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Synergistic Antioxidant Activity and Optimal Microwave-Assisted Extraction Condition of *Caesalpinia sappan* L., *Hibiscus sabdariffa* L., and *Clitoria ternatea* L. Combinations

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Abstract

The aims of this work were to evaluate the synergistic antioxidant activity and optimize microwave-assisted extraction of *Caesalpinia sappan* L., *Hibiscus sabdariffa* L., and *Clitoria ternatea* L. combinations. The simplex lattice design was applied to evaluate the synergistic antioxidant activity. The mass ratios of the 3 plants that exhibited synergistic antioxidant activity were further used to investigate the optimal condition of microwave-assisted extraction by Box-Behnken design. Results showed that the synergistic antioxidant activity among the 3 plants: Which was determined by the combination index, was observed at various mass ratios, especially at the equal mass ratio. The optimal microwave-assisted extraction condition providing the best antioxidant activity of the plant extract was microwave power of 300 W for 30 s and 3 cycles. This condition gave the extraction yield of 31.03 ± 0.26 %, total phenolic content of 72.03 ± 3.83 mg GAE/g extract, total flavonoid content of 15.18 ± 0.42 mg CE/g extract, half-maximal inhibitory concentration (IC_{50}) from DPPH assay of 178.16 ± 15.54 µg/mL and IC_{50} from FRAP assay of 75.26 ± 4.44 µg/mL. The optimal extract showed no toxicity on HepG2 cells although the tested concentration was up to 1 mg/mL with an IC_{50} value of 5.45 mg/mL. So, a mixture of the 3 colored plants could be used as a supplementary antioxidant product with a safety profile.

Keywords: Synergy, Microwave-assisted extraction, Herbal beverage, Cytotoxicity, Design of experiment

Introduction

Plant extraction is an important step for chemical isolation, chemical analysis, and evaluating the biological and pharmacological activities of plant compounds. Suitable extraction techniques and conditions can extract a high amount of desired plant active compounds, and prevent the decomposition of some sensitive compounds [1]. Thus, finding the optimal extraction conditions is a key step to maximizing extraction yield as well as maximizing the amount of active plant compounds.

Currently, several techniques both conventional and modern methods are applied for the extraction of herbal plant compounds. Microwave-assisted extraction (MAE) is a modern technique used in plant extraction. MAE has been recognized as an extraction technique with many advantages superior to other methods. It reduces the cost of production, time, solvent consumption, energy consumption, and carbon dioxide emissions. Therefore, it is accepted as a green extraction technique [2-4]. The efficiency of microwave energy depends primarily on the solid-to-solvent ratio, plant matrix properties, irradiation time, and irradiated microwave power [5].

Herbal beverages are good sources of antioxidant phytochemicals that may help in decreasing the risk conditions of some diseases and the management of non-communicable diseases [6]. Numerous Thai herbal beverages prepared from Thai herbal plants are widely consumed and they are already proven for their potential antioxidant activity [7]. Some colored plants: *Caesalpinia sappan* L. (Leguminosae), *Hibiscus sabdariffa* L. (Malvaceae), and *Clitoria ternatea* L. (Leguminosae), are used as ingredients in Thai herbal beverages. They possess several biological and pharmacological activities. *C. sappan* possesses antioxidant, anticancer, anti-inflammatory, immunosuppressive, antidiabetic, antimicrobial, vasorelaxing,

antiproliferative, antiplatelet, analgesic, and acaricidal activities [8,9]. *H. sabdariffa* shows anti-anemia, anti-carcinogenic, antihyperlipidemic, antihypertensive, antihyperuricemic, anti-inflammatory, antimicrobial, diuretic, hepatoprotective activities, etc. [10]. *C. ternatea* possesses anti-arthritic, antiasthmatic, antidiabetic, anti-inflammatory, analgesic, antilipidemic, antioxidant, antipyretic, diuretic, nootropic, and wound healing activities [11,12]. Among these activities, the antioxidant is interesting because it can be claimed as an indication for supplementary health products. The 3 colored plants used in Thai herbal beverages can be combined to prepare a new supplementary product. Furthermore, to the best of our knowledge, there is no research work investigating the antioxidant activity and cytotoxicity of the 3 colored plants in combination. Investigating antioxidant activity and cytotoxicity of the 3 colored plants' combinations can prove its efficacy and safety. In addition, the determination of total phenolic and total flavonoid contents can indicate its quality.

Synergistic effects occur among pharmaceuticals or natural products. It can enhance biological activities as well as therapeutic properties by some interactions of their chemical constituents. The synergistic effect of herbal plants has been reported in many studies. The synergistic effect can occur among herbs or other ingredients in the prescription, among effective parts of herbs and bioactive compounds of herbs [13]. In some cases, synergism could occur between 2 or more non-active compounds [14]. Traditional medicines usually used herbal combinations rather than a single herb to boost the therapeutic efficacy, decrease side effects, modulate immunity, and reduce drug resistance [15]. There are several methods used in the evaluation of the synergism of drug combination, *i.e.*, combination index (CI), isobole method, and system biology. However, the CI is the most practical and the most demonstrative method used for the investigation of synergistic effects [16-22]. This method has no limitation for the number of ingredients in the herbal combination. However, the dose-responses of an individual constituent and combination are required [16]. The application of response surface analysis in the investigation of synergistic effects can provide a complete description of the combined effect of pharmaceutical or herbal formulas [23].

Design of experiment (DOE) is a well-recognized collection of statistical and mathematical tools that have been widely exploited to improve and optimize the extraction process. Traditional optimization of extraction procedures is done by the 1-factor-at-time (OFAT) method, which is time and energy-consuming. Furthermore, it can lead to the loss of the effects caused by the interaction of the extraction parameters. Consequently, the DOE approach is considered a significant tool to seek a more rapid optimal procedure for experimental process development [24]. In addition, DOE exhibits several advantages over OFAT trialing, such as less time and financial consumption, less chemical substance use, interaction effects can be identified, and the response surface can be characterized [25].

The work aimed to evaluate the synergistic antioxidant activity of *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes, and *C. ternatea* flowers using the combination index method combined with response surface analysis. Furthermore, optimization of the MAE condition for extraction of the 3 plants was performed, and the *in vitro* cytotoxicity test of the extract obtained from the optimal condition was also evaluated to confirm its safety. The authors expected that the optimal MAE parameters could be used as a guide for the extraction process of the 3 colored plants in combination with a simple, rapid, and effective manner.

Materials and methods

Materials

Gallic acid monohydrate, (+)-catechin hydrate, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Methylthiazolyldiphenyltetrazolium bromide (MTT), and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich Pte Ltd., Ascent, Singapore. Sodium carbonate (Na_2CO_3) and aluminum chloride ($AlCl_3$) were purchased from Ajax Finechem Pty. Ltd., New South Wales, Australia. Sodium acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), and glacial acetic acid were purchased from Carlo Erba Reagents, Val-de-Reuil, France. Ferric (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from Merck KGaA, Darmstadt, Germany. Sodium nitrite ($NaNO_2$) was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. The other reagents and solvents were analytical grade.

Plant samples

Dried heartwoods of *C. sappan*, calyxes and epicalyxes of *H. sabdariffa*, and flowers of *C. ternatea* were purchased from Charoensuk Osod Herbal Store, Nakorn Pathom Province, Thailand. They were identified by Dr. Chaowalit Monton to ensure the correct plant species. The voucher specimens were kept

at the Drug and Herbal Product Research and Development Center. They were coded as CM-CS001-1-07-2020, CM-HS001-1-07-2020, and CM-CT001-1-07-2020, respectively.

Experimental design and plant extraction

The mass ratios of 3 plant powders that pass 60-mesh sieve were designed by simplex lattice design (**Table 1**). The independent variables, a mass ratio of *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes and *C. ternatea* flowers powder were coded as X_1, X_2 and X_3, respectively. Fourteen conditions were designed, while Conditions 10 - 14 were the replicate conditions at the center of the lattice. Ten g of plant powder mixture was mixed with 100 mL water contained in a 250-mL Erlenmeyer flask. It was extracted using a microwave-assisted extraction technique. The microwave power was set at 600 W and the microwave duration was 60 s. (Samsung, model: MS23F300EEK, Bangkok, Thailand). It was filtered using Whatman® filter paper (no. 1) by the vacuum filtration technique. The filtrate was cooled, frozen, and lyophilized by a freeze dryer (SCIenergy Co., Ltd., Nonthaburi, Thailand). The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity were evaluated as described below. The 5 dependent variables - extraction yield (Y_1), TPC (Y_2), TFC (Y_3), The half-maximal inhibitory concentration (IC_{50}) from DPPH assay (Y_4) and IC_{50} from FRAP assay (Y_5) were collected.

Table 1 Simplex lattice design.

Condition	Coded values			Experimental values (g)		
	X_1	X_2	X_3	Heartwoods of *C. sappan*	Calyxes and epicalyxes of *H. sabdariffa*	Flowers of *C. ternatea*
1	1	0	0	10	0	0
2	0	1	0	0	10	0
3	0	0	1	0	0	10
4	0.5	0.5	0	5	5	0
5	0.5	0	0.5	5	0	5
6	0	0.5	0.5	0	5	5
7	0.67	0.17	0.17	6.7	1.7	1.7
8	0.17	0.67	0.17	1.7	6.7	1.7
9	0.17	0.17	0.67	1.7	1.7	6.7
10	0.33	0.33	0.33	3.33	3.33	3.33
11	0.33	0.33	0.33	3.33	3.33	3.33
12	0.33	0.33	0.33	3.33	3.33	3.33
13	0.33	0.33	0.33	3.33	3.33	3.33
14	0.33	0.33	0.33	3.33	3.33	3.33

Determination of total phenolic content

The TPC of plant extracts was determined by the Folin-Ciocalteu method [26]. Twenty microliters of gallic acid in a concentration range of 6.25 - 200 µg/mL, was added to a 96-well plate (n = 3). After that, a hundred microliters of 0.2 N Folin-Ciocalteu reagent was added and mixed. Extracts were incubated for 6 min before adding 80 µL of 7.5 % Na_2CO_3 and mixed. They were further incubated at room temperature for 1 h before measuring the absorbance at 765 nm using a microplate reader (Biorad Laboratories, Inc, California, USA). The calibration curve of gallic acid was constructed. The sample group was performed similarly to the gallic acid group, but extracts in a concentration of 0.5 mg/mL were used. The TPC of plant extract was calculated from the calibration curve of gallic acid. The TPC of the samples was expressed as gallic acid equivalents (GAE) in mg per g extract.

Determination of total flavonoid content

The TFC determination was modified from the previous work [27]. Twenty-five microliters (+)-catechin hydrate in a concentration range of 5 - 250 µg/mL and water (100 µL) were added to a 96-well plate (n = 3). Ten microliters of 5 % $NaNO_2$ were then added. The plates were incubated in the dark at room temperature for 5 min, Then, 10 % $AlCl_3$ (15 µL) was added, mixed, and incubated in the dark at room temperature for 6 min. Then, 1 M NaOH (50 µL) and water (50 µL) were added and mixed before measuring the absorbance at 510 nm using a microplate reader. The calibration curve of (+)-catechin hydrate was constructed. The sample group was performed similarly to the (+)-catechin hydrate, except for those extracts that were in a concentration of 2.5 mg/mL. The TFC of plant extract was calculated from the calibration curve of (+)-catechin hydrate. The TFC was expressed as catechin equivalents (CE) in mg per g extract.

Antioxidant activity assay

DPPH assay

DPPH assay was slightly modified from the previous study [28]. First hundred microliters of the extracts (15.625 - 1,000 µg/mL) were added to the 96-well plate (n = 3). Then, 80 µM DPPH methanolic solution (100 µL) was added and mixed. The mixtures were kept in the dark at room temperature for 30 min before measuring the absorbance at 517 nm using a microplate reader. The DPPH radical without extract was used as blank. The capability to scavenge the DPPH radical was calculated using Eq. (1);

$$DPPH\ scavenging\ (\%) = \left(\frac{A_{blank} - A_{extract}}{A_{blank}}\right) \times 100 \quad (1)$$

where A_{blank} is the absorbance of the blank and $A_{extract}$ is the absorbance of the extract.

FRAP assay

FRAP assay was modified from the previous study [29]. Twenty microliters of the extracts in different concentrations (7.8125 - 500 µg/mL) were added to the 96-well plate (n = 3). Then, 180 µL of freshly prepared FRAP reagent (30 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in a volume ratio of 10:1:1, respectively) was added and mixed. Plates were incubated at 37 °C for 15 min before measuring the absorbance at 593 nm against a reagent blank using a microplate reader. The reducing powers were calculated using Eq. (2);

$$Reducing\ power\ (\%) = \left(\frac{A_{extract} - A_{blank}}{A_{extract}}\right) \times 100 \quad (2)$$

where A_{blank} is the absorbance of blank; $A_{extract}$ is the absorbance of the extract.

The curves between concentrations of the extract versus percent DPPH scavenging for DPPH assay and the curves between concentrations of the extract versus percent reducing power for FRAP assay were constructed. The IC_{50} was calculated from the equation of the curve.

Determination of combination index

The synergistic effect among the 3 plant extracts was investigated based on the response additivity approach [23]. The CI was used as a tool for the investigation of synergism Eq. (3);

$$CI = \frac{E_{combination}}{f_A E_A + f_B E_B + f_C E_C} \quad (3)$$

Where $E_{combination}$ was observed IC_{50} of plant mixture extracts. f_A, f_B, and f_C were the mass ratio or mass fraction of *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes and *C. ternatea* flowers. E_A, E_B, and E_C were the IC_{50} of an individual extract of *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes, and *C. ternatea* flowers, respectively. The CI values of less than 1, equal to 1, and higher than 1 were considered as the synergistic effect, additive effect, and antagonistic effect, respectively [23].

Construction of contour plots and overlay plots

The contour plots were constructed using Design-Expert® version 11.0.2.0 (Stat-Ease, Inc., Minnesota, USA). The mathematic models and equations were reported. The linear plots between predicted

values and experimental values, and coefficient of determination (R^2) were also reported. Response surface models were fitted by means of least-squares using the following second-order polynomial model Eq. (4);

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} {X_i}^2 \quad (4)$$

where Y is the dependent variable, X_i and X_j are the independent variables, β_0 is the constant coefficient, β_i is the coefficient of linear effect, β_{ij} is the coefficient of an interaction effect, β_{ii} is the coefficient of quadratic effect and n is the number of independent variables.

The overlay plots that the synergistic effect was observed, CI values of IC_{50} values which lower than 1 were constructed.

Experimental design and microwave-assisted extraction

The Box-Behnken design was applied in this work. The 3 independent variables were microwave power (X_1), time (X_2) and cycle (X_3). They were varied at low, medium, and high levels as shown in **Table 2**. The microwave power was varied as 300, 450 and 600 W; time was 10, 20 and 30 s; cycles were 1 - 3. The range of these values was selected based on the previous work [30]. These ranges did not make excessive boiling and bumping during extraction. So, the 17 conditions were obtained, while Conditions 7 - 11 were similar conditions that were replicated at the center point of the Box-Behnken design.

An equal mass ratio of *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes, and *C. ternatea* flowers powder (10 g) was added to a 250-mL Erlenmeyer flask and then 100 mL of water was added. Extraction was performed using a microwave oven at a specific power, time, and cycle as shown in **Table 2**. Then, the extract was filtered by the vacuum filtration technique. Finally, the filtrate was cooled, frozen, and lyophilized by a freeze dryer. The dependent variables—extraction yield (Y_1), total phenolic content (TPC) (Y_2), total flavonoid content (TFC) (Y_3), IC_{50} from DPPH assay (Y_4) and IC_{50} from FRAP assay (Y_5), were collected and reported.

Table 2 Box-behnken design.

Conditions	Coded values			Experimental values		
	X_1	X_2	X_3	Power (W)	Time (s)	Cycle
1	−1	−1	0	300	10	2
2	−1	0	−1	300	20	1
3	−1	0	+1	300	20	3
4	−1	+1	0	300	30	2
5	0	−1	−1	450	10	1
6	0	−1	+1	450	10	3
7	0	0	0	450	20	2
8	0	0	0	450	20	2
9	0	0	0	450	20	2
10	0	0	0	450	20	2
11	0	0	0	450	20	2
12	0	+1	−1	450	30	1
13	0	+1	+1	450	30	3
14	+1	−1	0	600	10	2
15	+1	0	−1	600	20	1
16	+1	0	+1	600	20	3
17	+1	+1	0	600	30	2

Optimization procedure

The data of each dependent variable was analyzed by Design-Expert®. The contour plots, mathematical model and regression coefficient, correlation plots between predicted values vs. actual values, the plots between internally studentized residuals vs. run were generated. The optimal condition providing the best antioxidant activity, the lowest IC_{50} from DPPH and FRAP assays, was selected based on the desirability function [31]. The accuracy of the prediction of the optimal condition by Design-Expert® was verified by re-extracting the plant combination and testing with the same topics. The experimental values were compared to the predicted values and percentage error was calculated as Eq. (5);

$$Error\ (\%) = \frac{(Experimental\ value - Predicted\ value)}{Experimental\ value} \times 100 \quad (5)$$

In vitro cytotoxicity test

In vitro cytotoxicity test of the extract obtained from the optimal condition on HepG2 cells was performed. HepG2 cells were seeded at a density of 10,000 cells/well onto a 96-well culture plate. They were incubated overnight before being treated for 24 h with different concentrations of the optimal plant combination extract (0 - 10 mg/mL) (n = 3). After that, culture media was removed and replaced with 500 µg/mL MTT solution and incubated at 37 °C for 3 h. MTT solution was then removed and 100 µL of dimethyl sulfoxide was added before being measured at 570 nm using the microplate reader. Relative cell viability of treatment was calculated as a percentage of a vehicle control group. Mean and standard deviation were reported. Hydrogen peroxide (200 µM) was used as a positive control. The IC_{50} was also calculated [32].

Statistical analysis

The difference of more than 2 groups was analyzed using One-way analysis of variance (One-way ANOVA) using SPSS Statistics 22.0 (IBM, New York, USA). Data was significantly different when the p-value was less than 0.05 at a 95 % confidence interval.

Results and discussion

Synergistic antioxidant activity of plants' combination

The previous works demonstrated the interaction of phenolic compounds contained in the herbal recipe, which might be related to the synergistic effect of the plant composed in the herbal recipe [33-35]. Therefore, this study introduced the evaluation of the synergistic antioxidant activity of herbal combinations used in herbal beverages.

Table 3 Mathematical models and equations of each dependent variable.

Dependent variables	Models	Actual equations
Extraction yield (%)	Special quartic	$Y_1 = 5.10X_1 + 43.81X_2 + 37.51X_3 + 3.06X_1X_2 + 1.66X_1X_3 - 0.56X_2X_3 + 84.96X_1^2X_2X_3 + 56.88X_1X_2^2X_3 + 188.28X_1X_2X_3^2$
TPC (mg GAE/g extract)	Special quartic	$Y_2 = 559.29X_1 + 36.71X_2 + 46.05X_3 - 792.92X_1X_2 - 733.90X_1X_3 - 40.83X_2X_3 - 6843.57X_1^2X_2X_3 + 4812.80X_1X_2^2X_3 + 4882.75X_1X_2X_3^2$
TFC (mg CE/g extract)	Special quartic	$Y_3 = 229.55X_1 + 5.85X_2 + 7.79X_3 - 348.46X_1X_2 - 302.76X_1X_3 - 3.35X_2X_3 - 2296.63X_1^2X_2X_3 + 1725.61X_1X_2^2X_3 + 1187.23X_1X_2X_3^2$
IC_{50} from DPPH assay (µg/mL)	Special cubic	$Y_4 = 32.28X_1 + 251.70X_2 + 249.16X_3 - 14.75X_1X_2 + 77.25X_1X_3 + 99.90X_2X_3 - 1418.05X_1X_2X_3$
IC_{50} from FRAP assay (µg/mL)	Quadratic	$Y_5 = 15.30X_1 + 94.58X_2 + 134.84X_3 - 61.67X_1X_2 - 135.26X_1X_3 - 19.44X_2X_3$

Mathematical models and equations of each dependent variable are shown in **Table 3**. The contour plots of extraction yield, TPC, TFC and IC_{50} values are shown in **Figure 1**. According to the equations in **Table 3** and the contour plots in **Figure 1**, the extraction yield was high when the mass ratio of *H. sabdariffa* increased. The highest extraction yield was found at the high mass ratio of *H. sabdariffa*. While the lowest extraction yield was found at the high mass ratio of *C. sappan* (**Figure 1(a)**). Oppositely, the highest TPC and TFC were achieved at the high mass ratio of *C. sappan* (**Figures 1(b)** and **1(c)**). The results revealed that *C. sappan* also exhibited the best antioxidant activity due to its low IC_{50} values (**Figures 1(d)** and **1(e)**).

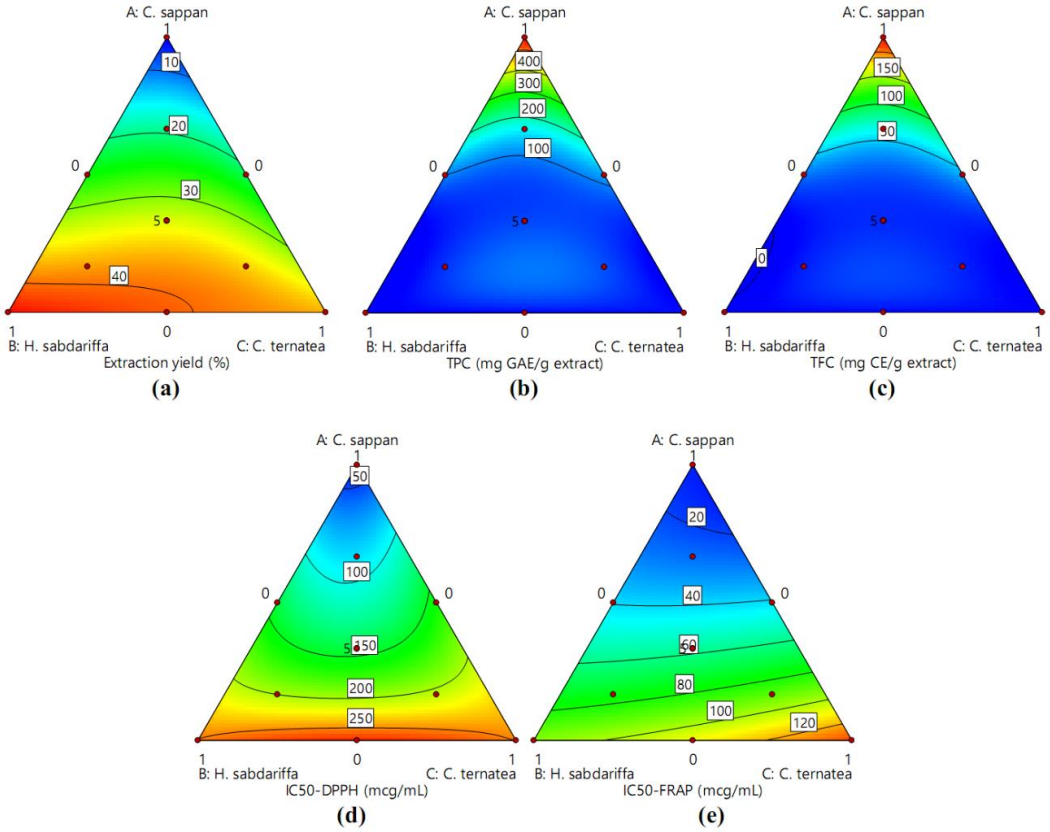

Figure 1 Contour plots of simplex lattice design of (a) extraction yield, (b) TPC, (c) TFC, (d) IC_{50} obtained from DPPH assay, and (e) IC_{50} obtained from FRAP assay.

Extraction yields of *C. sappan*, *H. sabdariffa* and *C. ternatea* from this study were 5.29, 44.00 and 37.70 %, respectively. The extraction yield of *C. sappan* when water was used as the extraction solvent seems low, similar to the previous work: 6.71 % [36]. Moreover, the extraction yield of hydroethanolic or ethanolic extract was also low: 7.34 % [37] and 2.10 - 9.44 % [36,38], respectively. According to extraction yield of *H. sabdariffa* was higher than the previous works: 4.0 % [39] or 12.8 % [39], when water or ethanol was used, respectively. In case of water extract of *C. ternatea* was high and closed to the previous work: 45.51 % [40].

The average TPC of *C. sappan*, *H. sabdariffa*, and *C. ternatea* from this study was 561.90, 39.14 and 48.66 mg GAE/g extract, respectively. The previous work reported the TPC of hydroethanolic and ethanolic extracts of *C. sappan* was 15.56 - 247.83 mg GAE/g [37,41] and 544.00 - 854.12 mg GAE/g [38,42], respectively, which the data from this study was in the range. However, the extraction yield of water extract in the prior work was not reported. In case of *H. sabdariffa*, when water was used as the solvent, the extraction yield was approximately 7 - 40 mg GAE/g [39, 41, 43, 44]. Similar data were found when ethanol was used: 13.02 - 27.6 mg GAE/g [39,45]. According to *C. ternatea*, water extract, ethanolic extract, and hydroethanolic extract gave extraction yields of 20.7 mg GAE/g [46], 50 mg GAE/g [47], and 10.91 mg GAE/g [48], respectively.

The average TFC of *C. sappan*, *H. sabdariffa*, and *C. ternatea* from this study was 230.10, 6.40 and 8.34 mg CE/g extract, respectively. The previous work reported the TFC of the hydroethanolic extract of *C. sappan* of 75.18 mg QE/g [37]. According to *H. sabdariffa*, water extract had a TFC of 3.53 - 33.3 mg QE/g [39,41], while the TFC of the hydroethanolic extract was 10.08 mg QE/g [41] and the ethanolic extract was 33.8 mg QE/g [39] or 4.98 CE/g [45]. The methanolic extract of *C. ternatea* had a TFC of approximately 75 mg QE/g [49].

The average IC_{50} values from the DPPH assay of *C. sappan*, *H. sabdariffa* and *C. ternatea* from this study were 31.51, 257.12 and 247.97 µg/mL, respectively. The average IC_{50} value of positive control quercetin was 6.07 µg/mL. The previous work reported the IC_{50} of water extract of *C. sappan* of 15.08 - 53.61 µg/mL [50], the hydroethanolic extract of 32.37 µg/mL [37], and ethanolic extract of 2 - 140 µg/mL [38,44,51]. In the case of *H. sabdariffa*, the IC_{50} values of water extract and hydroethanolic extract were 516 µg/mL [41] and 280 µg/mL [41]. According to *C. ternatea*, the water extract, hydroethanolic extract and methanolic extract had IC_{50} values of 467 µg/mL [52], 921.65 µg/mL [48] and 480 µg/mL [49], respectively. The above data revealed that *C. sappan* exhibited the best antioxidant activity compared with *H. sabdariffa* and *C. ternatea*.

The average IC_{50} values from the FRAP assay of *C. sappan*, *H. sabdariffa* and *C. ternatea* from this study were 11.30, 95.78 and 140.74 µg/mL, respectively. The average IC_{50} value of positive control ferrous sulfate was 84.90 µg/mL. The water extract of *C. sappan* from the previous work gave an IC_{50} value of 5.58 - 7.93 µg/mL [50]. However, the reported reducing power based on the FRAP assay of *H. sabdariffa* and *C. ternatea* was in different methods or different units, so it cannot be compared with the previous work.

The correlation plots between predicted values vs. experimental values of each dependent variable are shown in **Figure 2**. Results revealed that the R^2 values were relatively high: Higher than 0.90, which indicated that the prediction by the Design-Expert® program was precise and reliable [33].

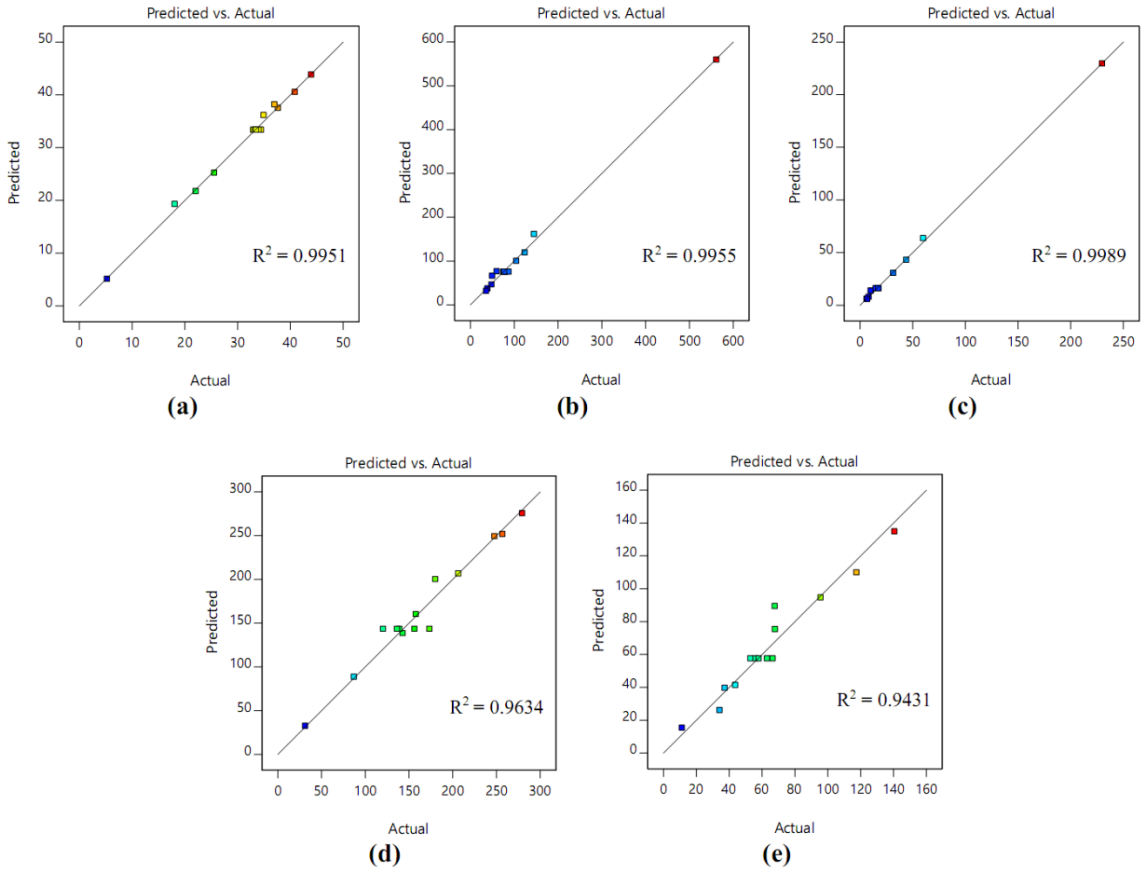

Figure 2 Correlation plots between predicted values vs. experimental values of (a) extraction yield, (b) TPC, (c) TFC, (d) IC_{50} obtained from DPPH assay, and (e) IC_{50} obtained from FRAP assay with their R^2 values.

Contour plots of CI of IC_{50} values, which indicated the synergistic effect are shown in **Figure 3**. The synergistic effect was recognized when the CI value was lower than 1.0 [23]. The pattern between data from the DPPH assay and FRAP assay was different due to the different principles of the assay. According to the DPPH assay, when DPPH free radical mixed antioxidant, the purple color of DPPH turns to the yellow color of the corresponding hydrazine. The reducing ability of antioxidants against DPPH can be detected in the decreasing of its absorbance at 515 - 528 nm. While FRAP assay relies on the reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ by the antioxidant. The binding of Fe^{2+} to the ligand makes a navy blue color [29]. The contour plot of CI of IC_{50} from FRAP assay (**Figure 3(b)**) gave lower CI values than that of DPPH assay (**Figure 3(a)**).

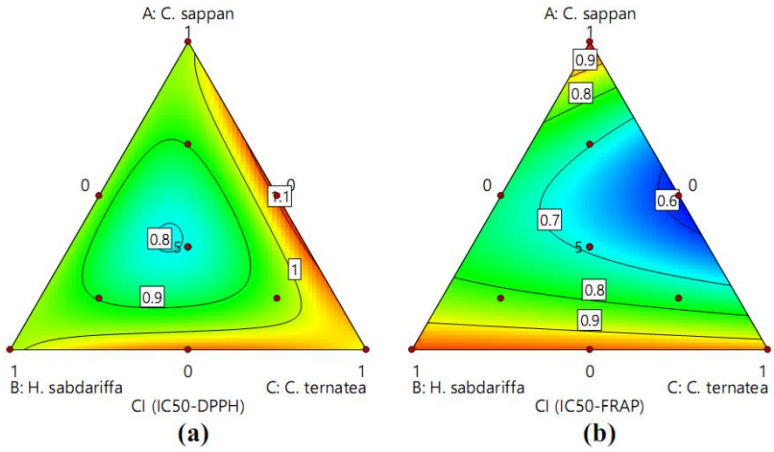

Figure 3 Contour plots of CI of IC_{50} values obtained from (a) DPPH assay and (b) FRAP assay.

Overlay plots of CI of IC_{50} values obtained from DPPH assay and FRAP assay are shown in **Figure 4**. Furthermore, their overlays are also shown. The areas in which the synergistic effect occurred (yellow area), which are broader for IC_{50} from FRAP assay (**Figure 4(b)**) than DPPH assay (**Figure 4(a)**). Because of the broad synergistic area of IC_{50} from FRAP assay, overlay synergistic area of IC_{50} from DPPH assay with IC_{50} from FRAP assay caused the overlay plots of the combination to be similar to the synergistic area of IC_{50} from DPPH assay (**Figure 4(c)**). The data seems that the synergistic effect occurred at the equal mass ratio, which can be selected to prepare supplementary health products.

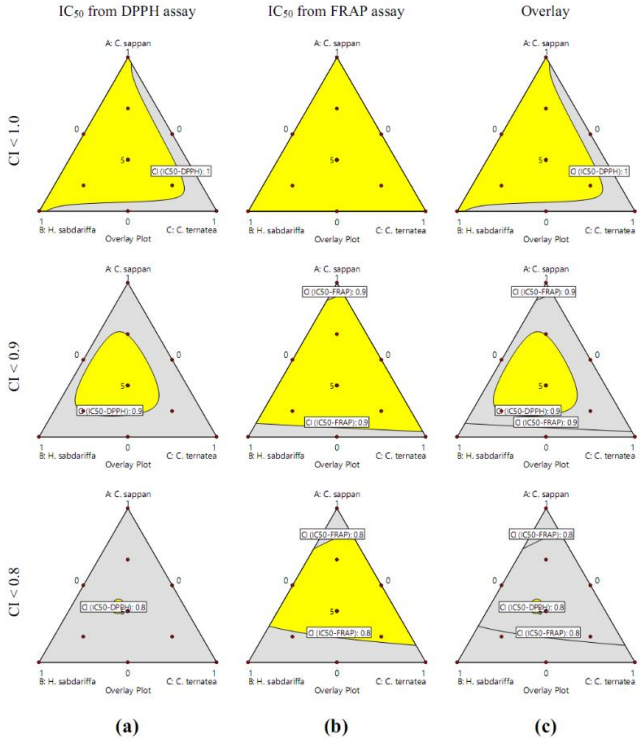

Figure 4 Overlay plots of CI of IC_{50} values obtained from (a) DPPH assay, (b) FRAP assay, and (c) overlay of both DPPH and FRAP assays, in which CI values were less than 1.0 (top), 0.9 (middle), and 0.8 (bottom).

This study indicated that the synergistic effect could occur among the plant compositions contained in the herbal formula. The synergistic antioxidant activity among the 4 plant compositions contained in Chatuphalathika herbal recipe: *Terminalia chebula* Retz. var. *chebula*, *Terminalia arjuna* Wight and Arn., *Terminalia bellirica* (Gaertn.) Roxb. and *Phyllanthus emblica* L., was also found in the previous work [53]. The results supported those synergistic effects can occur among herbs or bioactive compounds of herbs [13]. To the best of our knowledge, this is the first work that demonstrated the synergistic antioxidant activity of the combination of 3 colored plants: *C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes, and *C. ternatea* flowers. The synergistic area obtained from the overlay plots will be used as a guide for the selection of the mass ratio of the 3 plants to prepare the supplementary health products in our future work.

Effect of microwave power, time, and cycle on each dependent variable

The mathematical model was fitted and generated from the experimental data of 17 conditions. All data were used to construct contour plots. The Y_1 - Y_4 were fitted to the quadratic model, while the Y_5 was fitted to the 2FI (2-factor interaction) model.

The contour plots of extraction yield (Y_1) of 3 colored plants combination are shown in **Figure 5** showing that increasing X_1 - X_3 made Y_1 increase. The coefficient of each independent variable of Y_1 in **Table 4** also points out that the X_2, X_3, X_1X_3 and X_2^2 had significant effects, while other terms had insignificant effects on Y_1.

The contour plots of TPC (Y_2) of 3 colored plants combination extract are shown in **Figure 5** revealing that when X_3 was low, the maximum Y_2 was observed at the medium X_1 and X_2. While at medium and high X_3, the maximum Y_2 was found at the high X_1 and X_2. The coefficient of each independent variable of Y_2 in **Table 4** also indicates that the X_1, X_2, X_3, X_1X_3, X_2X_3, X_1^2 and X_2^2 had significant effects, while other terms had insignificant effects on Y_2.

The contour plots of TFC (Y_3) of 3 colored plants combination extract are shown in **Figure 5** indicting that when X_3 was varied in different levels, the maximum Y_3 was observed at the medium to high X_1 and X_2. The coefficient of each independent variable of Y_3 in **Table 4** also points out that the X_1, X_3 and X_2^2 had significant effects, while other terms had insignificant effects on Y_3.

The contour plots of IC_{50} from DPPH assay (Y_4) of 3 colored plants combination extract are shown in **Figure 5**. The low Y_4 was found when the X_3 was medium. The coefficient of each independent variable of Y_4 in **Table 4** also indicates that the X_3, X_1^2, X_2^2 and X_3^2 had significant effects on Y_4, while other terms had insignificant effects on Y_4.

The contour plots of IC_{50} from FRAP assay (Y_5) of 3 colored plants combination extract are shown in **Figure 5** revealing that when X_3 was high, the minimum Y_5 was observed. The coefficient of each independent variable of Y_5 in **Table 4** also points out that only X_3 had any significant effect, while other terms had insignificant effects on Y_5.

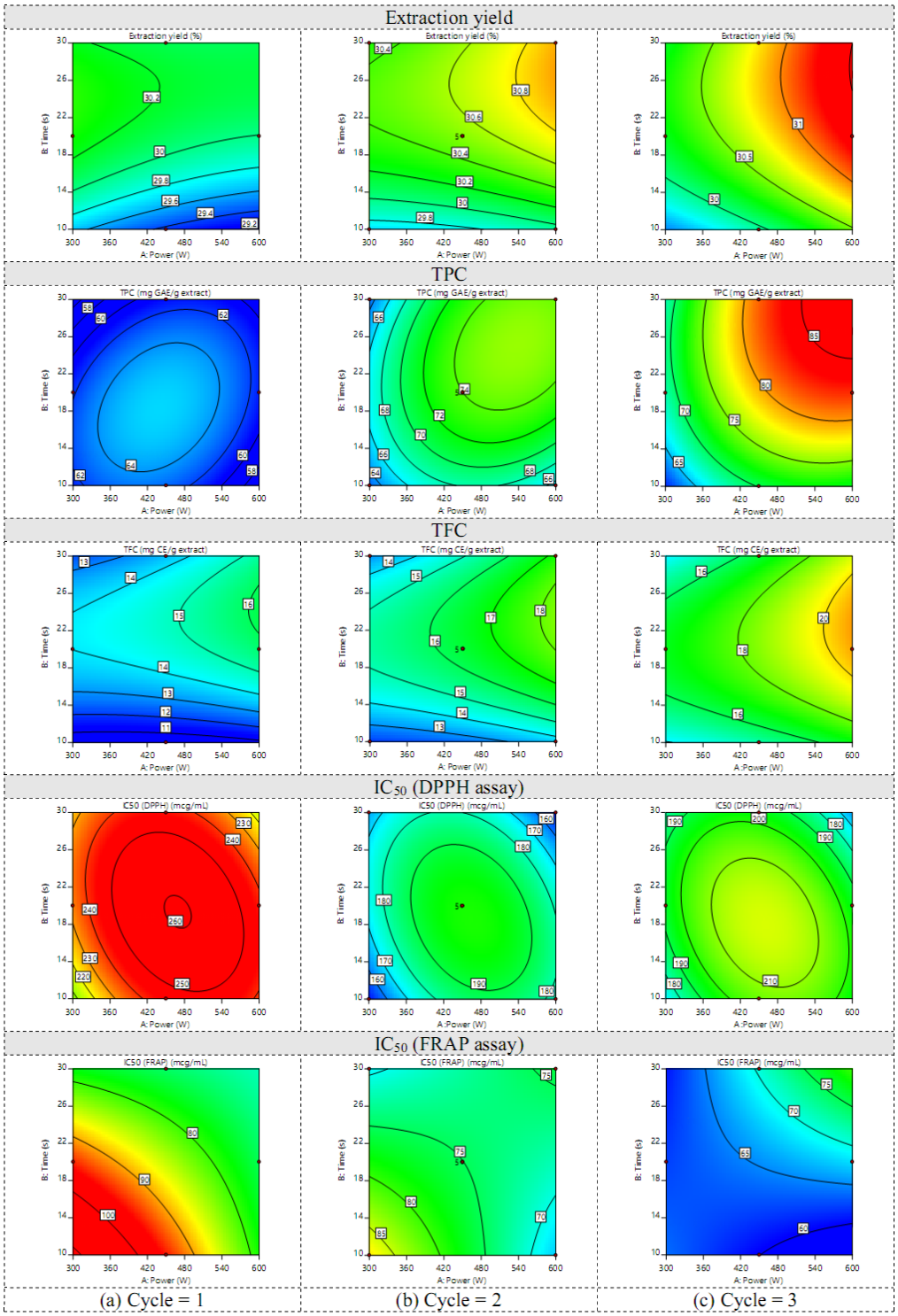

Figure 5 Contour plots of Box-Behnken design of extraction yield, TPC, TFC, IC_{50} from DPPH assay, and IC_{50} from FRAP assay of 3 colored plants combination when irradiation cycles were (a) low, (b) medium, and (c) high.

Table 4 The term of the significant model, regression coefficient (based on experimental values), and *p*-value for the responses of MAE.

Polynomial terms	Y_1		Y_2		Y_3		Y_4		Y_5	
	Coefficient	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**
Model	-	0.0221*	-	0.0013*	-	0.0472*	-	0.0045*	-	0.0148*
Intercept	29.82	-	37.92	-	3.57	-	47.21	-	218.44	-
X_1	-6.50×10^{-3}	0.0762	0.10	0.0068*	–0.02	0.0438*	1.10	0.5397	–0.20	0.1943
X_2	0.15	0.0048*	0.32	0.0344*	1.07	0.0752	9.57	0.5611	–3.80	0.3535
X_3	–0.34	0.0149*	–4.50	0.0001*	0.64	0.0127*	–170.65	0.0022*	–43.72	0.0032*
X_1X_2	5.80×10^{-5}	0.5503	1.46×10^{-3}	0.1315	4.09×10^{-4}	0.4726	-7.12×10^{-3}	0.1490	4.49×10^{-3}	0.0903
X_1X_3	2.35×10^{-3}	0.0394*	0.02	0.0230*	3.71×10^{-3}	0.5130	–0.01	0.7649	0.04	0.1152
X_2X_3	2.50×10^{-3}	0.8628	0.33	0.0364*	–0.04	0.5997	–0.22	0.7534	0.77	0.0586
X_1^2	2.22×10^{-6}	0.7239	-1.75×10^{-4}	0.0162*	1.20×10^{-5}	0.7517	-1.01×10^{-3}	0.0096*	-	-
X_2^2	-3.48×10^{-3}	0.0378*	–0.04	0.0266*	–0.03	0.0127*	–0.16	0.0460*	-	-
X_3^2	–0.11	0.4352	–1.59	0.2438	0.12	0.8828	39.81	0.0004*	-	-
Lack of Fit	-	0.5417	-	0.0254*	-	0.0114*	-	0.5564	-	0.9372

*Significant value

Independent variables investigated in this study were important parameters that affected the extraction yield, TPC, TFC, and antioxidant activity of the extract. The previous works reported that when microwave power or microwave time increased, the TPC of plants and antioxidant activity increased [54-57]. However, this seems incomplete. Some extraction conditions decomposed some bioactive compounds [54-56], which consequently affected antioxidant activity [58]. The other work demonstrated that 1-factor consideration found that when microwave power or extraction time were increased, TPC was decreased. While the interaction between microwave power and extraction time increased TPC [59].

MAE operates based on the direct effect on active compounds by dipole rotation and ionic conduction which leads to the power being dissipated inside the plant matrix and extraction solvent resulting in heating and molecular movement [60]. The plant matrix could absorb microwave energy and cause internal superheating resulting in cell disruption and active compounds leaching out from plant material [61]. However, some plant active compounds were destroyed by the extreme heat generated by microwave energy [54]. The mechanism of the phenomena that microwave power harmed plant active compound content was previously observed [62,63]. The very high microwave power induced the plant samples to be thermally compromised, leading to a decrease in the yield of active compounds.

Increasing microwave time could promote the decomposition of some plant active compounds; TPC was decreased when microwave time increased [54]. This phenomenon was also found in antioxidant activity [64]. Other work reported that with increasing microwave power, total curcuminoids were highly extracted at low and high rather than at medium microwave power. Increasing the irradiation cycle increased total curcuminoids content, however, curcuminoids could be destroyed from excessive irradiation time [65]. However, other work found that increased microwave time or irradiation cycle increased curcuminoids content of turmeric when vegetable oil was used as a solvent [66]. All of the above data indicated the importance of the optimization of MAE parameters for plant extraction.

The correlation plots between predicted values vs. actual values of each dependent variable are shown in **Figure 6**. **Figures 6(a)**, **6(c)**, and **6(e)** (top) show that the R^2 was relatively low, while **Figures 6(b)** and **6(d)** (top) were relatively high. **Figure 6** (bottom) shows that all data distributed within the red border lines indicated that they were within the 95 % confidence interval. The above data indicated that the prediction by the Design-Expert® software was precise, stable, and reliable [33, 67-70].

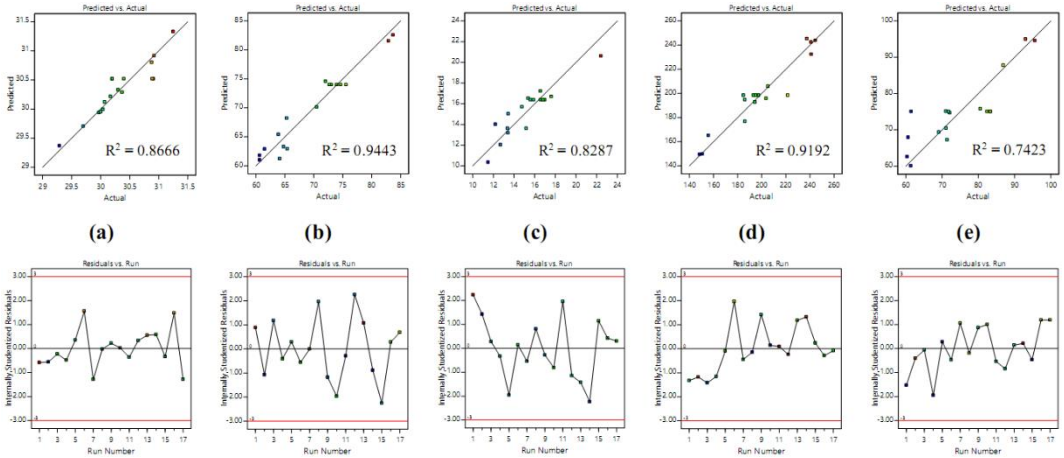

Figure 6 Correlation plots between predicted values vs. actual values (top) and residuals vs. run plots (bottom) of (a) extraction yield, (b) TPC, (c) TFC, (d) IC_{50} from DPPH assay, and (e) IC_{50} from FRAP assay.

Optimization, optimal MAE condition, and verification

The optimal condition provided the best antioxidant activity, simultaneously lowest IC_{50} from DPPH and FRAP assays: When independent variables—X_1 - X_3—were set as "is in range" and dependent variables—Y_1 - Y_3 were set as "none", and Y_4 and Y_5 were set as "minimize", microwave power was 300 W for 30 s and repeated 2.55 cycles. The desirability of this condition was 0.850. However, the authors increased the cycle to 3 cycles for easier performance. The Y_1 - Y_3 were not used in the optimization process because they gave a rather low desirability value. Verification of the predicted optimal condition was performed to warrant the accuracy of the prediction of the computer software. The percentage error of the prediction of all dependent variables was less than 10 %, except for Y_5. However, the authors mentioned that all actual values were within the 95 % confidence intervals (**Table 5**). Therefore, the results indicated that the prediction was verified accurately.

Table 5 The accuracy of the prediction that was presented as a percentage error.

Responses	Predicted values	Actual values (n = 3)	Percentage error (%)	95 % Confidence interval (lower - upper)
Y_1 (%)	30.84	31.03 ± 0.26	0.62	30.33 - 31.35
Y_2 (mg GAE/g extract)	68.23	72.03 ± 3.83	5.08	61.13 - 75.32
Y_3 (mg CE/g extract)	14.21	15.18 ± 0.42	6.36	9.75 - 18.67
Y_4 (µg/mL)	182.94	178.16 ± 15.54	−3.20	146.59 - 219.29
Y_5 (µg/mL)	61.05	75.26 ± 4.44	18.68	43.63 - 78.47

In vitro cytotoxicity of the extract obtained from the optimal MAE condition

According to ISO 10993-5, cytotoxicity is considered when cell viability is reduced by more than 30 % [71]. The extract in a concentration of at least 0.001 mg/mL significantly reduced the cell viability of HepG2 cells compared with the non-treated group. However, the extracts at concentrations up to 1 mg/mL, with cell viability of 71.14 %, did not report cytotoxicity due to cell viability being higher than 70 %. The cytotoxicity was observed at concentrations of 5 and 10 mg/mL, cell viability was decreased to 53.64 and 26.95 %, respectively, which was more toxic than a positive control 200 µM hydrogen peroxide (**Figure 7**). The IC_{50} value of the plant combination extract was 5.45 mg/mL. So, it could be concluded that optimized extract was safe at a concentration of less than or equal to 1 mg/mL. Calculation based on typical circulating blood volume of adult (approximately 5 L) [72] with an assumption that the whole extract was absorbed, the consumption dose of plant combination extract should not exceed 5.0 g, which is equivalent

to 16.11 g of plant combination powder (calculation based on the extraction yield of the optimal condition). Theoretically, whole extract could not be absorbed. The first-pass metabolism is the most important factor that results in a reduced concentration of the active drug upon reaching its site of action or the systemic circulation [73]. In addition, there are several factors affecting drug absorption when administered via oral route, including, physicochemical properties of active drug and drug formulation, gut content, surface area and blood flow of gastrointestinal tract, intestinal motility, destruction by gastrointestinal secretions, metabolism, pH, irritation of mucosa by the drug, vomiting, and concomitant administration of other drugs [74].

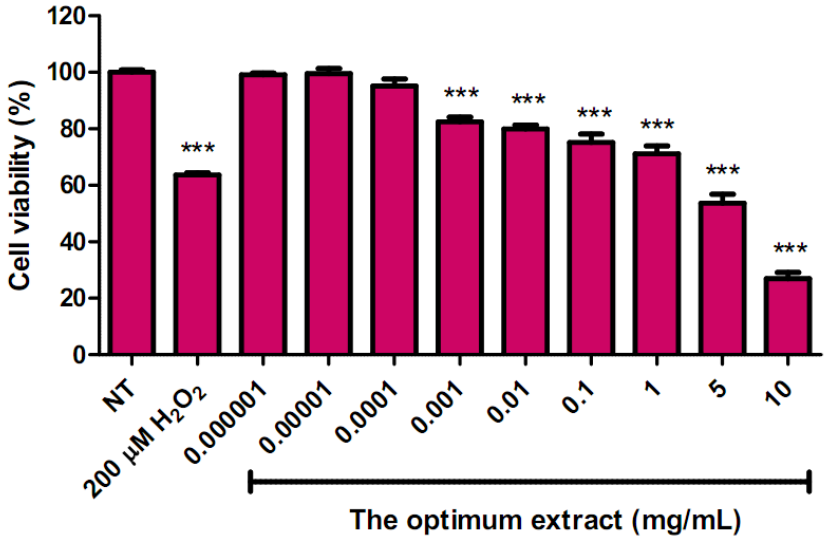

Figure 7 *In vitro* cytotoxicity in HepG2 cells of the extract of 3 colored plants combination obtained from the optimal MAE condition, compared with the non-treated (NT) group and positive control (200 μM hydrogen peroxide). The significance was presented as *, **, and *** when $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Conclusions

This work evaluated the synergistic antioxidant activity of the combined 3 plant extracts—*C. sappan* heartwoods, *H. sabdariffa* calyxes and epicalyxes and *C. ternatea* flowers. The synergistic effect was evaluated based on the CI of the IC_{50} values obtained from 2 assays — DPPH assay and FRAP assay. The synergistic effect among the 3 plants was observed at various mass ratios, especially at the equal mass ratio. This plants' combination was used as a sample to optimize the MAE using the Box-Behnken design. The optimal condition that provided the best antioxidant activity of the plant extract was a microwave power of 300 W for 30 s and 3 cycles. The optimum extract did not show any cytotoxicity with a test concentration up to 1 mg/mL. However, the plant combination extract at concentrations of 5 and 10 mg/mL exhibited a cytotoxic effect with the IC_{50} value of 5.45 mg/mL. Therefore, the colored plants' combination could be used as an antioxidant supplementary product. Furthermore, the cytotoxicity data could be used to set the safe dose of the supplementary health products for human consumption.

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