Robust optimization for the simultaneous enhancement of nitric oxide inhibition and reduction of hepatotoxicity from green tea catechins

Min Chae Kim¹ · Tuan-Ho Le² · Cheng Bao¹ · Jin Tae Kim¹ · Hyang Sook Chun¹ · Sangmun Shin² · Hong Jin Lee¹

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Abstract To provide a platform for evaluating significant interactions contributing to the enhanced physiological efficacy and reduced hepatotoxicity, we used a robust design to determine the optimal combination of six major green tea catechins, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallocatechin, and catechin. Based on the mixture design, 28 experiments were performed to inhibit nitric oxide (NO) in RAW 264.7 cells and hepatotoxicity in Chang liver cells. Significant candidates, EGCG, EC, gallocatechin and catechin, were selected after optimization. The combination showing simultaneous enhancement of NO inhibition and reduction of hepatotoxicity was EGCG and gallocatechin at a ratio of 0.65 to 0.35 by surface response methodology and desirability function, through which their co-treatment was validated. Here, we describe a platform for simultaneously determining the optimized combination of natural components exerting enhanced efficacy and reduced toxicity.

Min Chae Kim and Tuan-Ho Le these authors contributed equally to this work.

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✉ Sangmun Shin
sshin@dau.ac.kr

✉ Hong Jin Lee
hongjin@cau.ac.kr

¹ Department of Food Science and Technology, Chung-Ang University, Anseong 456-756, South Korea

² Department of Industrial and Management Systems Engineering, Dong-A University, Busan 604-714, South Korea

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Introduction

Accumulating evidence has demonstrated that natural resources possess health benefits for preventing the chronic diseases including obesity, inflammation, and cancer [1–3]. Natural resources contain a number of components, and studies have attempted to identify single molecules responsible for physiological activity using different extraction methods and/or instrumental analysis [4, 5]. Many studies have also evaluated the synergistic effects of natural product ingredients [6, 7]. Recently, combination of resveratrol and ellagic acid was found to synergistically inhibit 7,12-dimethylbenz[a]anthracene-induced epidermal hyperplasia [7]. However, studies of natural substances and their major active constituents have shown controversial results. For example, the consumption of soy food has been reported to be associated with a significant reduction of breast cancer risk [8–10], while some animal studies showed enhanced breast tumor growth following treatment with genistein, a major active isoflavone [11–14]. In addition, capsaicin, a major pungent constituent in hot pepper, was reported to act as a co-carcinogen during skin carcinogenesis [15]. These results suggest that the different interactions among food constituents lead to different physiological outcomes.

One concern related to pure ingredients from a natural product is toxicity. Herbal medicines used for a long period of time in Asia are generally considered as safe, but some were reported to induce hepatotoxicity [16]. However, PHY906 prepared from four herbs (*Scutellaria baicalensis*, *Paeonia lactiflora*, *Ziziphus jujube*, and *Glycyrrhiza uralensis*) reduced gastrointestinal toxicity induced by the chemotherapeutic

drug irinotecan and enhanced the anti-tumor activity of irinotecan in colorectal cancer [17, 18], supporting that combinations of natural resources can modulate toxicity.

It is important to identify optimal combinations of natural ingredients to enhance the efficacy of health benefits and reduce toxicity. However, it is difficult to test interactions among the constituents in natural resources because of the large number of tests required. In addition, conventional ‘one-at-a-time’ approximation may not reveal the optimal combination of natural ingredients because it cannot characterize possible interactions among input variables when considering the concentrations and different types of ingredients. Therefore, an approach for minimizing the number of tests to obtain the maximum number of significant results it is required.

Based on the reliability of statistical information, a small number of experiments may not provide reliable information containing all possible statistical interactions because the degree of freedom is not sufficient for statistical analysis. However, experimental design methods can reveal high dimensional correlations and interactions among many input variables by reducing the number of experimental runs. Based on the results of these experiments, it is often necessary to determine optimal input variable settings to minimize/maximize their associated output responses. Robust design (RD), which is a sequential design and optimization method, can provide the best settings for input variables while identifying functional relationships between input variables and output responses. In natural product experiments, RD can be used to determine the optimal combinations of natural ingredients. RD methods involve three main procedures: experimental design, parameter estimation, and optimization to obtain optimal variable settings [19]. A systematic RD procedure was developed by integrating both the mixture experimental design considering the combination of natural ingredients and response surface methodology (RSM) analyzing the functional relationship between input variables and their associated output responses. In addition, the optimal combination of natural ingredients can also be obtained using an RD optimization method based on a desirability function (DF).

In this study, we provided the significant platform using an RD approach for determining the optimal combination enhancing biological efficacy, nitric oxide (NO) inhibition, and reducing hepatotoxicity simultaneously with six catechins in green tea and validated.

Materials and methods

Reagents and cell culture

Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) and catechin

were purchased from Sigma (St. Louis, MO, USA) and gallicocatechin was from BOC Science (Shirley, NY, USA). Chang liver cells were obtained from ATCC (Manassas, VA USA) and cultured in Dulbecco’s modified eagle’s medium (DMEM, Biowest, Nuaillé France) with 10% fetal bovine serum (Biowest) and 1% penicillin/streptomycin (Life Technologies, Carlsberg, CA, USA). RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% HEPES (Life Technologies). The cells were incubated at 37 °C, and 5% CO₂ (Panasonic Healthcare, Wood Dale, IL, USA).

Inhibition of NO synthesis

RAW 264.7 cells were seeded in a 96-well plate and treated with 100 ng/mL lipopolysaccharide (LPS, Sigma) and different combination of green tea catechins for 15 h. NO production was quantified using Griess reagent system (Promega, Madison, WI, USA). Briefly, supernatants were collected and the concentration of NO was measured using sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) and naphthylethylenediamine solution (0.1% *N*-1-naphthylethylenediamine dihydrochloride in distilled water) for 10 min. The absorbance at 540 nm was measured using microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) assay

Chang liver cells were seeded in 96-well plates and treated with different combinations of green tea catechins for 24 h. Next, the activity of LDH released into the medium was measured using a PierceTM LDH Cytotoxicity Assay Kit (Pierce, Rockford, IL, USA).

Robust design methods

The proposed systematic design and statistical analysis procedure were sequentially implemented in natural ingredient experiments. The main purpose of these experiments was to identify the optimal combinations of six input ingredients (green tea catechins) in order to simultaneously minimize hepatotoxicity and maximize NO inhibitory activity. The overall procedure and analysis methods are shown in Fig. 1. In addition, the sequential RD methods including experimental design, estimation, and optimization are shown in Table S1.

Experimental design

The mixture design proposed by Scheffe [20] is often used to examine mixtures of ingredients and their

Fig. 1 Overall scheme of the application of robust design (RD) (experimental design, parameter estimation, and optimization) for determining the optimized combination that simultaneously increased NO inhibitory activity and reduced hepatotoxicity

associated interactions. The properties of the mixture are functions of the relative proportions in different ingredients rather than their absolute amounts. Here, the values of input variables were their proportions in the mixture, which can be between zero and one [21]. The proportions of all ingredients in each mixture experimental design point must sum to 100%. Therefore, the properties of a mixture were represented using the following formula:

$$\sum_{i=1}^k x_i = 1 \quad \text{and} \quad 0 \leq x_i \leq 1 \quad (1)$$

where x_i is the proportion of component i ($i = 1, 2, \dots, k$) and k is the number of components (i.e., input variables). Among the mixture designs, the simplex design is one of the most popular methods for conducting blending experiments. In this design, the polynomial regression model illustrates the response surface across the entire simplex region. There are standard simplex mixture designs for fitting standard models, such as simplex lattice designs and simplex centroid designs.

Regression analysis

Among the variety of ingredients in natural products, a large number of potentially significant ingredients can be considered as candidates in the mixture. The main objective of this experiment was to reduce the dimensionality associated with the large number of input ingredients by investigating the criticality and importance of mixture ingredients. A first-order or first-degree mixture design model is often used to construct screening designs and their corresponding models as follows:

$$E(y) = \alpha_1 x_1 + \alpha_2 x_2 + \dots + \alpha_i + \dots + \alpha_q x_q \quad (2)$$

where α_i denotes a non-negative estimated coefficient. The relative effects of the ingredients can be assessed by ranking the ratios of the estimated coefficients α_i and their associated relative standard errors. Small and large values of the estimated coefficients approximately represent insignificant and significant values, respectively.

Biological functions are often depended on a process variable (i.e., concentration) which is not a part of natural

mixture ingredients, but a conditional variable affecting the blending properties of the mixture. Experimental runs are exponentially increased when process variables are added. In order to resolve this large dimensionality problem, a regression analysis method (i.e., stepwise regression) can be applied by systematically conducting significant variable screening based on statistical criteria (i.e., correlation, R^2 , and p value). This regression analysis method is a semi-automated process used for model building by successively adding or removing variables solely based on the t -statistics of their estimated coefficients [22].

Response surface methodology (RSM)

RSM is typically used to identify the functional relationship between input variables and their associated output responses. In a natural product mixture experiment, the second order estimated response surface model for physiological efficacy and toxicity can be represented as follows:

$$\hat{y}(\mathbf{x}) = \hat{\beta}_0 + \sum_{i=1}^p \hat{\beta}_i x_i + \sum_{i=1}^p \sum_{j=1}^p \hat{\beta}_{ij} x_i x_j \quad (3)$$

where $\hat{\beta}$ denotes the estimated coefficient in the regression functions. The coefficient can be estimated by using least square method as follows:

$$\hat{\beta} = (\mathbf{X}^T \mathbf{X})^{-1} \mathbf{X}^T \mathbf{y} \quad (4)$$

where $\mathbf{X}$ is the design matrix of input variables $\mathbf{x}$.

Optimization

The main goal of the optimization stage was to identify the optimal settings of natural ingredients. Among the several optimization methods available, the DF-based optimization method was utilized to solve the simultaneous dual-objectives including enhancing physiological efficacy and minimizing hepatotoxicity where “the larger the better (L)-type” of the DF and “the smaller the better (S)-type” of the DF were applied, respectively. The L-type and S-type of individual desirability functions can be identified as;

$$d_{NO}(\hat{y}_{NO}) = \begin{cases} 0 & \text{if } \hat{y}_{NO}(x) < L_{NO} \\ \left(\frac{\hat{y}_{NO}(x) - L_{NO}}{U_{NO} - L_{NO}} \right)^r & \text{if } L_{NO} \leq \hat{y}_{NO}(x) \leq U_{NO} \\ 1 & \text{if } \hat{y}_{NO}(x) > U_{NO} \end{cases} \quad (5)$$

$$d_{tox}(\hat{y}_{tox}) = \begin{cases} 1 & \text{if } \hat{y}_{tox}(x) < L_{tox} \\ \left(\frac{U_{tox} - \hat{y}_{tox}(x)}{U_{tox} - L_{tox}} \right)^s & \text{if } L_{tox} \leq \hat{y}_{tox}(x) \leq U_{tox} \\ 0 & \text{if } \hat{y}_{tox}(x) > U_{tox} \end{cases} \quad (6)$$

where $\hat{y}_{NO}$ and $\hat{y}_{tox}$ denote the estimated functions between ingredients of a natural product. L_{NO} and U_{NO} denote the lower and upper values of physiological efficacy and L_{tox} and U_{tox} denote the lower and upper values of hepatotoxicity. The superscripts r and s represent the importance of individual DFs. The overall desirability, D , a geometric mean of all DFs, can be expressed as follows:

$$D = [d_{NO}(\hat{y}_{NO}) \times d_{tox}(\hat{y}_{tox})]^{\frac{1}{2}} \quad (7)$$

The optimal combinations of ingredients are obtained when the overall desirability D is maximized.

Results and discussion

Initial experiment of six different green tea catechins to investigate NO inhibition and hepatotoxicity

NO is generally produced by NO synthase in activated macrophages and plays a critical role in immune responses and inflammation where it is involved in the synthesis of different reactive nitrogen species [23]. In the initial experimental design to screen significant variables to evaluate NO inhibitory efficacy and hepatotoxicity simultaneously, the simplex lattice design with two lattices including center and axial points was performed in 28 runs (Table S2). The inhibition of NO synthesis induced by LPS was tested at two different concentrations, 10 $\mu\text{g/mL}$ (y_1) and 100 $\mu\text{g/mL}$ (y_2) with three replications based on different combinations of six green tea catechins, EGCG (x_1), EGC (x_2), ECG (x_3), EC (x_4), gallic catechin (x_5), and catechin (x_6) in RAW 264.7 cells. As shown in Table S2, single treatment with EGCG, EGC, ECG, EC, gallic catechin, and catechin exerted 32.6, 48.8, 21.9, 32.6, 19.3, and 7.0% of NO inhibitory activities at 10 $\mu\text{g/mL}$, respectively. However, a mixture of six catechins in which the major component was EC showed the strongest NO inhibition of 62.3%, suggesting that interactions among catechins may enhance physiological efficacy. Similarly, hepatotoxicity induced by combination treatment of green tea catechins was also investigated at 100 $\mu\text{g/mL}$ (y_3) and 1000 $\mu\text{g/mL}$ (y_4) with three replications in Chang liver cells.

Interactive combinations exerting enhanced NO inhibition and reduced hepatotoxicity among six green tea catechins based on regression analysis

The main effects on NO inhibition and hepatotoxicity of six green tea catechins at different concentrations were investigated using well-established regression analysis methods. In many experimental design and analysis cases, regression analysis methods can be utilized to perform significant ingredient selections based on the large number of main and interaction effects. As shown in Fig. 2(A and B) and Fig. S1 (A and B), 10 $\mu\text{g/mL}$ EC and 100 $\mu\text{g/mL}$ EGC and galliccatechin were most effective inhibitors of LPS-induced NO synthesis, respectively, whereas 100 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$ ECG showed the weakest hepatotoxicity. In addition, the interaction effects of six green tea catechins are shown in Fig. 2(C and D) and Fig. S1 (C and D). According to NO inhibition analysis, the combination of 10 $\mu\text{g/mL}$ of EGCG, EC, and catechin and 100 $\mu\text{g/mL}$ of EGCG and catechin showed the strongest enhancement of activity in RAW 264.7 cells, respectively, whereas the combination of 100 $\mu\text{g/mL}$ of ECG, EC, and catechin and 1000 $\mu\text{g/mL}$ of EGCG, galliccatechin, and catechin reduced

hepatotoxicity in Chang liver cells. However, because of the high complexity and dimensionality associated with a large number of interaction effects, we conducted optimization using the DF model as identified in Eqs. (5), (6), and (7) in order to identify ingredients that significantly affected NO inhibition and hepatotoxicity. The optimization results shown in Table 1 revealed four significant ingredients, EGCG, EC, galliccatechin, and catechin, which were selected for further experiments.

Simplex centroid design for further RSM and statistical analysis

In order to design additional detailed experiments for the selected four candidates, EGCG (x_1), EC (x_4), galliccatechin (x_5), and catechin (x_6), a simplex centroid design was employed and 19 runs of experiments examining NO inhibition and hepatotoxicity were performed. A more clearly optimized combination of selected catechins was investigated to maximize the inhibition of NO synthesis at lower concentration (10 $\mu\text{g/mL}$) and minimize hepatotoxicity at higher concentration (1000 $\mu\text{g/mL}$). As shown in Table 2, co-treatment with EGCG and galliccatechin at the

Fig. 2 A and B Main-effects of six green tea catechins on NO inhibition at 10 $\mu\text{g/mL}$ and hepatotoxicity at 1000 $\mu\text{g/mL}$, respectively. C and D Interactive effects of six green tea catechins on NO inhibition at 10 $\mu\text{g/mL}$ and hepatotoxicity 1000 $\mu\text{g/mL}$, respectively

Table 1 Optimization results for the screening experiment

Response	Target	Optimal value	Optimal setting					
			EGCG	EGC	ECG	EC	Gallicocatechin	Catechin
Hepatotoxicity	Min	−10.33	35.35	0 (Not significant)	0 (Not significant)	5.39	30.32	28.94
No inhibition	Max	96.00	(Significant)			(Significant)	(Significant)	(Significant)

Table 2 Simplex centroid experimental design results

SO ^a	EO ^a	Input variables				Output response (% NO production inhibition)						Output response (% Toxicity)					
		x				y ₁ (10 µg/mL)						y ₄ (1000 µg/mL)					
		x ₁	x ₄	x ₅	x ₆	y ₁₁	y ₁₂	y ₁₃	y ₁₄	y ₁₅	y ₁₆	y ₄₁	y ₄₂	y ₄₃	y ₄₄	y ₄₅	y ₄₆
17	1	0.12	0.62	0.12	0.12	12.6	23.0	23.0	21.1	8.4	21.1	26.0	26.6	28.8	22.3	24.7	25.1
5	2	0.50	0.50	0.00	0.00	28.7	26.2	26.2	21.3	23.7	23.7	31.2	30.8	31.6	25.3	26.1	26.7
9	3	0.00	0.50	0.00	0.50	6.0	13.9	13.9	−9.3	4.6	4.6	6.6	6.1	6.0	3.3	4.0	4.8
18	4	0.12	0.12	0.62	0.12	13.7	8.7	8.7	11.4	10.2	17.6	22.2	21.8	20.5	17.3	18.1	17.1
11	5	0.33	0.33	0.33	0.00	24.3	20.4	20.4	38.9	41.5	41.5	22.9	23.7	21.5	20.6	18.2	17.8
10	6	0.00	0.00	0.50	0.50	10.0	6.0	6.0	13.5	11.0	17.3	18.1	18.7	17.8	14.7	15.3	15.8
13	7	0.33	0.00	0.33	0.33	17.5	20.0	20.0	32.3	27.4	32.3	18.1	18.7	17.0	13.4	14.2	12.9
7	8	0.50	0.00	0.00	0.50	19.1	34.7	34.7	30.0	32.6	31.3	15.7	17.9	16.8	11.8	12.1	14.2
14	9	0.00	0.33	0.33	0.33	7.5	7.5	7.5	10.2	12.7	12.7	18.0	19.1	19.8	16.3	13.7	16.0
15	10	0.25	0.25	0.25	0.25	23.7	20.0	20.0	31.1	33.6	33.6	21.6	20.1	22.3	17.0	15.4	17.7
16	11	0.62	0.12	0.12	0.12	24.3	21.7	21.7	28.8	23.7	25.0	14.9	15.6	15.7	10.9	11.6	12.0
12	12	0.33	0.33	0.00	0.33	19.1	15.2	15.2	19.9	21.1	21.1	20.4	20.7	20.6	15.8	18.2	16.2
3	13	0.00	0.00	1.00	0.00	12.5	11.2	11.2	13.9	15.1	11.4	18.2	17.8	16.0	12.1	12.1	12.4
2	14	0.00	1.00	0.00	0.00	22.5	23.7	23.7	20.0	21.3	21.3	18.3	17.4	18.8	15.2	14.8	15.8
6	15	0.50	0.00	0.50	0.00	47.8	45.2	45.2	51.6	46.6	49.1	13.5	15.1	14.5	10.7	12.0	11.7
8	16	0.00	0.50	0.50	0.00	21.2	27.5	27.5	28.6	27.4	25.0	26.3	26.1	28.6	21.2	21.0	20.5
1	17	1.00	0.00	0.00	0.00	29.5	23.0	23.0	28.8	26.2	27.5	8.3	8.1	8.2	4.5	5.3	5.3
19	18	0.12	0.12	0.12	0.62	7.5	10.0	10.0	18.8	12.7	12.7	16.4	16.8	17.8	13.5	12.5	13.4
4	19	0.00	0.00	0.00	1.00	20.4	20.4	20.4	9.7	31.3	12.2	2.1	3.2	2.8	0.2	0.3	0.7

^a SO and EO represent standard and experimental orders for the proposed simplex centroid experiment design, respectively

same ratio inhibited NO synthesis to a larger extent than any other combinations or single treatment, whereas catechin and co-treatment with EC and catechin showed lower hepatotoxicity.

Functional relationships between output responses including NO inhibition and hepatotoxicity and their associated input variables including four green tea catechins estimated by RSM

The analysis of variance (ANOVA) results of RSM for the inhibition of NO synthesis and hepatotoxicity are

summarized in Tables 3 and 4, respectively, where small *p* values (i.e., less than 0.05) represent significant interaction effects. The ANOVA results revealed that six interaction effects (i.e., EGCG-gallicocatechin, EGCG-catechin, EC-gallicocatechin, EC-catechin, EGCG-EC-Gallicocatechin, and EGCG-gallicocatechin-catechin) associated with NO inhibition as well as six interaction effects (i.e., EGCG-EC, EGCG-Catechin, EC-gallicocatechin, EC-catechin, gallicocatechin-catechin, and EGCG-EC-gallicocatechin) related to hepatotoxicity were statistically significant. In addition, the estimated second-order response functions of RSM for the inhibition of NO synthesis and hepatotoxicity were respectively estimated as follows:

Table 3 ANOVA for inhibition of NO synthesis

Term	Degree of freedom	Sum of square	Mean square	F-value	<i>p</i> value
<i>Inhibition of NO synthesis (10 µg/mL)</i>					
Regression	14	10,138.90	724.21	20.19	0.000
Linear	3	925.21	308.40	8.60	0.000
Quadratic	6	4943.93	823.99	22.98	0.000
EGCG·EC	1	2.72	2.72	0.08	0.783
EGCG·Galliccatechin	1	3237.96	3237.96	90.28	0.000
EGCG·Catechin	1	201.91	201.91	5.63	0.020
EC·Galliccatechin	1	306.28	306.28	8.54	0.004
EC·Catechin	1	1027.51	1027.51	28.65	0.000
Galliccatechin·Catechin	1	96.12	96.12	2.68	0.105
3 variables interaction	4	375.75	93.94	2.62	0.039
EGCG·EC·Galliccatechin	1	142.90	142.90	3.98	0.049
EGCG·EC·Catechin	1	0.53	0.53	0.01	0.904
EGCG·Galliccatechin·Catechin	1	267.21	267.21	7.45	0.008
EC·Galliccatechin·Catechin	1	27.53	27.53	0.77	0.383
4 variables interaction	1	63.60	63.60	1.77	0.186
EGCG·EC·Galliccatechin·Catechin	1	63.60	63.60	1.77	0.186
Error	99	3550.54	35.86		
Total	113				
$S = 5.98866$	$R^2 = 74.06\%$				

· Represents interaction among components (i.e., input variables)

Table 4 ANOVA for hepatotoxicity

Term	Degree of freedom	Sum of square	Mean square	F-value	<i>p</i> value
<i>Toxicity (1000 µg/mL)</i>					
Regression	14	4565.63	326.12	43.13	0.000
Linear	3	1054.51	351.50	46.48	0.000
Quadratic	6	2172.63	362.10	47.89	0.000
EGCG·EC	1	1150.20	1150.20	152.11	0.000
EGCG·Galliccatechin	1	18.48	18.48	2.44	0.121
EGCG·Catechin	1	450.90	450.90	59.63	0.000
EC·Galliccatechin	1	275.92	275.92	36.49	0.000
EC·Catechin	1	60.70	60.70	8.03	0.006
Galliccatechin·Catechin	1	298.21	298.21	39.44	0.000
3 variables interaction	4	79.67	19.92	2.63	0.039
EGCG·EC·Galliccatechin	1	62.52	62.52	8.27	0.005
EGCG·EC·Catechin	1	0.01	0.01	0.00	0.974
EGCG·Galliccatechin·Catechin	1	10.22	10.22	1.30	0.248
EC·Galliccatechin·Catechin	1	3.95	3.95	0.52	0.472
4 variables interaction	1	6.87	6.87	0.91	0.343
EGCG·EC·Galliccatechin·Catechin	1	6.87	6.87	0.91	0.343
Error	99	748.62	7.56		
Total	113				
$S = 2.74987$	$R^2 = 85.91\%$				

· Represents interaction among components (i.e., input variables)

$$\begin{aligned}\hat{y}_{NO}(\mathbf{x}) = & 26.2x_1 + 22.5x_4 + 10.0x_5 + 18.6x_6 + 3.3x_1x_4 \\ & + 113.8x_1x_5 + 28.4x_1x_6 + 35.0x_4x_5 - 64.1x_4x_6 \\ & - 19.6x_5x_6 - 167.7x_1x_4x_5 - 10.2x_1x_4x_6 \\ & - 229.3x_1x_5x_6 - 73.6x_4x_5x_6 + 1085.6x_1x_4x_5x_6\end{aligned}\quad (8)$$

$$\begin{aligned}\hat{y}_{tox}(\mathbf{x}) = & 5.6x_1 + 17.8x_4 + 14.8x_5 + 2.1x_6 + 67.8x_1x_4 \\ & + 8.6x_1x_5 + 42.5x_1x_6 + 33.2x_4x_5 - 15.6x_4x_6 \\ & + 34.5x_5x_6 - 110.9x_1x_4x_5 + 1.2x_1x_4x_6 \\ & - 44.8x_1x_5x_6 + 27.9x_4x_5x_6 + 356.7x_1x_4x_5x_6\end{aligned}\quad (9)$$

As shown in Tables 3 and 4, the validity of the estimated response functions in Eqs. (8) and (9) was demonstrated by the small p values of regression and large values for R^2 (i.e., less than 0.05 p value and greater than 70% R^2) in the ANOVA results. In many RSM applications, second-order response functions are often utilized because of their coverage and quadratic representations with high precision. By using these second-order response functions, RD can be optimized in order to obtain the optimal ingredients while simultaneously maximizing NO inhibition and minimizing hepatotoxicity [21].

Optimal combinations of significant ingredients exerting enhanced NO inhibition and reduced hepatotoxicity simultaneously using the DF model

In order to calculate the two objectives of enhancing physiological efficacy and minimizing hepatotoxicity simultaneously, the DF-based optimization method identified in Eqs. (5), (6), and (7) was used, where the estimated

Table 5 Simultaneous optimization results associated with NO inhibition and hepatotoxicity

Criteria	Object ^a	Lower ^b	Target ^c	Upper ^d
NO inhibition	Maximum	20	55	–
Hepatotoxicity	Minimum	–	0	30
Component	EGCG	EC	Gallocatechin	Catechin
Optimal solutions	0.65	0	0.35	0

^a Objectives (i.e., minimize and maximize) of responses (i.e., NO inhibition and Hepatotoxicity)

^b Allowable minimum values

^c Desirable target values

^d Allowable maximum values

functions associated with NO inhibition and hepatotoxicity were transformed into same scale. The DFs in Eqs. (5) and (6) were integrated with the estimated response functions of RSM identified in Eqs. (8) and (9). The overall desirability D in Eq. (7) was then maximized to identify the optimal ingredient settings and associated values of inhibition of NO synthesis and hepatotoxicity using the MINITAB software package (Version 17, Minitab Inc. State College, PA, USA.). Using this software package, we performed the proposed RD methods with many statistical design and analysis tools, such as experimental design, regression, RSM, and optimization. This DF approach is also widely utilized in pharmaceutical science to simultaneously optimize multiple objectives associated with numerous input formulation components (24). As shown in Table 5, the optimization results for the four ingredients showed that the specific mixture of EGCG and gallocatechin at a ratio 0.65:0.35 simultaneously maximized NO inhibition and minimized hepatotoxicity.

Fig. 3 Validation of effects of the optimized combination (EGCG:gallic acid = 0.65:0.35) on NO inhibition (A) and hepatotoxicity (B). A RAW 264.7 cells (4×10^4 cells/well in 96-well plate) were treated with EGCG (10 μg/mL), GC (10 μg/mL), or EGCG (6.5 μg/mL) + GC (3.5 μg/mL) for 24 h, and NO in the culture media was quantified as described in Materials and Methods. B Change cells

(1×10^4 cells/well in 96-well plate) were treated with EGCG (1000 μg/mL), GC (1000 μg/mL), or EGCG (650 μg/mL) + GC (350 μg/mL) for 24 h, and cytotoxicity was measured by LDH assay. Values represent mean $\pm$ SD. ^{a-d} different letters indicate statistical differences at $p < 0.05$

Co-treatment of EGCG and gallic catechin was validated to enhance NO inhibitory activity and reduce hepatotoxicity

To validate the final optimization showing maximized NO inhibitory activity and minimized hepatotoxicity, we performed experiments with EGCG and gallic catechin at a ratio of 0.65 and 0.35. As shown in Fig. 3, co-treatment of EGCG (6.5 µg/mL) and gallic catechin (3.5 µg/mL) in LPS-induced RAW 264.7 cells exerted 1.5- and 1.6-fold enhancements in NO inhibitory activity compared to the single treatment of EGCG (10 µg/mL) and gallic catechin (10 µg/mL), respectively. In addition, the hepatotoxicity induced by co-treatment with EGCG (650 µg/mL) and gallic catechin (350 µg/mL) in Chang liver cells were reduced by 1.9- and 2.4-fold, compared to individual treatment with EGCG (1000 µg/mL) and gallic catechin (1000 µg/mL), respectively. These results suggest that co-treatment with EGCG and gallic catechin at a ratio of 0.65:0.35 simultaneously enhanced NO inhibition and reduced hepatotoxicity. In addition, RD and its statistical approach for identifying the optimized combination were well-matched with the actual biological phenotypes. Previously, an experimental design was employed to optimize the extraction conditions such as extraction temperature, ethanol concentration, and extraction time to improve anti-inflammatory activity in guava leaf extracts [25]. In pharmaceutical formulations, RD has been used to optimize the formulation ratios of lubricants, retinol nanoparticles, and hydrophilic polymers [24, 26, 27]. The results of previous studies as well as the current results suggest that a statistical approach employing RD to investigate the interaction between ingredients is reliable. Furthermore, our study is unique because we determined the optimal combination of green tea catechins considering health beneficial efficacy and hepatotoxicity together using a DF model. In conclusion, we determined the optimal combination of six major green tea components that simultaneously increased NO inhibition and reduced the hepatotoxicity through RD application. The biological data based on an experimental design were analyzed and optimized using RSM and DF, respectively. The final optimized combination, EGCG and gallic catechin at a ratio of 0.65:0.35, was also validated. To our best knowledge, it is the first report employing the systematic approach involving RD considering dual physiological efficacies and may provide a reliable and significant platform for increasing the efficiency of biological experiments with the optimized number of experimental runs.

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References

- Balaji M, Ganjaly MS, Hanuma Kumar GE, Parim BN, Mopuri R, Dasari S. A review on possible therapeutic targets to contain obesity: The role of phytochemicals. *Obes. Res. Clin. Pract.* 10: 363–380 (2016)
- Gullett NP, Ruhul Amin AR, Bayraktar S, Pezzuto JM, Shin DM, Khuri FR, Aggarwal BB, Surh YJ, Kucuk O. Cancer prevention with natural compounds. *Semin. Oncol.* 37: 258–281 (2010)
- Kaulmann A, Bohn T. Carotenoids, inflammation, and oxidative stress-implications of cellular signaling pathways and relation to chronic disease prevention. *Nutr. Res.* 34: 907–929 (2014)
- Arai MA, Akamine R, Sadhu SK, Ahmed F, Ishibashi M. Hedgehog/GLI-mediated transcriptional activity inhibitors from *Crinum asiaticum*. *J. Nat. Med.* 69: 538–542 (2015)
- Arai MA, Tateno C, Hosoya T, Koyano T, Kowithayakorn T, Ishibashi M. Hedgehog/GLI-mediated transcriptional inhibitors from *Zizyphus cambodiana*. *Bioorg. Med. Chem.* 16: 9420–9424 (2008)
- Axanova LS, Chen YQ, McCoy T, Sui G, Cramer SD. 1,25-dihydroxyvitamin D(3) and PI3 K/AKT inhibitors synergistically inhibit growth and induce senescence in prostate cancer cells. *Prostate* 70: 1658–1671 (2010)
- Kowalczyk MC, Kowalczyk P, Tolstykh O, Hanausek M, Walaszek Z, Slaga TJ. Synergistic effects of combined phytochemicals and skin cancer prevention in SENCAR mice. *Cancer Prev. Res. (Phila)* 3: 170–178 (2010)
- Hilakivi-Clarke L, Andrade JE, Helferich W. Is soy consumption good or bad for the breast? *J. Nutr.* 140: 2326S–2334S (2010)
- Shu XO, Zheng Y, Cai H, Gu K, Chen Z, Zheng W, Lu W. Soy food intake and breast cancer survival. *JAMA* 302: 2437–2443 (2009)
- Taylor CK, Levy RM, Elliott JC, Burnett BP. The effect of genistein aglycone on cancer and cancer risk: a review of in vitro, preclinical, and clinical studies. *Nutr. Rev.* 67: 398–415 (2009)
- Allred CD, Allred KF, Ju YH, Clausen LM, Doerge DR, Schantz SL, Korol DL, Wallig MA, Helferich WG. Dietary genistein results in larger MNU-induced, estrogen-dependent mammary tumors following ovariectomy of Sprague-Dawley rats. *Carcinogenesis* 25: 211–218 (2004)
- Allred CD, Ju YH, Allred KF, Chang J, Helferich WG. Dietary genistein stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis* 22: 1667–1673 (2001)
- Hsieh CY, Santell RC, Haslam SZ, Helferich WG. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. *Cancer Res.* 58: 3833–3838 (1998)
- Messina M, McCaskill-Stevens W, Lampe JW. Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *J. Natl. Cancer Inst.* 98: 1275–1284 (2006)
- Hwang MK, Bode AM, Byun S, Song NR, Lee HJ, Lee KW, Dong Z. Cocarcinogenic effect of capsaicin involves activation of EGFR signaling but not TRPV1. *Cancer Res.* 70: 6859–6869 (2010)
- Calitz C, du Plessis L, Gouws C, Steyn D, Steenekamp J, Muller C, Hamman S. Herbal hepatotoxicity: current status, examples, and challenges. *Expert Opin. Drug Metab. Toxicol.* 1–15 (2015)
- Kummar S, Copur MS, Rose M, Wadler S, Stephenson J, O'Rourke M, Brenckman W, Tilton R, Liu SH, Jiang Z, Su T,

- Cheng YC, Chu E. A phase I study of the chinese herbal medicine PHY906 as a modulator of irinotecan-based chemotherapy in patients with advanced colorectal cancer. *Clin. Colorectal Cancer* 10: 85–96 (2011)
18. Lam W, Bussom S, Guan F, Jiang Z, Zhang W, Gullen EA, Liu SH, Cheng YC. The four-herb Chinese medicine PHY906 reduces chemotherapy-induced gastrointestinal toxicity. *Sci. Transl. Med.* 2: 45ra59 (2010)
 19. Shin S, Cho BR. Studies on a biobjective robust design optimization problem. *IIE Transactions* 41: 957–968 (2009)
 20. Scheff H. Experiments with mixtures. *J. R. Stat. Soc. Series B Stat. Methodol.* 20: 344–360 (1958)
 21. Shin S, Truong NKV, Goethals PL, Cho BR, Jeong SH. Robust design modeling and optimization of a multi-response time series for a pharmaceutical process. *Int. J. Adv. Manuf. Tech.* 74: 1017–1031 (2014)
 22. Abderrahmane H, Abidine FZE, Hamenna B, Ammar B. Correlation, path analysis and stepwise regression in durum wheat (*Triticum durum* Desf.) under rainfed conditions. *J. Agric. Sustain.* 3: 122–131 (2013)
 23. Predonzani A, Cali B, Agnellini AH, Molon B. Spotlights on immunological effects of reactive nitrogen species: When inflammation says nitric oxide. *World J. Exp. Med.* 5: 64–76 (2015)
 24. Jung YJ, Truong NK, Shin S, Jeong SH. A robust experimental design method to optimize formulations of retinol solid lipid nanoparticles. *J. Microencapsul.* 30: 1–9 (2013)
 25. Jang M, Jeong S-W, Cho SK, Yang HJ, Yoon D-S, Kim J-C, Park K-H. Improvement in the anti-inflammatory activity of guava (*Psidium guajava* L.) leaf extracts through optimization of extraction conditions. *J. Func. Foods* 10: 161–168 (2014)
 26. Choi DH, Lim JY, Shin S, Choi WJ, Jeong SH, Lee S. A novel experimental design method to optimize hydrophilic matrix formulations with drug release profiles and mechanical properties. *J. Pharm. Sci.* 103: 3083–3094 (2014)
 27. Choi DH, Shin S, Khoa Viet Truong N, Jeong SH. A new experimental design method to optimize formulations focusing on a lubricant for hydrophilic matrix tablets. *Drug Dev. Ind. Pharm.* 38: 1117–1127 (2012)