Multi-Response Optimization of Antioxidant and Total Phenols-Flavonoids Content of Polyherbal Extract Drink from Turmeric, Java Tea, and Seed-under-leaf

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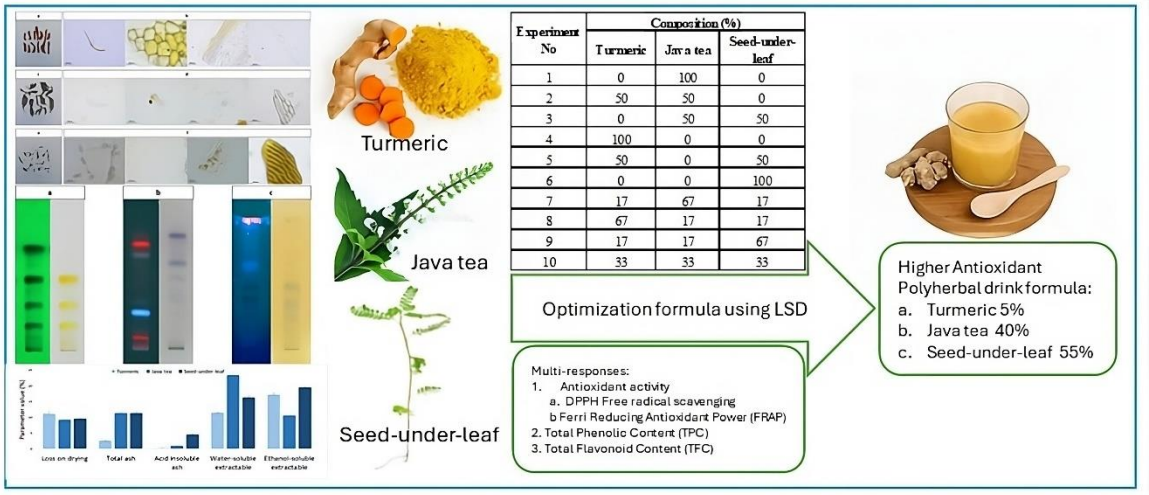
GRAPHICAL ABSTRACT



Experiment No	Composition (%)		
	Turmeric	Java tea	Seed-under-leaf
1	0	100	0
2	50	50	0
3	0	50	50
4	100	0	0
5	50	0	50
6	0	0	100
7	17	67	17
8	67	17	17
9	17	17	67
10	33	33	33

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The research aimed to evaluate quality profiles, optimize the antioxidant activity, and total phenolic and flavonoid content in a mixture of three crude drug: turmeric, java tea, and seed-under-leaf, using the Simplex Lattice Mixture Design. Ultimately, a quadratic model was used, as it best adjusted to the experimental behavior. It predicted the optimal composition of herbal mixtures antioxidant activity, determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and Ferri Reducing Antioxidant Power (FRAP) techniques, also in Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The crude drugs were of good quality according to selected pharmacognostic quality parameters of the official Malaysian herbal monograph (MHM). The optimum responses with the highest synergistic effect were composed of 5% (w/w) of turmeric, 40% (w/w) of Java tea, and 55% (w/w) of seed-under-leaf. The values were 1796 mM TE/g, 2230 mM TE/g, 355 mg GAE/g, and 177 mg QE/g for DPPH, FRAP, TPC, and TFC, respectively. The greatest contribution of antioxidant activity was shown in that mixture as potential as a functional beverage.

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Keywords: Polyherbal drink; Antioxidant; Turmeric; Java tea; Seed-under-leaf

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INTRODUCTION

Plants have been utilized by Indonesian people for medicinal purposes. The use of plants for traditional remedies is particularly popular for the management of diabetes (Pengpid and Peltzer 2018). The traditional antidiabetic plants, *i.e*., turmeric (*Curcuma longa* L.), Java tea (*Orthosiphon aristatus* (Blume) Miq.), and seed-under-leaf (*Phyllanthus niruri* L.), are used in multiple regions nationwide (Hartanti and Budipramana 2020). In patients with diabetic complications, there is a significant rise in reactive oxygen species, which surpasses the body's natural antioxidant defenses. This imbalance contributes to endothelial dysfunction, insulin resistance, and impairment of pancreatic β-cell function (Bandeira *et al.* 2013). Antioxidants might help with these oxidative stress conditions and, hence, delay the complications. Turmeric, Java tea, and seed-under-leaf have been reported to show antioxidant activities, and this might support their traditional antidiabetic uses (Navarro *et al.* 2017; Tanvir *et al.* 2017; Ashraf *et al.* 2020).

The antioxidant compounds of turmeric rhizomes are curcuminoids, phenolic compounds, flavonoids, and essential oils. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin show strong antioxidant activities, effectively scavenging free radicals and enhancing the body's antioxidant enzymes' activity (Shukla and Kushwaha 2024). The plant's oxidant-protective effects were also demonstrated by ferulic acid and vanillic acid, as well as flavonoids, which are commonly found in the leaves. Some volatile compounds, *i.e*. atlantone, turmerone, and zingiberene, also showed weak to moderate antioxidant effects (Lukitaningsih *et al.* 2020; Orellana-Paucar and Monserrath 2024). Phenolic acids, *i.e*., caffeic acid and rosmarinic acid, and flavonoids, *i.e*., cirsimaritin, eupatorine, myricetin, and sinensetin, are the antioxidant compounds of Java tea. Caffeic acid and its derivates scavenge free radicals, inhibit lipid peroxidation, and upregulate superoxide dismutase and catalase in the *in-vitro* and *in-vivo* models (Silva and Lopes, 2020). Similarly, sinensetin's antioxidant activity is mainly mediated by its capacity to scavenge reactive oxygen species, which subsequently reduces oxidative damage in cells. In addition, as in other flavonoids, it also chelates metal ions and activates antioxidant enzymes (Batubara *et al.* 2020; Han Jie *et al.* 2021).

Other than flavonoids and phenolic acids, antioxidant compounds in seed-under-leaf include tannins, lignans, and vitamin C. Major flavonoids in the plants are quercetin and kaempferol, while caffeic acid, ellagic acid, and gallic acid are the main phenolic acids. A substantial amount of antioxidant tannins, such as corilagin and geraniin, as well as lignans (phyllanthin and hypophyllanthin), can also be found in the leaves. Those compounds scavenge free radicals, reduce oxidative stress, inhibit lipid peroxidation, and modulate antioxidant enzyme activity to exhibit their antioxidant effects (Navarro *et al.* 2017; Ezzat *et al.* 2020).

The use of medicinal plants in polyherbal formulations, which involve multiple plant components, is a common practice in various traditional medicine systems. In particular, polyherbal preparations are more effective for treating a broader spectrum of diseases, offer a wider range of therapeutic benefits, and are more cost-effective and accessible compared to single-plant treatments in Ayurveda (Parasuraman *et al.* 2014). Additionally, polyherbal formulations are believed to enhance pharmacological efficacy through synergistic and polyvalent actions, potentially leading to improved therapeutic outcomes and reduced toxicity (Houghton 2009; Karole *et al.* 2019).

An herbal drink prepared from a mixture of 10% turmeric, 20% Java tea, and 70% seed-under-leaf showed considerable antioxidant activity and contained a high level of phenolic compounds (Hamad and Hartanti 2023). However, the report did not employ a mixture design, nor did it demonstrate any interaction effects when combining those three crude drugs toward their antioxidant activity. The primary aim of this study was to assess the impact of various ratio combinations of three crude drugs on antioxidant activity, total phenolic content (TPC), total flavonoid content (TFC), and color of the herbal drink. Antioxidant activity was measured using the DPPH free radical scavenging assay and the Ferric Reducing Antioxidant Power (FRAP) method. The formulation was optimized through the Lattice Square Design (LSD) approach, which is highly recommended for mixture formulation optimization.

EXPERIMENTAL

Materials

The reagents, *i.e.*, aluminum chloride, DPPH (2,2-diphenyl-1-picrylhydrazyl), ethanol, ferric chloride, Folin-Ciocalteu reagent, gallic acid, hydrochloric acid, quercetin, sodium hydroxide, sodium acetate, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), and Trolox, were purchased from Sigma-Aldrich (St. Louis, USA). Silica gel F254 plate (Merck Millipore, Darmstadt, Germany) and solvents (dichloromethane, ethyl acetate, formic acid, glacial acetic acid, methanol, n-hexane, and water (Sigma-Aldrich, St. Louis, MO, USA)) were utilized for Thin Layer Chromatography (TLC). The crude turmeric and Java tea crude drugs were purchased from Vejpong Herbal Pharmacy (Bangkok, Thailand) with the identifier codes FGTH0337A and FGTH6621A, respectively. Seed-under-leaf crude drugs were purchased from Wisata Kesehatan Jamu (WKJ) Kalibakung (Tegal, Indonesia).

Quality Evaluations of Crude Drugs

The quality profiles of crude drugs of turmeric, Java tea, and seed-under-leaf were evaluated according to the Malaysian Herbal Monograph (MHM), with parameters of macroscopic and microscopic morphology characters, Thin Layer Chromatography (TLC) profile, loss on drying, total ash, acid insoluble, and extractive values in water and ethanol (Malaysian Ministry of Health 2016). The microscopic evaluation utilized a camera-connected binocular microscope (Leica, Wetzlar, Germany). Separation of the crude drug extracts was conducted over a silica gel F_{254} plate, with mobile phases of dichloromethane:methanol (25:1) for turmeric, n-hexane:ethyl acetate (1:4) for Java tea, and ethyl acetate:glacial acetic acid:formic acid:water (25:3:3:5) for seed-under-leaf. TLC visualization was conducted utilizing TLC visualizer (Camag, Basel-Landschaft, Switzerland).

Preparation of the Herbal Drink

The powdered crude drugs of turmeric, Java tea, and seed-under-leaf were homogenously mixed in a ratio as a design of LSD in w/w (Table 1). The crude drug mixture was extracted in the water in a ratio of 1:20 w/v over a water bath (at a temperature of 100 °C) for 15 min. The extracts were filtered, and the fresh herbal drink was used for further experiments.

Evaluation of Extract Color

The herbal drink color was measured by a digital Lab colorimeter (Konica Minolta, Tokyo, Japan).

Evaluation of TPC and TFC

The TPC of the herbal drink was determined using a slightly modified version of the official method from the Indonesian Herbal Pharmacopeia (IHP) (Indonesian Ministry of Health 2017). One mL of either the drink or a standard gallic acid solution was mixed with 5 mL of 7.5% Folin-Ciocalteu reagent. After standing for 8 min, 4 mL of 1% NaOH was added to the mixture. Following a 60-min incubation at room temperature, the absorbance was measured using a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) at 730 nm. Gallic acid solutions (at 0 to 100 ppm) were used to generate the standard curve equation, and the TPC was expressed as mg gallic acid equivalent (GAE)/g dry weight (DW) crude drugs.

The TFC determination of the herbal drink was also modified from the official method in the IHP (Indonesian Ministry of Health 2017). A total of 0.5 mL of either the drink or standard quercetin solution was combined with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of water. After standing for 30 minutes at room temperature, the mixture absorbance was measured at 425 nm. Quercetin solutions (at 0 to 250 ppm) were used to obtain the standard curve equation, and TFC was reported as mg quercetin equivalent (QE)/g DW crude drugs.

Evaluation of Antioxidant Activities

The antioxidant activities of the herbal drink were measured using a slightly modified standard method (Thaipong *et al.* 2006). In the DPPH scavenging activity assay, 0.5 mL of either the drink or standard Trolox solution was mixed with 5 mL of 25 µg/mL DPPH solution and incubated at room temperature, protected from light, for 30 min. The absorbance was measured at 517 nm. Trolox solutions (at 0 to 400 µM) were used to create the standard curve equation, and the DPPH scavenging activity was expressed as µM Trolox equivalent (TE)/g dry weight (DW) crude drugs.

For the FRAP assay, the reagent was freshly prepared by homogenously mixing 10 parts of 300 mM sodium acetate buffer, 1 part of 10 mM TPTZ in hydrochloric acid, and 1 part of 20 mM ferric chloride, with a final pH of 3.6. A 0.21 mL of either the drink or Trolox solution was added to 0.4 mL of the FRAP reagent. After 30 min at room temperature, the absorbance was read at 594 nm. The standard curve equation was generated using Trolox solutions (at 0 to 225 µM), and FRAP values were reported as µM TE/g DW crude drugs.

Simple Lattice Mixture Design to Determine Optimum Antioxidant, TPC, and TFC Responses

A Simple Lattice Design was applied to discover the interactive effects of antioxidant activity from the extract mixture extracted from turmeric, java tea, and seed-under-leaf. The multi-responses variable was determined by FRAP and DPPH antioxidant activity assays, TPC, and TFC. Mixture design experiments were conceived and analyzed employing Design Expert Software (Design-expert 6.0.8 Stat-Ease Inc, Minneapolis, MN, USA) software. The entire 10 mixtures are shown in Table 1. It is important to encourage that, although not all possible combinations were made with replicates and not all possible mixtures or combinations were represented in the study, precisely the advantage of this type of design is that the data can be processed statistically with a minimum number of points (Cornell 2011).

Selection and Validation of Simplex Lattice Design Model

The evaluation of data for the optimization process using Simplex Lattice Design was carried out by Design expert software Design-expert 6.0.8, Stat-Ease, Minneapolis, MN, USA). The statistical attributes of the models were considered in the selection. The adjusted and predicted R^2 coefficient values were determined. The weight for each experimental run was obtained, and the appropriateness of the model was calculated by analysis of variance (ANOVA) (Gorman and Hinman 1962). The comparison of means was performed using the ANOVA F-test and Duncan's multiple comparison test, while Pearson's correlation test was used to evaluate the correlation between the antioxidant capacity and the phenolic compound content. All statistical tests were done at 95% significance. The 'synergistic effect' was calculated as the percentage of increase shown

concerning the algebraic sum of the proportion, taking into consideration the individual contribution of each crude drug.

Statistical Data Analysis

All the analyses were performed in the duplicates. The experimental design and formulation optimization were performed with Design-Expert 6.0.8 software (Stat Ease Inc., Minneapolis, MN, USA). Duncan's post hoc test was applied for the one-way analysis of variance (ANOVA) for analysis data of Color, Antioxidant, TPC and TFC using SPSS 16.0 (IBM SPSS Statistics, Armonk, NY, USA) with a significance level of 0.05.

RESULTS AND DISCUSSION

Pharmacognostic Specification of the Polyherbal Drink

Turmeric crude drugs were thin fragments in outside-brown and inside-bright orange color with a strong pungent odor and bitterish-earthy taste. Java tea appeared as wrinkly lanceolate, dark green leaves with a slightly aromatic odor and somewhat bitterish-salty taste.

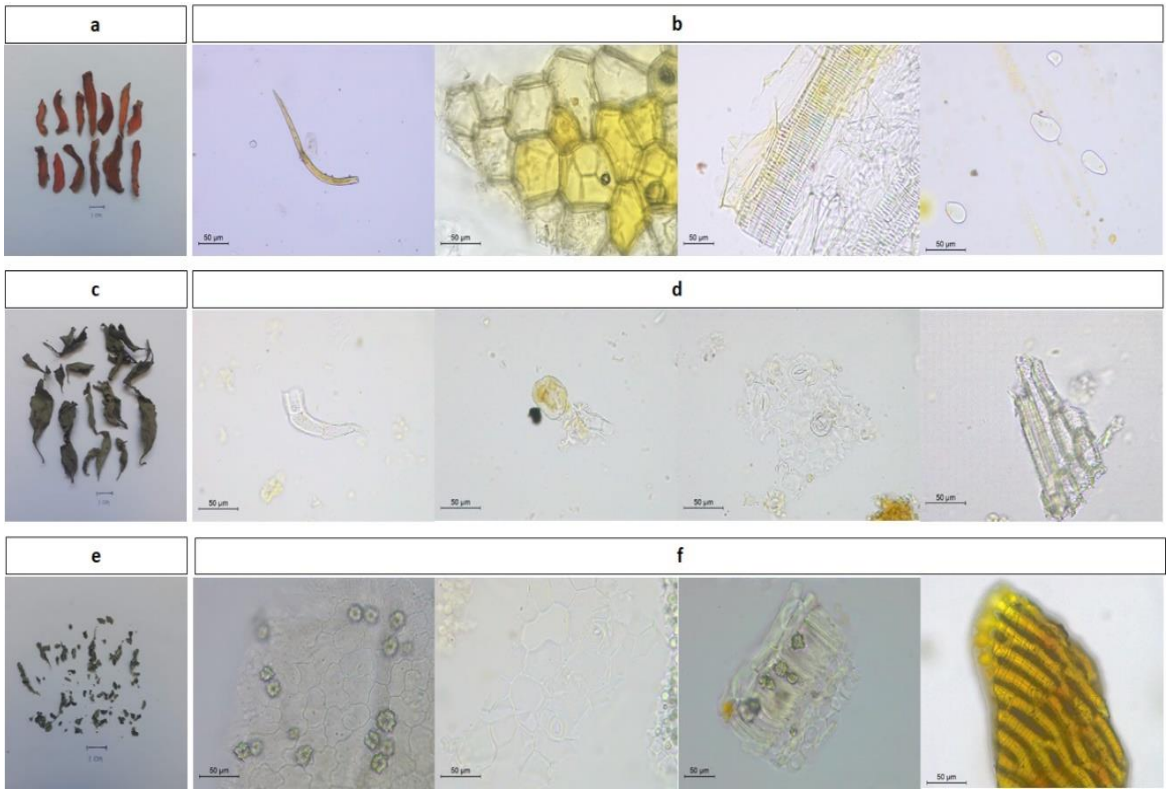

Fig. 1. The macroscopic morphology of the crude drugs of (a) turmeric, (c) Java tea, and (e) seed-under-leaf; and their diagnostic fragments showed (b) unicellular trichome, cork in surface view, spiral vessels, and starch granules of turmeric; (d) covering trichome, glandular trichome, lower epidermis with glandular trichome and stomata, and pitted vessels of Java tea; and (f) upper epidermis with underlying palisade containing calcium oxalate crystal in surface view, lower epidermis with stomata, epidermis with palisade containing crystal and sponge parenchyma in sectional view, and epicarp of seed-under-leaf.

The seed-under-leaf were small roundish, brownish-green leaves with an aromatic pleasant odor and bitter taste. Trichomes, cork, spiral vessels, and starch granules are described in the official monograph as turmeric diagnostic fragments. Similarly, diagnostic fragments of Java tea powdered crude drugs covered glandular trichomes, glandular trichomes and stomata-dense epidermis, and pitted vessels. Accordingly, the numerous rosette calcium oxalate crystals in the leaf mesophyll and fruit epicarp defined seed-under-leaf crude drugs (Fig. 1). The morphological characters of crude drugs represented the identity aspect of their quality. The macroscopic and microscopic morphology appearances of all three crude drugs were similar to their respective description in the official monograph (Malaysian Ministry of Health 2016). Hence, the identity of turmeric, Java tea, and seed-under-leaf crude drugs was confirmed.

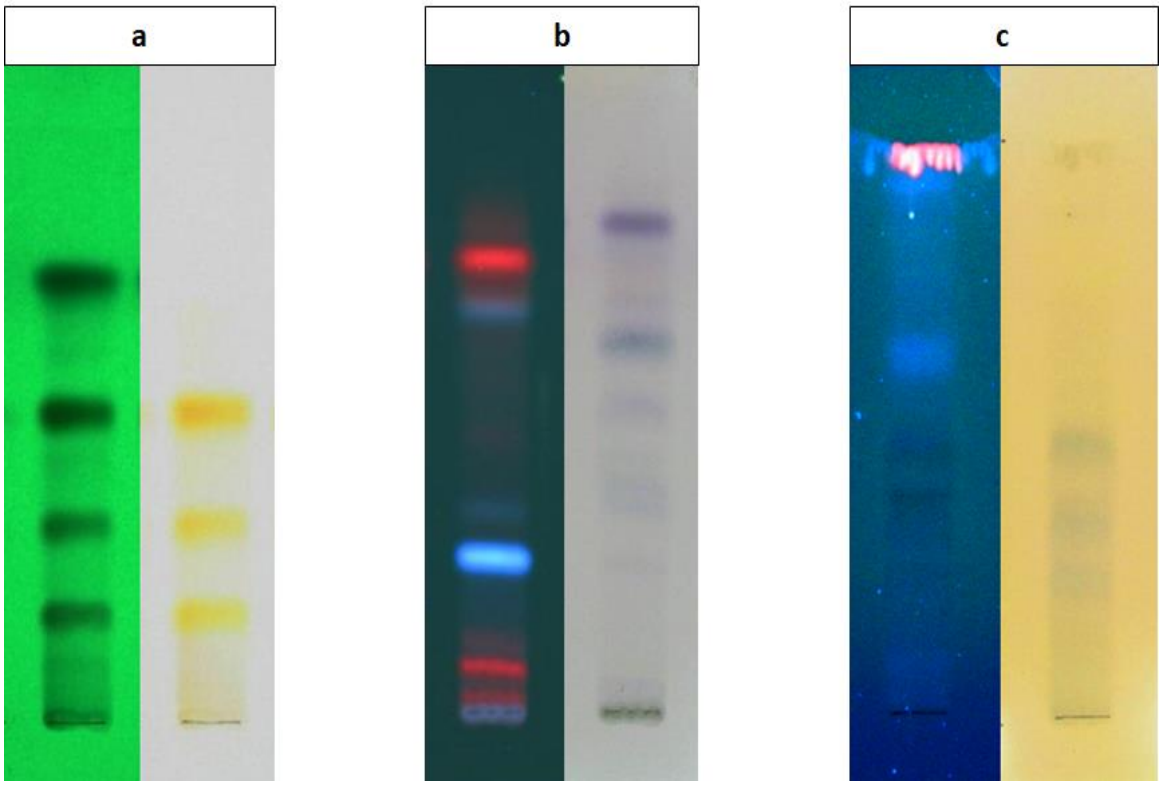

Fig. 2. TLC profile of (a) turmeric, observed under UV 254 lamp and visible light, (b) Java tea, observed under UV 366 lamp and visible light after sprayed with anisaldehyde-sulphuric acid, and (c) seed-under-leaf observed under UV 366 lamp and visible light after sprayed with 3% ferric chloride in ethanol

The TLC profile of turmeric showed the presence of curcuminoids that were shown as three bright yellowish-orange spots under the visible light. Java tea was separated into five spots under a UV 366 light. The spot with bright blue fluorescence at an Rf value of 0.21 was likely sinensetin. Seed-under-leaf showed three distinctive spots (**Fig. 2**). In this study, the TLC profile represented the identity and purity aspects of crude drug quality. Considering the morphological character and chromatographic evaluation results as well as comparison to the previously reported TLC profiles, the identity of all crude drugs was confirmed, and they were contamination-free (Faramayuda *et al.* 2021; Zahiruddin *et al.* 2021; Kartini *et al.* 2024).

The profile of the physicochemical properties of the crude drugs is presented in Fig. 3. Loss on drying, total ash, and acid insoluble ash represented the purity aspect of quality. Turmeric was the only crude drug that did not meet the moisture requirement of the official monograph, with a loss on drying value of 11.15 ± 0.82%. High loss on drying enables microbial growth on crude drugs, which might degrade the active compounds, diminish their therapeutic potency, or in some cases, produce mycotoxins that pose health risks during use. Excess moisture can cause significant variations in the weight of the crude drug and subsequently affect the dosage accuracy. In addition, it affects crude drug size reduction and extraction process efficiency. The moisture might result from the improper drying method and absorption during long-term storage (Gempo *et al.* 2024). The total ash (11.37 ± 0.20%) and acid-insoluble ash (4.57 ± 0.02%) of seed-under-leaf also exceeded the standard (Malaysian Ministry of Health, 2016). These values were much higher than those collected in Dhaka, Bangladesh, and Java, Indonesia (Jayani *et al.* 2020; Islam *et al.* 2022). Both ash contents represented inorganic mineral impurities, which directly related to the safety of crude drugs during use (Al-Harrasi *et al.* 2022). The water-soluble and ethanol-soluble extractable defined the content aspects of the crude drugs, and all three crude drugs met the standard for these parameters. The extractable values are closely linked with the efficacy of the crude drugs during use (Al-Harrasi *et al.* 2022). Considering all quality evaluation results, Java tea was the only crude drug that met the standard quality of MHM.

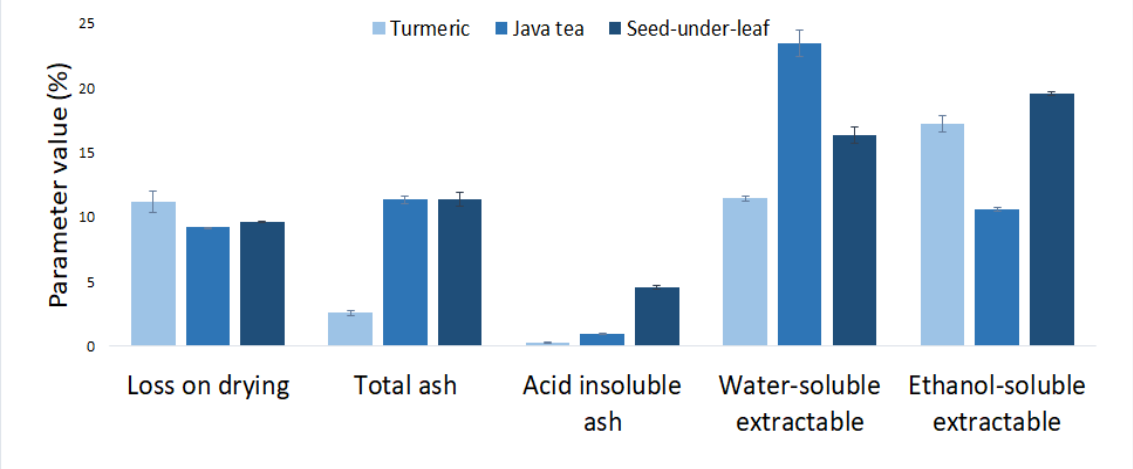

Fig. 3. Physicochemical properties of the crude drugs of turmeric, Java tea, and seed-under-leaf

Optimization of the Formulation of Polyherbal Drink

Simplex lattice mixture design and antioxidant response

The SLD allowed for the discovery of a combination of the three herbals studied that guarantees a synergic potentiation of the antioxidant activity. This could have a practical use in the food industry for functional beverages. Using mixtures of different herbals, in addition to potentiating antioxidant activity, guarantees other important organoleptic properties for consumers, for example, increased smell and taste. Using a Simplex Lattice Mixture Design, a Quadratic model was obtained for both methods in DPPH and FRAP with R^2 values of 0.9845 and 0.9656, respectively.

Table 1. Design Layout and Experimental LSD Results for Color, Antioxidant Properties, TPC, and TFC of the Polyherbal Drink

Run No.	Composition (%)			Color Parameter			DPPH (mM TE/g)	FRAP (mM TE/g)	TPC (mg GA/g)	TFC (mg QE/100 g)
	Turmeric	Java Tea	Seed-under-leaf	*L**	*a**	*b**				
1	0	100	0	21.49 ± 1.82^{cd}	0.73 ± 0.17^{bcd}	1.78 ± 0.20^{g}	1765.67 ± 8.39^{a}	1340.34 ± 121.33f^{g}	220.43 ± 1.56^{e}	233.88 ± 18.71^{a}
2	50	50	0	24.98 ± 0.71^{bcd}	0.19 ± 0.07^{cd}	8.69 ± 1.12^{e}	1386.10 ± 139.79^{c}	1008.80 ± 42.62^{h}	196.12 ± 4.69^{e}	237.74 ± 23.65^{a}
3	0	50	50	20.75 ± 0.14^{d}	2.51 ± 0.23^{a}	3.41 ± 0.51^{fg}	1800.27 ± 15.38^{a}	2355.57 ± 58.33^{a}	344.21 ± 17.19^{a}	185.37 ± 1.56^{cd}
4	100	0	0	35.30 ± 2.04^{a}	-0.85 ± 0.47^{g}	26.59 ± 0.88^{a}	664.53 ± 27.96^{d}	290.20 ± 33.65^{i}	118.76 ± 4.69^{f}	56.37 ± 5.20^{f}
5	50	0	50	24.48 ± 6.03^{bcd}	-0.07 ± 0.08^{cde}	15.26 ± 1.87^{c}	1799.28 ± 13.98^{a}	1840.02 ± 168.25^{cd}	277.90 ± 20.32^{d}	164.24 ± 1.30^{de}
6	0	0	100	22.13 ± 0.48^{cd}	0.74 ± 0.35^{bcd}	4.22 ± 0.13^{f}	1773.56 ± 35.55^{a}	2135.87 ± 185.08^{ab}	332.06 ± 12.50^{ab}	152.48 ± 9.62^{e}
7	17	67	17	30.28 ± 3.05^{abc}	-0.50 ± 0.18^{fg}	2.44 ± 0.28^{fg}	1758.75 ± 23.76^{a}	1684.56 ± 159.28^{de}	258.01 ± 7.81^{e}	205.76 ± 9.10^{c}
8	67	17	17	32.49 ± 0.06^{ab}	1.02 ± 0.15^{b}	20.66 ± 0.54^{b}	1516.58 ± 41.94^{b}	1134.12 ± 17.95^{gh}	203.86 ± 6.25^{e}	152.29 ± 3.12^{e}
9	17	17	67	26.45 ± 0.15^{bcd}	0.20 ± 0.02^{cde}	10.66 ± 0.18^{d}	1775.56 ± 2.80^{a}	2058.14 ± 59.45^{bc}	306.64 ± 10.94^{bc}	167.73 ± 7.28^{de}
10	33	33	33	25.68 ± 0.88^{bcd}	0.24 ± 0.11^{cde}	9.62 ± 1.51^{de}	1764.68 ± 12.58^{a}	1384.75 ± 159.28^{fg}	272.38 ± 21.88^{d}	212.38 ± 1.82^{ab}
Optimum model	5	40	55	-	-	-	1796.00 ± 3.86^{a}	2228.57 ± 80.52^{a}	354.89 ± 13.39^{a}	177.26 ± 14.97^{de}

The different alphabet in the same column represented significantly different values for a given parameter, evaluated by one-way ANOVA at $p < 0.05$ (n = 3)

The expected R^2 was also in agreement with the adjusted R^2; the difference was lower than 0.2. Concerning the ANOVA (Table 2), the lack of fit (F value) was not significant. Additionally, a non-significant lack of fit indicated that the calculated model was suitable for an experimental run. The model was deemed satisfactory because it generated results higher than four (Bangdiwala 2016).

Table 2. ANOVA of Fitted Model for Mixture Quadratic Model for Antioxidant Activity Responses

Response:	Antioxidant Using DPPH Radical Free Scavenging					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	1863073	5	372614.5	88.92789	< 0.0001	significant
Linear Mixture	1464307	2	732153.5	174.7352	< 0.0001	
AB	40423.62	1	40423.62	9.647468	0.0172	
AC	909.841	1	909.841	0.217142	0.6554	
BC	326235.1	1	326235.1	77.859	< 0.0001	
Residual	29330.53	7	4190.075			
Lack of Fit	24494.12	4	6123.529	3.798393	0.1508	not significant
Pure Error	4836.411	3	1612.137			
Cor Total	1892403	12				
Response:	Antioxidant using FRAP Method					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5188143	5	1037629	39.27114	< 0.0001	significant
Linear Mixture	4799225	2	2399612	90.81817	< 0.0001	
AB	464.9983	1	464.9983	0.017599	0.8982	
AC	160286.1	1	160286.1	6.06635	0.0433	
BC	187440.8	1	187440.8	7.094073	0.0323	
Residual	184955.1	7	26422.16			
Lack of Fit	165656.1	4	41414.02	6.437731	0.0790	not significant
Pure Error	19299.05	3	6433.015			
Cor Total	Resulted 5373098	12				

The antioxidant response values expected for the quadratic model and the real ones obtained from the experiment are shown in Table 1. A coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The regression quadratic model for the experiment is shown in Eqs. 1 and 2,

$$DPPH = 1742.21\ X_1 + 722.96\ X_2 + 1756.94\ X_3 + 799.76\ X_1X_2 + 119.98\ X_1X_3 + 2272.01\ X_2X_3 \quad (1)$$

$$FRAP = 1442.86\ X_1 + 340.29\ X_2 + 2194.65\ X_3 + 85.77\ X_1X_2$$

$$+ 1592.54\ X_1X_3 + 1722.17X_2X_3 \quad (2)$$

where DPPH and FRAP are in mM Trolox Equivalent/g; X_1 is the composition of Java tea; X_2 is the composition of turmeric, and X_3 is the composition of seed-under-leaf.

The highest antioxidant value of pure herbal was obtained by the crude drug of seed-under-leaf, followed by java tea, and then turmeric. The DPPH free radical scavenging activity and FRAP values of the optimum composition of the mixture of three herbals (40% of java tea, 5% turmeric, and 55% seed-under-leaf) were 1796.00 ± 3.86 mM TE/g and 2228.57 ± 80.52, respectively. The activities were higher than the pure of herbal. The DPPH free radical scavenging values of pure java tea, turmeric, and seed-under-leaf were 1765.67 ± 8.39, 664.53 ± 27.96 and 1773.56 ± 35.55 mM TE/g, respectively. Whereas the FRAP values of the pure crude drug were 1340.34 ± 121.33, 56.37 ± 5.20, and 2135.87 ± 185.08 mM TE/g, respectively.

It can be concluded that the optimal composition had a synergistic effect on antioxidant activity both in DPPH free radical scavenging and FRAP methods (Table 3). The synergistic effect is when the experimental value of a given parameter is statistically higher than its theoretical counterpart, evaluated by paired t-test at $p < 0.05$ ($n = 3$). Theoretical is calculated by the sum of composition in a particular formulation using a single crude drug. The experiment is based on the results of the model. The antioxidant polyherbal drink in all compositions showed a statistically strong correlation between TPC and DPPH, TPC and FRAP, TFC, and DPPH, and TFC and FRAP in a positive manner ($R = 1.000$, $p = 0.000$).

Table 3. Interaction Effects of the Combination of the Plant Components toward the Antioxidant Activity

Run No.	DPPH (mM TE/g)			FRAP (mM TE/g)		
	Experimental	Theoretical	Interaction	Experimental	Theoretical	Interaction
1	1765.67 ± 8.39	-	-	1340.34 ± 121.33	-	-
2	1386.10 ± 139.79	1224.00	Synergistic	1008.80 ± 42.62	866.43	Synergistic
3	1800.27 ± 15.38	1757.76	Synergistic	2355.57 ± 58.33	1804.33	Synergistic
4	664.53 ± 27.96	-	-	290.20 ± 33.65	-	-
5	1799.28 ± 13.98	1240.80	Synergistic	1840.02 ± 168.25	1253.88	Synergistic
6	1773.56 ± 35.55	-	-	2135.87 ± 185.08	-	-
7	1758.75 ± 23.76	1588.32	Synergistic	1684.56 ± 159.28	1375.63	Synergistic
8	1516.58 ± 41.94	1071.35	Synergistic	1134.12 ± 17.95	825.18	Synergistic
9	1775.56 ± 2.80	1605.12	Synergistic	2058.14 ± 59.45	1763.08	Synergistic
10	1764.68 ± 12.58	1393.44	Synergistic	1384.75 ± 159.28	1295.13	Synergistic
Optimum result	1796.00 ± 3.86	1707.75	Synergistic	2228.57 ± 80.52	1788.03	Synergistic

The synergistic effects achieved from the combination of turmeric, Java tea, and seed-under-leaf in the herbal drink have been regarded as beneficial for enhancing overall antioxidant activity. A previous study demonstrated that this polyherbal mixture provided the optimal antioxidant profile when subjected to a 15-minute extraction process (Hamad and Hartanti 2023). Similarly, another report highlighted the synergistic interaction of a mixture of candy leaf, Java tea, *pecah beling*, and seed-under-leaf from Malaysia in enhancing DPPH scavenging activity (Rahim *et al.* 2018). Moreover, a formulation composed of celery, Javanese turmeric, Java tea, *pegagan*, seed-under-leaf, and turmeric, was shown to effectively reduce blood pressure in patients with stage 1 hypertension as well as hypertensive Wistar rats (Triyono *et al.* 2018b). This antihypertensive preparation was also clinically verified to be safe for kidney function, with tea formulations proving more efficacious than infusions (Triyono and Novianto 2015; Triyono *et al.* 2018a).

LSD: TPC, TFC responses

Phenolic compounds and flavonoids are directly associated with antioxidant properties. Those compounds can donate hydrogen atoms or electrons, neutralize free radicals, and further prevent oxidative stress. The TPC and TFC of a given plant material can be correlated with their overall antioxidant capacity. The polyherbal formulation evaluated in this study demonstrated a strong and positive correlation between TPC – TFC and DPPH scavenging activity – FRAP (Hamad and Hartanti 2023). A strong correlation between TPC and antioxidant activity and a moderate one between TFC and antioxidant activity was observed in turmeric rhizome extracts (Muflihah *et al.* 2021). Similarly, a strong correlation was observed between total phenolic flavonoid contents and the total antioxidant activity in Java tea extracts, but the activity was not attributable to rosmarinic acid and sinensetin (Bovani *et al.* 2024). However, there was no correlation between total antioxidant activity and the flavonoid content in the seed-under-leaf originating from Taraba, Nigeria (Yakubu *et al.* 2021).

The TPC and TFC values expected for the quadratic model and the real ones obtained from the experiment are also shown in Table 1. A coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The regression quadratic model for the experiment is shown in Eqs. 3 and 4,

$$TPC = 253.27\ X_1 + 119.75\ X_2 + 329.99\ X_3 + 12.18\ X_1X_2 + 164.06\ X_1X_3 + 180.79\ X_2X_3 \quad (3)$$

$$TFC = 222.13\ X_1 + 59.12\ X_2 + 150.98\ X_3 + 351.37\ X_1X_2 - 39.08\ X_1X_3 + 211.88\ X_2X_3 \quad (4)$$

where the TPC unit is in mg Gallic Acid Equivalent (GAE)/g and the TFC unit in mg Quercetin Equivalent (QE)/g; X_1 is the composition of Java tea, X_2 is the composition of turmeric, and X_3 is the composition of seed-under-leaf.

Using a Simplex Lattice Mixture Design, a Quadratic model was obtained for both methods in TPC and TFC with R^2 values of 0.9597 and 0.9733, respectively. The expected R^2 were also in agreement with the adjusted R^2 ; the difference was lower than 0.2. With respect to the ANOVA (Table 4), the lack of fit (F value) was not significant. Additionally, a non-significant lack of fit indicated that the calculated model was suitable for an experimental run. The model was deemed satisfactory because it generated results higher than four (Bangdiwala 2016).

Table 4. ANOVA of Fitted Model for Mixture Quadratic Model for TPC and TFC Responses

Response:	TPC (Total Phenolic Compound)					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	65756.44	5	13151.29	33.36405	< 0.0001	significant
Linear Mixture	61526.23	2	30763.11	78.04423	< 0.0001	
AB	9.380056	1	9.380056	0.023797	0.8818	
AC	1701.143	1	1701.143	4.315701	0.0764	
BC	2065.894	1	2065.894	5.241052	0.0559	
Residual	2759.228	7	394.1754			
Lack of Fit	557.6662	4	139.4165	0.189979	0.9288	not significant
Pure Error	2201.562	3	733.8539			
Cor Total	68515.66	12				
Response:	TFC (Total Flavonoid Content)					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	38346.76	5	7669.351	50.95004	< 0.0001	significant
Linear Mixture	26832.36	2	13416.18	89.12813	< 0.0001	
AB	7802.741	1	7802.741	51.83619	0.0002	
AC	96.52472	1	96.52472	0.641246	0.4496	
BC	2837.107	1	2837.107	18.84784	0.0034	
Residual	1053.688	7	150.5269			
Lack of Fit	818.6444	4	204.6611	2.612206	0.2281	not significant
Pure Error	235.044	3	78.348			
Cor Total	39400.45	12				

CONCLUSIONS

1. The following mixture of 5% turmeric, 40% Java tea, and 55% seed-under-leaf was found to be the best herbal drink composition formulation, resulting in the optimum values of antioxidant activities, TPC, and TFC. The best composition formulation will be important to be analyzed in vitro and in vivo to be claimed as a functional food for further research.
2. A synergistic relationship between polyherbal drinks with three mixtures of turmeric, java tea, and seed-under-leaf regarding their antioxidant activity, TPC, and TFC was predicted by a quadratic regression model using Simplex Lattice Design and was observed at various proportions.

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