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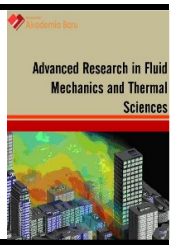

Optimization of the Antioxidant Properties of the Polyherbal Formulations

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ABSTRACT

The secondary metabolites from plants have important biological and pharmacological activities such as anti-oxidative, anti-carcinogenic, and antibiotic. Plant extracts which are rich in polyphenol can be beneficial both towards product development and human health. They are well known of having high content of antioxidant compounds and have been recognized as having potential in reducing disease risk as antioxidant. The objective of this study is to optimize polyherbal extracts with antioxidant properties. It was conducted by involving leaves of Strobilanthes crispus (pecah beling), Phyllanthus niruri (dukung anak), Orthosiphon aristatus (misai kucing) and Stevia rebudiana through in-vitro assay. Polyherbal formulation was developed with 22 different formulations of various aqueous extracts proportion designated by using commercial statistical software package which is Design Expert 6.0.4. Antioxidant activities of the plants depend on the concentration of the antioxidant and also the structure and interaction between the antioxidant. Antioxidant properties of polyherbal formulation were identified spectrophotometrically through 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reduction Antioxidant Potential Assay (FRAP), and total phenolic content (TPC) assay. Most of the assay showed that O. aristatus has higher antioxidant properties than S. crispus, P. niruri and S. rebudiana but most of the polyherbal combination exhibit synergistic effect. Through optimization process, examination of the fit summary output indicates that quadratic model was statistically significant for the FRAP and TPC meanwhile DPPH was statistically significant for cubic model. From a series of formulation, the optimum mixture for antioxidant properties was revealed to be the extract mixture of 23.96% of S. crispus, 0.62% of P. niruri and 75.42% of O. aristatus with desirability of 0.985. Hence, through all the results obtained, the present study justifies these polyherbal formulations have promising antioxidant properties. This identification would be able to let the traditional medicine to be fully utilized and become much beneficial to human being.

Keywords:
Antioxidant properties, DPPH, FRAP, Optimization, TPC

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1. Introduction

In this fast-paced world, the development of herbal supplement involving traditional medicinal plants have been increased compliance with other functional food that has been established. Traditional medicine can offer several advantages over modern medicine. It is mostly due to its high margin of safety, cost effective, eco-friendly and readily availability in developing herbal supplement [1]. Knowledge on properties and usage of the plants are usually passed down from generation to generation according to custom. It is reported that herbal medicinal products contain a combination of botanicals which is each of these comprises a number of chemical compounds that might contributed to expected activity in combination [2]. Antioxidant is significantly a compound which act as a health protecting factor where may lower the danger of oxidative stress-related diseases and able to give health-enhancing effect on the human organism [3]. The main criterion of an antioxidant is its ability to trap free radicals which referred to the oxygen-centered molecules that contain a single electron at the outermost orbit. A wide-ranging of sources is presented with highly reactive free radicals and oxygen species that are exist in biological systems [4]. Plant rich in phytochemicals are well known of having high content of antioxidant compounds and have been recognized as having potential in reducing variety of diseases risk. Thus, the study of biological activity and chemical composition of medicinal plant extracts as a potential source of natural antioxidants are becoming trends in development of product.

S. crispus or also known as "*pecah beling*" comes from the *Acanthaceae* family, a well-known herbal medicine plant in Malaysia that has been reported to have various properties including antioxidant effect [5]. Various phytochemical has been revealed in the plant contain catechins, polyphenols, tannins and alkaloids compounds which capable to possess multiple health beneficial effects. In addition, *S. crispus* contained high amount of mineral content including potassium (51%), calcium (24%), sodium (13%), iron (1%) and phosphorus (1%) besides high in vitamin C, B1 and B2 [6]. It is an annual plant that grows easily in the forest, riverbanks, and abandoned fields [7]. Traditionally, *S. crispus* has been used as antidiabetic, antilytic, laxative, anticancer, and as a diuretic agent [8]. Meanwhile, *P. niruri* is from the family of *Euphorbiaceae* and the plant extract was widely used in the preparation of many ayurvedic formulations [9]. The whole plant is used as remedies for many conditions for over 2000 years. Locally known as "*Dukung anak*" it is used internally for diarrhoea, kidney disorders, gonorrhoea and coughs [10]. Another herb, *O. aristatus* is locally known as "misai kucing" belongs to the family *Lamiaceae*. It is found that the leaves exhibit dynamic pharmacological properties such as strong antioxidant potency and total phenolic content [11]. In addition, the herb has been shown to be exceptionally safe with no toxicity in vitro and in vivo [12]. In Malaysia, the whole plant other than roots is used for controlling high blood pressure, rheumatic fever, gout, arthritis, and diabetes [13]. The use of *S. rebudiana* from the family *Compositae* in various formulations was mainly to improve the palatability of the formulation in which it acts as natural sweetener [14]. Stevia is odorless and approximately 300 times sweeter than sucrose. Due to its specialty for calorie free, people tend to consume stevia for health purpose such as for losing weight. Besides, it is also has been commonly used as a non-caloric sugar substitute for people with diabetes. In addition, this herb has been shown to act as a contraceptive drug and to have cardiovascular and metabolic effects [15]. Nevertheless, the extract indicates that it has antioxidant with radical scavenging activity as it has the ability to reduce the DPPH radical [16].

Basically, plant extracts are natural component that might employ synergistic, antagonistic, additive and indifferent effect depending on the interaction on the phytochemicals [17]. Those effects should be taken into account during development of supplement or food product from natural sources, or when it is use as replacement of synthetic antioxidant which can contribute to

harmful effects on human health [18]. Previous study reported that commercial statistical software was used to discover the experimental conditions which produce the best possible analytical performance [19]. Therefore, in this present study, commercial statistical software package that is Design Expert 6.0.4 was used to determine the optimum antioxidant properties of polyherbal formulations of the aqueous extract of leaves of *S. crispus* (pecah beling), *P. niruri* (dukung anak), *O. aristatus* (misai kucing) and *S. rebudiana* using *in vitro* method.

2. Materials and Methods

2.1 Chemicals and Instruments

DPPH (2,2-diphenyl-1-picrylhydrazyl), methanol, Folin-Ciocalteu reagent, sodium carbonate, acetate buffer, glacial acetic acid and TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from Merck Germany. The reagents were analytical grade procured from local sources. The instrument used was UV-Vis spectrophotometer (T60u, PG Instrument, USA) located in Food Analysis Laboratory.

2.2 Collection and Preparation of Plant Materials

Figures, Leaves of *S. crispus*, *P. niruri, O. aristatus* and *S. rebudiana* were used in polyherbal formulations. The samples were purchased from local company of Ethno Resources in dried form. Referring to Muryanto *et al.,* the preparation of leaves extracts was done with slight modifications [20]. The dried leaves of selected extracts were grinded into powdered form using a standard laboratory blender. Next, 100 g of the powder was heated slowly to be boiled in 1000 mL of distilled water, until the volume of the mixture reduced to about a third of the original volume. It was immersed in the hot water at range of 80°C to 90°C for 15 minutes. The extracts were filtered separately using sterile Whatman no 1 filter paper to get the supernatant mixture from the extraction solution while the residues were stored in cool condition for further use.

2.3 Formulation Development of Plant Materials

For the polyherbal formulations development, by using the Design Expert software, the optimization calculations were performed to find an optimum mixture proportions to evaluate the antioxidant properties. Plant extracts were mixed in various proportions of 22 formulations which have been designated using Design Expert 6.0.4. at which the factors were *S. crispus*, *P. niruri, O. aristatus* and *S. rebudiana* while the responses of the experimental design were antioxidant properties (DPPH, FRAP, and TPC). The mixture was standardized at 20 g/100 mL (100% w/v) of formulation. The simplex-centroid mixture design was chosen for the experiments because all the components have the same range of 0 to 100 and there were no constrains on the design space [21]. The relationship between independent variables were illustrated by three dimensional response surface plots by fixing one variable constant at its optimal level [22]. For the validation, one point is selected from the sample space of the data, and the response is predicted using the model. Then, predicted is analyzed and conducted in experiment. The percentage of errors was calculated using the formula below.

$$\textit{Percentage of errors (\%)} = [(\textit{experimental value} - \textit{predicted value})/\textit{predicted value}] \times 100 \quad (1)$$

2.4 DPPH Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl or DPPH is a free radical that produces a violet solution and in the presence of the antioxidant it will change to yellowish solution. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it was reduced. Radical scavenging activity of polyherbal formulations against stable DPPH was determined spectrophotometrically. The changes in colour were measured at 517 nm by UV-Vis spectrophotometer. The radical scavenging activities of each extract and polyherbal combination were measured according to previous method with slight modification [23,24]. The DPPH solution (5.9 mg in 100 mL methanol) was prepared daily before UV measurement. Three (3) mL of DPPH solution was mixed with 77 μL of sample in cuvettes. The mixed samples are kept in the dark for 15 minutes at room temperature and then the decrease in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH was prepared and measured. The experiment was done in triplicate. The radical scavenging activity was calculated by the following formula.

$$Percentage\ of\ inhibition\ (\%) = [(AB - AA)/AB] \times 100 \quad (2)$$

Where,
AB-absorption of blank sample (t=0 min),
AA-absorption of tested extract solution (t=15 min).

2.5 FRAP Assay

The FRAP assay was carried out according to Benzie & Strain with minor modification [25]. Reagents included are 300 mmol/L acetate buffer, pH 3.6, and 16 mL glacial acetic acid per liter of buffer solution; 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L HCl; 20 mmol/L $FeCl_3 \cdot 6H_2O$. Working FRAP reagent was prepared as required by mixing 25 mL acetate buffet, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution. Three (3) mL freshly prepared FRAP reagent was warmed to 37°C and a reagent blank reading was taken at 593 nm. 100 μL of sample was then added along with 300 μL H_2O. Absorbance reading was taken after four (4) minutes. The experiment was done in triplicate. The change in absorbance between the final reading and the blank reading was calculated for each formulation and compared to standard curve. A standard of known Fe (II) concentrations was carried out using several concentrations from 100 to 1000 µM. A standard curve was plotted by plotting the FRAP value of each standard against its concentration. The FRAP values for the samples were determined using this standard curve. The results obtained were calculated using equation below:

$$FRAP\ value\ of\ sample\ (\mu M) = (a/b) \times FRAP\ value\ of\ standard\ (1000\ \mu M) \quad (3)$$

Where,
a – change in absorbance of sample from 0 to 4 min,
b – change in absorbance from 0 to 4 min.

2.6 TPC Assay

The content of total phenolic compounds in polyherbal formulations was determined by Folin-Ciocalteu colorimetric method with slight modification [24]. For the preparation of calibration curve, one (1) mL aliquots of 10, 20, 40 and 80 µg/mL gallic acid solutions was mixed with five (5) mL Folin-

Ciocalteu reagent (diluted ten-fold) and four (4) mL (75 g/L) sodium carbonate. The absorption was read after 30 minutes at 20°C at 750 nm and the calibration curve was drawn. 100 μL of sample was mixed with two (2) mL sodium carbonate two (2) g in 100 mL distilled water and will be left for 2 minutes at room temperature. Then, it was mixed with the same Folin-Ciocalteu reagent and was left for 30 minutes. The experiment was done in triplicate. The absorbance reading was taken at 750 nm. The absorbance values were compared to standard curve.

2.7 Statistical Analysis

The Design Expert® 6.0.4 software was used in determining the optimum polyherbal formulation. Values expressed are means of the three replicate determinations. Variance analysis (one-way ANOVA) was applied on mean data obtained from each antioxidant assay using SPSS 16.00 for Windows of Turkey-LSD test. Differences were considered significant when $p < 0.05$ ($\alpha = 0.05$).

3. Result and Discussion

3.1 Model Fitting

Table 1 presents the results of mixture design studies of antioxidant properties. The independent variables and the runs were suggested and arranged randomly by the Design Expert software for optimization of antioxidant properties. Both the independent and dependent variables were fitted to linear, quadratic, special cubic and cubic models and residuals plots were generated to check the goodness of model fit. Antioxidant assays were run on the formulation and the results were expressed in percentage value. Table 2 presents the important values that were examined to determine the adequate model for each dependent variable. Since the standard deviation is important for checking data distribution, the predicted sum of squares for measuring the model's predictive ability and the predicted R-squared were calculated and compared. The best model has low standard deviation and predicted sum of squares, and high predicted R-squared [25]. Therefore, examination of the fit summary output (Table 2) revealed that the quadratic model was statistically significant for the FRAP and TPC. Meanwhile, special cubic model was statistically significant for DPPH. Thus, this model was used to represent each of the response for further analysis.

3.2 Modeling of Antioxidant Properties

The following equation is the final empirical models in term of coded factors of DPPH, FRAP and TPC, where A for *S. crispus*, B for *P. niruri*, C for *O. aristatus* and D for *S. rebudiana.*

a) DPPH = 51.28*A + 70.59*B + 78.95*C + 64.81*D + 111.39*A*B + 5.32*A*C + 5.61*A*D + 10.35*B*C + 93.23*B*D - 14.47*C*D - 250.93*A*B*C - 613.54*A*B*D - 189.84*A*C*D + 26.46*B*C*D (4)

b) FRAP = 65.34*A + 131.67*B + 141.20*C + 146.74*D + 100.51*A*B + 139.73*A*C + 138.05*A*D + 33.18*B*C - 6.02*B*D - 30.84*C*D (5)

c) TPC = +34.12*A + 108.69*B + 131.60*C + 128.53*D + 182.02*A*B + 184.21*A*C + 194.58*A*D + 41.80*B*C + 49.93*B*D - 10.98*C*D (6)

The equation is necessary for determining the type of interaction of the factors by comparing the factor coefficients such as the synergistics and antagonistics effect. By default, the low levels of the

factors are coded as -1 while the high levels of the factors are coded as +1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 1
Design layout and experimental results for antioxidant properties

Run	Factor 1 (%)	Factor 2 (%)	Factor 3 (%)	Factor 4 (%)	Antioxidant assay		
No	*S. crispus*	*P. niruri*	*O. aristatus*	*S. rebudiana*	DPPH	FRAP	TPC
1	0	0	0	100	64.160^{def}	152.636^{a}	132.608^{ab}
2	100	0	0	0	48.321^{g}	60.805^{d}	39.255^{d}
3	33.33	33.33	33.33	0	86.776^{ab}	142.740^{ab}	131.349^{ab}
4	0	33.33	33.33	33.33	86.716^{ab}	142.882^{ab}	132.453^{ab}
5	50	50	0	0	88.648^{a}	120.578^{abc}	117.976^{c}
6	50	0	0	50	57.860^{efg}	142.676^{ab}	131.794^{ab}
7	62.5	12.5	12.5	12.5	69.837^{cde}	129.961^{ab}	126.484^{ab}
8	12.5	12.5	62.5	12.5	77.147^{abcd}	142.450^{ab}	133.131^{ab}
9	12.5	12.5	12.5	62.5	67.964^{cde}	141.874^{ab}	133.558^{ab}
10	0	0	0	100	65.379^{def}	141.627^{ab}	124.856^{ab}
11	0	100	0	0	87.340^{a}	131.462^{bcd}	107.414^{bc}
12	0	0	100	0	72.987^{bcd}	142.265^{ab}	132.201^{ab}
13	100	0	0	0	53.373^{fg}	67.3683^{cd}	23.810^{d}
14	0	0	100	0	80.713^{abc}	140.640^{ab}	131.329^{ab}
15	0	50	0	50	91.233^{a}	135.825^{ab}	134.410^{ab}
16	50	0	50	0	90.134^{a}	141.380^{ab}	132.046^{ab}
17	0	0	50	50	67.310^{cdef}	140.619^{ab}	132.705^{ab}
18	33.33	33.33	0	33.33	66.389^{def}	142.862^{ab}	134.875^{ab}
19	33.33	0	33.33	33.33	70.639^{cde}	137.286^{ab}	135.166^{ab}
20	12.5	62.5	12.5	12.5	69.242^{cde}	143.315^{ab}	132.182^{ab}
21	0	50	50	0	77.860^{def}	143.912^{ab}	135.224^{ab}
22	25	25	25	25	65.468^{def}	142.368^{ab}	136.678^{a}

Table 2
Model summary statistics for DPPH, FRAP and TPC

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	Predicted sum of squares
DPPH					
Linear	9.77	0.4094	0.3110	0.0769	2687.18
Quadratic	7.42	0.7728	0.6024	-0.0995	3200.81
Special Cubic	4.84	0.9356	0.8309	-2.1993	9313.34
Cubic	4.48	0.9656	0.8554	-9.0316	29202.84
FRAP					
Linear	14.70	0.6561	0.5988	0.4260	6489.10
Quadratic	5.09	0.9725	0.9519	0.8936	1203.08
Special Cubic	4.82	0.9835	0.9568	0.5539	5043.23
Cubic	4.20	0.9922	0.9673	0.7647	2660.42
TPC					
Linear	21.37	0.5626	0.4897	0.2187	14683.48
Quadratic	6.96	0.9691	0.9459	0.8732	2383.16
Special Cubic	7.73	0.9746	0.9332	0.2085	14876.04
Cubic	5.88	0.9908	0.9614	0.6184	7172.79

3.3 Mixture Proportion Optimization

Based on Figure 1 for DPPH model plots, it can be shown that the contour decreasing as it approaches to factor *S. crispus* and increasing while approaching the line in factor *P.niruri*. It determined that the scavenging activity is also increases which DPPH value was 71.17%. However, increasing of *P.niruri* while decreasing *S. crispus* decrease the DPPH values. On the other hand, for FRAP (Figure 2), it was obviously shown that the contour is declines as it closer to factor *S. crispus*. However, it was increasing while approaching the line in factor *P. niruri* and *O. aristatus*. On top of that, the same pattern was applied in the model plot for TPC test (Figure 3). Meanwhile, factor *S. rebudiana* acts as constant of 25% in the model plot. For response optimization there are four solutions found when conducting the optimization process. However, only one formulation is being chosen to be proceeding with validation process. The formulations chosen were 23.96% of *S. crispus*, 0.62% of *P. niruri* and 75.42% of *O. aristatus* because it gave the highest composite desirability that is 0.985 than other acceptable optimum combinations. The predicted responses and the composite desirability approaching to value 1 represent that the optimum antioxidant properties were met. On the other hand, zero composite desirability indicates that one or both responses are outside their acceptable limits [20].

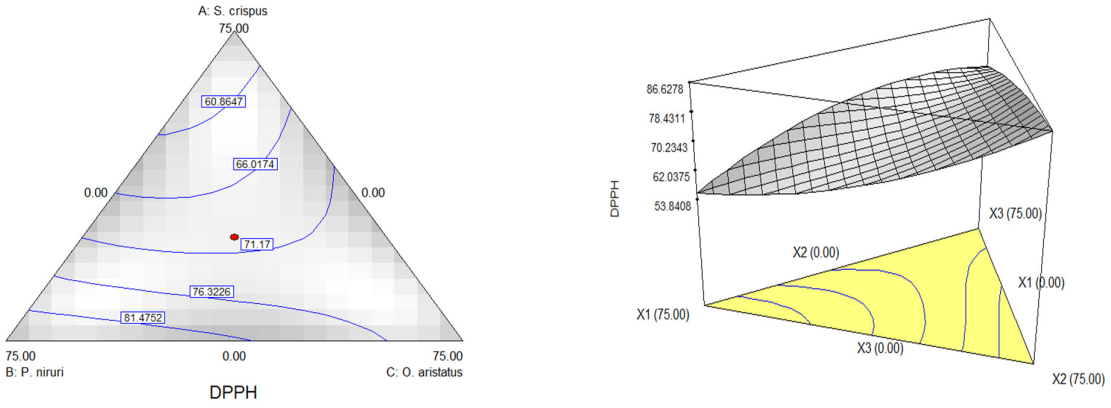

Fig. 1. Mixture contour (left) and surface three dimensional (right) plots for DPPH

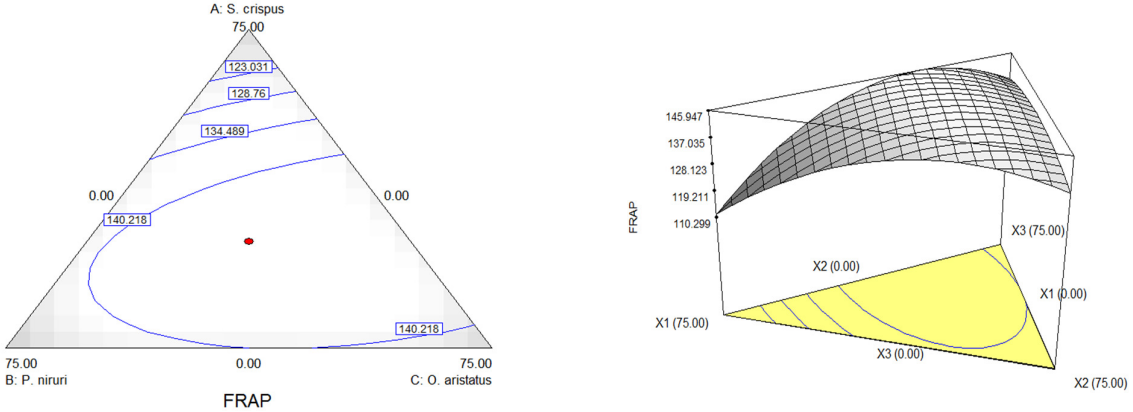

Fig. 2. Mixture contour (left) and surface three dimensional (right) plot for FRAP

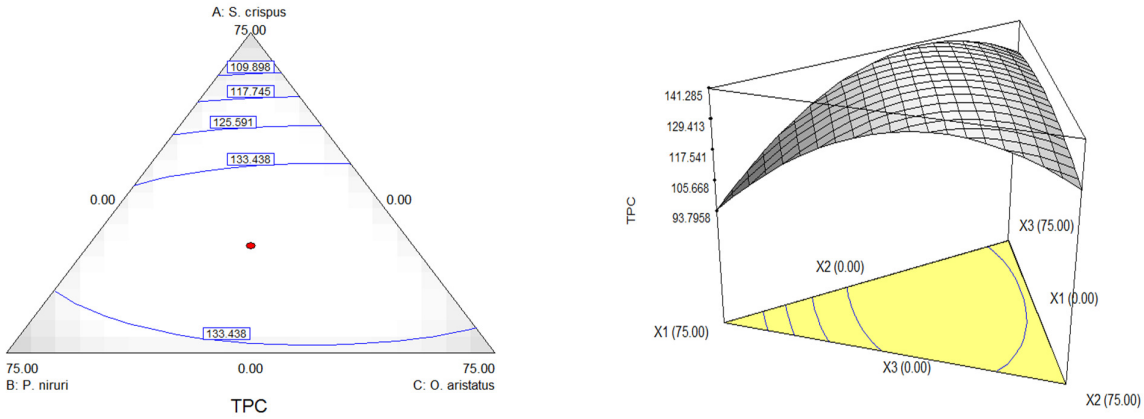

Fig. 3. Mixture contour (left) and surface three dimensional (right) plots for TPC

3.4 Validation of the Model

The experimental data were fitted into the equation (4) to (6) and the optimum proportions were found to be 23.96% of *S. crispus*, 0.62% of *P. niruri* and 75.42% of *O. aristatus.* At these optimum formulations, the experimental value obtained for DPPH was 93.95% which is close to the predicted value which is 91.23% while the experimental value for FRAP was 149.12% and also reasonably close to the predicted which is 148.52%. Whereas, the predicted value for TPC is 141.85% and the value obtained for the validation of TPC was 145.42%. All of the responses obtained the percentage of errors of less than 10% indicates the formulations was validated [26]. These results were comparable with the evaluation of antioxidant properties for each assay. *O. aristatus* is in higher percentage in the polyherbal formulations as it contributes strong potential of antioxidant properties as stated in previous study [27]. Therefore, these proportions of the polyherbal formulations were optimum conditions for antioxidant properties.

3.5 Evaluation of Interaction Effect

The equation (4) which presented empirical model for DPPH assay showed that the combinations of plant extracts *S. crispus* and *O. aristatus, S. crispus* and *S. rebudiana, P. niruri* and *O. aristatus* as well as *P. niruri* and *S. rebudiana* presented the synergistic effects. However, the highest synergistic effect was shown in the combination of plant extracts of *S. crispus* and *P. niruri*. This might be because *S.cripus* has terpene compounds which reported to have synergistic effect in antioxidations with other antioxidants [28]. Besides that, the presence of bioactive compounds in *P. niruri* extracts helps in contributing synergistic effects as it was stated to have antioxidant capabilities in the free radical scavenging activity [29]. Meanwhile, the equation (5) which is the FRAP assay showed that only two of the polyherbal formulations presented the antagonistic interactions which are combination of *P. niruri* and *S. rebudiana* as well as *O. aristus* and *S. rebudiana*. Both of the combinations showed no significant different ($p < 0.05$) in the Tukey-LSD test. The highest effect on synergistic interaction was presented in the combination of the extracts of *S. crispus* and *O. aristatus*. The interaction effect of total phenolic content was showed in the equation (6). It presented that only one formulation had antagonistic interactions which is combination of *O. aristatus* and *S. rebudiana*. In general, most of the polyherbal formulations showed synergistic effect among the three assays of antioxidant which are DPPH, FRAP and TPC.

4. Conclusion

In conclusion, the mixture design was used to provide the optimum mixture of polyherbal formulation at which the most preferred solution containing the combination of aqueous extract of 23.96% of *S. crispus*, 0.62% of *P. niruri* and 75.42% of *O. aristatus* on antioxidant properties which shown synergistic effect. The proportion of each extract in the polyherbal formulation is important to create a better antioxidant activity as a whole. Different amounts of *S. crispus, P. niruri, O. aristatus* and *S. rebudiana* gave different interactions on antioxidant properties since their contents are different.

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