

Improvement in β -glucan extraction from *Ganoderma lucidum* with high-pressure steaming and enzymatic pre-treatment

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Abstract In this study, the high-pressure steaming and enzymatic pre-treatment (SET) was used to improve β -glucan extraction from *Ganoderma lucidum* (*G. lucidum*), an oriental medicinal mushroom. Response surface methodology and central composite design were used to determine the optimum pre-treatment conditions: high-pressure steaming, enzymatic hydrolysis, and Viscozyme L concentrations. The optimal conditions were 15.51 min for high-pressure steaming, 0.84 g/100 mL of Viscozyme L, and 4.16 h for hydrolysis. The predicted β -glucan content in *G. lucidum* extract at optimal conditions, approximately twofold (8.05 g/100 g) of the control treatment value, was consistent with the empirical value. The total sugar and protein contents through SET were higher than those values of the control treatment. The cell migration assay showed that SET-processed *G. lucidum* extracts significantly suppressed B16F10 murine melanoma cell growth. SET process using Viscozyme L could be utilized for β -glucan extraction from *G. lucidum* to develop the functional food.

Keywords Anti-metastatic effect · *Ganoderma lucidum* · β -Glucan · Steaming · Viscozyme L

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Introduction

Ganoderma lucidum, which is an oriental mushroom belonging to Basidiomycetes, has several medicinal properties and has been used in traditional medicine in many Asian countries, including Korea [1]. The mushroom is rich in carbohydrates, proteins, lipids, minerals, and vitamins [2], and its medicinal effects are attributed to its polysaccharide, triterpene, polyphenol, nucleoside, and steroid contents [3]. The polysaccharides of *G. lucidum* have antioxidant [4], anti-aging [5], and anti-tumor effects [6, 7]; they also boost the immune system [8] and reduce blood sugar and lipid levels [9, 10]. β -Glucan, in particular, inhibits cancer cell growth by activating the immune response in normal cells [11]. Studies have shown that β -glucan promotes the production of interferon gamma and interleukin-12 (IL-12) in lymphocytes [12] and induces the T helper 1 reaction by increasing the production of interleukin-1 (IL-1), tumor necrosis factor alpha (TNF- α), and nitric oxide [13].

β -Glucan consists of β -D-glucose polysaccharides, which occurs naturally in the cell walls of mushrooms and fungi group [14]. The most common form of β -glucan is D-glucose with β - 1 \rightarrow 3 linkages, occasionally with β - 1 \rightarrow 6 branching side chains in certain structures [15]. The extent of branching in β -glucan affects its molecular weight, solubility, and physiological functions [16]. Generally, hot water extraction is used for extracting β -glucan for subsequent use as a healthcare product [17]. Various strains of *G. lucidum* contain 12.4–19.0 g/100 g of β -glucan [11]. However, the yield of β -glucan obtained by hot water extraction is considerably low because of the solid woody tissue of *G. lucidum*. The yield of extracted β -glucan increases with temperature [18], supercritical fluids

[19], and pressurized hot water [20, 21] from mushrooms. However, there is a paucity of research on the β -glucan extraction from *G. lucidum* by the combined pre-treatment with the high-pressure steaming and hydrolyzing enzymes which facilitate the extraction of polysaccharides by weakening the hydrogen bonding within plant tissues and cell walls [22, 23].

In this study, we improved the yield of β -glucan extracted from *G. lucidum* using SET. Response surface methodology (RSM) and central composite design (CCD) were used to determine the optimal conditions of extraction, and the physicochemical properties and anticancer activities of the extracts were determined.

Materials and methods

Materials and chemicals

Ganoderma lucidum mushrooms were obtained from the area of cultivation (Chilgok, Korea) and were stored at 4 °C. The sample for β -glucan extraction was pulverized and screened through a 25-mesh sieve to obtain a powder. Viscozyme L (*Aspergillus aculeatus*, 100 FBG/g, Novozymes A/S, Bagsvaered, Denmark) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Citric acid, sodium hydroxide, sodium acetate, potassium hydroxide, and hydrochloric acid were purchased from Duksan Pure Chemical Co. Ltd (Seoul, Korea). SET was performed using an autoclave (ILSHIN autoclave Co. Ltd, Daejeon, Korea) and a shaking incubator (SW-90R, Sang Woo Scientific Corp., Seoul, Korea).

Experimental design for response surface methodology

RSM was used to determine the optimum conditions for the pre-treatment of *G. lucidum*. Experiment and statistical analyses were performed using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). CCD with a quadratic model was employed [24]. Three independent variables, namely high-pressure steaming time (X_1), enzyme concentration (X_2), and enzymatic hydrolysis time (X_3), were selected. Each independent variable had five levels, namely -2 , -1 , 0 , $+1$, and $+2$. Sixteen different combinations (including duplicates of the center point, each assigned with the coded value 0) were selected in random according to a CCD configuration for three factors [25]. The variance for each factor was partitioned into linear, quadratic, and interactive components and was represented as:

$$Y = \beta_0 + \sum_{i=1}^j \beta_i X_j + \sum_{i=1}^j \beta_{ii} X_i^2 + \sum_{i \neq j=1}^j \beta_{ij} X_i X_j,$$

where β_0 is a constant, β_i is a linear coefficient, β_{ii} is a quadratic coefficient, β_{ij} is a cross-product coefficient, and X_i and X_j are the levels of the independent variables. Three-dimensional plots were constructed by keeping one variable constant at the center point and varying the other two variables within the experimental range. Analysis of variance (ANOVA), which is a partial F -test for individual terms, and an analysis of residuals were performed. ANOVA tables were generated, and the effects and regression coefficients of individual linear, quadratic, and interaction terms were determined. The degrees of significance of all the terms in the polynomial were determined statistically by calculating the F value at a probability (p) of 0.001, 0.01, 0.05, or 0.1.

β -Glucan extraction

A mixture of 1 g of *G. lucidum* powder in 20 mL of distilled water was subjected to different conditions of high-pressure steaming (121 °C and 15 lb psi) and enzymatic hydrolysis using the autoclave and shaking incubator (50 °C and 120 rpm), respectively. The enzymatic hydrolysis was carried out by cooling to 50 °C after the high-pressure steaming. The ranges of the variables were based on the preliminary experiment. The independent variables for the conditions of β -glucan extraction were high-pressure steaming time X_1 (0–20 min), enzyme concentration X_2 (0–1 g/100 mL), and enzymatic hydrolysis time X_3 (1–5 h). The temperatures of the enzymatic treatment were adjusted using a water bath at constant temperature. The hydrolyzed samples were adjusted to pH 10 with 1 N sodium hydroxide, followed by reflux extraction at 90 °C for 6 h. Control treatment was carried out only under reflux extraction. The final extracts were used for physicochemical analyses and analysis of inhibitory effects on cancer cells after lyophilization.

Chemical analyses

The β -glucan contents in the extracts were determined using the β -glucan assay kit (yeast & mushroom, Megazyme, Ireland) [26]. The extraction yields were determined by a drying method using an oven at 105 °C. Purity was expressed as β -glucan content per total glucan content in percentage. The protein contents were determined by the bicinchoninic acid assay [27]. The total sugar contents were determined by the phenol–sulfuric acid method [28]. After hydrolysis with hydrochloric acid, the monosaccharide composition of the extracts was analyzed using a high-

performance liquid chromatograph (HPLC, 1260 Infinity Quaternary LC System, Agilent Technologies, Waldbronn, Germany) equipped with a refractive index detector and a carbohydrate analysis column (3.9 × 300 mm, Waters, Milford, MA, USA) with 80% acetonitrile at a flow rate of 1.5 mL/min [29]. Glucose, mannose, galactose, and fructose were used as standard.

Cell culture

The B16F10 murine melanoma cell line was obtained from the American Type Culture Collection (American Type Culture Collection, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Life technologies) and a mixture (Sigma-Aldrich, St. Louis, MO, USA) of 100 units/ml penicillin and 100 µg/ml streptomycin sulfate in a humidified, 5% CO₂ atmosphere.

Proliferation assay

MTT assay was carried out to evaluate the viability of the cells cultured with the *G. lucidum* extracts [30]. In brief, the cells were plated in 96-well plates and incubated for 24 h in 100 µL of DMEM. The *G. lucidum* extract at various concentrations (1, 3, 10, 30, and 100 µg/mL) was added to the cells, and the cells were incubated for an additional 24 h. Then, 10 µL of the MTT solution (5 mg/mL MTT in phosphate-buffered saline) was added to each well, followed by incubation at 37 °C for 1 h. After removing the MTT solution, 100 µL of dimethyl sulfoxide was added to each well with vigorous mixing. The absorbance at 470 nm was measured using a Victor multi-label counter (Wallac; Turku, Finland).

Cell migration assay

The B16F10 cells, which were cultured in a 6-well plate (3.0 × 10⁵ cells/mL), were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. Then, the cells were scratched with a 200-µL pipette tip and further incubated with different concentrations of the *G. lucidum* extracts (1, 10, and 100 µg/mL) and resveratrol (10 µg/mL, positive control), which served as a positive control, for 12 h at 37 °C. Cell migration on the slit of the confluent well was assessed at 0, 6, and 12 h in each condition. The plates were photographed at 16, 24, and 40 h, and the exact width of the wound was calculated using a micro-ruler [31].

Table 1 Analysis of variance of the regression parameters for the predicted response surface quadratic models of the conditions of enzymatic hydrolysis and β-glucan contents of the *Ganoderma lucidum* extracts obtained under optimal conditions

Response	Polynomial equations	R ²	Significance	Optimum condition			Predicted value (A)	Empirical value (B)	B/A × 100
				X ₁	X ₂	X ₃			
β-Glucan (g/100 g)	$Y_1 = 1.52125 + 0.129875X_1 + 8.9875X_2 + 0.893125X_3 - 0.015X_1X_2 - 0.00765X_1X_3 - 0.185X_2X_3 - 0.0032X_1^2 - 4.78X_2^2 - 0.07625X_3^2$	0.9561	0.0020	15.51	0.84	4.16	8.22	8.05	97.93

The predicted value was calculated using the predicted equation for the response variables X₁, high-pressure steaming time (min); X₂, enzyme concentration (g/100 g); X₃, enzymatic hydrolysis time (h)

Statistical analysis

The data are expressed as mean \pm standard deviation (SD) of triplicate experiments. One-way ANOVA and Duncan's new multiple range test were performed to determine significant differences between the means with SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). p values < 0.05 were considered statistically significant.

Results and discussion

Modeling of the pre-treatment conditions for β -glucan extraction

Generally, the yield of β -glucan from *G. lucidum* is low because of the rigidity of the woody tissue. Therefore, we employed high-pressure steaming and enzymatic hydrolysis treatment to overcome this drawback. We used RSM to evaluate the interaction between the various treatment parameters and their relationship with the dependent variable [32]. Using RSM, we also generated a mathematical model with minimal experimentation and investigation for determining the optimal conditions [33]. Therefore, we used RSM to determine the optimum pre-treatment conditions in the range of the suggested variables. A duration of 10 min of high-pressure steaming, a Viscozyme L-to-substrate ratio of 0.5 g/100 mL, and a duration of 3 h of enzymatic hydrolysis were selected from the preliminary experiments as the main conditions of the central composite rotatable design. The β -glucan contents differed significantly with the treatment conditions, ranging from 4.08 g/100 g to 8.14 g/100 g. R^2 , which is the coefficient of determination, is defined as the ratio of the explained variation to the total variation, and is a measure of the degree of fitness [34]. The empirical model fits the actual data better as R^2 approaches unity. As shown in Table 1, the R^2 value for the response variable (β -glucan contents) was 0.9561, which indicated the fitness of the regression model for the pre-treatment reactions.

Improvement in β -glucan extraction from *G. lucidum* by SET

Viscozyme L is composed of a multi-enzyme complex with a wide range of hydrolyzing enzymes [35, 36]. Therefore, it is assumed that Viscozyme L might decompose cellulose and pectin substances in the *G. lucidum* cell wall and could increase the extraction yield of β -glucan. The scores of significance of the effects of high-pressure steaming time, enzyme concentration, and enzymatic hydrolysis time on β -glucan contents are shown in Table 2. Figure 1 illustrates the four-dimensional response surfaces for β -glucan contents at constant values of 5, 7, and 8 g/100 g constructed using the regression equation of the response

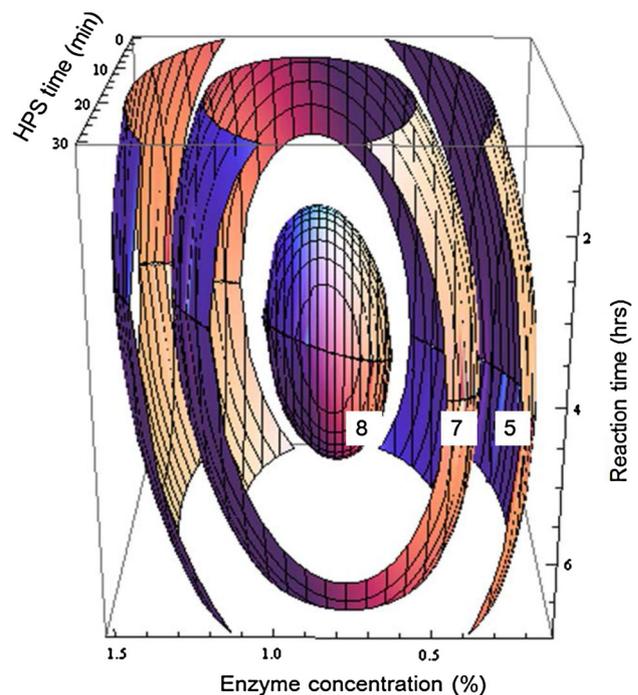


Fig. 1 Four-dimensional response surfaces for β -glucan contents (at constant values, 5, 7, 8 g/100 g) of the *Ganoderma lucidum* extracts as functions of high-pressure steaming time, ratio of enzyme concentration-to-substrate concentration, and enzymatic hydrolysis time in β -glucan extraction

Table 2 Analysis of variance of the overall effects of the pre-treatment conditions for the extraction of β -glucan from *Ganoderma lucidum*

	F value	p value	Critical value
High-pressure steaming time (X_1)	2.12	0.1962	15.51
Enzyme concentration (X_2)	27.93****	0.0005	0.84
Enzymatic hydrolysis time (X_3)	2.72	0.1319	4.16

****Significant at $p < 0.001$

surface analysis. It graphically demonstrates the effect of variables individually and in combination, and shows the nature of the stationary point of the fitted surface. As shown in Table 2 and Fig. 1, the β -glucan contents were significantly affected by enzyme concentration ($p < 0.001$), whereas high-pressure steaming time and enzymatic hydrolysis time had no significant effect. The β -glucan contents increased with enzyme concentration to the maximum value (high-pressure steaming for 15.51 min, enzyme concentration of 0.84 g/100 mL, and hydrolysis with Viscozyme L for 4.16 h) predicted by the stationary point (8.22 g/100 g). The graphical approach allowed for the study of the influence of each parameter and determination of the optimal operating conditions required to obtain β -glucan. The results suggested that the optimum pre-treatment conditions might enhance the yield of extracted β -glucan. Similar studies reported that optimum pre-treatment conditions may enhance the efficiency of antioxidant extraction, whereas excessive treatment had a negative effect [23, 37]. Based on practical considerations, the optimal high-pressure steaming time, enzyme concentration, and enzymatic hydrolysis time were determined to be 15.51 min, 0.84 g/100 mL, and 4.16 h, respectively, based on which the β -glucan contents of the *G. lucidum* extracts were predicted to be 8.22 g/100 g (Table 1). This value was in agreement with the empirical value (8.05 g/100 g) of the optimum pre-treatment condition, which reflected the fitness of the optimization. The extraction of β -glucan from *G. lucidum* by SET ($p < 0.05$) increased twofold (19.89 g/100 g) (Table 3). These results were consistent with those of previous studies on steaming and enzymatic pre-treatments [37]. The extraction yield and contents of β -glucan, total sugar, monosaccharide, and protein in the SET processes of *G. lucidum* extract were compared with those of the control treatment (Table 3). The total sugar and protein contents of SET also increased twofold compared with those values of the control treatment. These results were closely associated with the enhanced yield of extraction through the pre-treatment conditions. The increase in soluble solid contents of persimmon peel through enzymatic treatment [37] and total sugar and protein contents of *G. lucidum* through pressurized extraction [39] was also reported. In contrast to glucose, mannose, and galactose which were detected in both control and the SET-processed extracts, fructose was only detected in the SET-processed extracts (Table 3). The free sugar contents of the SET-processed extract were higher than those of the control extract ($p < 0.05$). Glucose was the main sugar component of the *G. lucidum* extract, followed by mannose, galactose, and fructose ($p < 0.05$). Although the sugar composition varied depending on the growth environment, the hot water extract of *G. lucidum* contained glucose, galactose, mannose, and xylose [40].

Table 3 Extraction yield and contents of β -glucan, total sugar, monosaccharide, and protein of the *Ganoderma lucidum* extracts obtained by high-pressure steaming and enzymatic pre-treatment (g/100 g)

Sample	Yield	β -Glucan	Purity (%)	Total sugar	Monosaccharide			Protein	
					Glucose	Mannose	Galactose	Fructose	
Control	9.69 \pm 1.12 ^b	4.02 \pm 0.02 ^b	41.58 \pm 0.25 ^a	5.16 \pm 0.07 ^b	2.93 \pm 0.05 ^{Ab}	1.24 \pm 0.12 ^{Bb}	0.159 \pm 0.01 ^{Cb}	–	3.32 \pm 0.07 ^b
SET	19.89 \pm 1.34 ^a	8.05 \pm 0.16 ^a	39.57 \pm 0.12 ^b	9.31 \pm 0.06 ^a	5.85 \pm 0.03 ^{Aa}	2.25 \pm 0.05 ^{Ba}	0.25 \pm 0.00 ^{Ca}	0.14 \pm 0.01 ^D	5.58 \pm 0.40 ^a

SET, high-pressure steaming and enzymatic treatments

The results are expressed as mean \pm standard deviation ($n = 3$). Values with different superscript letters in the columns (a, b) or rows (A, B, C, D) are significantly different at $p < 0.05$, as determined by Duncan's multiple range test

Increase in sugar and protein contents also indicated improvement in β -glucan yield by SET. Thus, we propose that high-pressure steaming in combination with Viscozyme L pre-treatment could be adopted for the extraction of β -glucan from *G. lucidum*.

Anti-metastatic effect of the *G. lucidum* extracts

To evaluate the anticancer activities of the *G. lucidum* extracts, we performed an MTT assay and a cell migration assay on the murine melanoma cell line B16F10. As shown in Fig. 2(A), all the samples exhibited a significant degree of cell viability of over 80% at all the extract concentrations. Therefore, it was confirmed that the *G. lucidum*

Fig. 2 Effect of the *Ganoderma lucidum* extracts obtained by high-pressure steaming and enzymatic pre-treatment on the viability (A) and the migration (B) of B16F10 cells. CON, control; RES, resveratrol (10 μ g/mL); NPT, non-pre-treatment; SET, steaming and enzymatic pre-treatment; *, **, ***, **** significant difference from the control at $t < 0.1$, $t < 0.05$, $t < 0.01$, $t < 0.001$, respectively

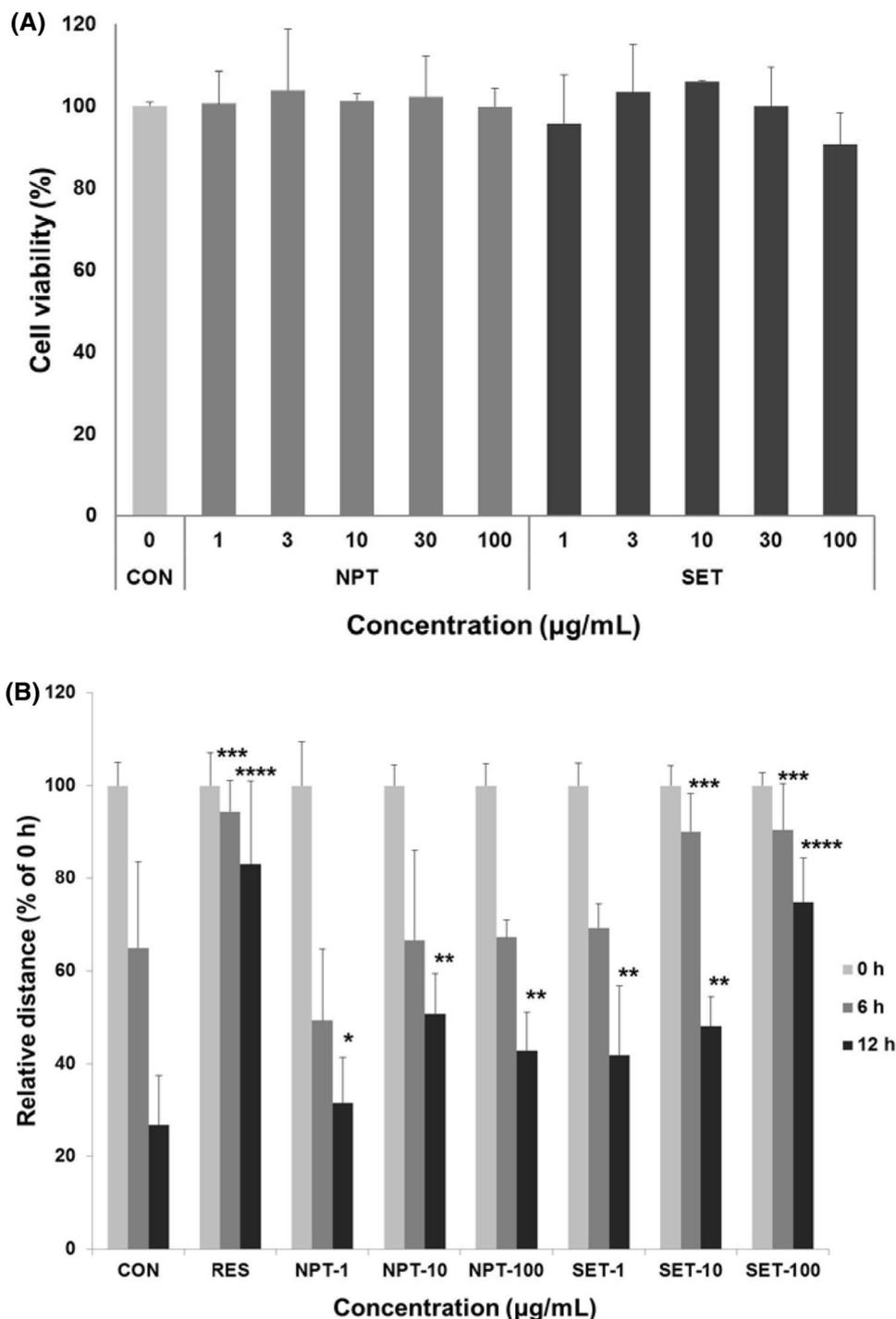
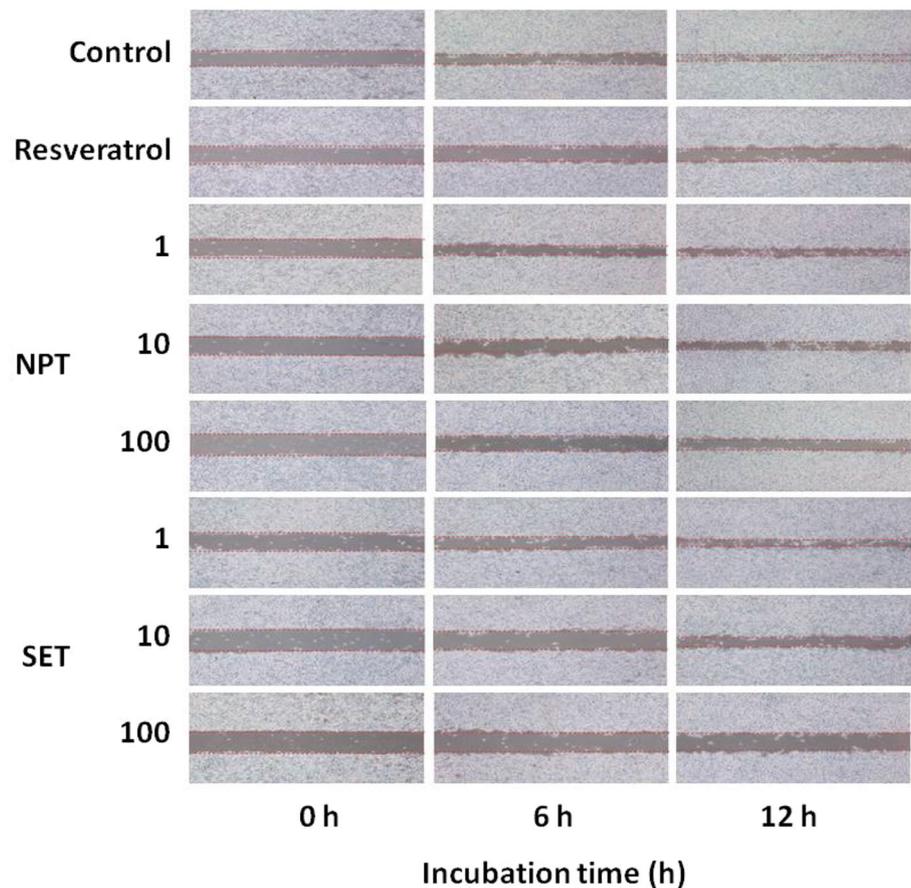


Fig. 3 Cell migration assay of the *Ganoderma lucidum* extracts obtained by high-pressure steaming and enzymatic pre-treatment with B16F10 cells. Resveratrol (10 $\mu\text{g}/\text{mL}$); NPT, non-pre-treatment; SET, steaming and enzymatic pre-treatment



extract was harmless to the cells, consistent with previous research using human prostate cell [41]. The cell migration on the slit of the confluent wells was photographed after 0, 6, and 12 h in each condition. As shown in Figs. 2(B) and 3, there was a significant dose-dependent inhibitory effect on cell migration. The SET showed high inhibitory effects similar to the positive control (10 $\mu\text{g}/\text{mL}$, resveratrol). It was also reported that *G. lucidum* extracts suppress the growth of cancer cells [42, 43]; moreover, their biological activity and solubility were enhanced through sulfation [41]. In conclusion, the SET-processed *G. lucidum* extract showed the increase in the β -glucan extraction yield with the enhanced anti-metastatic effect on B16F10 cells. Therefore, it is suggested that SET process could be an effective way to develop the functional food from *G. lucidum* with anticancer activity.

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