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Application of Statistically Simplex-Centroid Mixture Design to Optimize the TPC and TFC on the Proportion of Polyherbal Formulation Used by Jakun Women

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Abstract: The objectives of this study were to model the extraction of Total Phenolic (TPC) and Flavonoid Content (TFC) from mixtures of *Cnestis palala*, *Urceola micrantha*, *Labisia pumila* and *Microporus xanthopus* and to determine the optimal formulation of the herbal mixture. The dried plant materials were extracted using hot boiling water extraction procedure. Samples (0.1 g) were infused in 200 mL boiling distilled water (100°C), stirred for 3 min using a magnetic stirrer, left to cool for 5 min and filtered using filter paper. Folin-Ciocalteu method and aluminium colorimetric method were used for measurement of TPC and TFC. About 24 formulations of herbal mixture were generated by using Design-Expert 9.0 Software. Quadratic and linear model were found to be the best model for describing the relationship between the proportion of polyherbs with the TPC and TFC. The optimal formulations were validate experimentally. The highest TPC and TFC were shown by single formulation of *C. palala*. The experiments reported in current study demonstrated that the application of statistical mixture design is an efficient tool to analyse the optimal formulation of herbal mixture.

Key words: Herbal mixture, simplex centroid design, *Cnestis palala, Urceola micrantha, Labisia pumila, Microporus xanthopus*

INTRODUCTION

Indigenous people in Malaysia have their own traditional health care system. Jakun people (aborigine of Endau Rompin forest) still depends on medicinal plants to treat illnesses. Few researchers have documented the precious ethnobotanical knowledge of Jakun community (Taylor and Wong, 1987; Ismail, 2015; Kardooni *et al*., 2014). Their reliance and dependency on the medicinal plants may provide baseline information on the potential value of the plants to be an active medicinal principles (Fatima *et al*., 2016). The medicinal plants have been administered orally (in the form of infusion, decoction or maceration) or used topically (for bath and paste). Herbal infusion or widely known as herbal tea is a commonly consumed beverage that were infused with leaves, flowers, seeds, fruits, stems or roots of plant species other than *Camellia sinensis* which usually taken for health care maintenance and disease prevention worldwide (Zhao *et al*., 2013).

The medicinal plants are traditionally boiled or infused in water and the extracts are used for consumption. The boiling water method eliminates the use of organic solvents. The boiling water extracts (herbal infusions) of medicinal plants have been consumed for several thousands of years in China and they proved to be non-toxic. Herbal practitioners and skillful healers have learned from their ancestors that herbal mixtures can be useful to increase the medicinal properties, to decrease some kinds of toxicity and to improve the taste of some oral forms (Guimaraes *et al*., 2011). In some studies, it has been reported that the polyherbal formulation exhibit more therapeutic effects than single herbal formulation (Guimaraes *et al*., 2011; Jain *et al*., 2011). Among Jakun community, other than taking the medicinal plant in the form of single herb, they also consume it in the form of mixtures. *Cnestis palala* (Connaraceae), *Urceola micrantha* (Apocynaceae), *Labisia pumila* (Myrsinaceae) and a type of fungi, *Microporus xanthopus* (Polyporaceae) are among the medicinal plants that were used by Jakun women for post-partum recovery and as an energy booster.

The decoction and infusions of the concomitant were drunk by the women during the confinement period.

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The bioactivities of some of the aforementioned herbs have been investigated in the form of single component by previous researchers (Dej-adisai *et al*., 2015; Chua *et al*., 2011; Balashanmugam *et al*., 2013). The root of *Cnestis palala* has been used traditionally for the treatment of stomach ache, malaria, urinary track disorder and snakebite (Dej-adisai *et al*., 2015). *C. palala* leaf and seed ethanol extract had shown the potential as anti-bacterial activity while its hexane leaf and seed extract and petroleum ether bark and root extract showed strong inhibition towards MCF-7. There are about 7 compounds that have been isolated from this plants such as β-sitosterol-glucoside, hydroquinone, β-sitosterol, mixture of fatty acids and ethyl caffeate, scopoletin and 2-nonenal.

Labisia pumila, the most popular medicinal plants among Malay women has been used traditionally to facilitate childbirth and for post-partum recovery (Abdullah *et al*., 2013). *L. pumila* or locally known as *Kacip Fatimah*, has been reported to contain high total phenolics and flavonoid content. Nine flavonols (quercetin, myricetin and kaempferol), two flavanols (catechin and epigallocatechin) and nine phenolic acids were identified from active fraction of *L. pumila* (Abdullah *et al*., 2013). Despite research on bioactivities of single herb, no scientific evidence was found to support the medicinal claims of the mixture for the four medicinal plants.

The statistical mixture design using individual or formulations contains binary, ternary and quaternary mixture of the medicinal plants is a well establish approach in formulation research. In mixture design, the effects of combination of two or more components in different proportions (independent variable) are analysed based on the resulting products, the responses (dependent variables) (Orives *et al*., 2014). If one component is increased, the proportion of other component must decrease if the total weight (or amount) of the mixture remains the same (Smith, 2005). In this mixture design, the synergistic effect as well as antagonistic effects of the blending component could be analyzed (Smith, 2005). (Fatima *et al*., 2016) has I nvestigated the synergistic potential of mixture of β-tocopherol, β-carotene and lycopene. Hence, compounds in the form of mixtures have been shown to be more effective for antioxidant capacities compared to the individual compound.

The simplex-centroid mixture design is the most suitable approach to be used in the current study. In addition, the use of statistical mixture designs using individual or formulations contain binary (mixture of two medicinal plants), ternary (mixture of three medicinal plants) or quaternary mixture (mixture of four medicinal plants) of Jakun's medicinal plants has not yet been reported in the literature. Therefore, the objectives of the present study were to evaluate the Total Phenolic (TPC) and Total Flavonoid Content (TFC) of the herbal infusions of four medicinal plants, *Cnestis palala* bark, *Urceola micrantha* bark, *Labisia pumila* root and *Microporus xanthopus* fruiting body in the form of single basis and mixture as well as to determine the optimal combination of the mixture using the simplex-centroid design that would maximize the TPC and TFC.

MATERIALS AND METHODS

Plant preparation: The plant samples were obtained in Feb. 2015 from Endau Rompin National Park under a permit approved by Johor National Parks Corporation (JNPC). Voucher specimens of each plant were identified by Mr. Abu Hussin from Forest Research Institute Malaysia.

Preparation of sample infusion: The fresh plant materials collected were dried according to the traditional method used by Jakun people.. The dried plant materials were grounded and sieved into 500 mesh-size. Herbal tea was prepared using hot boiling water extraction procedure. Sample (0.1 g) was infused in 200 mL boiled distilled water (100°C) and stirred for 3 min using a magnetic stirrer. The infusion left to cool for 5 min and filtered using filter paper (Whatman No. 1).

Total Phenolic Content (TPC) The Folin-Ciocalteu method was adapted from Velioglu *et al*. (1998). About 100 μL of sample infusion was mixed with 0.75 mL of Folin Ciocalteu reagent (10% v/v). The mixture was vortexed for 15 sec. After 5 min, 0.75 mL of sodium bicarbonate (60 g/L) solution was added to the mixture and allowed to stand at 22°C for 90 min. The mixture was placed into cuvette and absorbance value was measured at 725 nm by using spectrophotometer. Gallic acid was used as standard and the total phenolic content was expressed as Gallic Acid Equivalent Per Litre of infusion (mg GAE/L). Standard was prepared by diluting 0.01 g gallic acid into 100 mL distilled water with different concentration (0-100 μg/mL) and the calibration equation of $y = 0.0032x+0.0169$ ($R^2 = 0.9969$) was generated.

Total Flavonoid Content (TFC): Method for the determination of total flavonoid content was adapted from Zhishen *et al*. with slight modification. Briefly, 1 mL extract was added to the beaker with 4 mL distilled water. At 0 min, 0.3 mL of 5% sodium nitrite were added

Table 1: Simplex centroid design and response values

	Mixture ratio				Responses	
Formulations	*Cnestis palala* x_1	*Urceola micrantha* x_2	*Labisia pumila* x_3	*Microporus xanthopus* x_4	TPC (mg GAE/L)	TFC (mg CE/L)
1	1.000	0.000	0.000	0.000	154.72	69.64
2	0.000	1.000	0.000	0.000	126.91	69.41
3	0.000	0.000	1.000	0.000	17.74	3.02
4	0.000	0.000	0.000	1.000	5.52	1.17
5	0.500	0.500	0.000	0.000	107.91	67.39
6	0.500	0.000	0.500	0.000	73.16	33.93
7	0.500	0.000	0.000	0.500	65.66	38.77
8	0.000	0.500	0.500	0.000	58.36	31.22
9	0.000	0.500	0.000	0.500	59.27	35.86
10	0.000	0.000	0.500	0.500	7.53	0.19
11	0.333	0.333	0.333	0.000	71.73	39.98
12	0.333	0.333	0.000	0.333	71.52	44.60
13	0.333	0.000	0.333	0.333	46.87	20.48
14	0.000	0.333	0.333	0.333	38.26	21.69
15	0.250	0.250	0.250	0.250	59.79	32.70
16	0.625	0.125	0.125	0.125	90.76	51.44
17	0.125	0.625	0.125	0.125	78.85	50.23
18	0.125	0.125	0.625	0.125	41.87	15.54
19	0.125	0.125	0.125	0.625	30.41	13.64
20*	1.000	0.000	0.000	0.000	152.46	64.23
21*	0.000	1.000	0.000	0.000	125.03	60.41
22*	0.000	0.000	1.000	0.000	21.91	3.44
23*	0.000	0.000	0.000	1.000	5.17	0.38
24*	0.500	0.500	0.000	0.000	102.98	67.27

*replicated points

into the mixture. Then, 0.6 mL of 10% $AICI_3.6H_2O$ were added after 5 min. After 6 min, 2 mL NaOH (1M) were added to the solution. The mixture was vortexed before placing it into cuvette. The absorbance value was measured at 510 nm by using spectrophotometer. Catechin (20-100 μg/mL) was used as a standard. Results were compared with standard curve equation ($y = 0.0045x+0.0063$; $R^2 = 0.9953$) and represented as mg catechin equivalent/L (mg CE/L) sample infusion.

Mixture design and statistical analysis: To assess the quaternary phytochemicals and antioxidant activity of the medicinal plants, 4-component simplex-centroid design was used. The factors represent the proportions of herbs in the mixture which ranges from 0-1. As shown in Table 1, the experimental design consisted of 24 experiments with five replicated points. The dependent variables were total phenolic content and flavonoid content. A reduced model was obtained by analysing the coefficients regression model using ANOVA ($p<0.05$) and removing the non-significant coefficients from the initial model. This analysis was carried out using Design-Expert 9 Software.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content: Considering the well-known biological properties of the polyphenols, current analysis may provide the baseline information about the potential bioactivities in the samples (Royer *et al*., 2013). Phenolics are the largest group of phytochemicals in plants. The phenolic compounds have exhibited an antioxidant activity by acting as free radical scavengers that able to control various ailments due to oxidative stress (Sharma *et al*., 2016).

Table 2: Average value of TPC and TFC based on type of formulations

Formulations	TPC (mg GAE/L)	TFC (mg CE/L)
Single	76.18	33.96
Binary	67.84	39.23
Ternary	72.01	36.60
Quaternary	60.34	32.71

In the current study, hot water extraction was used to extract the samples. The extraction method of the herbs were intentionally employed to mimic the traditional preparation of medicinal plants by the Jakun people. The usage of water as solvent is most likely to be less harmful to the researcher (Li *et al*., 2013a, b). According to Royer *et al*. (2013), hot water extract of Canadian forest species bark exhibited higher yield, total phenolic and flavonoid content compared to ethanol extract.

The TPC and TFC data are listed in Table 1. The total phenolic and flavonoid content varied from 5.17-154.7 mg GAE/L and from 0.19-69.64 mg CE/L. Overall, *Cnestis palala* bark infusion showed the highest TPC and TFC. The lowest value for TPC has been shown by *M. xanthopus* whereas the lowest TFC value was shown by binary formulation of *L. pumila* and *M. xanthopus*.

Based on Table 2, the average value of TPC and TFC were calculated on single component and mixture basis.

By comparing the average of TPC, the single component displayed the highest amount of phenolic (76.18 mg GAE/L), followed by ternary mixture (72.10 mg GAE/L), binary mixture (67.84 mg GAE/L) and quaternary mixture (60.34 mg GAE/L). The average amount of TFC was increasing in the following order: quaternary mixture (32.71 mg CE/L), single herb (33.91 mg CE/L), ternary mixture (36.57 mg CE/L) and binary mixture (39.23 mg CE/L).

Among the quaternary formulations, the formulation with the highest TPC and TFC were shown by combination of 0.625 *C. palala*: 0.125 *U. micrantha*: 0.125 *L. pumila* : 0.125 *M. xanthopus*.

The lowest value of TPC was shown by *M. xanthopus* and the binary mixture of *L.pumila* and *M. xanthopus*. Tamrakar *et al.* (2016) demonstrate that *M. xanthopus* (order: Polyporales) contain 14.5±0.33 mg GAE/g and the antioxidant activities of this order is attributed to the phenolics content based on correlation analysis (Ferreira *et al.*, 2009).

Model fitting: Table 1 displayed the mixture design generated by Design-Expert 9 Software. The experimental responses data were subjected to the simplex-centroid design analysis where linear, quadratic, special cubic and cubic model were allowed to fit the data. To minimize the variance, the TPC and TFC for each run were triplicated. For TPC, the quadratic model was found to be more suitable to describe the relationship between the response variables and factors (Table 2). Backward elimination was used to remove the insignificant variables from the TPC quadratic model. However, for TFC, only linear model was found to be significant to describe the response. An Analysis Of Variance (ANOVA) was performed using $\alpha = 0.05$ to determine the accuracy of the models (Li *et al.*, 2013). The model is said to be significant when the p-value for the model is <0.05. Lack of fit test was evaluated to measure the adequancy of the data. If $p>0.05$, the developed model is adequate, if $p<0.05$, the model is not adequate to describe the experimental data. The coefficient of determination (R^2) and the adjusted R^2 value was used to determine whether the selected models displayed a good fit of the data. The higher value of R^2 indicates that the developed model can predict the result better. Based on this criteria, the two models were adequately fitted to the two responses (Table 3). In current study, the R^2 value for TPC and TFC are 0.9970 and 0.9890, respectively which is very close to 1. The predicted R^2 is in agreement with adjusted R^2. Two regression Eq. 1 and 2 for TPC and TFC were generated from the model:

$$Y_{TPC} = 152.95x_1+125.50x_2+19.67x_3+4.40x_4-136.45x_1x_2-49.51x_1x_3-51.71x_1x_4-56.26x_2x_3-24.81x_2x_4 \quad (1)$$

Table 3: ANOVA table for phytochemical and antioxidant activity

Source	Sum of squares	df	Mean square	F-values	p-values	Prob>F
TPC[a]-Quadratic model (backward elimination)						
Model	44563.36	8	5570.42	630.41	<0.0001	Significant
Linear mixture[b]	42205.41	3	14068.47	1592.15	<0.0001	
x_1x_2	1822.02	1	1822.02	206.2	<0.0001	
x_1x_3	168.08	1	168.08	19.02	0.0006	
x_1x_4	183.31	1	183.31	20.75	0.0004	
x_2x_3	217	1	217	24.56	0.0002	
x_2x_4	42.21	1	42.21	4.78	0.0451	
Residual	132.54	15	8.84			
Lack of fit	107.31	10	10.73	2.13	0.2095	Not significant
Pure error	25.23	5	5.05			
Cor total	44695.9	23				
SD	2.97		R-squared	0.9970		
Mean	67.27		Adj R-squared	0.9955		
C.V. (%)	4.42		Pred R-squared	0.9946		
PRESS	241.32		Adeq precision	81.611		
TFC–Linear model						
Model	13277.53	3	4425.84	598.94	<0.0001	Significant
Linear mixture	13277.53	3	4425.84	598.94	<0.0001	
Residual	147.79	20	7.39			
Lack of fit	92.25	15	6.15	0.55	0.8279	Not significant
Pure error	55.54	5	11.11			
Cor total	13425.32	23				
SD	2.72		R-squared	0.9890		
Mean	34.86		Adj R-squared	0.9873		
C.V. (%)	7.80		Pred R-squared	0.9824		
PRESS	236.22		Adeq precision	60.124		

[a]Backward elimination regression with alpha equals to 0.05 [b] x_1, x_2, x_3 and x_4 represent the proportion of *C. palala, U. micrantha, L. pumila and M. xanthopus* in mixtures, respectively

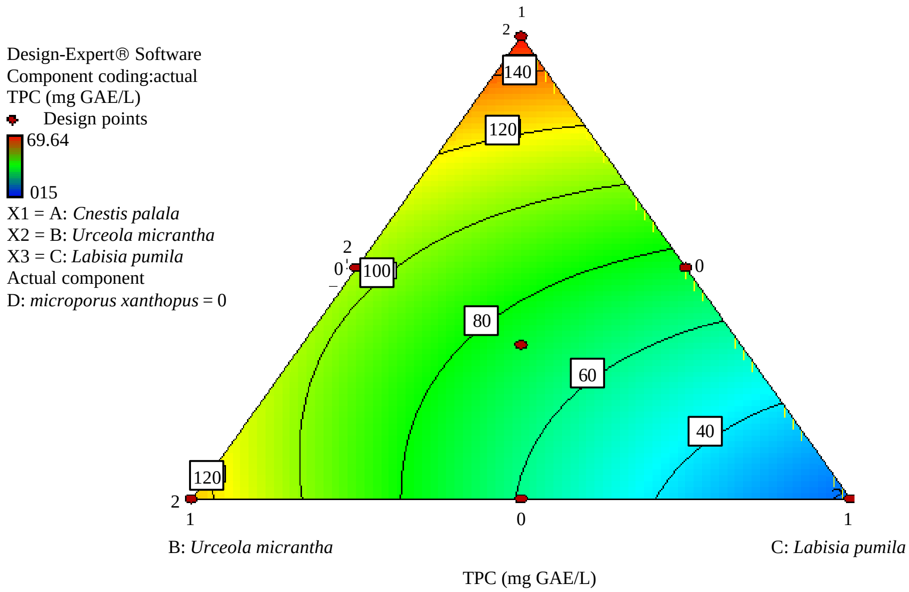

Fig. 1: Mixture contour plot showing the effect of *C. palala, U. micrantha, L. pumila* and *M. xanthopus* on TPS

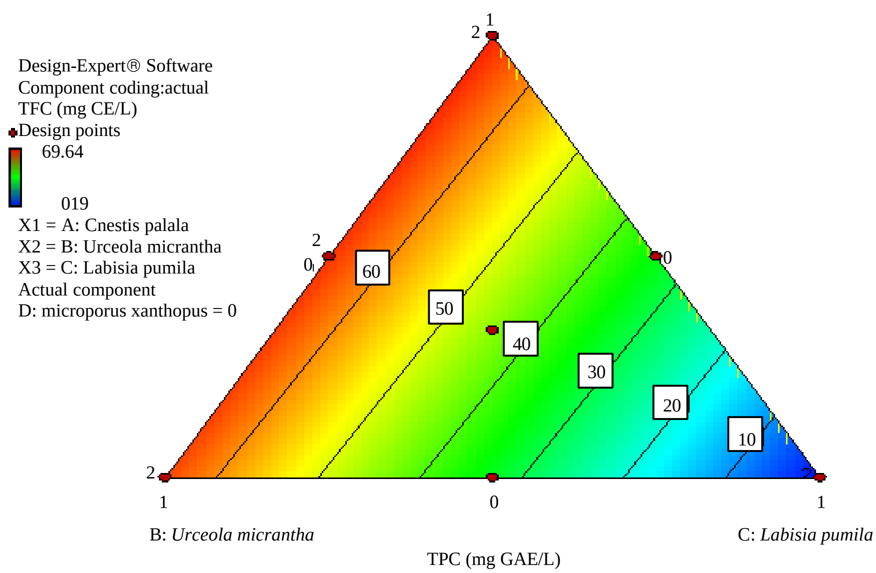

Fig. 2: Mixture contour plot showing the effect of *C. palala*, *U. micrantha*, *L. pumila* and *M. xanthopus* on TFC

where Y_{TPC} and Y_{TFC} are the predicted responses of total phenolic content and total flavonoid content and x represent the four medicinal plants. (x_1, *Cnestis palala*, x_2, *Urceola micrantha*, x_3, *Labisia pumila*, x_4, *Microporus xanthopus*)

Interpretation of contour and line plots: From 2D contour plots of TPC (Fig. 1), the zones for the maximum variables were located towards the vertices of *C. palala* and *U. micrantha*. In this contour plot, *Microporus xanthopus* was set as zero due to the low amount of TPC and TFC showed by this species. This indicate the single formulation of *C. palala* and *U. micrantha* contain more TPC compared to any mixtures. For TFC contour plot (Fig. 2), the maximum response variable is the located at the side of triangle which include the mixture of *C. palala* and *U. micrantha*. This showed that the single and mixture of *C. palala* and *U. micrantha* could give high TFC value.

Synergism between mixtures: Combination of plants may possess additive, synergistic or antagonistic effects due

Table 4: Optimum proportion of herbal mixture

				TPC		TFC		
C. palala	*U. micrantha*	*L. pumila*	*M. xanthopus*	Predicted	Observed	Predicted	Observed	Desirability
1.0	0.0	0.0	0.0	152.782	125.19	67.46	64.71	0.978
0.0	1.0	0.0	0.0	134.732	115.29	64.86	61.94	0.898
0.5	0.5	0.0	0.0	105.756	114.93	66.161	61.60	0.799

to the interaction between the different phytochemicals. An additive effect means a combination of plants that will result in the sum of effects of individual components whereas synergistic effects happen when the combination effect is greater than the sum of individual component (Wang *et al*., 2011). Generally, a coefficient with negative sign in the fitted model equations (Eq. 1 and 2) indicates the ability of its associated factor to decrease the response while the positive sign display the ability of a factor to increase the response variable (Ouedrhiri *et al*., 2016). In current study, the goal was to increase the TPC and TFC of the formulations. Therefore, the positive sign of a coefficient (Eq. 1) exhibit the ability of its associated factor to increase the responses. For TPC, all the binary mixture showed the antagonistic effects. As for the single component coefficient, the highest was shown by *C. palala* and the smallest coefficient was *M. xanthopus*. As been investigated by Tshivhandekano *et al*. (2014), the single component of special tea contain higher total polyphenol compared to bush tea and binary combination of special and bush tea. For TFC, the linear model could not detect the synergism effects between the mixtures.

Mixture proportion optimization and validation: Optimization of mixture proportion was performed to determine the optimum mixture. The goal for TPC and TFC were targeted for maximum. The optimized percentage of the herbal mixtures are shown in Table 4. For validation purposes, the optimum formulation was done in triplicate. The infusions were subjected to the TPC and TFC analysis. The experimental value was lower than the predicted value. However, *C. palala* still have the highest TPC and TFC. The validation studies showed that a formulation composed of single medicinal plants was the best to achieve higher TPC and TFC.

CONCLUSION

The highest TPC and TFC were obtained from the single medicinal plants formulation which is *C. palala*. The experiments reported in current study demonstrated that the application of statistical mixture design is an efficient tool to analyse the optimal formulation of herbal mixture.

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