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Article

An Extreme Vertices Mixture Design Approach to Optimization of Tyrosinase Inhibition Effects

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Abstract. The objective of this study is to optimize the tyrosinase inhibition effects of three mixtures included Emblica extract, L-Ascorbic acid, and Kojic acid. Tyrosinase is a copper-containing oxidase, which has activity for both catechol and cresol. It is responsible for browning reactions. Mushroom tyrosinase was used in this study as the tyrosinase source and L-DOPA was used to activate the browning activity. The seventeen formulations were conducted by extreme vertices mixture design. To specify the lower and upper limit, prior study for each ingredient was experimented. Ten and thirty-three percentage of inhibition were proposed to specify the lower and upper limit of each ingredient. The result of experiment shown the difference tyrosinase inhibition effects of seventeen formulations. The analysis of variance for result was significant for all pairs and all of components with 92.89 percentage of R-square adjusted. To optimize the best combination, used of response optimization by setting up the target to 80, 85, 90, and 95 percentage of tyrosinase inhibition effect. The optimum combination with 90%inhibition target is 3.1541 mg./ml. of Emblica extract, 0.1420 mg./ml. of L-Ascorbic acid, and 0.0297 mg./ml. of Kojic acid, this formula unlike the formula that was experimented on.

Keywords: extreme vertices mixture designs, optimization, tyrosinase inhibition, Emblica extract, L-Ascorbic acid, Kojic acid.

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1. Introduction

Concentrating on personal health and well-being has become a main objective for people living in wealthy societies, one of the world megatrend [1]. Wellness areas not only to have a healthy body but also to have a healthy mind, the skincare, massage, spa, salon, gym, and yoga are a part of wellness way [2]. Skin whitening is a sub-group of skincare products refers to the practice of using natural or synthetic substances to lighten the skin tone or reducing the melanin concentration in the skin [3]. Skin whitening products account for more than 60% of Thailand's annual US$100 million facialskincare market [4]. Although most of the raw materials used in the production, imported from abroad. Most local cosmetic raw materials are inefficient enough to compete with foreign raw materials like the materials from Japan [5]. This research is aimed to development of raw materials for cosmetic skin care extracted from local natural sources to be effective equivalent to raw materials imported from abroad. The concept is to use synthetic substances used in high-performance skin care products to enhance the effect of natural extracts [6].

In this research was chosen to develop the natural raw materials from Emblica extract, and synergistically with synthetic substances from the L-Ascorbic acid and Kojic acid [7]. Emblica extract or a Phyllanthus extract, used for topical cosmetic applications, particularly to lighten skin [8]. Phyllanthus emblica contain of many phenolic compounds include gallic acid and ellergic acid [9]. Gallic acid was oxidized by tyrosinase as substrates, yielding yellow oxidation products, but the long alkyl (>C 0) chain esters inhibited the enzyme with- out producing the pigmented products, indicating that the carbon chain length was related to their tyrosinase inhibitory activity [10]. The skin lightening effects of ellergic acid may due to chelating copper at the active site of tyrosinase to reduce its activity and inhibition of proliferation of melanocytes and melanin synthesis [11]. Vitamin C, or L-ascorbic acid, is the most abundant antioxidant in skin and used for skin lightening agent for many years [12]. For Kojic acid, it is used in cosmetics for its excellent whitening effect, inhibits catecholase activity of tyrosinase in a non-classical manner [13]. By performing tests on the inhibition of enzyme tyrosinase that it is a process to synthesize skin pigmentation or melanin. Melanins high molecular weight brown-pigments are synthesized by polymerization of quinine because of their high reactivity and react with amino acids and proteins to enhance brown color of the pigment [14]. In vitro test, modified L-DOPA was used to evaluate the performance of each combination.

Minitab17 was used for planning the experiments, data analysis, contour diagrams and response optimizations. The extreme vertices designs, the measures response is assumed to depend on only the proportions of the ingredients present in the mixture and not on the amount of the mixture [15]. Like this experimental, the amount of three combination; Emblica extract, L-Ascorbic acid, and Kojic acid, up to 4 mg./ml. in phosphate solution or 0.4% in the whole of formulation. After experiment, Minitab software will create a regression equation that can predict the response result [16]. Because experimental formula may not be the best formula yet. The final decision may be determined by the cost factor of the raw materials involved. Because of the diversity of situations in which multi-objective decision problems can arise and because of the multiplicity of factors that are involved [17].

2. Tyrosinase Inhibition Experiments

Nowadays mushroom tyrosinase has become popular because it is readily available and useful in a number of applications [18]. Mushroom Tyrosinase was used in this study as the Tyrosinase source. Briefly, 40 µl of aqueous solution of mushroom Tyrosinase (480 units/ml) was added to a 96-wellmicor-plate, in a total volume of 520 ml. assay mixture containing 40 µl. (0.16 mg./ml) of L-DOPA solution, and 20 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 20 min. Following incubation, dopachrome produced in the reaction mixture was deter-mined spectrophotometrically at 492 nm. in a micro-plate reader.

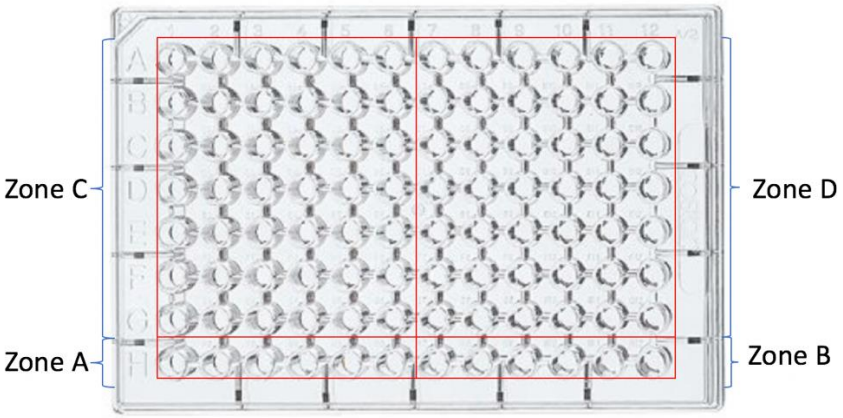

Fig. 1. 96-wellmicro-plate.

Figure 1 shows zone A, B, C, and D, zone A are the mixture of solvent, buffer, Tyrosinase, and L-DOPA. Zone B are the mixture of solvent, buffer, and L-DOPA. Zone C are the mixture of active ingredients, buffer, Tyrosinase, and L-DOPA. Zone D are the mixture of active ingredients, buffer, and L-DOPA. Every zone, 6 replicates on rows were conducted. After deter-mined by spectrophotometrically, the percentage of inhibition were calculated by the following equation.

$$\%\text{Inhibition} = (C\text{-}D) / (A\text{-}B) \quad (1)$$

The individual agent was prior study to explore the amount of each and to specify the upper and lower bound. The 33.33% inhibition was selected as upper bound (because total of three upper bound should not be over 100%) and 10% inhibition was selected as lower bound of each agent. Calculated by below equations that it analyzed from Microsoft Excel.

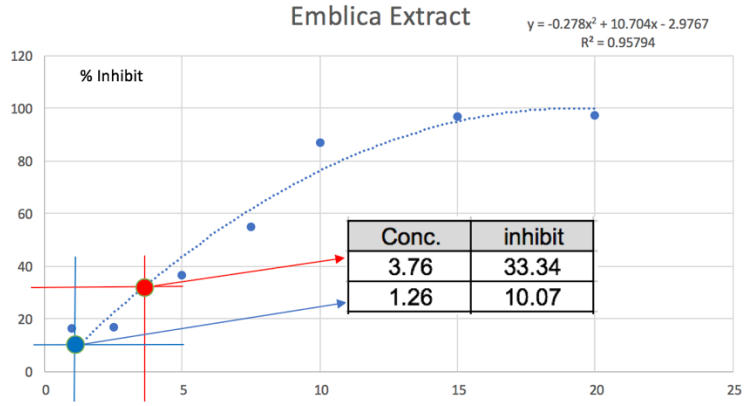

Fig. 2. Emblica Extract individual test. (Conc.=Concentration mg./ml.)

From Fig. 2, Emblica Extract inhibition equation with $R^2 = 0.95794$ is:

$$y = -0.278x^2 + 10.704x - 2.9767 \quad (2)$$

Thus, if x = 33.34%inhibiton then y = 3.76 mg./ml., if x = 10%inhibiton then y = 1.26 mg./ml.

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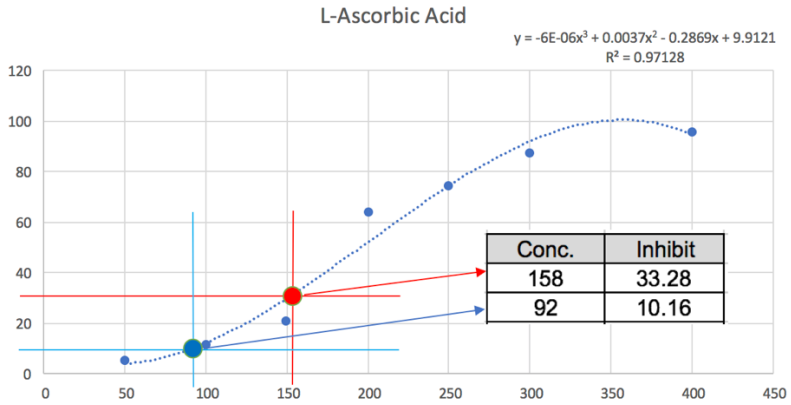

Fig. 3. L-Ascorbic acid individual test. (Conc.=Concentration µg./ml.)

From Fig. 3, L-Ascorbic acid inhibition equation with $R^2 = 0.97128$ is:

$$y = -6E\text{-}06x^3 + 0.0037x^2 - 0.2869x + 9.9121 \quad (3)$$

Thus, if x = 33.34%inhibiton then y = 0.158 mg./ml., if x = 10%inhibiton then y = 0.092 mg./ml.

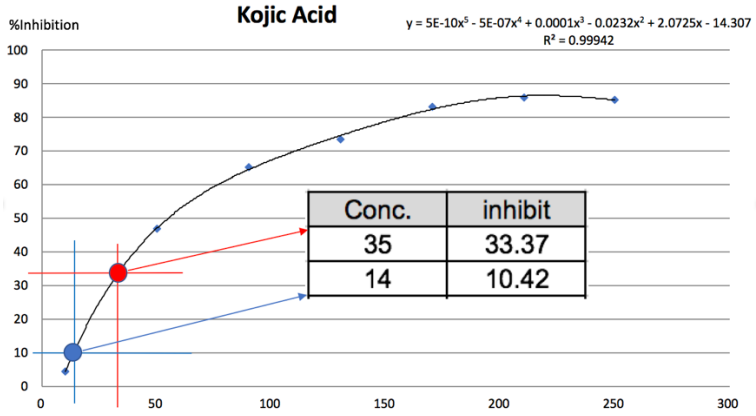

Fig. 4. Kojic acid individual test. (Conc.=Concentration µg./ml.)

From Fig. 4, Kojic acid inhibition equation with $R^2 = 0.99942$is:

$$y = 5E\text{-}10x^5 - 5E\text{-}07x^4 + 0.0001x^3 - 0.0232x^2 + 2.0725x - 14.307 \quad (4)$$

Thus, if x = 33.34%inhibiton then y = 0.035 mg./ml., if x = 10%inhibiton then y = 0.014 mg./ml.

From above equations, the upper bound and the lower bound of each ingredient were calculated that shown on Table 1.

Table 1. Upper and lower bound for mixture design.

Agents	Inhibtion	Emblica Extract	L-Ascorbic acid	Kojic Acid
Upper Bound	33.33%	3.76 mg./ml.	0.158 mg./ml.	0.035 mg./ml.
Lower Bound	10%	1.26 mg./ml.	0.092 mg./ml.	0.014 mg./ml.

3. Extreme Vertices Design

Mixture experiments are a special class of response surface experiments in which the product under investigation is made up of several components or ingredients [19]. Designs for these experiments are useful because many product design and development activities in industrial situations involve formulations or mixtures. Extreme vertices designs are mixture designs that cover only a sub-portion or smaller space within the simplex. These designs must be used when your chosen design space is not an L-simplex design. The presence of both lower and upper bound constraints on the components often create this condition. The goal of an extreme vertices design is to choose design points that adequately cover the design space [20].

Table 2. Experiments result.

Formula	Emblica (A)	Vit.C (B)	Kojic (C)	Buffer (D)	% Inhibition
1	**3.76**	**0.158**	**0.035**	**996.047**	**96.28619287**
2	3.76	0.092	0.035	996.113	92.16080402
3	1.885	0.1085	0.01925	997.98725	70.13161043
4	1.26	0.158	0.014	998.568	73.15147164
5	1.26	0.158	0.035	998.547	94.35271596
6	1.885	0.1415	0.01925	997.95425	73.09882747
7	2.51	0.125	0.0245	997.3405	80.36850921
8	3.135	0.1415	0.02975	996.69375	90.44268964
9	3.76	0.158	0.014	996.068	83.68987796
10	1.885	0.1415	0.02975	997.94375	84.60397224
11	3.76	0.092	0.014	996.134	72.66331658
12	3.135	0.1415	0.01925	996.70425	81.76118689
13	3.135	0.1085	0.01925	996.73725	79.01411821
14	3.135	0.1415	0.02975	996.69375	87.98755683
15	**1.26**	**0.092**	**0.014**	**998.634**	**56.29576454**
16	1.885	0.1085	0.02975	997.97675	83.2160804
17	1.26	0.092	0.035	998.613	80.54079923
18	2.51	0.092	0.0308	997.3672	81.90954774
19	1.7	0.157	0.02952	998.11348	91.63436229

The Minitab software generated 17 different combinations of Emblica Extract, L-Ascorbic acid, and Kojic acid. The results of 17 formula were illustrated on the Table 2. The combinations had inhibited effect between 56.29% to 96.28%. This result referred to maximum and minimum of ingredients usage. The maximum used should be inhibited 100% but it had only 96.9% (formula1). On the other hand, the minimum used should be inhibited 30% but it had 56.29% (formula15).

The result of analysis with Minitab17 shown the ANOVA table for %inhibition in the Table 3 with R-square adjusted 92.89% regression coefficients for %inhibition. By set up analysis criteria as DOE>Mixture Design>Analysis components in Proportions>Terms A, B, C, D, AB, AC, AD, BC, ABC, ABD, ACD, BCD. Where A=Emblica Extract, B=L-Ascorbic acid, C=Kojic acid, D=Phosphate buffer.

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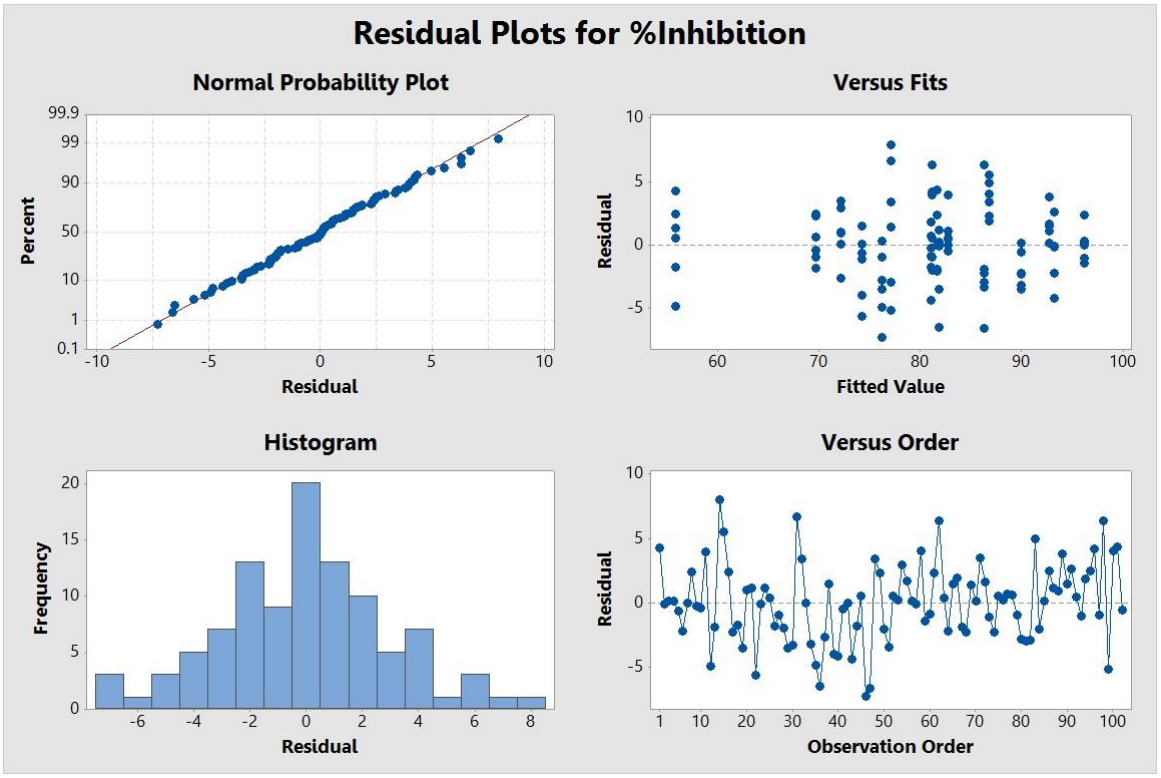

Fig. 5. Residual Plots for %inhibition.

Figure 5 shows 4 in 1 residual plots for % inhibition. The normal probability plot, versus fits, histogram, and versus order explained normal distribution of the data.

Table 3. Analysis of Variance for %Inhibition. (component proportions).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	11	9768.2	9768.22	888.020	121.00	0.000
Linear	3	9053.7	380.36	126.788	17.28	0.000
Quadratic	4	451.8	248.73	62.181	8.47	0.000
Emblica *L-Ascorb	1	195.3	33.00	32.997	4.50	0.037
Emblica *Kojic ai	1	162.8	42.81	42.807	5.83	0.018
Emblica *Phosphat	1	0.0	51.96	51.963	7.08	0.009
L-Ascorb*Kojic ai	1	93.7	42.22	42.221	5.75	0.019
Special Cubic	4	262.8	262.75	65.688	8.95	0.000
Emblica *L-Ascorb*Kojic ai	1	14.0	42.23	42.229	5.75	0.019
Emblica *L-Ascorb*Phosphat	1	199.1	33.00	32.996	4.50	0.037
Emblica *Kojic ai*Phosphat	1	7.5	42.81	42.808	5.83	0.018
L-Ascorb*Kojic ai*Phosphat	1	42.2	42.22	42.221	5.75	0.019
Residual Error	90	660.5	660.49	7.339		
Lack-of-Fit	5	33.9	33.90	6.781	0.92	0.472
Pure Error	85	626.6	626.59	7.372		
Total	101	10428.7				

Table 3 illustrates the analysis of variance for %inhibition effect. P-value of all combination terms were more than 0.05 that there were significant at 95% confidence level. Although, for AB (Emblica and L-Ascorbic acid) and ABD (Emblica and L-Ascorbic acid and Phosphate buffer were nearly 0.05 of P-value.

For residual error term shown lack-of-fit was not significant at 0.472 that means no more term of combination need to analyze.

Table 4. Estimated Regression Coefficients for %Inhibition (component proportions).

Term VIF	Coef	SE Coef	T	P
Emblica Extract 1333835097	-9719791	3646008	*	*
L-Ascorbic acid 207152	3145188	957240	*	*
Kojic acid 30041144	164621081	68205929	*	*
Phosphate Buffer5620	-45	20	*	*
Emblica Extract*L-Ascorbic acid 2.96992E+11	-9.03533E+11	4.26109E+11	-2.12	0.037
Emblica Extract*Kojic acid 1.18921E+12	-1.01673E+13	4.20975E+12	-2.42	0.018
Emblica Extract*Phosphate Buffer 1341015933	9760071	3667907	2.66	0.009
L-Ascorbic acid*Kojic acid 5.54788E+15	1.45573E+16	6.06911E+15	2.40	0.019
Emblica Extract*L-Ascorbic acid* 40323901236 Kojic acid	-1.45517E+16	6.06627E+15	-2.40	0.019
Emblica Extract*L-Ascorbic acid* 2.98157E+11Phosphate Buffer	9.08312E+11	4.28365E+11	2.12	0.037
Emblica Extract*Kojic acid* 1.19338E+12Phosphate Buffer	1.02191E+13	4.23119E+12	2.42	0.018
L-Ascorbic acid*Kojic acid* 5.52155E+15Phosphate Buffer	-1.45616E+16	6.07093E+15	-2.40	0.019
S = 2.70902 PRESS = 833.125				
R-Sq = 93.67% R-Sq(pred) = 92.01% R-Sq(adj) = 92.89%				

Table 4 illustrated the estimated regression coefficients for %inhibition effect with 2.70902 standard error and R-square predict was 92.01%. This regression model was used to predict the %inhibition effect by vary the amount of each ingredients and used in the algorithm for optimization process.

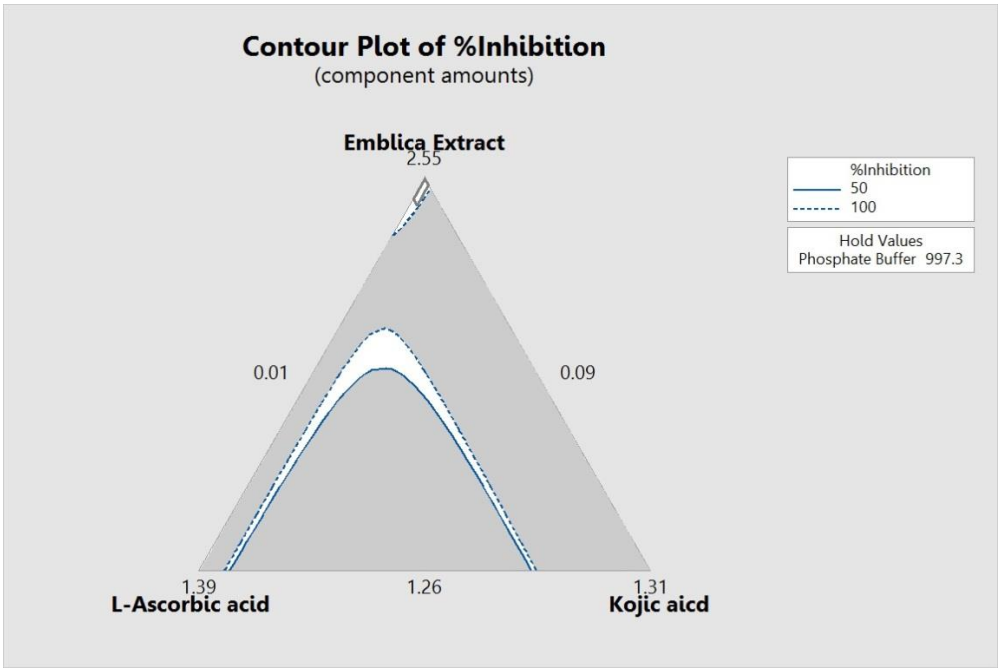

Fig. 6. Overlaid contour plot of % inhibition.

Figure 6 shows the overlaid contour plot of %inhibition, setting up the feasible area for 50 to 100% inhibition effect. Because of the data in Table 2 indicating the range between 56.29% to 96.28%, the white area in the figure is the possible area for the combinations that can make 50 to 100% inhibition effect.

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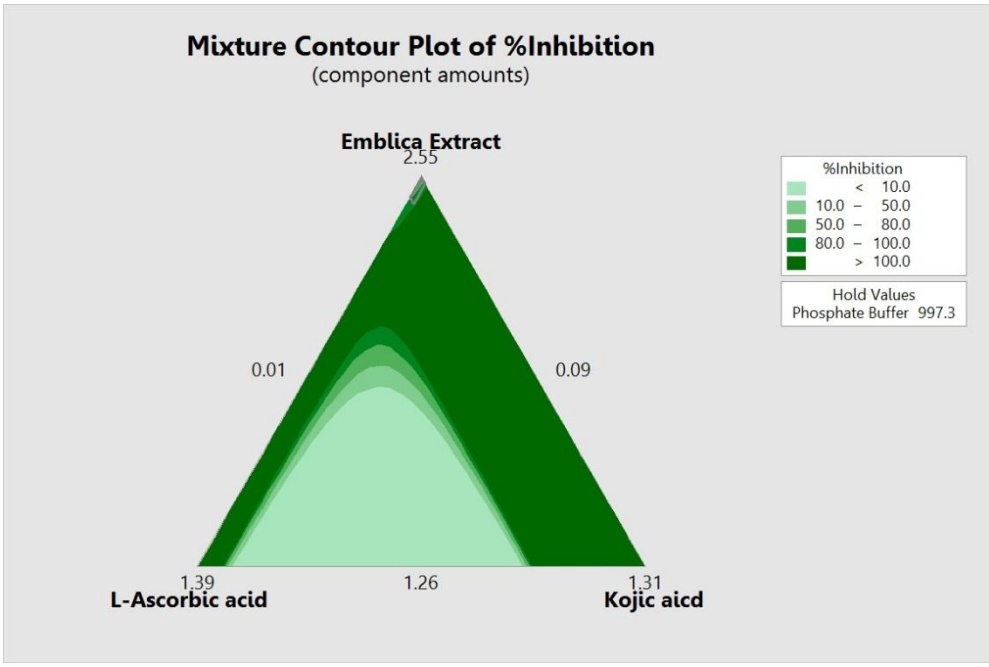

Fig. 7. Contour plot of % inhibition.

Figure 7 shows the contour plot of %inhibition, separated feasible area to 5 ranges for inhibition effect, less than 10%, 10% to 50%, 50% to 80%, 80% to 100%, and more than 100%. This plot make contrast from Fig. 6. The darkest area is impossible area (more than 100%) and lighter area is possible area for each range.

4. Optimization the Tyrosinase Inhibition Effects

Consider Fig. 8 that shows optimization result; set it to the maximum value. Dotted horizontal line is a line that cuts through every ingredient in the recipe. For vertical solid lines of three inhibitors that this concentration is required only to get the desired result. For the solid line of Phosphate buffer, more the volume increases, it will tend to decrease inhibition effect. Software predicts the value of the component that is used most. And the results close to the real experiment (formula1) result on the Table 2.

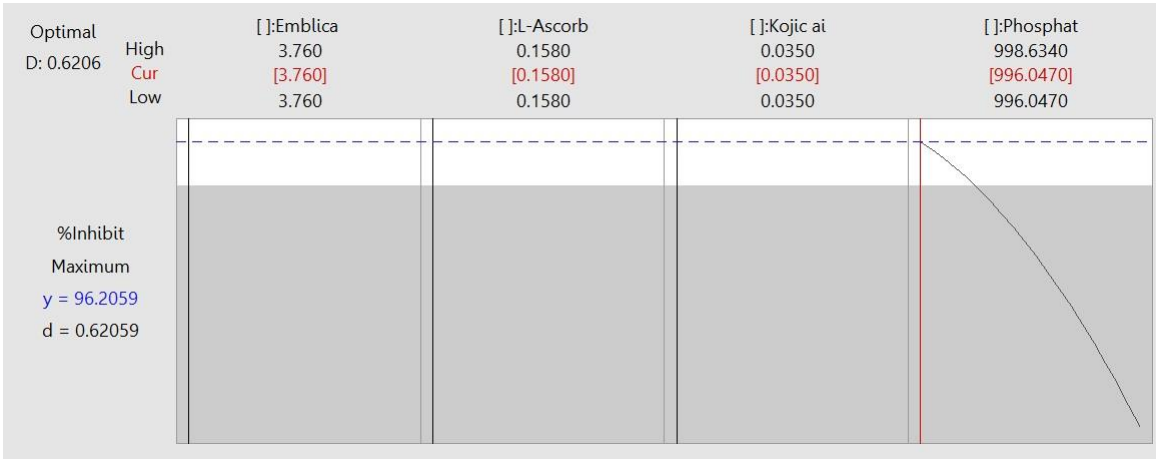

Fig. 8. Optimization the tyrosinase inhibition effect with maximum target.

Therefore, in setting the goal of this experiment should be more than 80% inhibition, so set to optimize with the target of 80%, 85%, 90%, 95%, and Max%. Then compare the values to make the decision to choose the best formula. Figure 9 is an example of optimization at the target of 90%.

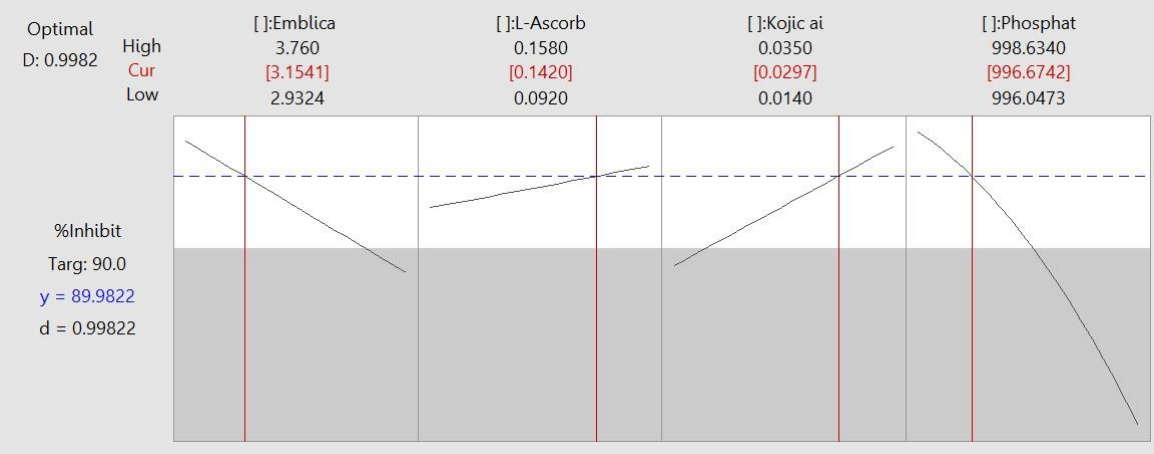

Fig. 9. Optimization the tyrosinase inhibition effect with 90% target.

Consider Fig. 9 that shows optimization result; set it to 90% inhibition effect. Dotted horizontal line is a line that cuts through every ingredient in the recipe like Fig. 8. But the solid lines of three inhibitors were different from Fig. 8. For the solid line of Emblica extract and Phosphate buffer, more the volume increases, it will tend to decrease inhibition effect. On the other hand, the solod line of L-Ascorbic acid and Kojic acid, more the volume increases, it will tend to increase inhibition effect too.

Table 5. Optimization the tyrosinase inhibition effect with target of 80%, 85%, 90%, 95%, and Max%.

Reponse	80%	85%	**90%**	95%	100%
Predicted	80.0000	85.0000	**89.9822**	94.9992	96.2059
Emblica	1.2600	2.5100	**3.1541**	3.6070	3.7600
L-Ascorbic	0.1250	0.1250	**0.1420**	0.1554	0.1580
Kojic acid	0.0284	0.0286	**0.0297**	0.0337	0.0350

Table 5 shows the result from Optimization the tyrosinase inhibition effect with target of 80%, 85%, 90%, 95%, and Max%. Of course, the more effective formula, the higher cost of raw materials, as well. Figure 10 illustrated the multiple choice can be used for decision support data.

Fig. 10. Multiple choice for decision making.

5. Conclusion and Discussion

This study aim to test the efficacy of three types of whitening compounds, Emblica, L-Ascorbic acid, and Kojic acid, by designing an extreme lactices mixture design, suitable for chemical ingredients, which could not calculate the reaction to each other. The results show that the proportion of different ingredients is effective for different inhibition tyrosinase. The results of the contour plot show that the formula for each performance level can make a variety of formulas on possible graph areas. So, choosing the most appropriate formulas by Minitab17 optimizer can be found using the costing calculator to make that decision support need of performance at any level. Because, based on the graphs in the single-agent test, 80% of the inhibit level is linear. It means that if it is over 80%, the concentration increase will start to slow down. For the conclusion, middle target of 90%inhibtion effect was selected. The optimum combination is 3.1541 mg./ml. of Emblica extract, 0.1420 mg./ml. of L-Ascorbic acid, and 0.0297 mg./ml. of Kojic acid,

This research will not only to test the efficiency of raw materials but also to development of natural ingredients to be as effective as synthetic materials. Contribution from this research will guide researchers to develop new raw materials from nature by using extreme vertices mixture design. The benefits of this technique are make reduce time and saving experiment cost. For the further research, the other optimization techniques can be applied for the optimization process. Like this study, the Minitab optimizer could not be calculated the multi-objective task. The solution may be fall in local result (consider wide area on contour plot). Evolutional programing such as Genetic Algorithm, Particle Swarm, or other meta heuristic may be apply for this project, to make decision both effective and value altogether.

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