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The interaction effect and optimal formulation of selected polyherbal extracts towards antioxidant activity

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Abstract

Past study showed that lemongrass (*Cymbopogon citratus*), curry leaves (*Murrya koenigii*), turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) contain phytochemicals associated with antioxidant properties. However, all the herbs are tested individually and rarely mix together. This study was conducted to examine the antioxidant properties and interaction effect when combined. The plants studied were decocted with distilled water. Eighteen formulations of aqueous extracts were established using simplex lattice mixture design that was generated by Design Expert software. The antioxidant properties were analyzed by 2-diphenyl-2-picryl hydrazyl radical scavenging (DPPH), ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and total flavonoid content (TFC) assays. The result showed that the mixture of lemongrass and curry leaves extracts gave the highest reading in DPPH assay (91.14%), FRAP assay (215.66 mM) and TFC (22.62 µg Rutin/mL). In the DPPH assay, the ratio of one to one (1:1) mixture of lemongrass with other plants extracts showed antagonistic interaction. There were five (5) formulations that showed synergistic interaction in all assays. However, there were two (2) formulations that showed antagonistic interactions on both DPPH and FRAP assays. No additive effect was observed in all formulations. The suggested optimum formulation contains 53.7% lemongrass, 43.4% curry leaves, 2.9% ginger and 0.0% turmeric. Most of the mixtures presented synergistic interactions. This indicated the potential of plant extract mixtures to be developed into nutraceutical products in the future by conducting *in-vivo* study.

1. Introduction

In recent years, antioxidants are raising interest especially in those intended to prevent the oxidative damage to the human body and prevent deterioration of food products. In industry, synthetic antioxidant was widely applied in food manufacturing (Thorat *et al.,* 2013). However, the synthetic antioxidant might cause an unexpected effect on the human body. Hence, the idea of using antioxidants from natural rather than from synthetic sources was proposed (Abdalla and Roozen, 1999). Medicinal plant was studied to support the idea that plant contains antioxidant which was capable to prevent oxidative stress in biological system (Cao *et al.,* 1996). Besides, some of the known natural antioxidants was implemented in the food industry as antioxidant additives or nutritional supplement. Plant-based antioxidants such as vitamin C, vitamin E, phenolic acid and flavonoid have been tested to have the potential to reduce oxidative stress (Kasote *et al.,* 2015).

Oxygen is a highly reactive molecule which could damage living organisms by producing reactive oxygen species (ROS). Consequently, oxidative stress-related diseases were caused by the formation of free radicals and the chain reactions (Ningappa *et al*., 2008). Synthetic antioxidant seems to be more effective but their unexpected toxicity and side effects fear the consumers (Karmegam *et al.,* 2008). Thus, the interest moved on to natural products such as plants and herbs. Most of plants and herbs contain natural antioxidants which can give synergistic interaction effect. However, as the interaction of phytochemicals is not inevitable, they might also exert antagonistic, additive and indifferent effect (Karmegam *et al*., 2008). Synergistic interaction was defined as positive interaction when combining two or more substances and the mixture show higher mechanism than the sum of the substances. The

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idea of synergism is the practice by researchers or scientist in the study of antioxidant, antimicrobial, antifungal and formulation of a new drug (Blesson *et al.,* 2015). In opposite, antagonism was the reduced effect when mixed together. Meanwhile, the result show neither good nor bad is defined as indifferent and additive is the sum effect of the mixture.

In this research, the interaction effect of the mixture of curry leaves (*Murraya koenigii*), ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citratus*) and turmeric (*Curcuma longa*) was studied in term of antioxidant properties that are mainly phenolic and flavonoid compounds. The extraction process of the plant extracts was prepared using water decoction. Meanwhile, the antioxidant properties were assessed by using 2,2-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging and ferric reducing antioxidant power (FRAP) assay whereas quantitative phytochemical contents for phenolic and flavonoid compounds were done by Folin-ciocalteu colorimetric and aluminium chloride colorimetric method respectively. Optimization was then carried out based on the best-fitting model by using Design Expert Software® 6.0.4 to obtain an optimum formulation of the plant extracts mixture.

2. Materials and methods

2.1 Chemical and instruments

DPPH (2,2-diphenyl-1-picrylhydrazyl), methanol, Folin-Cioclateu reagent, sodium carbonate (Na_2CO_3), sodium acetate, acetate buffer, hydrochloric acid (HCl), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous sulphate hexahydrate ($FeSO_4.6H_2O$), glacial acetic acid, TPTZ (2,4,6-tripyridyl-s-triazine), aluminium chloride-6-hydrate and rutin were analytical grade procured from local sources. The instrument used was UV-Vis spectrophotometer (T60u, PG Instrument, USA) located in Food Analysis Laboratory, Universiti Tun Hussein Onn Malaysia (UTHM).

2.2 Plant materials

Curry leaves and lemongrass were obtained from Taman Waja, Parit Raja located in Batu Pahat, Johor. Ginger and turmeric were from Agricultural Market Parit Raja. The plant materials were identified by the botanist expert in Universiti Tun Hussein Onn Malaysia (UTHM). All of the plant materials were picked fresh and decocted with distilled water. The ratio of plant and distilled water was 1:10. The plant samples were boiled until water is half of the original volume. Then the solution was filtered with Whatman No. 1 filter paper three times to ensure no impurities of the extracts (Maizura *et al*., 2011).

2.3 Sample preparation

The filtered samples were then introduced into aluminum foil covered test tubes before further analyzed (Maizura *et al.,* 2011). Formulations were designed by Design Expert Software® 6.0.4 from State-Ease Inc, United State. A total of 18 formulations of the plants' extracts were being analyzed in the experiment. Table 1 shows the design layout of the corresponding samples.

Table 1. Formulation layout

Number of formulations	Lemongrass (mL)	Curry Leaves (mL)	Turmeric (mL)	Ginger (mL)
1	10	0	0	0
2	5	5	0	0
3	5	0	5	0
4	5	0	0	5
5	0	10	0	0
6	0	5	5	0
7	0	5	0	5
8	0	0	10	0
9	0	0	5	5
10	0	0	0	10
11	6.25	1.25	1.25	1.25
12	1.25	6.25	1.25	1.25
13	1.25	1.25	6.25	1.25
14	1.25	1.25	1.25	6.25
15	2.5	2.5	2.5	2.5
16	0	10	0	0
17	0	0	10	0
18	0	0	0	10

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging activity

An antioxidant is able to prevent oxidation of the molecule by donating hydrogen. Radical scavenging activity with DPPH is a technique that widely used in the industry to evaluate the antioxidant based on its reduction of stable free radical DPPH. The decrease in absorbance value by spectrophotometer delegated the antioxidant activity as the DPPH solution will turn from deep violet to light yellow colour when it is reduced. The colour changes indicate the antioxidant properties of the samples. In this study, $6x10^{-5}$ M DPPH solution was prepared daily and 3 mL of DPPH solution was mixed with 77 µL of sample. Then the mixture was tested with spectrophotometer at 515 nm after 15 mins. Blank sample was prepared with 3 mL DPPH solution and tested with 515 nm. The percentage of inhibition was calculated as the following formula (Dudonné *et al.,* 2009; Muhammad *et al.,* 2012).

$$\% \text{ inhibition} = [(AB-AA)/ AB] \times 100$$

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Where AB is the absorption of blank, AA is the absorption of sample

2.4.2 Ferric reducing antioxidant power (FRAP)

The ferric reducing power of plant was determined based on reduction of Fe^{3+}-tripyridyltriazine (colourless ferric complex) to Fe^{2+}-tripyridyltriazine (blue colour ferric complex) at low pH. The reduction occurred due to the action of electron-donating antioxidants. The working FRAP solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution. The working FRAP solution was then place in 37°C water bath. A solution of 300 mmol/L acetate buffer was prepared at pH 3.6 with 3.1 g sodium acetate and 16 mL glacial acetic acid per liter of buffer solution. TPTZ was prepared by mixing 10 mmol/L TPTZ (3.1 g) in 40 mmol/L HCl where 3.4 mL HCl in 1L distilled water. Meanwhile, $FeCl_3 \cdot 6H_2O$ solution was prepared by introducing 5.4 g $FeCl_3 \cdot 6H_2O$ in 1L distilled water. A blank was done with inserted 3 mL of working FRAP solution into a cuvette and ran with a spectrophotometer with absorbance at 593 nm. Then, 100 µL of sample was mixed with 300 µL distilled water and inserted into previous cuvette filled with working FRAP solution. Absorbance at 593 nm was taken with spectrophotometer after 4 mins. The difference between sample absorbance and blank was calculated and the value was compared with standard curve. Meanwhile, a standard curve developed with $FeSO_4.6H_2O$ ranging from 0 to 100µM. FRAP values were expressed in mM of sample (Dudonné *et al.,* 2009).

2.5 Phytochemical content

2.5.1 Total phenolic content (TPC)

The total phenolic of plant extract mixture was done by Folin-Ciocalteau method. An amount of 2.5 mL of 10% Folin-Ciocalteu reagent was mixed with 0.5 mL of mixture. Then the solution was let stand for 2 mins and 7.5% Na_2CO_3 was added. The mixture was incubated at room temperature for 1 hour and absorbance was taken at 765 nm. Meanwhile, blank was prepared by replacing mixture with 0.5 mL of distilled water. Standard curve was calibrated with gallic acid at different concentrations. Absorbance values were then substituted into equation from the standard curve and expressed as gallic acid equivalent (µg of GAE/mL of extract) (Borkataky, 2015).

2.5.2 Total flavonoid content (TFC)

Total flavonoid content was determined based on formation of flavonoid-aluminum content. One (1) mL of sample was mixed with 1 mL of aluminium chloride-6-hydrate solution and let incubate for 15 mins. Then absorbance was tested at 430 nm with spectrophotometer. Absorbance values were then substituted into equation from the standard curve and expressed as rutin equivalent. Rutin with different concentration were measured with 430 nm and plotted to get standard curve (Othman *et al.,* 2014).

2.6 Interaction effect

The interaction effect of the plant was calculated according to Hugo *et al.* (2012) with slight modification. The predicted values of the response were calculated by combining the percentage of each response of each single plant extract according to the proportion of the mixtures. Thus the predicted inhibition was used to compare with experimental percentage to determine the interaction effects.

2.7 Model fitting

The most significant model was chosen by determining F-P-ratio where the larger the F-value and the smaller the P-value indicate the more significant of the corresponding coefficient (Khan *et al.,* 2010) In addition, standard deviation of a significant model with the lowest values compared to other models was selected (Cornell, 2002). Besides, R-squared values nearest to 1 and lack of fit of the model with not significant were selected (Iman Kamaludin *et al.,* 2016).

2.8 Statistical analysis

The statistical analysis was performed using the Design Expert Software® 6.0.4 from State-Ease Inc, United State. Simplex lattice was used to design the formulations with the factors were lemongrass, curry leaves, turmeric root and ginger roots while the responses were antioxidant activities which expressed in percentages and values. The data were analyzed as one-way analysis of variance (ANOVA), surface contours plot and triangular contours diagram. The statistical significant difference ($p < 0.05$) was then evaluated with SPSS 16 (Laus *et al.,* 2013).

3. Results and discussion

3.1 Interaction effect of the plant extracts mixture

Tables 2 and 3 show the result of the antioxidant activity and phytochemical content respectively. Based on the results obtained, there were eight (8) synergistic and three (3) antagonistic interactions found in the DPPH assay. The mixture of turmeric and ginger at the same proportion showed a huge synergistic interaction as it dramatically increases in antioxidant activity from 66.22% to 93.64% among the other formulations in DPPH assay. This was supported by the previous study (Maizura *et al.,* 2011). Besides, FRAP assay showed seven (7) synergistic and four (4) antagonistic interaction

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Table 2. Antioxidant activity and interaction effect of studied extracts

No	DPPH (%)			FRAP (Mm)		
	Experimental	Predicted	Interaction	Experimental	Predicted	Interaction
1	95.90±0.39^{a}	95.90±0.39	-	191.65±0.44^{e}	191.65±0.44	-
2	91.14±2.00^{b}	93.95±0.47	Antagonistic	215.66±0.44^{a}	201.94±0.04	Synergistic
3	79.05±0.27^{f}	82.46±0.42	Antagonistic	140.32±0.13^{g}	162.59±1.30	Antagonistic
4	77.95±0.22^{g}	79.66±0.31	Antagonistic	184.64±0.04^{f}	189.84±0.22	Antagonistic
5	90.64±0.71^{c}	90.64±0.71	-	207.23±0.57^{b}	207.23±0.57	-
6	94.65±0^{g}	80.51±0.51	Synergistic	140.29±0.17^{g}	172.89±0.91	Antagonistic
7	94.12±0^{h}	77.71±0.44	Synergistic	201.09±0.26^{c}	200.14±0.17	Synergistic
8	68.59±0.14^{i}	68.59±0.14	-	131.28±3.06^{i}	131.28±3.01	-
9	93.64±0.41^{l}	66.22±0.36	Synergistic	142.76±0.44^{g}	160.79±1.08	Antagonistic
10	63.60±0.73^{j}	63.60±0.73	-	191.37±0.04^{e}	191.37±0.04	-
11	93.49±0.73^{d}	88.00±0.39	Synergistic	195.38±0.22^{f}	186.50±0.50	Synergistic
12	94.41±0.82^{e}	86.04±0.49	Synergistic	205.60±0.96^{b}	196.80±0.11	Synergistic
13	86.24±0.65^{i}	74.55±0.43	Synergistic	185.44±0.48^{f}	157.46±1.36	Synergistic
14	88.38±0.95^{j}	71.75±0.35	Synergistic	197.98±0.48^{cd}	184.70±0.24	Synergistic
15	92.07±0.77^{g}	80.08±0.41	Synergistic	200.23±0^{c}	181.37±0.56	Synergistic
16	93.34±0.52^{b}	93.34±0.52	-	217.23±1.27^{a}	217.23±1.27	-
17	69.42±0.79^{k}	69.42±0.79	-	135.81±1.27^{h}	135.81±1.27	-
18	63.21±0.61^{j}	63.21±0.61	-	184.70±0.04^{f}	184.70±0.04	-

Values are presented in mean±standard deviation (n=3). Different alphabet superscript in the same column are significantly different ($p < 0.05$).

Table 3. Phytochemical content and interaction effect of studied extracts

No	TPC (µg/mL)			TFC (µg/mL)		
	Experimental	Predicted	Interaction	Experimental	Predicted	Interaction
1	558.65±21.58^{ef}	558.65±21.58	-	16.46±0.13^{e}	16.46±0.13	-
2	1526.40±84.53^{b}	1098.07±31.66	Synergistic	22.62±0.08^{b}	22.33±0.12	Synergistic
3	626.96±53.00^{ef}	605.39±20.46	Synergistic	13.19±0.05^{f}	12.82±0.06	Synergistic
4	535.35±68.16^{ef}	515.30±9.81	Synergistic	8.85±0.04^{g}	9.09±0.06	Antagonistic
5	1653.09±26.58^{ab}	1653.09±26.58	-	28.37±0.47^{a}	28.37±0.47	-
6	1524.14±56.89^{b}	1144.81±30.53	Synergistic	21.69±0.04^{c}	18.69±0.09	Synergistic
7	927.15±85.60^{d}	1054.72±13.89	Antagonistic	17.92±0.01^{d}	14.99±0.08	Synergistic
8	646.59±21.11^{ef}	646.59±21.11	-	9.33±0.01^{g}	9.33±0.01	-
9	695.01±18.66^{e}	562.04±2.68	Synergistic	5.31±0.01^{h}	5.48±0.01	Antagonistic
10	471.74±17.01^{f}	471.74±17.01	-	1.37±0.01^{j}	1.37±0.01	-
11	1261.74±57.95^{c}	694.35±19.38	Synergistic	16.14±0.01^{e}	15.18±0.09	Synergistic
12	1760.99±14.35^{a}	1233.77±29.45	Synergistic	22.20±0.04^{bc}	21.05±0.11	Synergistic
13	1264.74±5.85^{c}	741.09±18.24	Synergistic	13.02±0.01^{f}	11.55±0.03	Synergistic
14	1168.87±40.41^{c}	650.99±1.61	Synergistic	9.52±0^{g}	7.84±0.02	Synergistic
15	1750.46±2.66^{c}	830.05±17.17	Synergistic	16.13±0.04^{e}	13.91±0.06	Synergistic
16	1621.89±56.89^{ab}	1621.89±56.89	-	28.02±0.32^{a}	28.37±0.47	-
17	657.68±14.54^{e}	657.68±17.54	-	9.04±0.01^{g}	9.04±0.01	-
18	472.15±44.93^{f}	472.15±4.93	-	2.20±0.03^{i}	2.20±0.01	-

Values are presented in mean±standard deviation (n=3). Different alphabet superscript in the same column are significantly different ($p < 0.05$).

effects. In the midst of formulations with antagonistic interaction effect, there were three (3) formulations (number 3, number 6, number 9) showed no significant difference ($p < 0.05$) amongst three of them for the FRAP assay. The previous study stated that some of the flavonoid-flavonoid mixture responsible for antagonistic interactions in FRAP assay (Hidalgo *et al*., 2010). In this study, the interaction calculation showed that the formulations contained 50% turmeric extract gave lower ferric reducing activity of the mixture of the extracts. However, formulation number 13 which contained 62.5 % of turmeric showed synergistic interaction effect. This might be due to the flavonoid interaction of turmeric with flavonoid of other sample plant extracts. There were found nine (9) synergistic interaction and two (2) antagonistic interaction effect in total flavonoid content assay (Ojiako *et al*., 2016) The two (2) antagonistic interactions of the TFC assay which was showed to have antagonistic effect in FRAP assay. Lastly, there were ten (10) synergistic and one (1) antagonistic interaction

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effect in total phenolic content assay which is the combination of curry leaves and ginger.

3.2 Model fitting of plant mixture

The regression coefficients for all the terms in the optimized models are summarized in Table 4. In this study, optimization of the plant extract mixture is a process of the search for the mixture that satisfying all the required parameter such as DPPH, FRAP, TPC and TFC. The statistical analysis lattice design batches were done by one way ANOVA at a significant level ($p<0.05$). From the analysis, there were two parameters that statistically significant for the quadratic model which were DPPH and TFC. Meanwhile, FRAP was tested statistically significant for the linear model while TPC was statistically significant for the special cubic model. The best model for each assay was determined by referring low standard deviation and predicted sum of squares as well as high predicted R-squared (Abdullah and Chin, 2010). Then the selected models were used for optimization.

Table 5 shows the optimized mixture formulation of DPPH, FRAP, TPC and TFC with their suggested model where A is lemongrass, B is curry leaves, C is turmeric and D is ginger. Based on the result obtained, lemongrass was exhibited with the highest percentage of DPPH while ginger was the lowest one individually. The previous study reported ginger had 79.0% of DPPH radical scavenging activity which are slightly higher than obtained in this study (63.62%) (Maizura *et al*., 2011). Since FRAP assay fitted the linear model, the coefficients for mixture extracts of AB, AC, AD, BC, BD and CD as well as ABC, ABD, ACD and BCD did not exist (Abdullah and Chin, 2010). As for TPC, special cubic was fitted into the model that enabled the analyzation of the interaction effect among three types of extracts studied and it showed the mixture of lemongrass, curry leaves and ginger have the highest phenolic compounds when combined. Meanwhile, TFC established quadratic effect and ginger generated low TFC values individually as well as its combination with lemongrass.

3.3 Optimization

The optimized formulation has done with design expert software by setting criteria to maximize the DPPH, FRAP, TFC and TPC. Many studies have proven that extract with a high amount of polyphenol content was able to exhibit high antioxidant activity (Mohd Noor *et al.,* 2020). Desirability close to one was selected indicated the optimized formula has the highest satisfaction on the parameters chosen. In this study, the desirability selected was 0.759 with the optimized formulation shown in Figure 1. The formulation contains 53.7% lemongrass, 43.4% of curry leaves and 2.9% of ginger and 0% of turmeric. This proves that to obtain the best antioxidant properties, turmeric was not suggested to be added into the formulation.

Table 4. Model summary statistics for DPPH, FRAP and TPC assay

Response	Model	F-value	Prob > F	Standard Deviation	R^2	Adjusted R^2	Predicted R^2	Lack of Fit	Probability
DPPH	Linear	4.65	0.0186	8.99	0.499	0.392	0.1671	-	0.0022
	Quadratic	47.95	<0.0001	1.96	0.987	0.9712	0.8255	No	0.1449
	Special Cubic	2.86	0.1664	1.41	0.997	0.9851	-1.3702	-	0.1897
FRAP	Linear	16.38	<0.0001	18.11	0.778	0.7308	0.6477	No	0.6791
	Quadratic	0.3	0.9205	21.65	0.819	0.6153	-2.2138	-	0.4514
	Special Cubic	1.69	0.3123	18.68	0.933	0.7137	-11.767	-	0.5435
TPC	Linear	8.35	0.002	322.13	0.642	0.5647	0.4296	-	<0.0001
	Quadratic	2.58	0.1079	248.83	0.878	0.7402	-2.3793	-	0.0001
	Special Cubic	368.47	<0.0001	18.31	1	0.9986	0.7292	No	0.1288
TFC	Linear	264.39	<0.0001	1.17	0.983	0.9789	0.9723		0.0345
	Quadratic	12.08	0.0012	0.49	0.998	0.9963	0.9869	No	0.3067
	Special Cubic	2.74	0.1759	0.36	1	0.998	0.9192		0.5605

Table 5. Optimized mixture formulation of DPPH, FRAP, TPC and TFC assay

Parameter	Model	Final Equation
DPPH	Quadratic	DPPH (%) = 96.60A + 91.70B + 68.79C + 63.62D - 10.42AB - 12.34AC - 2.98AD + 54.72BC + 66.36BD + 110.87CD
FRAP	Linear	FRAP(Mm) = 198.48A + 215.81B + 114.93C + 191.63D
TPC	Special Cubic	TPC (µg/mL) = 559.87A + 1638.10B + 652.74C + 472.55D + 1719.38AB + 92.33AC + 86.25AD + 1524.61BC -502.99BD + 539.16CD + 768.20ABC + 16298.95ABD + 0522.23ACD + 8675.10BCD
TFC	Quadratic	TFC(µg/mL) = 16.46A + 28.06B + 9.17C + 1.81D + 1.40AB + 2.41AC + 0.083AD + 12.12BC + 12.06BD + 0.35CD

A is lemongrass, B is curry leaves, C is turmeric and D is ginger.

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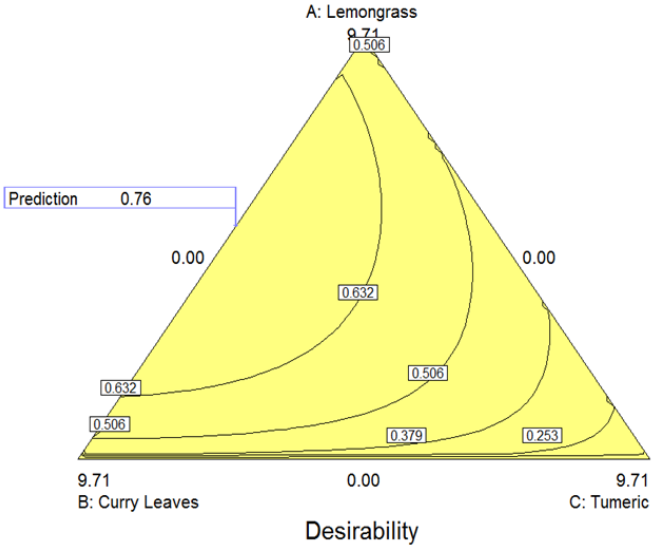

Figure 1. Triangular-dimensional contours diagram for optimized diagram at the desirability of 0.759

3.4 Validation

The optimized formulation was then calculated for the percentage of error compared to the predicted value by design expert software. The result was shown in Table 6. DPPH and TPC assays showed a higher percentage of error compared to FRAP and TFC assays, but they are still in the range of ideal percentage error which is 5% (Che Sulaiman *et al.,* 2017).

Table 6. Percentage error of predicted and experimental value of antioxidant assays

Parameter	Predicted Value	Experimental Value	Percentage Error (%)
DPPH	91.8724	88.01±0.27	4.20
FRAP	205.82	202.76±0.32	1.45
TFC	21.5663	22.04±0.74	2.87
TPC	1532.53	1456.85±4.70	4.90

Values are presented in mean±standard deviation (n=3).

4. Conclusion

There were a total of eighteen formulations of plant extracts designed with Design Expert software. The result showed lemongrass exert the highest percentage of radical scavenging activity inhibition in DPPH assays while curry leaves exert highest ferric reducing antioxidant power. Meanwhile, the mixture of the lemongrass and curry leaves extracts gave the highest reading in DPPH, FRAP and TFC assays. Nonetheless, one to one (1:1) mixture of turmeric and ginger showed highest synergistic interaction in DPPH but the antagonistic effect in FRAP. The mixture was also showed synergistic interaction in TPC assay but antagonistic interaction effect in TFC assay. The suggested optimum formulation contains of 53.7% of lemongrass, 43.4% of curry leaves and 2.9% of ginger and 0.0% of turmeric. In conclusion, most of the plants extract mixture contributed to synergistic interaction in the formulations. This indicates the potential of the mixture of plant extracts to be developed into food product in the future.

Conflict of interest

The authors declare no conflict of interest.

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