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Optimization of extraction process for enhancement of antioxidant activity of *Acer mono* bark

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Summary

Response surface methodology (RSM) was used to determine the optimum extraction condition of *Acer mono* bark for its crude extract to have better antioxidant activities. Twenty experimental conditions were set based on three key variables such as temperature, time and pressure by signalling reaction variables with 5 levels of - 2, - 1, 0, 1, 2 in accordance with central composite design for proceeding extraction and antioxidant tests. The optimized condition for the highest extraction yields was 13.10% at 83.48 °C, 54.36 MPa for 13.08 minutes. For DPPH radical scavenging ability, an optimal condition was 92.89% at 88.50°C, 49.69 MPa for 15.08 minutes, and for SOD-like activity 40.69% at 85.21°C, 53.28 MPa for 15.83 minutes. The optimized condition for total polyphenol content was 4.23 mg/g at 81.51 °C, 52.92 MPa for 14.79 minutes. The most optimized extraction condition was determined to be at 85 °C, 52 MPa for 14 minutes for considering both extraction yield and its biological activities of this plant.

Introduction

Acer mono in the Aceraceae (also called the Maple family) is an arbor of fallen leaves, which grows wild in 100~1,800 m altitude in wide areas of Korea, Japan, China and Manchuria. However, most stu-dies conducted in accordance with *Acer mono* have been focused on either utilizing its sap rather than tree itself (Kim et al., 1991), or only cultivating various types of the trees (Seiwa, 1998). Particularly, in Japan, there have been a wider range of studies of analyzing the saps from birch trees (Terazawa et al., 1984; Iguchi et al., 1985), but not investigating the biological activities of the trees such as antioxidant or anticancer activities, etc. Therefore, it would be beneficial to seek a possibility of utilizing *Acer mono* by investigating the biological activities of the tree itself since in recent years, people have been trying to lead a healthier lifestyle and improve their nutrition.

However, even with the healthier lifestyle, modern people are exposed to various adult diseases, in addition to aging. Active oxygen is considered as a cause of many serious diseases. A variety of factors such as normal metabolism, photochemistry reaction, drug metabolism or other abnormal cell metabolism will produce more active oxygen. The reactivity of such active oxygen is so strong that free radical reactions of which destroy lipid, protein, sugar and DNA by non-selective and irreversible reactions (Morita et al., 1980; Morita et al., 1982), which is believed to cause cancers and many other diseases such as brain disorders, heart diseases, ischemia, arteriosclerosis, skin diseases, digestive troubles, infection, rheumatic arthritis or autoimmune diseases, etc. (Halliwell and Gutteridge, 1990; Davies, 1995). In general, phenolic synthetic compounds such as BHA (butylated hydroxyl anisol) and BHT (butylated hydroxyl toluene) have been widely used for the treatment of such diseases because of their effective and economic factors as well as reliability; however, recently natural components from natural plant resources, not synthetic ones, have been more interested in applying for many areas of food, cosmetics, and pharmaceuticals, etc. (An et al., 2003; Kinghorn et al., 1996; Yoon et al., 2012) because excessive use of chemical substances could cause more severe toxic reactions in the liver, mucous membrane of stomach, lung, kidney and circulatory system (Surh, 1993).

Therefore, it is necessary to develop safe and effective natural antioxidants (Branen, 1975; Choe and Yang, 1982), and *Acer mono* could be one of promising resources because this tree has been proved to have antioxidant activities by water and/or ethanol extractions (Kwon et al., 1997; Jin et al., 2008). However, there have been some difficulties in extracting the plants with those solvents through conventional extraction processes, compared to the cases for most commonly used herbs such as *Panax ginseng*, *Artemisia iwayomogi*, and *Rubus coreanus*, etc. since the plants have relatively hard barks that caused to significantly reduce and limit the extraction of active components from them (Senorans et al., 2003). To obtain economic and biological feasibility of extracting these plants, proper extraction processes should be developed along with optimizing these extraction parameters because this unique process would contain particular process variables such as pressure and temperature, etc., not for the heat labile substances to be more damaged during the extraction at high temperature even with high extraction efficiency under these conditions (Pasquini et al., 2005; Butz et al., 2004). Optimization of the extraction processes has been considered to be critical not only for maintaining or improving their biological activities, but also enhancing the extraction yields because there must be very close correlation between extraction conditions and biological activities of the extracts especially under extreme extraction conditions of high pressure and high temperature for hard plants (Zhang et al., 2007; Saldana et al. 2002). Therefore, for this case, the interactions of several factors should be carefully considered, not only one or two variables of extraction parameters. However, in most studies, the one-factor-at-time method has been most widely used for extracting natural resources, in which variables such as temperature, extraction solvent, or time are correspondingly set based on a certain variable (Gontard et al., 1992). Such simple methodology would not consider the cause and effect interactions among those variables, which results in limiting the reliability of the results. This process could not determine a really optimized condition to enhance the biological activities, either. On the other hand, RSM is an effective statistical analyzing method that minimizes the number of tests and evaluates reaction changes in wider ranges, in particular, when multiple independent variables interact in complexities affecting dependent variables (Lee et al., 2000; Myers, 1971). Thus, it is able to find the most optimized condition by consecutively eliminating non significant interaction terms associated with RSM (Yoon et al., 1997), which cannot be likely found by the one-factor-at-time method (Wang et al., 2008; Cho et al., 2004; Yoon and Cho, 2007). In addition, as shown in this study, optimizing the extraction process correlated to antioxidant activity has not been carried out for trees, and increasing the significance of the model by removing the non-significant terms either. Therefore, three key extraction variables were selected as extraction time, temperature and pressure to find an

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optimized extraction process for enhancing antioxidant activity of *Acer mono* bark by using central composite design and RSM, then a variety of extraction conditions was performed in order to obtain the most optimized condition with most significant confidence.

Materials and methods

Materials

The *Acer mono* collected in May, 2010 was provided by Korea Forestry Research Institute (KFRI), Korea. The trees were dried in the shade in room temperature. Then, only bark parts of the plants were peeled and grinded by a grinder, then they were extracted under various conditions.

Extraction conditions

To determine most proper extraction conditions for antioxidant activities of *Acer mono*, 100 g of dried *Acer mono* bark was extracted by reflux condensing extractor (pyrex, Seoul, Korea) with various extraction solvents such as distilled water at 100 °C, 70% ethanol with water at 85 °C, hexane at 85 °C, chloroform at 85 °C or butanol at 85 °C for 24 hours, then the extracts were concentrated by a rotary vacuum evaporator (Eyela, Tokyo, Japan) before being freeze-dried. Electron Donating Ability (EDA) of the freeze dried extracts were estimated by measuring DPPH free radical scavenge, to determine the most proper extraction solvent for this plant. After selecting the extraction solvent, three most important extraction parameters for *Acer mono* were set as temperature for 40~120 °C, process time for 5~25 minutes, and pressure for 0~100 MPa. The ranges of these variables were chosen from the preliminary experimental results.

Experimental Design for optimizing the extraction condition

As shown in Tab. 1, to determine the most optimized extraction condition, 20 experimental extraction conditions were defined by Central Composite Design (CCD) in a combination of variables such as extraction temperature (40, 65, 80, 100 and 120 °C), time (5, 10, 15, 20 and 25 minutes) and pressure (0, 25, 50, 75 and 100 MPa) for RSM. A high pressure liquefying extractor (Ilshin Autoclave Co., Daejeon, Korea) was used to maintain each extraction condition: 20 g of dried *Acer mono* bark was extracted with water in a 1 L high pressure extraction vessel under various extraction conditions from central composite design. Then, the extracts were filtered by a membrane filter paper and concentrated a rotary vacuum evaporator (rotary vacuum evaporator N-N series, Eyela, Tokyo, Japan). After that, the concentrated liquid was freeze-dried as a powder before being used. The extraction yield (%, w/w) from each condition was estimated by the ratio of the weight of dry matter of *Acer mono* bark after being extracted to the weight of total dry matters before extraction because higher extraction yield should contain more biologically active substances at first.

To determine the most optimized model, after running the above experimental designs, non significant factors were consecutively eliminated based on p-values of curves, square, or interaction terms in proposed equations (i.e. $p > 0.05$) obtained from Analysis of variance (ANOVA) for re-designing the models.

Measurement of antioxidant activities of DPPH and SOD-like

The electron donating effect for DPPH (a,a-diphenyl-picrylhydrazyl) of each extract was measured as follows: 0.5 mL of 0.5 mM DPPH liquid (Abs. EtOH soln.) was put into a test tube in which 1 ml

Tab. 1: Extraction yields, DPPH scavenging activity, SOD-like activity and total polyphenol contents of bark of *Acer mono* by the central composite experimental design for response surface analysis.

Exp. No†	Independent variables			Response variables‡			
	Extraction temp. (°C)	Extraction time (min)	Extraction pressure (MPa)	Yields (%, w/w)	DPPH (%)	SOD (%)	Polyphenol (mg/g)
1	40	15	50	5.67 ± 0.06	65.25 ± 0.54	32.87 ± 0.45	2.11 ± 0.04
2	60	10	25	8.41 ± 0.04	82.15 ± 1.22	33.11 ± 0.11	2.53 ± 0.06
3	60	10	75	8.16 ± 0.08	83.22 ± 0.45	33.28 ± 0.93	2.43 ± 0.06
4	60	20	25	8.55 ± 0.06	81.23 ± 1.20	33.21 ± 0.41	2.34 ± 0.02
5	60	20	75	8.47 ± 0.04	80.42 ± 0.06	34.25 ± 0.34	2.41 ± 0.14
6	80	5	50	11.15 ± 0.16	92.45 ± 0.07	36.69 ± 0.51	3.08 ± 0.08
7	80	15	0	7.49 ± 0.01	93.22 ± 1.60	37.58 ± 0.43	2.39 ± 0.21
8	80	15	50	13.42 ± 0.16	93.45 ± 0.43	41.25 ± 0.28	4.25 ± 0.08
9	80	15	50	13.24 ± 0.07	92.54 ± 0.91	39.46 ± 0.84	4.36 ± 0.02
10	80	15	50	12.42 ± 0.18	95.62 ± 0.35	42.15 ± 0.31	4.46 ± 0.21
11	80	15	50	13.56 ± 0.16	93.24 ± 0.05	40.28 ± 0.54	4.25 ± 0.10
12	80	15	50	13.28 ± 0.05	92.46 ± 1.84	41.11 ± 0.26	4.36 ± 0.06
13	80	15	50	13.33 ± 0.03	94.25 ± 1.37	40.29 ± 0.49	4.25 ± 0.02
14	80	15	100	11.25 ± 0.19	93.11 ± 0.51	38.44 ± 0.64	3.12 ± 0.04
15	80	25	50	10.28 ± 0.04	92.97 ± 1.64	38.45 ± 0.22	2.94 ± 0.08
16	100	10	25	10.24 ± 0.05	84.21 ± 0.88	37.45 ± 0.10	2.88 ± 0.01
17	100	10	75	10.38 ± 0.14	83.44 ± 2.41	37.11 ± 0.57	2.79 ± 0.04
18	100	20	25	8.36 ± 0.03	85.42 ± 1.64	37.69 ± 0.64	2.56 ± 0.05
19	100	20	75	8.42 ± 0.02	85.21 ± 2.15	37.42 ± 0.55	2.64 ± 0.03
20	120	15	50	6.89 ± 0.11	83.36 ± 1.24	31.25 ± 0.64	2.24 ± 0.02

†The number of experimental condition by central composite design.
‡Results are expressed as mean±SD of data obtained from three independent experiments.

ethanol, 10 μL sample and 990 μL of 100 mM sodium acetate buffer (pH 5.5) were agitated, which remained in a darkroom for 5 minutes in order to induce responses. After that, a UV spectrometer was used to measure the concentration of the remaining radical in 517 nm (OH et al., 1996).

$$EDA\ (\%) = (1 - As/Ac) \times 100 \quad (1)$$

Then, EDA was calculated by Equation (1), where "As" represents the optical density values of sample group (with the extract) and "Ac" represents the optical density of control group (without the extract). The activity of an antioxidant, Butylated Hydroxyl Anisole (BHA) was also examined as a positive control to compare with the extracts.

To confirm the antioxidant activities of DPPH, the amounts of pyrogallol generated as a catalyst for conversion reaction to H_2O_2 have also been estimated as SOD-like activity by a Marklund method (BLOIS, 1958): 0.2 mL of 7.2 mM pyrogallol and 2.6 mL tris-HCI buffer (pH 8.5) were added to 0.2 ml sample in a certain concentration, which remained in 25 °C for 10 minutes. After that, a spectrophotometer was used to measure the optimal density at 420 nm to calculate the amount of pyrogallol oxidized from the response liquid.

$$SOD\text{-}like\ (\%) = (1\text{-}As/Ac) \times 100 \quad (2)$$

Then, SOD-like activity was calculated by Equation (2), where "As" represents the optical density values of sample group (with the extract) and "Ac" represents the optical density of control group (without the extract).

Measurement of total polyphenol contents

The total polyphenol contents of the extract from each extraction condition were estimated by a method of DEWANTO et al. (2002), where Folin-Ciocaltea phenol reagent measures total phenols of polyphenolic compounds by being coloured with molybdenum blue reaction. In detail, 2 ml of 2% Na_2CO_3 was added to each of 100 μL extract and remained for 3 minutes, to which 100 μL of 50% Folin-Ciocalteu reagent was added. After 30 minutes treatment, optimal density was measured at 750 nm. A standard for phenol content in the plants, garlic acid (Sigma, USA) was used to obtain a calibration curve. Total polyphenol content was expressed as the weight of garlic acid (mg) in each reagent (*g*).

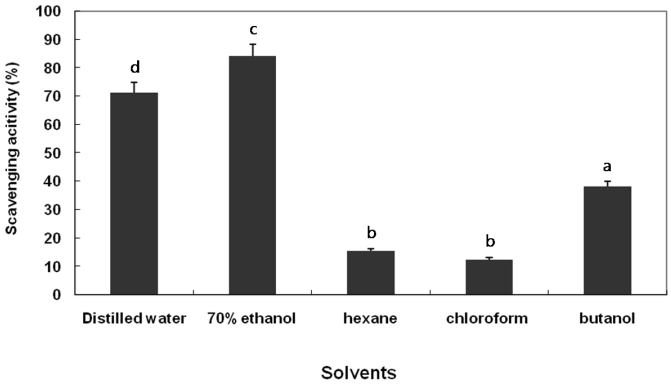
Statistical Analysis

All experiments were carried out in triplicates and the results were expressed as mean±SD. Data were analysed by the generalised linear model (GLM) procedure of the Statistical Analysis System (SAS, version 9.1, SAS Institute, Cary, NC). Data were analyzed by the analysis of variance and the mean values were considered significantly different with $p<0.05$. The optimal extraction condition was obtained by regression analysis.

Results

Selection of the extraction solvents and measurement of extraction yields

Fig. 1 shows the effect of five different extraction solvents on DPPH electron donating ability of *Acer mono*, and 70% ethanol appeared to be the best extraction solvent with 84.15% of EDA, compared to others. It was found that hexane or chloroform extracts had low antioxidant activities since in general non polar solvent could not effectively extract bioactive components from plant resources than polar solvents. This result was well consistent with the others reported by



Mean values ± SD from triplicate separated experiments are shown. Mean with difference letter (a-d) within different solvents are significantly different at $p < 0.05$.

Fig. 1: Scavenging effect of the extract from bark of *Acer mono* according to the several solvents on DPPH.

LEE et al. (2004) that water or ethanol extracts of Dulcis showed higher DPPH scavenging activity than those extracted by hexane or butanol as well as high total phenol contents (WANG et al., 2008). Based on this result, for all other experiments, 70% ethanol was used to extract the bark of *Acer mono*.

The central composite design was represented in Tab. 1, to optimize the extraction condition of increasing antioxidant activities of *Acer mono* bark by setting 20 extraction conditions with experimental values of extraction yields, polyphenol contents and antioxidant activities. The extracts at 80 °C for 15minutes with 50 MPa pressure were appeared to have the best extraction yield as 13.56% as well as the best antioxidant activities as 95.6 % of DPPH and 42.15 % of SOD. The lowest yields, on the other hand, were observed at 40 °C for 15 minutes with 50 MPa pressure as 5.67%, which also resulted in low antioxidant activities. These results indicate that low antioxidant activities would be caused by low extracting active components at low temperature, but at too high temperature the antioxidant activities were also observed to be low because of degrading or breaking biologically active components. Therefore, it is absolutely necessary to optimize the extraction condition for increasing biological activities especially from hard barks of the plants by considering all four response variables primarily based on extraction yield since high extraction yield could contain more biologically active substances at relatively lower extraction temperature that that of conventional hot water extraction.

Tab. 2 shows the results of estimating P-values of each response variable for extraction yields in the full quadratic model. P-values of constant and square in the model are 0.000 which falls into $p<0.05$; however, p-values of linear and interaction terms are 0.427 ($p>0.05$), which led to decrease the significance of the model. Thus, these response variables were removed to determine a more optimized model, and Tab. 2 is the result of re-estimating P-value after deleting interaction terms. The model equation without the interaction term was appeared to be more suitable, having p-value of the model was 0.000, which is very significant. The second order polynomial of the extraction model was determined with 0.8720 of R-square (adj) as follows:

$$Y_{Yield} = 13.0357 + 0.7812X_1 - 0.6412X_2 + 0.7837X_3 - 7.2736X_1^2 - 2.8386X_2^2 - 4.4636X_3^2 \quad (1)$$

In the model equation (1), p-value of the pressure term was appeared to affect the most based on p-value of 0.098, followed by temperature and time terms. It was also found that linear term of those response variables was proved to be non significant: however, on the other

Tab. 2: Estimation of p-value of the full quadratic model from the multi-regression analysis of extraction yields before and after deletely most non-significant values.

Term	P-value†	P-value‡	Term	P-value†	P-value‡
Constant	0.000	0.000	Temp	0.105	0.099
Linear	0.093	0.080	Time	0.175	0.168
Square	0.000	0.000	Press	0.104	0.098
Interaction	0.428	-	$Temp^2$	0.000	0.000
			$Time^2$	0.002	0.001
			$Press^2$	0.000	0.000
R-Square (adj)	0.8720	0.8720	Temp*Time	0.115	-
			Temp*Press	0.835	-
			Time*Press	0.972	-

†Estimated value before deletely most non-significant values based on P-value.
‡Estimated value after deletely most non-significant values based on P-value.

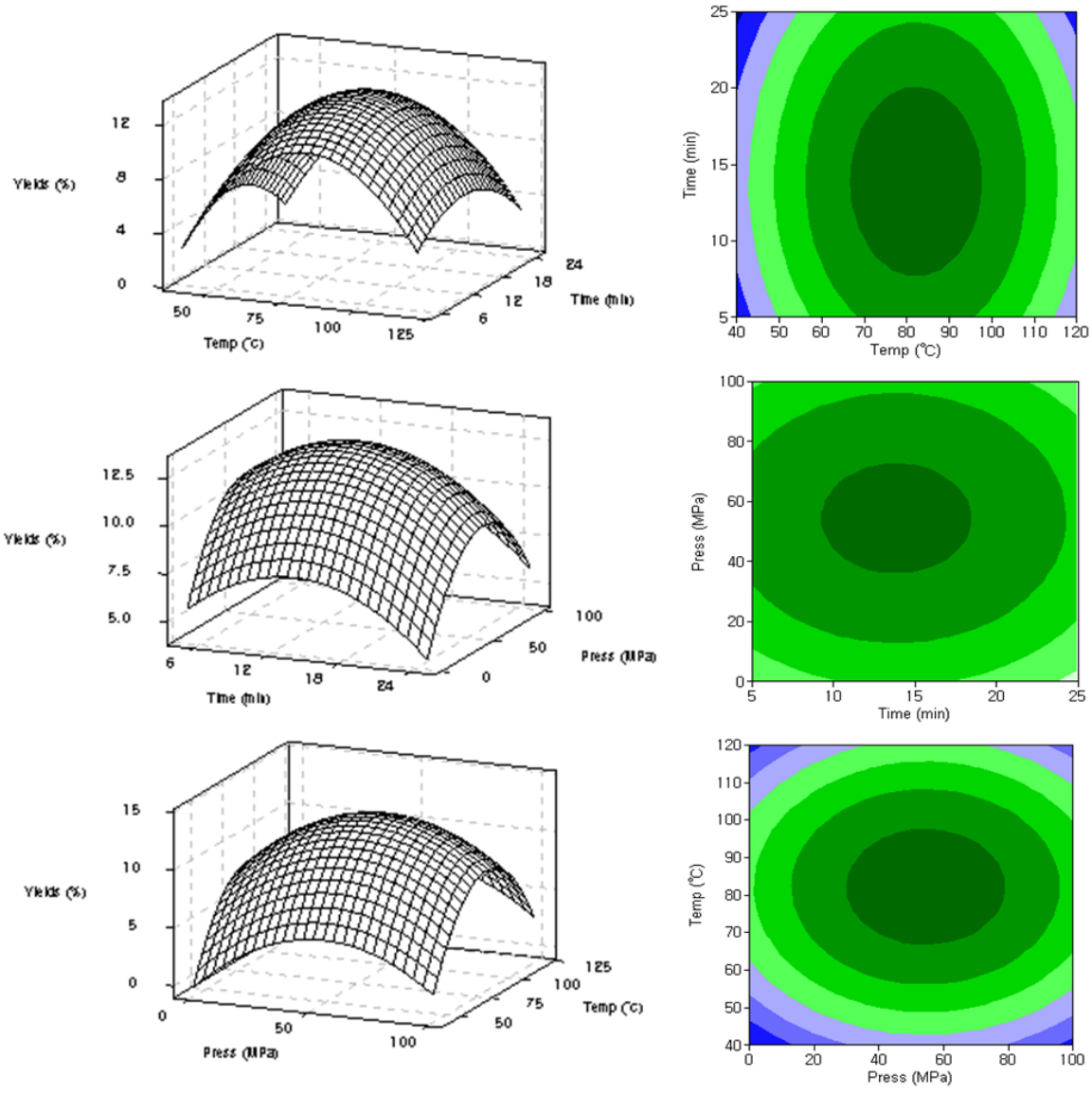


Fig. 2: Effect of extraction temperature, time and pressure on yields of extracts from bark of *Acer mono*.

hand, square terms of temperature, time and pressure seemed to be very significant ($p<0.05$), which also supports that the polynomial equation (1) would be most proper for extraction yield. In Fig. 2, effects of temperature, time and pressure on extraction yields based on each response variable are illustrated in surface and contour line plots by only reflecting linear and square terms, not interaction term. This figure also shows that pressure appeared to significantly affect to extraction yields, which is similar of the p-value results in Tab. 2.

Measurement of antioxidant activities

Two different DPPH radical scavenging and SOD-like activities were measured to make sure the antioxidant activities of *Acer mono* bark since these two activities represent different antioxidant action mechanisms. Electron donating abilities of natural resources feature donating electrons to active radical and controlling oxidation of fat in food, and restrain aging in human body by active radical. Radical scavenging reactions take an important role to prevent diseases or aging in human body (Kim et al., 2001). Active oxygen kinds in live bodies are derived from oxygen, which is produced by secure structured molecules such as triplet oxygen ($^3O_2$) through ultra violet rays, radioactive rays, chemical reaction, or metabolism (Trush et al., 1982).

The most favorable extraction condition for obtaining both high DPPH and SOD-like antioxidant activities was observed at 80 °C for 15 minutes with 50 MPa pressure from Tab. 1 as 95.62% and 42.15%, respectively. However, it was interesting that for DPPH activity, low temperature of 40 °C had the lowest antioxidant activity as 65.25% while for SOD-like activity, at 120 °C the lowest activity was observed as 31.20% under same pressure of 50 MPa and extraction time of 15 min.

Tab. 3 are to compare the p-values of the quadratic models for two antioxidant activities before and after sequentially deleting non-significant terms in the model equations, to determine more accurate equations reflecting higher antioxidant activities of *Acer mono* bark. It was interesting that interaction terms for both models of DPPH radical scavenging and SOD-like activities were found to be most non-significant, based on p-values such as 0.895 and 0.964, respectively. It was same pattern for extraction yield in Tab. 2, but the interaction terms of the models for antioxidant activities were more non-significant than the case of extraction yield, whose trend and comparison have not been reported elsewhere even though it is necessary to improve biological activities by considering all the factors together, not separately. In Tab. 3, it was clearly shown that re-designing the models by deleting non-significant variables seemed to be a proper methodology to have more suitable models by showing better regression coefficients for both antioxidant activities than the R-square values before deleting the terms as well as significant improvement of the linear terms. The re-designed polynomial equations for DPPH radical scavenging and SOD-like activities were obtained as follows:

$$Y_{DPPH} = 92.7893 + 4.2175X_1 - 0.0375X_2 - 0.1175X_3 - 24.3314X_1^2 - 2.4914X_2^2 - 2.0364X_3^2 \quad (2)$$

$$Y_{SOD} = 40.5336 + 2.4375X_1 + 0.6425X_2 + 0.2900X_3 - 10.8727X_1^2 - 3.6327X_2^2 - 3.1927X_3^2 \quad (3)$$

For DPPH radical scavenging activity, the extraction temperature seemed to be most effective as 0.012 ($p<0.05$), followed by pressure and time that were not much significant based on p-values. Such results can also be illustrated in Fig. 3, in which models are shown in surface plots as expecting the effects for linear and square terms, but not for interaction term, showing that DPPH radical scavenging ability was not significantly changing in accordance with time or pressure, but temperature was appeared to be the significant variable which changes the DPPH radical scavenging.

For SOD-like activity in Tab. 4, the extraction temperature also seemed to be most effective as 0.007 ($p<0.05$), and followed by time and pressure that were not much significant either. However, it was interesting that two variables of pressure and time were not much non-significant for SOD-like activity even though they are still higher than p-value of 0.05, compared to the cases of DPPH radical scavenging activity in Tab. 4. When extraction time and pressure were set at 15 minutes and 50 MPa respectively, but extraction temperature was either 40 °C or 80 °C, then extraction at 80 °C appeared to have higher SOD-like activity compared to extraction at 40 °C. In addition, when extraction time and pressure were fixed at 10 minutes and 25 MPa, SOD-like activity of extracts at 100 °C was 37.45% and 33.11% was at 60 °C. Thus, it appears that approximately 5% was increased in SOD-like activity at 100 °C compared to 60 °C. As drawn in surface and contour line plots, it was also confirmed by indicating big changes of SOD-like activity indicates in accordance with temperature changes in Fig. 4. It should also indicate that extraction temperature must be more important factors for enhancing antioxidant activities while extraction pressure for improving extraction yield of *Acer mono* bark.

Measurement of total polyphenol contents

4.46 mg/g of the highest polyphenol content was obtained under the extraction condition of 80 °C for 15 minutes with 50 MPa, and 2.11 mg/g of the lowest concentration at 40 °C for 15 minutes with 50 MPa in Tab. 1. For other studies of extracting daisy (small flowered chrysanthemum) (Park et al., 1998) or mushroom (*Flammulina velutipes*) (Kim et al., 2003), the total polyphenol contents increased in using 70% ethanol, not water, which was same result with our preliminary tests for *Acer mono* bark. That was why this study had used 70% ethanol as an extraction solvent with variables of extraction temperature, time and pressure. Tab. 5 was the results of estimating p-values of the full quadratic model from the multi-regression analysis of total polyphenol content before and after deleting most non-significant variable such as interaction term in the proposed model. After reconstructing the model, most suitable second polynomial was determined as follows:

$$Y_{Polyphenol} = 4.2320 + 0.1775X_1 - 0.1200X_2 + 0.1775X_3 - 2.3259X_1^2 - 1.4909X_2^2 - 1.7459X_3^2 \quad (4)$$

This model seemed to be better fitting with higher regression coefficient as 0.8590 of R-square(adj) and smaller p-values (more statistically significant) than the cases of not deleting the interaction terms. It was also found that extraction pressure and temperature were considered mostly affecting the total polyphenol contents of *Acer mono*, whereas time was considered less influenced. These results can also be found in Fig. 5, in which the graphs were well fitted to the data as temperature or pressure changed, implying that extraction temperature was most significant variable for extracting polyphenols from the plant. Similar results were also reported in other work that the extraction yields of polyphenol from *Inga edulis* leaves were increased 20% by increasing the extraction temperature from 40 °C to 65.2 °C (Silva et al., 2007). This result indicates that the temperature mostly affected to the total polyphenol content and that as extraction temperature increased total polyphenol content also increased.

Validation of the optimal extraction condition with experimental results

To evaluate the predictive models, the optimal extraction conditions were determined to obtain high extraction yield with high antioxidant

Tab. 3: Estimation of p-value of the full quadratic model from the multi-regression analysis of DPPH radical scavenging activity before and after deletely most non-significant values.

Term	P-value†	P-value‡	Term	P-value†	P-value‡
Regression	0.000	0.000	Temp	0.024	0.012
Linear	0.134	0.077	Time	0.982	0.980
Square	0.000	0.000	Press	0.943	0.936
Interaction	0.895	-	$Temp^2$	0.000	0.000
			$Time^2$	0.349	0.297
			$Press^2$	0.441	0.390
R-Square (adj)	0.8300	0.8620	Temp*Time	0.474	-
			Temp*Press	0.893	-
			Time*Press	0.886	-

†Estimated value before deletely most non-significant values based on P-value.
‡Estimated value after deletely most non-significant values based on P-value.

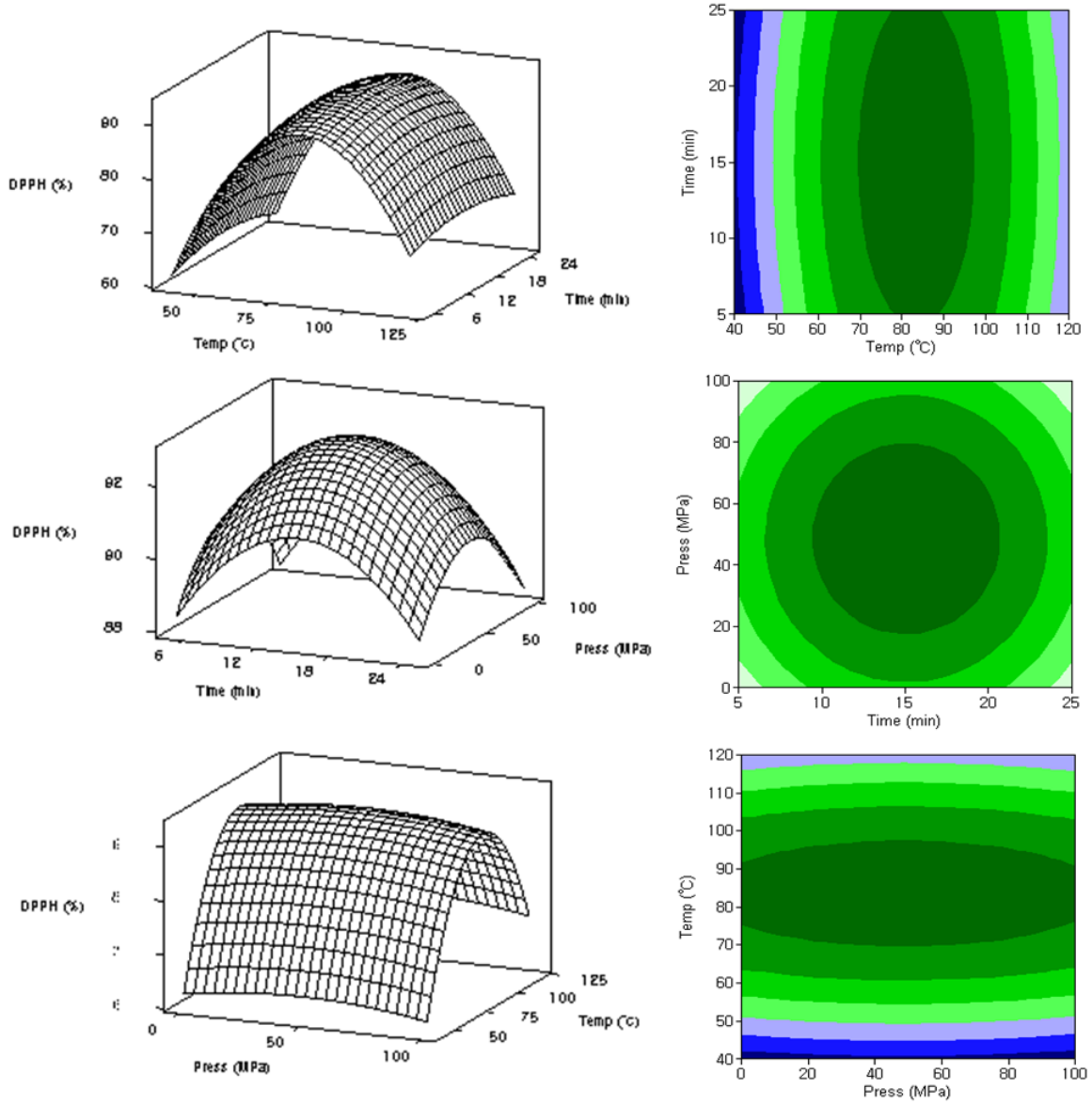


Fig. 3: Effect of extraction temperature, time and pressure on DPPH scavenging activity of extracts from bark of *Acer mono*.

Tab. 4: Estimation of p-value of the full quadratic model from the multi-regression analysis of SOD-like activity before and after deletely most non-significant values.

Term	P-value†	P-value‡	Term	P-value†	P-value‡
Regression	0.000	0.000	Temp	0.007	0.002
Linear	0.037	0.013	Time	0.392	0.332
Square	0.000	0.000	Press	0.695	0.657
Interaction	0.964		$Temp^2$	0.000	0.000
			$Time^2$	0.010	0.003
			$Press^2$	0.019	0.008
R-Square (adj)	0.8340	0.8690	Temp*Time	0.901	-
			Temp*Press	0.663	-
			Time*Press	0.822	-

†Estimated value before deletely most non-significant values based on P-value.
‡Estimated value after deletely most non-significant values based on P-value.

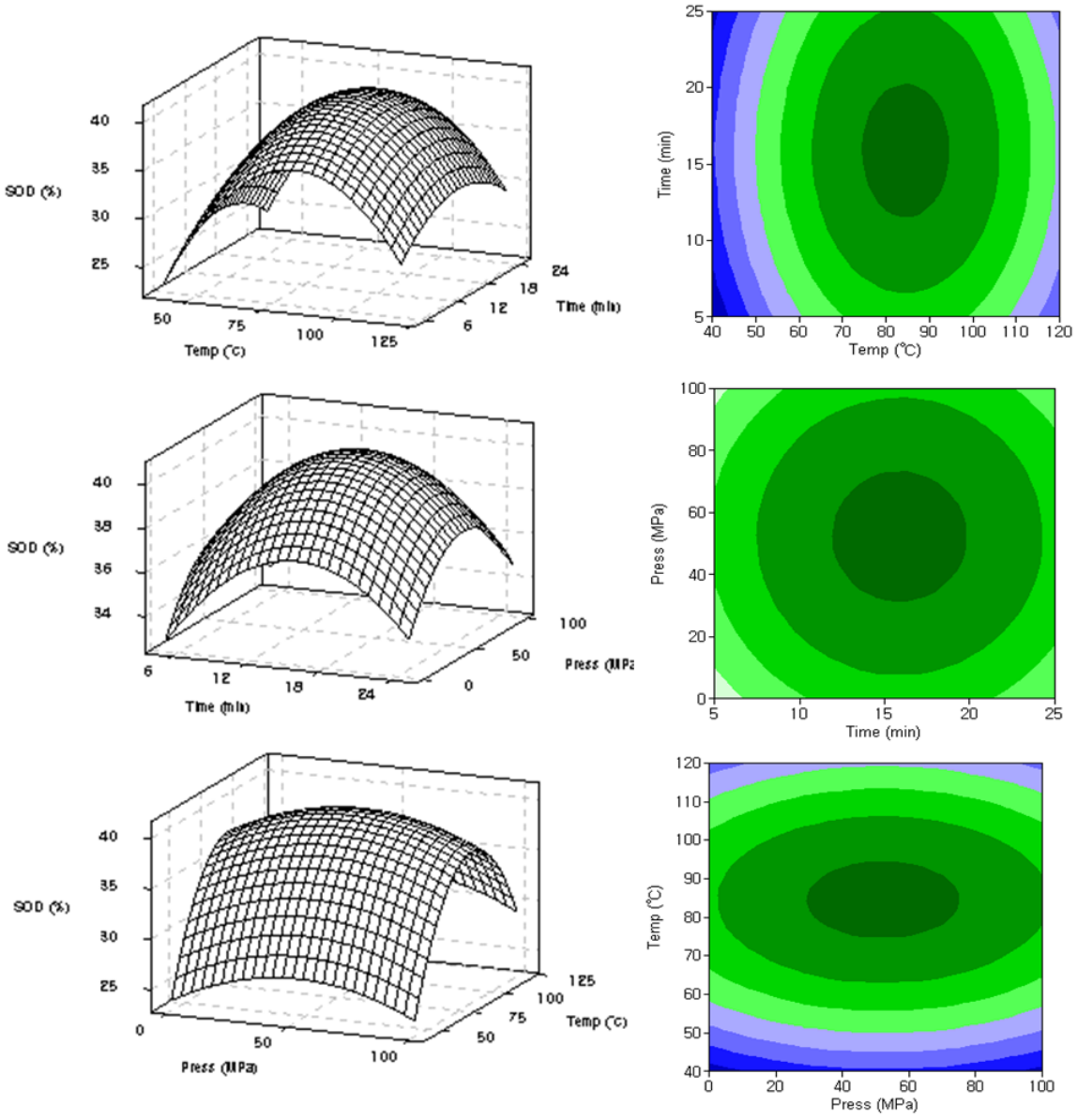


Fig. 4: Effect of extraction temperature, time and pressure on SOD-like activity of extracts from bark of *Acer mono*.

Tab. 5: Estimation of p-value of the full quadratic model from the multi-regression analysis of total polyphenol before and after deletely most non-significant values.

Term	P-value†	P-value‡	Term	P-value†	P-value‡
Regression	0.000	0.000	Temp	0.348	0.286
Linear	0.524	0.418	Time	0.521	0.465
Square	0.000	0.000	Press	0.348	0.286
Interaction	0.980	-	$Temp^2$	0.000	0.000
			$Time^2$	0.000	0.000
			$Press^2$	0.000	0.000
R-Square (adj)	0.8200	0.8590	Temp*Time	0.804	-
			Temp*Press	0.985	-
			Time*Press	0.746	-

†Estimated value before deletely most non-significant values based on P-value.
‡Estimated value after deletely most non-significant values based on P-value.

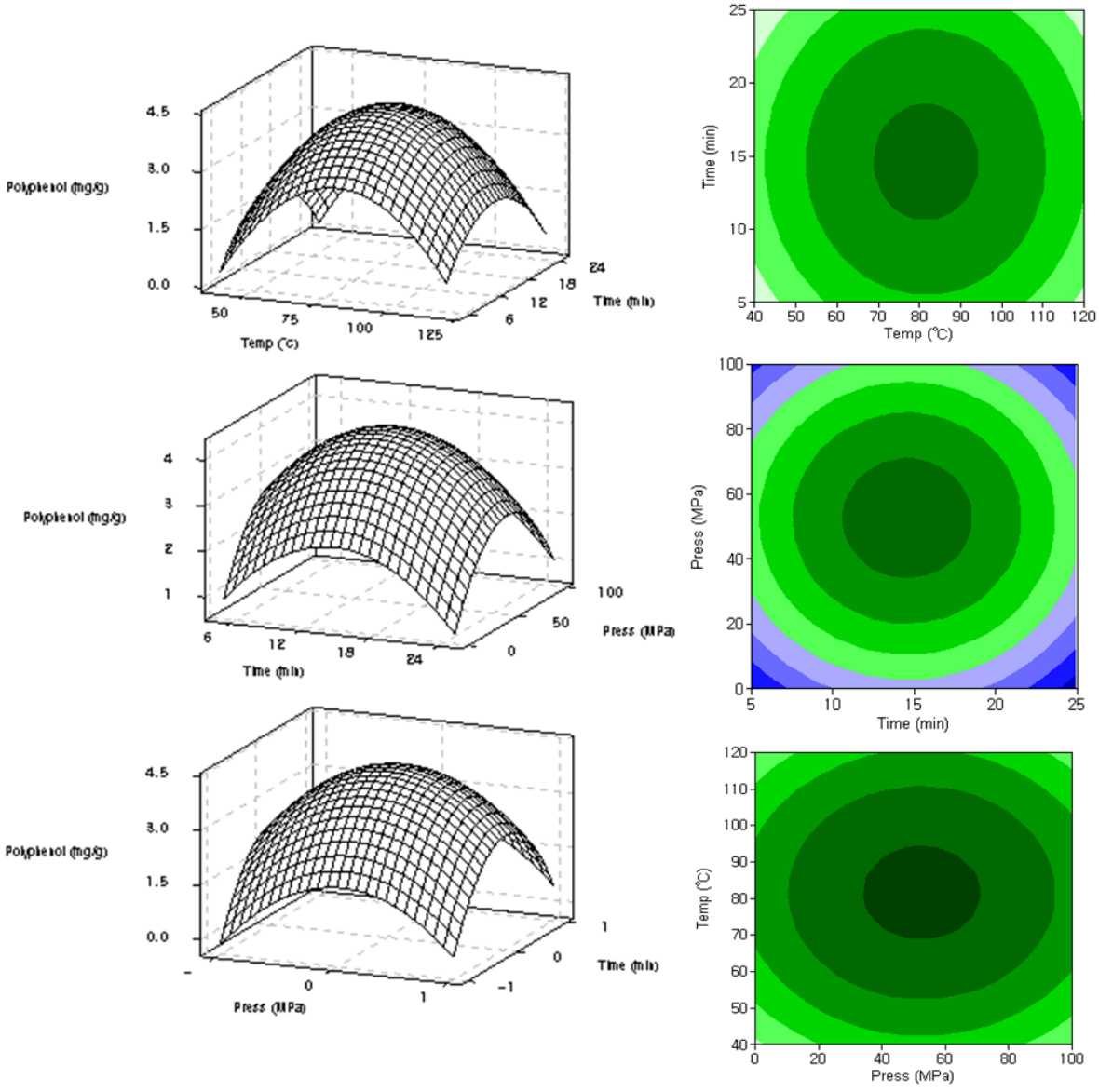


Fig. 5: Effect of extraction temperature, time and pressure on polyphenol content of extracts from bark of *Acer mono*.

Tab. 6: Polymonial equations calculated by response surface analysis program for extraction condition of bark of *Acer mono*.

Response variables†	Second order polynomials‡	R^2
Yields	$Y_{Yield} = 13.0357 + 0.7812X_1 - 0.6412X_2 + 0.7837X_3 - 7.2736X_1^2 - 2.8386X_2^2 - 4.4636X_3^2$	0.8720
DPPH	$Y_{DPPH} = 92.7893 + 4.2175X_1 - 0.0375X_2 - 0.1175X_3 - 24.3314X_1^2 - 2.4914X_2^2 - 2.0364X_3^2$	0.8620
SOD	$Y_{SOD} = 40.5336 + 2.4375X_1 + 0.6425X_2 + 0.2900X_3 - 10.8727X_1^2 - 3.6327X_2^2 - 3.1927X_3^2$	0.8690
Polyphenol	$Y_{Polyphenol} = 4.2320 + 0.1775X_1 - 0.1200X_2 + 0.1775X_3 - 2.3259X_1^2 - 1.4909X_2^2 - 1.7459X_3^2$	0.8590

†DPPH: DPPH scavenging activity, SOD: SOD-like activity, Polyphenol: Total polyphenol.
‡X_1, Temperature (°C); X_2, Time (min); X_3, Pressure (MPa).

Tab. 7: Predicted and experimented value of response variables at a given condition within the range of optimum extraction conditions.

Response variables	Independent variables†			Prediected value	Experimental value
	X_1	X_2	X_3		
Extraction yield (%, w/w)	83.48	13.08	54.36	13.10	13.24
DPPH scavenging activity (%)	88.50	15.08	49.68	92.89	90.11
SOD-like activity (%)	85.21	15.83	53.28	40.69	39.48
Polyphenol (mg/g)	81.51	14.79	52.92	4.23	3.94

†X_1, Temperature (°C); X_2, Time (min); X_3, Pressure (MPa)

activities by the ridge analysis with means of Minitab release 14.12.1 (Minitab Inc., USA) (Silva et al., 2007), and this operating condition was also used for extraction experiments as shown in Tab. 7. For extraction yields, the most optimized condition was at 83.48 °C, 54.36 MPa for 13.08 minutes, having 13.10% of the maximum response with 13.24% of experimental results carried out under this condition. The maximum response of DPPH radical scavenging activity was observed 92.89% at 88.50 °C, 49.68 MPa for 15.08 minutes while 40.69% of the maximum SOD-like activity at 85.21 °C, 53.28 MPa and 15.83 minutes, comparing to 90.11% and 39.48% of experimental results, respectively. The optimized condition for total phenolic compounds was at 85 °C, 52 MPa for 14 minutes, having 4.23 mg/g of the maximum response of total polyphenol content by comparing with 3.94 mg/g of experimental data. The experimental data were within 95% confidence interval of the predicted values for all of independent variables, which confirms that the proposed model could be used to optimize the extraction of relatively hard plants such as *Acer mono* bark. Accordingly, antioxidant activities of *Acer mono* bark was considered as it mostly depends on the total polyphenol content.

Discussion

The RSM was used to optimize extraction parameters that could have high antioxidant activities along with high contents of phenolic compounds from *Acer mono* bark. Statistical analysis revealed that the effect of pressure was most significant in extraction yield whereas the effect of temperature was significant in antioxidant activities. More specifically, in considering to extract relatively harder resources of the plant, *Acer mono* bark than other medicinal herbs, more than most commonly used variables of temperature, solvents ratio or ethanol concentration, etc. should be employed, which would be pressure response since this variable was hardly found in RSM (Karacabey and Mazza, 2010; Liu et al., 2010). From our results, the pressure ($p = 0.098$) appeared to be most significant in extraction yield, followed by temperature and time, respectively. However, this was different from other works that the temperature was most significant in extraction yield of roasted wheat germ (Gelmez et al., 2009). For polyphenol contents with high antioxidant activities, both temperature and pressure appeared to be important, which was similar to other studies that as temperature rose under less than 150 MPa, polyphenol content increased (Choi et al., 1998; Murga et al., 2002). Overall antioxidant activity shows that the highest antioxidant activity appeared at 80 °C, 50 MPa for 15 minutes, which was similar to the condition regarding total polyphenol content.
Quadratic models were used in predicting all the responses, and the optimal extraction conditions were determined based on combination all responses. The second-order polynomial models were derived by consecutively deleting most non-significant terms in the models, maintaining better significance with lower p-values of many interaction terms in the models. It was proved that this model well predicted satisfactory results, compared to the experimental data. This study would also be a standard methodology considering relationship among variables such as temperature, pressure and time to extract resources from wood substances, based on the fact that considering multiple variables is required for enhancing activity of extracts.

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