Physiological Properties of *Viscum album* var. *coloratum* Extracts by Response Surface Methodology

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The extraction characteristics and physiological properties of *Viscum album* var. *coloratum* (mistletoe) were monitored by response surface methodology (RSM). The maximum electron donating ability 88.05% was obtained at 42.10 W of microwave power, 36.99% of ethanol concentration, and 7.17 min of extraction time. The maximum inhibition effect on tyrosinase was 62.88% at microwave power, and its condition was 84.02 W, 92.46%, and 7.07 min of ethanol concentration, and extraction time, respectively. Superoxide dismutase (SOD) showed a maximum pseudo-activity of 53.55% at microwave power, 142.69 W, ethanol concentration, 32.69%, and extraction time, 3.58 min. The maximum total polyphenol content was 72.82% and it was under 122.57 W, 16.81%, and 2.94 min of microwave power, ethanol concentration, and extraction time, respectively. Based on superimposition of four-dimensional RSM regarding electron-donating ability, inhibition effect on tyrosinase, pseudo-activity of SOD, and total polyphenol content, the optimum ranges of extraction conditions are as follows; microwave power, 0-135 W, ethanol concentration , 64-70%, and extraction time , 4.15 min.

Key words: physiological property, response surface methodology, total polyphenol content, *Viscum album* var. *coloratum*

In recent times, how to prevent diseases such as cancer, arteriosclerosis, and heart disease has become one of the most important problems in our society. These diseases have been diffusing more and more because of the change in eating habits following with the national income increase. According to the WHO, World Health Organization, 13% of human death in 2006 was owing to cancer. Especially, change in modern life style like smoking increase, high-lipid diet, and lack of exercise dramatically cause cancer in developing countries [Lacombe *et al*., 2002]. Cancer is a class of disease in which a group of cells displays uncontrolled growth and invasion, and spreads to other location in the body through lymph or blood [Hains *et al*., 2009]. Most cancers are occurred by abnormalities in the genetic material of the transformed cells. These abnormalities are probably caused due to the effects of carcinogens such as chemicals, ionizing radiation, bacterial infection, immune system dysfunction, and heredity. As the concerns about keeping health from many diseases increase, people are more looking for better health aids. That is the reason why they prefer to have organic foods and spend much time on doing excise. From the nutritional point of view, herbal plants such as basil and mistletoe are rich in vitamins and minerals, as well as D-menthol which help to prevent cancer outbreaks in human body.

Viscum album var. *coloratum*, common name for the Loranthaceae which belongs to the family of chiefly tropical hemiparastic herbs, is shrub with leathery evergreen leaves and waxy white berries. *Viscum album* var. *coloratum* leaves and young twigs have been used by herbalists for treating circulatory and respiratory system problems. In addition, diluted *Viscum album* is widely used for alternative cancer treatments in Europe and it has already been over than 75 years as a treatment for tumors [Bohling *et al*., 2003]. *Viscum album* extracts are used to stimulate the immune system and kill cancer cells [Capernaros, 1994]. The active ingredients in *Viscum album* can kill cells by damaging cellular membranes, stopping protein synthesis, and stimulating the immune system [Oisnes *et al*., 1982].

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The methods of extraction, pressing, steam distillation, and supercritical fluid extraction have been used as methods of extracting effective components from medicinal herbs. However, these methods met some limitations in terms of extraction time, energy solvent consumption, and cost. Thus, a micro-wave-assisted process (MAP^{TM}) was developed as a new extraction technique. MAP has been used to extract essential oil, flavor precursor from grape juice, and ginseng components. The overall objective of this study was to establish the optimum conditions for physical properties from the samples applying response surface methodology (RSM). Response surface analysis was used to monitor physiological properties and extraction characteristic of the effective components of *Viscum album* var. *coloratum* under various experimental conditions.

Materials and Methods

Preparation of ground *Viscum album* var. *coloratum*. The *Viscum album* var. *coloratum* was purchased from the Kyungdong market in Seoul, Korea. After cleaning, the *Viscum album* var. *coloratum* was cut into 0.1 cm pieces and dried at 40°C in a hot-air drier (Jeio Tech, OF-02, Korea). The dried *Vitis coignetiae* pieces were then ground less than 0.5 mm pieces and stored in a sealed 0.2-mm polyethylene film bag at −20°C. Then, after the *Viscum album* var. *coloratum* sample (1 g) was washed and crushed, they were extracted with 20 mL of solvents such as water, 0~100% ethanol by MAP (microwave, Prolabo, soxwave, France) 100-120 Power (30-150 W) for 1~9 min, respectively.

Experimental design for response surface methodology. To optimize the extraction condition, RSM [Shin *et al.*, 2005] was applied to monitor the extraction characteristics affected by various extraction conditions. Experimental design was used for extraction conditions from central composite design [Lee and Kwon, 2000]. The SAS program (SAS Institute, INC., Cary, NC) was used for analysis of a standard deviation and a regression coefficient calculation. The models were modified for making a four-dimensional response surface that was generated by the Mathematica program [Martha and James, 1992]. Independent parameters in the extraction, namely, the microwave power (30-150 W, X_1), ethanol concentration (0-100%, X_2), and extraction time (6-18 min, X_3) were assigned numbers (−2, −1, 0, 1, 2), and 16 intervals were set on the basis of the central composite design for the extraction experiment (Table 1). The dependent parameters (Y_n) such as electron-donating ability, inhibitory effect on tyrosinase, pseudo-activity of superoxide dismutase, and polyphenol content were determined three times, and their average values were used for the regression analysis.

Determination of electron-donating ability. Electron-donating ability (EDA) of the *Viscum album* var. *coloratum* extracts was determined in terms of reducing power of α, α-diphenyl-picrylhydrazyl (DPPH) in each extract according to a modified method of Kang *et al.* [1996]. One milliliter of each extract was mixed with 1 mL of 4×10^{-4} M DPPH dissolved in 99.9% ethanol to make total volume of 2 mL. After shaking the mixtures on a vortex mixer for 10 s and holding the room temperature for 30 min, the absorbance was measured at 525 nm using a UV/VIS spectrophotometer (Jasco, Hachioji, Japan). EDA was found in percent using the following equation:

$$\text{EDA (\%)}=\left(1-\frac{A}{B}\right)\times100$$

A, B: 525 nm absorbance with/without test sample

All data represent means of three values measured separately.

Determination of the inhibitory effect on tyrosinase. The inhibitory effect on tyrosinase was measured by a method reported by Wong *et al.* [1971]. A crude tyrosinase solution was prepared by dissolving mushroom tyrosinase (Sigma, T7755, 110 U/mL) in a 50 mM sodium phosphate buffer (pH 7.0). Subsequently, 0.2 mL of the crude tyrosinase solution and 0.1 mL of *Viscum album* var. *coloratum* extract were added to 2.8 mL of a 10 mM catechol solution. The absorbance of the resulting mixture was determined at 420 nm by a UV/VIS spectrometer in order to measure the tyrosinase activity. The inhibitory effect on tyrosinase was calculated by measuring changes in the absorbance per unit of time, as follows:

$$\text{Inhibitory effect (\%)}=\frac{1-(A-B)}{C}\times100$$

A: the absorbance of samples treated by the enzyme solution

B: the absorbance of samples treated by a buffer solution in lieu of an enzyme solution

C: the absorbance of samples treated by distilled water in lieu of extracts.

Antioxidant activity of superoxide dismutase. Pseudo-activity of superoxide dismutase (SOD) was measured by a modified method of Kim *et al.* [2001]. After vacuum concentration of each extract, pH of each sample was adjusted to 8.5 using tris-HCl buffer (50 mM tris [hydroxymethyl] amino-methane+10 mM EDTA, pH 8.5). Three milliliters of the tris-HCl buffer and 0.2 mL of 7.2 mM pyrogallol were added to 0.2 mL of each sample.

The mixtures were held at 25°C for 10 min before stopping the reaction by adding 1 mL of 1 NHCl, and the absorbance was determined at 420 nm using a UV/VIS spectrometer. Pseudo-activity of SOD was found in percent using the following equation:

$$\text{Pseudo-activity of SOD (\%)} = \left(1 - \frac{A}{B}\right) \times 100$$

A: the absorbance difference between treated sample and control

B: the absorbance difference between untreated sample and control.

Determination of total polyphenol content. Total polyphenol content was measured by the Folin and Denis method [1912]. *Viscum album* var. *coloratum* extract (0.1 mL), 8.4 mL distilled water, and 0.5 mL of 2 N Folin regents was set for 3 min before adding 1 mL of 20% Na_2CO_3 solution. After holding the mixed solution for 1 h, absorbance was measured at 765 nm using a UV/VIS spectrophotometer (Jasco, SSE-343, Hachioji, Japan). Total polyphenol content was determined from the standard curve obtained using (+)-catechin.

Prediction of optimum extraction condition. The optimum ranges of extraction conditions were predicted by superimposing the response surfaces regarding extraction EDA, inhibitory effect on tyrosinase, pseudo-activity of SOD, and total polyphenol content. Random points selected within the optimum ranges were applied to repression equation to determine optimum extraction values.

Results and Discussion

Changes in electron-donating ability. EDA provides electron to free radical, so that it can protect lipid oxidation in foods as well as delay aging process in human body [Kang *et al*., 1996]. Removal of free radicals plays an important role in preventing chronic diseases and cancers by aging of our body. DPPH method has been used to measure hydrogen-donating ability, which acts as an antioxidant by reducing physiological activity compounds such as tocopherol, ascorbate, flavonoid compounds, aromatic amines, maillard-type browning materials, and some peptides [Blois., 1958]. Table 1 shows electron donating ability of *Viscum album* var. *coloratum* under 16 extraction conditions set by the central composite design, and Fig. 1 shows four-dimensional response surface. We found the highest value of 86.74% of EDAs at 90 W of microwave power, 50% ethanol concentration, and 5 min of extraction time in experimental run 9. The regression equation of changes in EDA calculated by RSM program for various extraction conditions (microwave power, ethanol concentration, and extraction time) is shown in Table 2 with R^2 being 0.9129 with less than 2% significance level recognized. EDA of

Table 1. Experimental data on electron donating ability, tyrosinase inhibitory activity, SOD-like activity, and total polyphenol content of extracts of *Viscum album* var. *coloratum* under central composite design for response surface analysis

Experiment Number[1)]	Microwave power (Watt)	Ethanol Concentration (%)	time (min)	Electron donating ability (%)	Tyrosinase inhibition (%)	SOD-like activity (%)	Polyphenol contents (mg %)
1	60(-1)	25(-1)	3(-1)	82.053	54.16	40.30	316.4
2	60(-1)	25(-1)	7(1)	86.512	48.42	36.13	200.3
3	60(-1)	75(1)	3(-1)	66.710	43.91	28.12	378.8
4	60(-1)	75(1)	7(1)	78.637	65.92	50.42	328.6
5	120(1)	25(-1)	3(-1)	79.274	38.90	43.38	668.5
6	120(1)	25(-1)	7(1)	79.653	40.35	46.75	407.9
7	120(1)	75(1)	3(-1)	74.991	48.27	43.76	537.1
8	120(1)	75(1)	7(1)	76.551	53.31	38.77	451.8
9	90(0)	50(0)	5(0)	86.743	53.97	44.79	393.2
10	90(0)	50(0)	5(0)	84.601	45.71	27.75	380.2
11	30(-2)	50(0)	5(0)	78.499	47.19	51.84	129.0
12	150(2)	50(0)	5(0)	77.354	34.38	49.42	547.7
13	90(0)	0(-2)	5(0)	67.855	41.78	43.38	599.4
14	90(0)	100(2)	5(0)	41.017	55.63	39.87	490.0
15	90(0)	50(0)	1(-2)	65.676	45.29	38.91	479.6
16	90(0)	50(0)	9(2)	79.450	39.79	44.59	448.8

[1)]The number of experimental condition by central composite design.

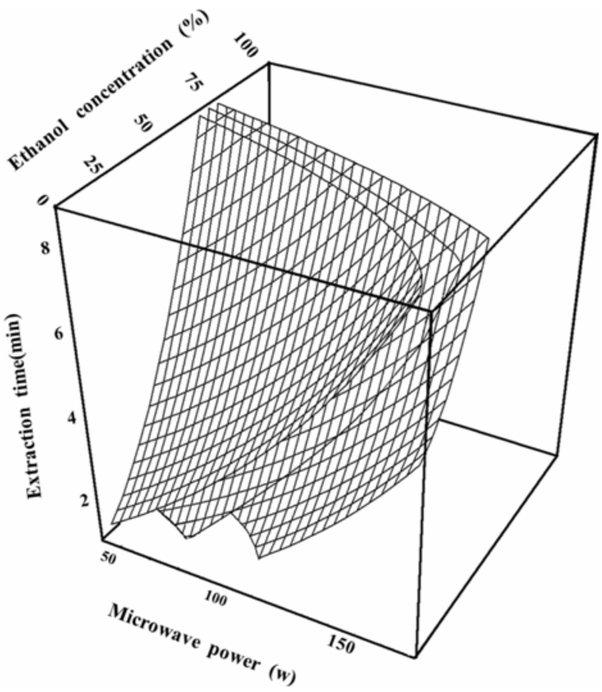

Fig. 1. Response surface plot for electron donating ability (at constant value, 10-50-90%) of extracts from *Viscum album* var. *coloratum* as physical properties of microwave power, ethanol concentration, and extraction time in a microwave-assisted process.

Viscum album var. *coloratum* extracts was at the maximum level of 88.05% with the microwave power, ethanol concentration, and extraction time that are 42.10 W, 36.99%, and 7.17 min, respectively. These predictions were made by using ridge analyses (Table 3). EDA of *Viscum album* var. *coloratum* extracts was strongly influenced by the extraction conditions and also affected by microwave power, while the effect of extraction time was less significant (Table 4). In the similar study by Jeong [2004], it was reported that the EDA was more easily affected by extraction temperature than extraction time. In addition, similar result was reported by Yoon *et al*. [2003] that are the optimum ethanol concentration ranging is from 30 to 40%.

Inhibitory effect on tyrosinase. Tyrosinase (Dihydroxy-L-phenylalanine oxygen oxidoreductase, EC 1.14.18.1) is a browning enzyme which uses phenol compounds as a substrate. In particular, several prenylated flavonoids have shown that they strongly inhibit tyrosinase. Therefore, the prenyl residues in flavonoid molecule are probably responsible for the potent inhibition activity [Lee *et al.,* 2003]. By controlling tyrosinase, it is able to recognize the characteristics of foods such as color, flavor, and nutrition change. This study used RSM to find optimal extraction condition for the highest effects of tyrosinase

Table 2. Polynomial equations calculated by RSM program for extraction conditions of *Viscum album* var. *coloratum*

Responses variables	Second order Polynomials[1)]	R^2	Significance
Electron donating ability	$Y=27.411875-0.0393875X_1+0.692950X_2+11.252188X_3+0.002151X_1^2+0.002638X_1X_2-0.012492X_2^2-0.030104X_1X_3+0.021625X_2X_3-0.819063X_3^2$	0.9129	0.0140
Tyrosinase inhibition	$Y=42.225000+0.309792X_1-0.429350X_2+2.846250X_3-0.002515X_1^2-0.002513X_1X_2-0.000454X_2^2-0.020375X_1X_3+0.078350X_2X_3-0.456250X_3^2$	0.7490	0.2077
SOD-like activity	$Y=62.010313-0.404583X_1-0.326200X_2-1.114687X_3+0.003989X_1^2-0.001618X_1X_2+0.002142X_2^2-0.041146X_1X_3+0.45275X_2X_3+0.045275X_3^2$	0.5289	0.6661
Total polyphenol content	$Y=10.894375+1.010375X_1-0.545100X_2-6.284375X_3-0.001343X_1^2-0.004637X_1X_2+0.006320X_2^2-0.037417X_1X_3+0.060300X_2X_3+0.484375X_3^2$	0.9217	0.0104

[1)]X_1: microwave power (W), X_2: ethanol concentration (%), X_3: extraction time (min)

Table 3. Predicted levels of extraction condition for the maximum responses of variables by the ridge analysis

Responses variables	Independent variables[1)]			Maximum	Morphology
	X1	X2	X3		
Electron donating ability (%)	42.10	36.99	7.17	88.05	Maximum
Tyrosinase inhibition (%)	84.02	92.46	7.07	62.88	Saddle point
SOD-like activity (%)	142.69	32.69	3.68	53.55	Minimum
Total polyphenol content (mg%)	122.57	16.81	2.94	728.2	Saddle point

[1)]X_1: microwave power (W), X_2: ethanol concentration (%), X_3: extraction time (min)

Table 4. Regression analysis for regression model of physiochemical properties in extraction condition of *Viscum album* var. *coloratum*

Extraction condition	F-Ratio			
	Electron donating ability	Tyrosinase inhibition	SOD-like activity	Total polyphenol content
Microwave power	7.11*	3.56*	0.40	17.37***
Ethanol concentration	13.01***	1.02	1.25	4.32*
Extraction time	0.82	1.39	0.60	1.84**

*Significant at 10% level; **significant at 5% level, ***significant at 1% level

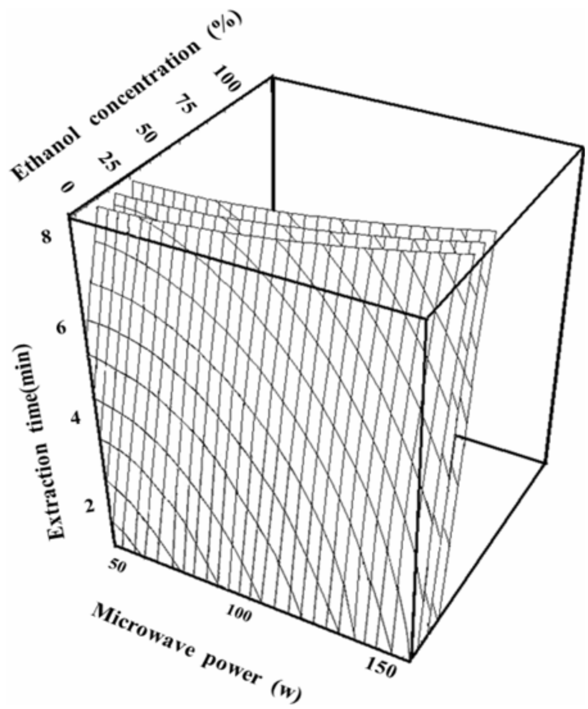

Fig. 2. Response surface plot for tyrosinase inhibitory activity (at constant value, 40-50-60%) of extracts from *Viscum album* var. *coloratum* as physical properties of microwave power, ethanol concentration, and extraction time in a microwave-assisted process.

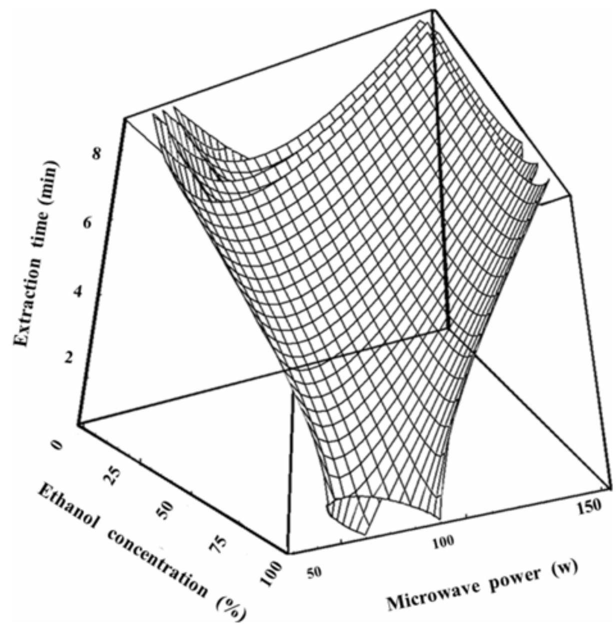

Fig. 3. Response surface plot for SOD-like activity (at constant value, 28-30-32%) of extracts from *Viscum album* var. *coloratum* as physical properties of microwave power, ethanol concentration, and extraction time in a microwave-assisted process.

inhibition. The regression equation for the inhibitory effect on tyrosinase showed R^2 as 0.7490 (Table 2). The estimated maximum inhibitory effect on tyrosinase was 62.88% under the conditions of microwave power 84.02 W, 92.46% ethanol concentration, and 7.07 min of extraction time (Table 3).

Antioxidant activity of superoxide dismutase. SOD is an enzyme in human body which inhibits superoxide anion activity [Kim *et al.*, 2001]. The SOD-like activity material is normally called SOD since they perform a similar in the living body nevertheless have different operation mechanism. SOD-like activity of *Viscum album* var. *coloratum* under various extraction conditions are listed in Table 1 and Fig. 3. R^2 of the regression equation for SOD-like activity results 0.5389 and its significance was not confirmed. Predicted morphology was minimum value, and the maximum SOD-like activity was 53.55% when the microwave power, ethanol concentration, and extraction time were 142.69 W, 32.69%, and 3.68 min, respectively (Table 3). Response surface regarding SOD-like activity is presented in Fig. 3. SOD is related with removal of Superoxide in the living body. Numerous materials having SOD-like active oxygen formed in the body are considered as factors to prevent oxidation. Therefore, this optimal extract condition of SOD result can be used for other studies about antioxidative materials ability of the controlling reactivity superoxide.

Changes in total polyphenol contents. Total polyphenol contents (TPC) under various extraction conditions are listed in Table 1. The highest value of 73.82% of TPC was shown at microwave power of 122.57 W, ethanol concentration of 16.81%, and extraction time of 3 min in experimental run 5. Fig. 4 shows four-dimensional response surface for TPC. The regression equations for

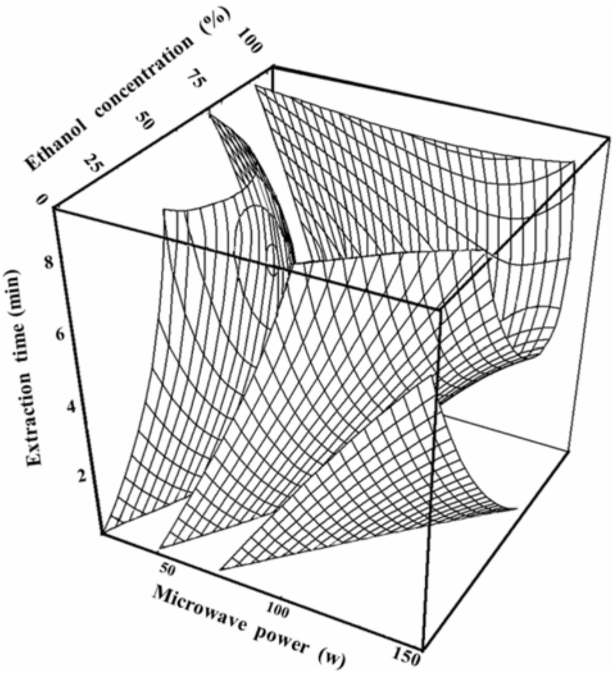

Fig. 4. Response surface plot for total polyphenol content (at constant value, 300-500-700 mg%) of extracts from *Viscum album* var. *coloratum* as physical properties of microwave power, ethanol concentration, and extraction time in a microwave-assisted process.

response surface are listed in Table 2. R^2 for the regression equation was 0.9217 with significance of less than 2% being recognized. The predicted peak point led to the highest total polyphenol content of 728.2 mg% with corresponding independent parameters; microwave power, 122.57 W, ethanol concentration, 16.81%, and extraction time, 2.94 min (Table 3). The most predominant effect was observed as the microwave power, also the other independent parameters, the extraction time and ethanol concentration, were also effective but less significant. In a similar research by Lee *et al.*, 2003 polyphenol has also been more affected by short extraction time. However, the study predicted both ethanol concentration and microwave power affected total polyphenol content although this study has shown that extraction time and ethanol concentration were less significant than microwave power [Lee *et al.,* 2003].

Prediction of optimum extraction conditions. The optimum ranges for extraction conditions of *Viscum album* var. *coloratum* was predicted by superimposing the four-dimensional response surfaces about EDA, tyrosinase inhibition, SOD-like activity, and TPC obtained under various conditions. As a result, the optimum extraction ranges to maximize the quality characteristics of *Viscum album* var. *coloratum* were established at 30-135 W of microwave power, 64-70% ethanol concentration, and 4.15 min of extraction time (Fig. 5, Table 5).

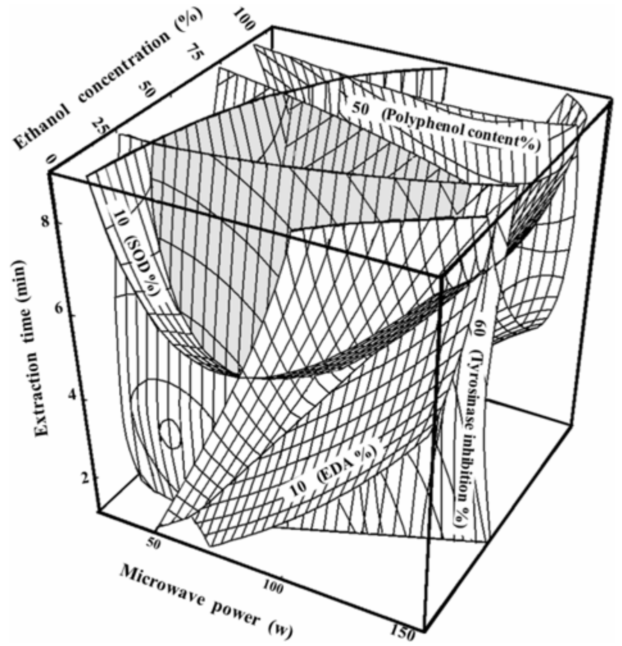

Fig. 5. Superimposed response surface plot for optimization of electron donating ability (10%), tyrosinase inhibitory activity (60%), SOD-like activity (32%), and total polyphenol content (500 mg%) of extract from *Viscum album* var. *coloratum.*

Table 5. Optimum extraction condition for response variables yielding the optimum response by superimposing of the 4-dimensional response surface

Extraction condition	Range of predicted condition
Microwave power (W)	30-135
Ethanol concentration (%)	64-70
Extraction Time (min)	4.15

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