0 (80)	0 (4)	8.79
2	−1	1 (10)	0	0	9.58
3	1 (120)	−1	0	0	9.51
4	1	1	0	0	10.69
5	0 (100)	0 (9)	−1 (70)	−1 (3)	8.91
6	0	0	−1	1 (5)	9.54
7	0	0	1 (90)	−1	8.95
8	0	0	1	1	9.83
9	−1	0	0	−1	8.82
10	−1	0	0	1	9.51
11	1	0	0	−1	9.49
12	1	0	0	1	10.68
13	0	−1	−1	0	9.01
14	0	−1	1	0	9.07
15	0	1	−1	0	9.55
16	0	1	1	0	9.63
17	−1	0	−1	0	8.83
18	−1	0	1	0	8.89
19	1	0	−1	0	8.88
20	1	0	1	0	9.62
21	0	−1	0	−1	8.86
22	0	−1	0	1	9.43
23	0	1	0	−1	9.46
24	0	1	0	1	10.63
25	0	0	0	0	10.99
26	0	0	0	0	10.93
27	0	0	0	0	10.91

ing Eq. (1), the natural (uncoded) independent variables ($X_1, X_2, \ldots, X_k$) are coded according to the following transformation:

$$\frac{x_i(X_i - X_0)}{\Delta X_i} \quad (2)$$

where x_i is dimensionless coded value of the ith independent variable, X_i is the uncoded value of the ith independent variable, X_0 is the uncoded ith independent variable at the center point, and ΔX_i is the step change value.

2.5. Cells and culture conditions

Lovo cell lines were obtained from the Shanghai Institute of Cell Biology, Shanghai, China. The cells were incubated in DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere with 5% CO_2 in air at 37 °C. Then, cells in the logarithmic growth phase were collected for the following experiments.

2.6. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay

Cells in the logarithmic growth phase were plated at a density of 6×10^4 cells/mL in 96-well plates. Twenty-four hours later, the cells were incubated with various concentrations of extracts. At times of 24, 48, and 72 h after addition of cinobufacini, 20 μL of MTT (5 mg/mL) was added to each well and then the plates were incubated for another 4 h at 37 °C. Then, water-insoluble formazan was dissolved by adding 100 μL dimethyl sulfoxide (DMSO) to each well. Finally, optical density (OD) was monitored at 490 nm with 570 nm as a reference wavelength using a Bio-Rad Model 680 micro plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The inhibitory rate (IR) were then calculated (Xu et al., 2008).

2.7. Flow cytometry analysis

The Annexin V Apoptosis kit was used to assay apoptosis according to the manufacturer's instructions. LOVO cells were seeded in 6-well plates (2×10^5 cells/well) and incubated with various concentrations of extracts for 72 h. To collect the cells, 2 mL of trypsin–EDTA solution (0.05% trypsin and 0.53 mM EDTA; GIBCO Invitrogen) was added for another 5 min at 37 °C. After centrifuging at 400 × *g* for 5 min, the cell pellets were obtained. The cell pellets were washed twice with cold Hanks' Balanced Salt Solution (HBSS; GIBCO Invitrogen) and then resuspended in Solution A at 1×10^6 cells/mL (Solutions A, B, and C were obtained from the Annexin V Apoptosis kit). The supernatant was discarded after centrifuging at 200 × *g* for 5 min at 25 °C. The cell pellets were suspended by adding 100 μL of Solution A, 2 μL of Solution B, and 5 μL of Solution C. The cells were gently agitated and incubated at 25 °C for 15 min. Finally, 400 μL of Solution A was added to each tube, and the cells were analysed by flow cytometry (CyFlow®SL, Partec, Germany).

3. Results and discussion

3.1. Monosaccharide compositions of Fraction I and II

Carbohydrates and their derivatives are not normally electroactive at carbon electrodes, the most commonly used working electrode in ED. Thus, a variety of metal electrodes made of copper, nickel, gold, and platinum have been employed for the electrochemical detection of carbohydrates and their derivatives (Ye and Baldwin, 1994; Luo, Prabhu, & Baldwin, 1990; Chen and Huang, 1997; You et al., 2003). Among them, copper and nickel are most widely utilized. Carbohydrates and their derivatives can be detected by copper electrodes at a constant applied potential in strongly alkaline media based on electrocatalytic oxidation (Ye and Baldwin, 1994; Luo et al., 1990). The average recoveries and the corresponding R.S.D. were 96.7% and 3.5% for paeoniflorin, 98.1% and 2.7% for sucrose, 97.9% and 3.1% for paeonoside, 101.3% and 3.3% for glucose, and 97.7% and 2.7% for fructose, respectively (Chen, Zhang, & Zhu, 2006).

Fraction I or II of Cortex moutan heteroglycan were analysed using capillary electrophoresis. In comparison with the retention time of standards, the monosaccharide composition was identified.

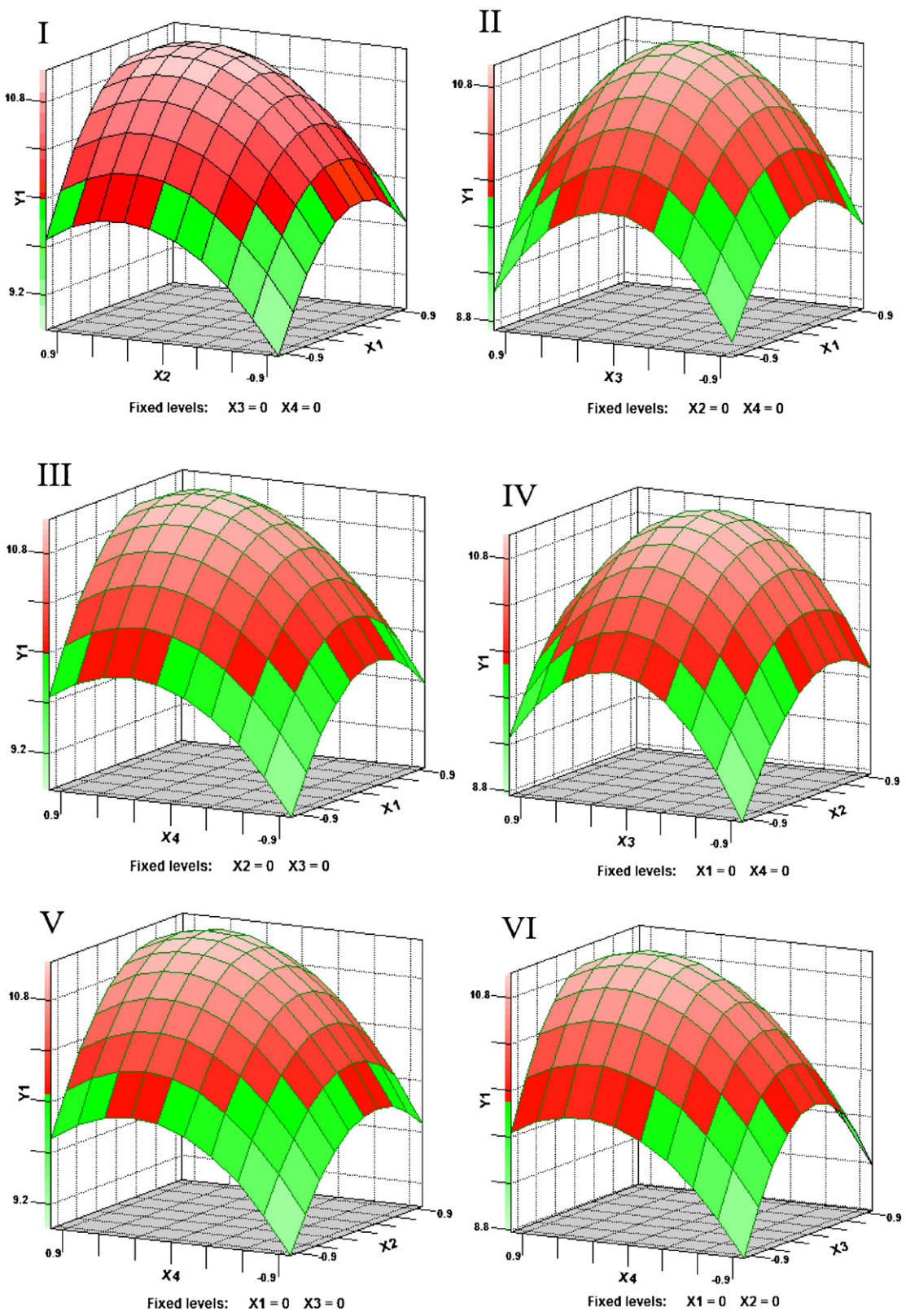

Fig. 3. The response surface plots.

Four monosaccharides, including arabinose, fructose, glucose and galactose, were identified for Fraction I, while arabinose, fructose, glucose, galactose and xylose were detected for Fraction II. Molecular weight of Fraction I was 5×10^5. Molecular weight of Fraction II was 4×10^3.

3.2. Optimization for extraction yield

RSM was employed to check the best operating parameters and decide optimum operating conditions of the extraction process. Four variables (extraction temperature ($X1$), extraction time ($X2$),

Table 2
Coefficients and *t*-values for extraction production using Box–Behnken design.

Effect	Estimate	Std. error	*t* ratio	*P* value
*X*1	0.37083	0.058131	6.3793	<0.0001
*X*2	0.40583	0.058131	6.9814	<0.0001
*X*3	0.10583	0.058131	1.8206	0.0937
*X*4	0.4275	0.058131	7.3541	<0.0001
*X*1*X1	−0.73375	0.087196	−8.415	<0.0001
*X*1*X2	0.0975	0.10069	0.96837	0.3520
*X*1*X3	0.17	0.10069	1.6884	0.1171
*X*1*X4	0.125	0.10069	1.2415	0.2381
*X*2*X2	−0.61875	0.087196	−7.0961	<0.0001
*X*2*X3	0.005	0.10069	0.04966	0.9612
*X*2*X4	0.15	0.10069	1.4898	0.1621
*X*3*X3	−1.0563	0.087196	−12.114	<0.0001
*X*3*X4	0.0625	0.10069	0.62075	0.5464
*X*4*X4	−0.63125	0.087196	−7.2394	<0.0001
	Master model	Predictive model		
Mean	9.592222	9.592222		
R-square	96.51%	96.51%		
Adj. *R*-square	92.43%	92.43%		
RMSE	0.20137	0.20137		
CV	2.099308	2.099308		

ratio of liquid to solid (*X*3) and particle size (*X*4)) were studied by Box–Behnken design at three levels and their interactions on extraction production were determined. The highest output of lactic acid production observed was 10.99 g present in 1000 g of raw material as seen at run 25 (Table 1). The Student's *t*-distribution and the corresponding values along with the estimated parameters are given in Table 2. The probability (*P*) values were used as a tool to check the significance of each of the coefficients. The results show that among the independent variables, extraction temperature (*X*1), extraction time (*X*2), and particle size (*X*4) have significant effect as they have positive coefficients (Table 2) according to which, increase in their concentrations can increase the production yield. Among the interactions, none are highly significant as indicated by the positive coefficients (Table 2). In order to determine the maximum extraction yield corresponding to the optimum levels of extraction temperature (*X*1), extraction time (*X*2), ratio of liquid to solid (*X*3) and particle size (*X*4), a second order polynomial model was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, a second order polynomial model (I) explains the role of each variable and their second order interactions in producing.

$$\begin{aligned} Y1 &= 10.94333 + 0.370833 * X1 + 0.405833 * X2 \\ &+ 0.105833 * X3 + 0.4275 * X4 - 0.73375 * X1 * X1 \\ &+ 0.0975 * X1 * X2 + 0.17 * X1 * X3 + 0.125 * X1 * X4 \\ &- 0.61875 * X2 * X2 + 0.005 * X2 * X3 + 0.15 * X2 * X4 \\ &- 1.05625 * X3 * X3 + 0.0625 * X3 * X4 - 0.63125 * X4 * X4 \end{aligned} \quad (3)$$

ANOVA results of the quadratic model presented in Table 2 indicated that the model equation sufficiently describes the response surface of extraction yield in the interval of investigation. Parameters like *F*-value, probability > *F*, lack of fit, *R*-squared are statically obtained values, which are the measure of how the predicted model fit the experimentally monitored data. It should be noted that the effect of each variable on the response is the combination of coefficients and variable values as well as the contribution of joint effect of variables, which cannot be observed by traditional optimization methods.

The three-dimensional response surfaces estimate extraction yield over independent variables are shown in Fig. 3I–VI. These figures depict the relative effects of two variables on the extraction yield when the third variable was kept constant in their zero level.

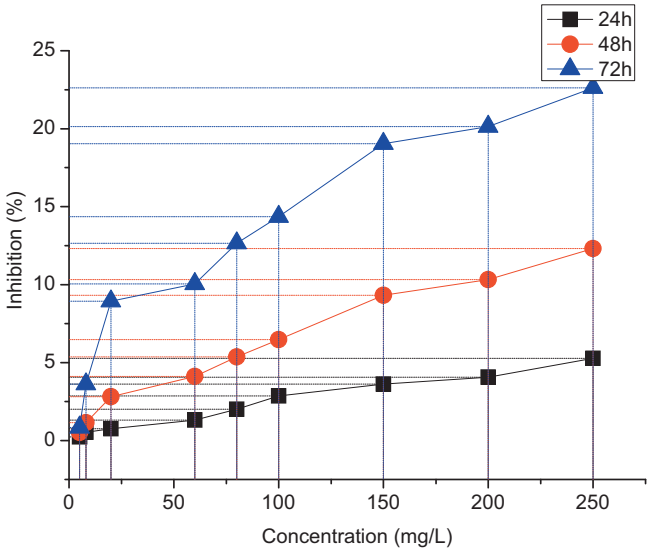

Fig. 4. Antitumor activities of Cortex moutan heteroglycan.

In Fig. 3I, The extraction yield increased with the increasing time or ratio of liquid to solid to its peak, but then decreased with a further increase in time or ratio. Fig. 3II also indicates that the variation of extraction time remarkably affects the extraction yield, while the extraction temperature is less important. As shown in Fig. 3VI, the variation of extraction number is relatively more important than that of extraction temperature on extraction yield. Overall, the important degree of three variables on extraction yield is: extraction time > ratio of liquid to solid > extraction number > extraction temperature.

3.3. Inhibition of LOVO cells growth

To determine the antitumor activity of all extracts against LOVO cells, cytotoxicity MTT assay was carried out, and net growth inhibition was calculated comparing to a negative control growth. Of all extracts tested in Cortex moutan heteroglycan at maximum concentration (250 mg/L), Cortex moutan heteroglycan showed the highest activity (22.56% of control growth). Antitumor activities of Cortex moutan heteroglycan increased with increasing incubation

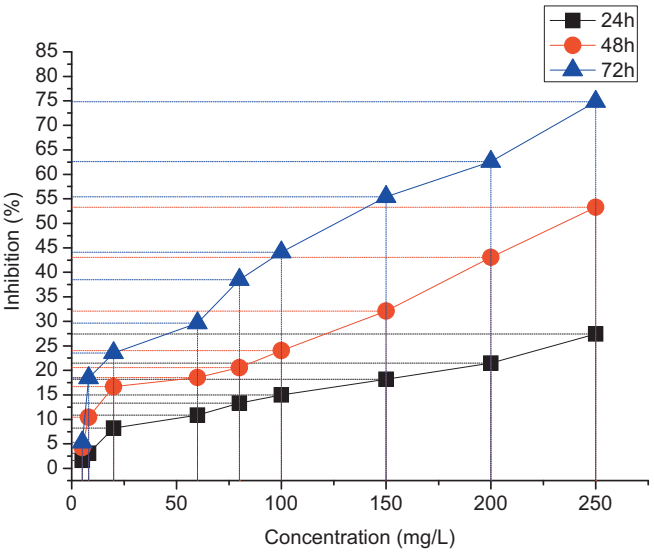

Fig. 5. Antitumor activities of Curcumin.

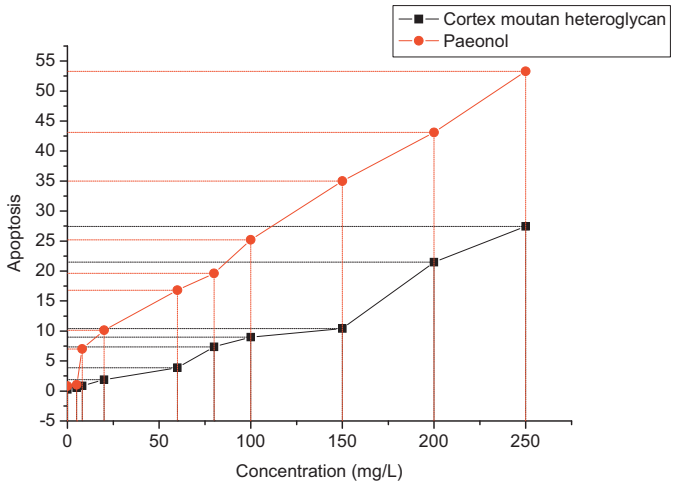

Fig. 6. Effect of Cortex moutan and Curcumin on LOVO apoptosis.

time (Fig. 4). No significative antitumor activity was shown at the lowest concentration of curcumin tested (10 μg/mL), but curcumin at high concentration (250 mg/L) showed strong antitumor activity (75.77%) (Fig. 5).

3.4. Apoptosis assay

Fig. 6 shows the effect of curcumin on apoptosis of LOVO cells. The three independent measurements made after 72 h of exposure to curcumin revealed that the percentages of apoptotic cells (annexin V+/PI− Fraction) increased with increasing concentration of curcumin and Cortex moutan heteroglycan (Fig. 6). At the dose of 250 mg/L, apoptosis were 25.43 and 53.01%, respectively.

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