STUDY ON INTERACTION ANALYSES FOR CYTOTOXICITY OF NATURAL PRODUCTS BY USING EXPERIMENTAL DESIGN

[1]HYUN JEONG LEE, [2]BOGEUN LEE, [3]SANGMUN SHIN

[1,2,3]Department of Industrial & Management Systems Engineering, Dong-A University,
37, Nakdong-daero 550beon-gil, Saha-gu, Busan 49315, Republic of Korea
E-mail: [1]gpfapt1223@gmail.com, [2]bglee@dau.ac.kr,[3]sshin@dau.ac.kr

Abstract - Most existing approaches for cytotoxicity analysis of multi-components natural products may often not consider interactions among a number of components in a natural product without using a designed experiment. This analysis problems include a number of difficulties associated with a dimensionality, such as simultaneous consideration of many ingredients, a huge number of experimental runs, statistical errors related to high dimensions, etc. To this end, this study is to perform systematic statistical designed experimental methods (i.e., mixture experimental design). In order to consider both a number of runs and their associated statistical efficiencies, a simplex lattice design was utilized in this study. In addition, this research also investigates a tradeoff between hepatotoxicity and synergy effects based on a cell viability. The primary goal of this research is to obtain an optimal combination of multi-components by maximizing the cell viability with low cytotoxicitand high synergy effects. To verification purposes, this paper demonstrates an experimental design, its associated statistical analysis results, and an optimization approach and its related results.

Keywords: Design of experiment, Mixture design, Multi-components, Natural product, Cytotoxicity

I. INTRODUCTION

Oriental medicines includingmany representative traditional natural substances have been used for more than a couple of hundred years in manyeastern countries. According to a survey about the use and consumption of the oriental medicine by Ministry of Health and Welfare in South Korea in 2017, 84.2% of the respondents answered that they would use oriental medicineswhen they might need to takea medical service. Overall satisfaction with the treatment of oriental medicines was considerably high around 76.2% [1].In South Korea, pharmaceutical industries continuously consider and perform a new drug development in terms of chemical, bio, and natural product drugs.

To perform a natural product drug discovery, both cytotoxicity and synergy effects should be investigated simultaneously. In much literature, the cytotoxicity can be significantly reduced while maintaining its synergy effect when a number of natural products are mixed together, although each natural product itself may provide a high cytotoxicity. For this reason, correlation and interaction effect analyses instead of single natural product investigation should be conducted in this natural product drug development. In order to perform interaction effect analyses with a large number of natural products, designed experimental methods and their associated statistical analysis methods must be applied [2]. According to a multicenter study for the development and detection of a nationwide reporting system for toxic liver injuries, 26 of the 110 cases collected were reported to be caused by natural product drugs.In this study, natural products known as medical herbs (i.e., Xanthium fruit, ToosendanFructus, Ephedra, Pinellia ternate, Scutellariabaicalensis, VisciHerba, Glycyrrhizaurarlensis,andCorydalis remota)caused significant hepatotoxicity [3]. In addition, a component (i.e., monocrota line which is a pyrrolizidine alkaloid) in natural products provide a considerably high hepatotoxicity [3]. A prescription of natural product drugs is mixedby a number of different medicinal herbs (i.e., natural products). Each natural product includes many components with different medical functionalities and metabolisms. In order to consider these medical functionalities and metabolisms, both cytotoxicity and synergy effects should be integrated simultaneously. In many cases, there is a tradeoff (i.e., minimization of the cytotoxicity and maximization of the synergy effect) issue between cytotoxicity and synergy effects. However, the primary approach can be identified by maximizing the synergy effect while maintaining considerably low cytotoxicity in most natural product drug development problems. For this reason, it is difficult to identify the cause of the cytotoxicity and synergy effects among those multiple components and their interaction effects clearly because there exist complex interactions (i.e., synergism and antagonism) among many natural products as well as among their associated many components [2]. This study is to perform a systematic statistical designed experiment methods (i.e., mixture experimental design). Based on the proposed method, the four substances in the alkaloid group of compounds derived from the corydalis remota were mixed at various rates. In order to consider both a number of runs and their associated statistical efficiencies, a simplex lattice design was utilized in this study. In addition, this research also investigates tradeoff between hepatotoxicity and synergy effects based on cell viability. The primary goal of this research is to obtain an optimal combination of multi-components by maximizing cell viability with low cytotoxicity and high synergy effects. To verification purposes, this paper de-

monstrates an experimental design, its associated statistical analysis results, and an optimization approach and its related results.

II. DETAILS EXPERIMENTAL

2.1 Mixture Design

The mixture design proposed by Scheffe [4] is often used to examine mixtures of ingredients and their associated interactions. The properties of the mixture are functions of the relative proportions in different ingredients rather than their absolute amounts. The values of input variables are their proportions in the mixture, which can be between zero and one [5]. The proportions of all ingredients in each mixture experimental design point must sum to 100%. Therefore, the properties of a mixture is represented by the following formula:

$$\sum_{i=1}^{k} x_i = 1 \; and \; 0 \le x_i \le 1 \qquad (1)$$

where x_i is the proportion of component and k is the numberof components (i.e., input variables)[2].In a case where there are three components, a graphical representation of design points are illustrated inFig.1.

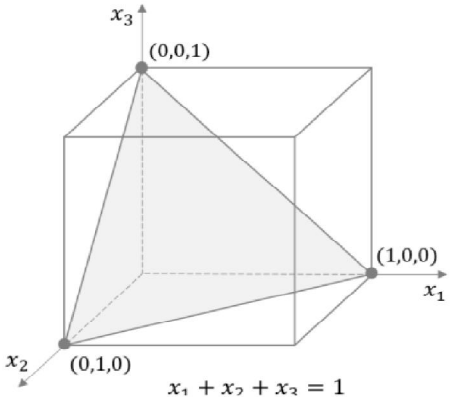


Fig.1. Constrained factor space for mixtures with k=3

2.1.1 Simplex lattice design

A simplex lattice design can be used to determine the effects of mixture components on the response variable. A {k, m} simplex lattice design for k components consist of points defined by the following coordinate settings: the proportions assumed by each component take the m + 1 equally spaced values from 0 to 1, $x_i = 0, \frac{1}{m}, \frac{2}{m}, \cdots, 1 \; (i = 1, 2, \cdots, k)$ and all possible combinations (mixture) of the proportions are used to determine the design points. In general, the number of points in a {k, m} simplex lattice design is $n = \frac{(k+m-1)!}{m!(k-1)!}$. If more precise predictions are desired, additional axial points and center points may be added to the design [6]. In this paper, we analyzed the main and interactions effects for fouringredients of natural products in order to estimate the effect on cell number. To this experiment, a {4, 2} simplex lattice design by adding four axial points and one center point is utilized as shown inFig.2.

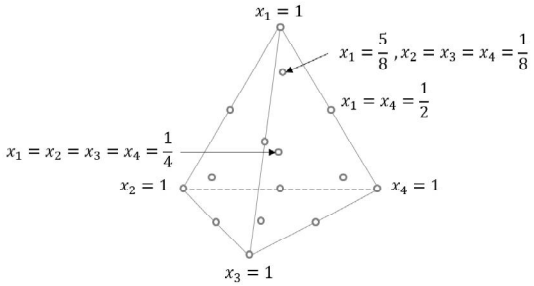


Fig.2. Simplex lattice design for k=4, m=2 with axial point and center point

2.2 Regression analysis

In general, supposing that there is a single dependent variable or response y that depends on k independent or input variables, for example, $x_1, x_2, \ldots, x_k$, the relationship between these variables is characterized by a mathematical model called a regression model. The response variable y may be related to kinput variables. The model is called a multiple linear regression model with kinput variables and its corresponding model can beas follows:

$$y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \cdots + \alpha_k x_k + \epsilon. \qquad (2)$$

Theparameter α_j (where, j=1, 2, …,k)are called the regression coefficients. This model describes a hyperplane in the k-dimensional space of the input variables $\{x_j\}$. The parameter α_j represents the expected change in response y per unit change in x_j when all the remaining independent variables $x_i (i \neq j)$ are held constant[6].

2.3 Cox Response Trace Plot

Based on the results of the mixture experimental design (i.e., simplex lattice design), Cox response trace plot can demonstrate the estimated change of output responses with respect to the changed constitution of input ingredients. In addition, the main effect of each input ingredient can be graphically determined by this Cox response trace plot although analysis of variance (ANOVA) may not directly provide statistical hypothesis results associated with the main effects of input ingredients in the mixture design and analysis as shown in Figures 3 and 4.

2.4 Optimization

The main goal of the optimization stage is to identify the optimal settings of input ingredients (i.e., components of a natural product).In order to conduct this optimization problem, a desirability function is used to identify the optimal solutions. The desirability of each response value depends often on its specification limits [8].Three different types (i.e., larger the better, smaller the better, normal the best) of desirability functions can be applied by three different optimization approaches, such as maximization, minimization,

minimization of the deviation form the target value, respectively.

The desirability function $d_i(Y_i)$ of each response can have a value within the scale [0 1], where $d_i(Y_i) = 0$ and $d_i(Y_i) = 1$represent completely undesirable and completely desirable, respectively [8]. In this paper, since only smaller the better type of desirability function is used, its associated desirability function can be identified as follows:

$$d_i(\hat{Y}_i) = \begin{cases} 1 & if \hat{Y}_i(x) < L_i \\ \left(\dfrac{\hat{Y}_i(x) - U_i}{L_i - U_i}\right)^r & if L_i \le \hat{Y}_i(x) \le U_i \\ 0 & if \hat{Y}_i(x) > U_i \end{cases} \quad (3)$$

whereL_i and U_i represent the lower and upper values, respectively. In this desirability function, different weights can be assigned by changing a weight parameter represented by the superscript r. For example,

when the superscript r=1, the desirability function can be a linear. When the superscript$r > 1$and $0 < r < 1$, the desirability functions can then be changed to convex and concave nonlinear functions with high and low importance, respectively [6].

III. RESULTS AND DISCUSSION

3.1 Experimental design

Aderived from the corydalis remota, such asRotundine(x_1), Allocryptopine(x_2), Corynoline(x_3), Dehydrocorydalin(x_4), were selected. The significant issue of this experiment is to design mixtures of these four ingredients. In addition, both low and high densities of this mixture were also prescribed into the cells, such as 10μg/mL and 100μg/mL, in order to determine cell viabilities.In this experimental design, cell viability candefined by the deviation from cell numbers ofa controlgroup and an experimental group.

Therefore, a small value of cell viability represents a low cytotoxicity and/or a high synergy effect (i.e., positive cell viability increased by a number of interactions among mixtures of components).

In this experiment, a{4, 2} simplex lattice design with a center point and four axial points was utilized. This simplex lattice design provides 15 design points. The proposed experimental design and its associated output results (i.e., values of cell viability)for bothdensities (i.e., 10μg/mL and 100μg/mL) are demonstrated in Table 1.

3.2 Experimental results

As shown in Tables 2 to 5, ANOVA for the cell viability is performed in order to identify the interaction effects on two different densities. As explained in section 2.3, since the main effects cannot demonstrated in this ANOVA, the main effects can be predicted by using Cox response trace plot as shown in Fig.3 and Fig.4. When negative (-) and positive (+) slopes in a Cox response trace plot represent reduction and increment effects of cytotoxicity, respectively. In density 10μg/mL as shown in Fig.3, the slopes of all four substances are close to zero. Therefore, it can be predicted that the influence of all main effectsto the response (i.e., cell viabability)may be small.In density 100μg/mL as shown in Fig.4, two components (i.e., Allocryptopine$(x_2)$and Dehydrocorydalin(x_4)) and the other components (i.e., Rotundine(x_1) and Corynoline(x_3)) providehigh cytotoxicity effects and relatively low cytotoxicity effects, respectively.

The levels of both main and interaction effects can be indirectly determined by the regression coefficients in Tables 2 and 4. In order to minimize the cell viability (i.e., minimize cytotoxicity effects and maximize synergy effects) simultaneously, the proposed desirability function is utilized for two different densities.

This simultaneous optimization results can provide the optimal mixture of ingredients (i.e., components) as shown in Tables 6, 7, and 8.

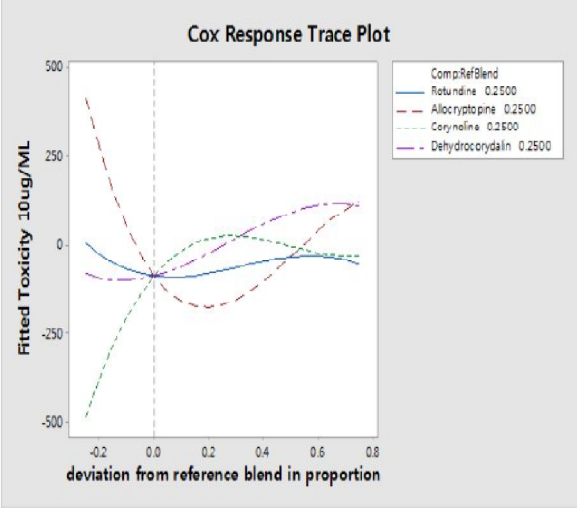


Fig.3. Cox Response Trace Plot for main effects (10μg/mL)

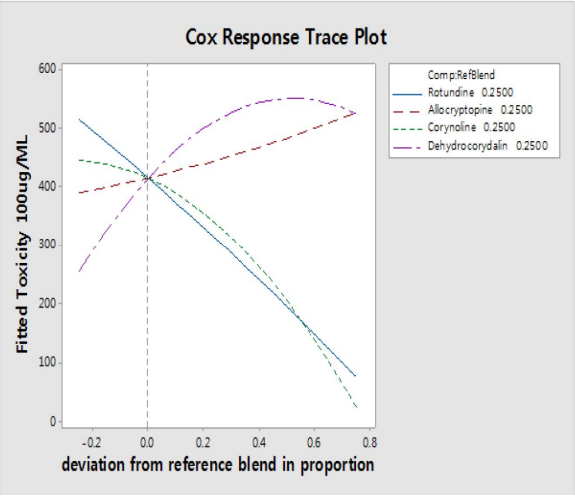


Fig.4. Cox Response Trace Plot for main effects (100μg/mL)

Table 1. Simplex lattice experimental design results

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SO	EO	Input factors				Output responses(Cell viability)					
		x_1	x_2	x_3	x_4	$y_1(10\mu g/mL)$			$y_2(100\mu g/mL)$		
		Rotundine	Allocryptopine	Corynoline	Dehydrocorydalin	y_{11}	y_{12}	y_{13}	y_{21}	y_{22}	y_{23}
1	9	100	0	0	0	-262.25	56.75	-26.25	44.75	102.75	29.75
2	12	50	50	0	0	-18.25	-31.25	-63.25	327.75	311.75	339.75
3	13	50	0	50	0	-58.25	-62.25	88.75	-141.25	41.75	161.75
4	8	50	0	0	50	286.75	107.75	327.75	396.75	457.75	540.75
5	14	0	100	0	0	262.75	51.75	110.75	533.75	461.75	563.75
6	11	0	50	50	0	-173.25	-105.25	8.75	341.75	399.75	347.75
7	4	0	50	0	50	8.75	129.75	135.75	435.75	504.75	515.75
8	6	0	0	100	0	-17.25	-116.25	10.75	-155.25	176.75	62.75
9	3	0	0	50	50	53.75	18.75	40.75	519.75	551.75	545.75
10	1	0	0	0	100	221.75	60.75	117.75	528.75	573.75	582.75
11	7	25	25	25	25	-37.25	-65.25	-83.25	327.75	480.75	399.75
12	5	62.5	12.5	12.5	12.5	-56.25	-58.25	-18.25	258.75	249.75	309.75
13	10	12.5	62.5	12.5	12.5	-133.25	-150.25	-126.25	444.75	509.75	542.75
14	15	12.5	12.5	62.5	12.5	-212.25	115.75	92.75	328.75	273.75	279.75
15	2	12.5	12.5	12.5	62.5	72.75	33.75	-53.25	418.75	506.75	522.75

The ANOVA results of mixture design for the cell viability at densities 10μg/mL and 100μg/mL are demonstrated in Table 3 and Table 5, respectively, and where small p values (i.e., less than 0.05) represents that the interaction effects are significant.The ANOVA results revealed that four interaction effects (i.e., Rotundine · Dehydrocorydalin, Allocryptopine · Corynoline, Rotundine · Allocryptopine · Dehydrocorydalin, Rotundine · Corynoline · Dehydrocorydalin) associated with cell viability at 10μg/mL as well as three interaction effects(i.e., Rotundine · Dehydrocorydalin, Allocryptopine · Corynoline, Corynoline · Dehydrocorydalin) related to cell viability at toxicity 100μg/mL.

Coefficient is an estimate of the change in the mean response as the unit of the input factor increases when all other predictors are fixed in constants [7]. Thus, the levels of both main and interaction effects can be indirectly determined by the regression coefficients in Tables 2 and 4. In density 10μg/mL, the most significant interaction is Rotundine · Allocryptopine · Dehydrocorydalin. Because the coefficient of itsinteraction is large and negative, the cell viability is increasedby the interaction of the ingredients.In the case of Rotundine · Corynoline · Dehyorine, on the other hand, its coefficient is positive value so that the cell viability is decreasedby the interactions. In density 100μg/mL, the coefficient value of all terms is positive so that the cell viability is expected to decrease over all.

Table 2Estimated regression coefficients for Cell viability at Toxicity10μg/mL

Terms	Coefs.	SE Coefs.	T	p-value	VIF
Rotundine	-56.3	26.9	*	*	1.74
Allocryptopine	122.9	27.5	*	*	1.83
Corynoline	-31.2	25.4	*	*	1.55
Dehydrocorydalin	107.4	24.7	*	*	1.47
Rotundine·Allocryptopine	-281	145	-1.94	0.100	1.77
Rotundine· Dehydrocorydalin	852	144	5.90	0.001	1.76
Allocryptopine· Corynoline	-568	143	-3.97	0.007	1.73
Rotundine·Allocryptopine· Dehydrocorydalin	-16461	3136	-5.25	0.002	5.61
Rotundine·Corynoline· Dehydrocorydalin	8474	3108	2.73	0.034	5.51

*Coef.: Least square estimator of regression coefficient, T: Test statistic, P: p-value, VIF: variance inflation factor

Table 3 ANOVA for Cell viability at Toxicity10μg/mL

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Terms	Degree of freedom	Sum of square	Mean square	F-value	p-value
Regression	8	140274	17534.3	18.74	0.001
Linear	3	41507	13835.8	14.79	0.004
Quadratic	3	49416	16472.1	17.61	0.002
Rotundine·Allocryptopine	1	3535	3534.9	3.78	0.100
Rotundine· Dehydrocorydalin	1	32606	32606.2	34.85	0.001
Allocryptopine· Corynoline	1	14719	14719.1	15.73	0.007
3 variable interaction	2	35084	17541.8	18.75	0.003
Rotundine·Allocryptopine· Dehydrocorydalin	1	25784	25784.0	27.56	0.002
Rotundine·Corynoline· Dehydrocorydalin	1	6955	6955.0	7.43	0.034
Error	6	5614	935.6		
Total	14				
S = 30.5879	$R^2 = 96.15$				

Table 4 Estimated regression coefficients for Cell viability at Toxicity100μg/mL

Terms	Coefs.	SE Coefs	T	p-value	VIF
Rotundine	74.2	28.9	*	*	1.42
Allocryptopine	525.1	28.9	*	*	1.42
Corynoline	22.7	32.0	*	*	1.74
Dehydrocorydalin	524.1	32.0	*	*	1.74
Rotundine·Dehydrocorydalin	622	167	3.72	0.006	1.67
Allocryptopine· Corynoline	405	167	2.42	0.042	1.67
Corynoline· Dehydrocorydalin	1018	167	6.12	0.000	1.65

*Coef.: Least square estimator of regression coefficient, T: Test statistic, P: p-value, VIF: variance inflation factor

Table 5 ANOVA for Cell viability at Toxicity100μg/mL

Terms	Degree of freedom	Sum of square	Mean square	F-value	p-value
Regression	6	479189	79865	60.31	0.000
Linear	3	398352	132784	100.27	0.000
Quadratic	3	71652	23884	18.04	0.001
Rotundine·Dehydrocorydalin	1	18347	18347	13.85	0.006
Allocryptopine· Corynoline	1	7775	7775	5.87	0.042
Corynoline· Dehydrocorydalin	1	49615	49615	37.47	0.000
Error	8	10594	1324		
Total	14				
S = 36.3097	$R^2 = 97.84$				

When it value has the smaller, we find the toxic effect of interactionis smaller and the synergy effect is bigger. Therefore, optimal combination of components is searched by using the desirability function of the STB(smaller the better) characteristics to minimize cytotoxicity and maximize the synergy effects.The optimal combinations of input factors to minimize toxicity and maximize synergy effect is derived and shown in Table 6, 7, and 8, respectively. As shown in Table 6, the optimization resultsfor toxicity 10&100μg/mL for the substances showed that the specific mixture of Corynoline at ratio 100% simultaneously minimized cytotoxicity and maximized synergy effect.

Table 6.Optimization result for Toxicity10 & 100μg/mL

Criteria (Cell viability)	Object[a]	Lower[b]	Target[c]	Upper[d]
10μg/mL	Minimum	-	0	144.27
100μg/mL	Minimum	-	0	249.48
Components	Rotundine	Allocryptopin	Corynoline	Dehydrocorydalin
Optimal solution	0	0	100	0

[a] Objectives (i.e., minimize and maximize) of responses
[b] Allowable minimum values
[c] Desirable target values

CONCLUSIONS

In this paper, we proposed an analytical method for understanding the toxic effects and synergy effects among the constituents of toxic substances in traditional natural products.Based on this, four substances of corydalis remota were investigated systematically based on the proposed customized statistical design and analysis methods. Major conclusions are as follows:

1. The Mixture simplex lattice design, cox response trace plot, and analysis of variance (ANOVA) were proposed to investigate interaction effects.
2. The main effects as well as the main interaction effects of toxicity can be determined by using the proposed analysis of variance, cox response trace plot and regression coefficient.
3. By using the MINITAB software package, the optimal combination of all the components that minimize toxicity and maximize synergy effect can be obtained by using the desirability function.
4. The optimal combinations to minimize toxicity and maximize synergy effects areCorynoline (100%) for Toxicity 10 & 100μg / mL and also Toxicity 100μg/mL. In the case of Toxicity 10μg/mL, the optimal combinations are Rotundine(34.5672%), Allocryptopin(36.1399%), Dehydrocorydalin(29.2929%).

The methods proposed in this paper can be utilized as a key technology for rapid evaluation of new toxic components.

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