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Crude Drug Standardization, Formula Optimization, and Interaction Effects of a Five-Component Antioxidant Polyherbal Formulation

[Standardisasi Simplisia, Optimasi Formula, dan Efek Interaksi dalam Ramuan Jamu Kaya Antioksidan]

Tri Kusuma Wardani[1], Elfa Arianingsih[1], Arini Syarifah[1], Alwani Hamad[2], Dwi Hartanti[1]*

[1]*Faculty of Pharmacy, Universitas Muhammadiyah Purwokerto, Purwokerto, Central Java, 53182, Indonesia*
[2]*Department of Chemical Engineering, Faculty of Engineering and Science, Universitas Muhammadiyah Purwokerto, Purwokerto, Central Java, 53182, Indonesia*

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ABSTRACT

A polyherbal formulation was developed from java tea (*Orthosiphon aristatus* (Blume) Miq.), turmeric (*Curcuma longa* L.), seed-under-leaf (*Phyllanthus niruri* L.), cinnamon (*Cinnamomum verum* J. Presl), and ginger (*Zingiber officinale* Roscoe). This study aimed to standardize crude drugs, optimize polyherbal formulations, and evaluate the interaction effect of the crude drug mixture. Standardization followed these methods and compared them with the Indonesian Herbal Pharmacopeia (IHP) standards. The crude drugs were mixed in 26 different ratios, and each formulation was extracted using the decoction method. The antioxidant activities of the extracts were evaluated using 2-diphenyl-2-picryl hydroxyl scavenging (DPPH) radical scavenging activity and ferric-reducing antioxidant power (FRAP) assays. The optimum formula was obtained by antioxidant activity-based prediction using the simplex lattice design (SLD) method. The interaction effects of crude drug mixtures were determined using a statistical comparison method for the predicted and obtained antioxidant activities. Crude java tea, turmeric, cinnamon, and ginger were of good quality. The five-component formulation with optimum antioxidant activity consisted of java tea, turmeric, seed-under-leaf, cinnamon, and ginger crude drugs in a ratio of 20-20-40-10-10 with interaction effects of additive toward DPPH radical scavenging activity (7.05 ± 0.94 μmol TE/g) and antagonistic toward FRAP (19.37 ± 0.94 μmol TE/g). Formula 21 (mixture of java tea and seed-under-leaf crude drugs) showed the highest DPPH scavenging activity (6.39 ± 0.16 μmol TE/g) with synergistic effects, while the synergistic, highest FRAP (23.74 ± 0.03 μmol TE/g) was shown by Formula 13 (mixture of seed-under-leaf and ginger crude drugs).

Kata kunci:
Aktivitas antioksidan
Standarisasi simplisia
Optimasi formula
Efek interaksi
Ramuan jamu

ABSTRAK

Telah dilakukan upaya pengembangan ramuan kaya antioksidan yang tersusun oleh daun kumis kucing (*Orthosiphon aristatus* (Blume) Miq.), rimpang kunyit (*Curcuma longa* L.), herba meniran (*Phyllanthus niruri* L.), kulit kayu manis (*Cinnamomum verum* J.Presl), dan rimpang jahe (*Zingiber officinale* Roscoe). Penelitian ini bertujuan untuk melakukan standarisasi simplisia, optimasi formulasi ramuan, dan mengevaluasi efek interaksi simplisia tersebut terhadap aktivitas antioksidan ramuan tersebut. Standarisasi dilakukan dengan metode dan dibandingkan dengan standar dalam Farmakope Herbal Indonesia (FHI). Kelima simplisia tersebut dicampur menjadi 26 formula ramuan dengan rasio yang berbeda. Masing-masing ramuan diekstraksi dengan metode rebusan. Aktivitas antioksidan ekstrak dievaluasi dengan aktivitas penangkapan radikal *2-diphenyl-2-picryl hydroxyl* (DPPH) dan *ferric reducing antioxidant power* (FRAP). Optimasi formula berdasarkan aktivitas antioksidan diprediksi menggunakan *simplex lattice design* (SLD). Efek

interaksi pencampuran simplisia dalam ramuan ditentukan secara statistik dengan membandingkan aktivitas antioksidan teoritis dan hasil pengujian. Simplisia daun kumis kucing, rimpang kunyit, kulit kayu manis, dan rimpang jahe berkualitas baik dan memenuhi standar mutu FHI. Ramuan optimum dengan lima komponen tersusun oleh daun kumis kucing, rimpang kunyit, herba meniran, kulit kayu manis, dan rimpang jahe dalam perbandingan 20-20-40-10-10 dengan efek interaksi aditif terhadap aktivitas penangkapan radikal DPPH (7.05 ± 0.94 µmol TE/g) dan antagonis terhadap FRAP 19.37 ± 0.94 µmol TE/g). Formula 21 (kombinasi daun kumis kucing dan herba meniran) menunjukkan aktivitas penangkapan radikal DPPH tertinggi (6,39 ± 0,16 µmol TE/g) dengan efek sinergis. Efek sinergis dengan aktivitas FRAP tertinggi (23,74 ± 0,03 µmol TE/g) teramati pada Formula 13 (kombinasi herba meniran dan rimpang jahe).

*Corresponding author:
Dwi Hartanti (dwihartanti@ump.ac.id)

1. INTRODUCTION

The prevalence of degenerative diseases in Indonesia in 2018 was 65.7%. Not only affected the elderly, but degenerative diseases were also found in young adults, particularly those with obesity (Kemenkes RI, 2018; Mihardja & Soetrisno, 2012). Oxidative damage caused by external factors contributes to the prognosis of degenerative diseases and the intake of antioxidant supplements can slow the development of these disorders (Iakovou & Kourti, 2022). The use of plants as natural antioxidant sources is of great interest globally. In Indonesia, those commonly used in traditional medicine, such as java tea (*Orthosiphon aristatus* (Blume) Miq.), turmeric (*Curcuma longa* L.), seed-under-leaf (*Phyllantus niruri* L.), cinnamon (*Cinnamomum verum* J. Presl.), ginger (*Zingiber officinale* Roscoe.) are promising candidates for developing such supplements (Fauziah et al., 2023; Sukweenadhi et al., 2020).

Many factors, such as genetics, environment, cultivation, post-harvest processing, and storage conditions, can greatly affect the quality of crude drugs. The quality of crude drugs is determined by three aspects: identity, purity, and content. The safety and efficacy of crude drugs are directly correlated with their purity and content (Al-Harrasi et al., 2022; Das et al., 2019). Therefore, standardizing their quality is crucial to guarantee the safety and effectiveness of crude drugs. Furthermore, the standardization of crude drugs is required for those meant to be used as raw materials for phytomedicines (*fitofarmaka*) and standardized herbal medicines (*obat herbal terstandar*).

Jamu, a traditional Indonesian medicine, is commonly used as a polyherbal formulation in which multiple plant components are mixed and utilized as a concoction. The interaction between plant components may occur in a plant mixture, leading to antagonistic, synergistic, or additive effects. A synergistic effect is expected from a polyherbal formulation, in which the therapeutic activity of a formulation is higher than the sum of that of individual components or exerts an overall lower toxic effect (Houghton, 2009). These effects are enabled by various compounds available in the polyherbal formulation, which may interact with multiple receptors and produce biological results through numerous mechanisms (Caesar & Cech, 2019). Hence, a synergistic effect can only be achieved with a polyherbal formulation. However, additive and antagonistic effects on the antioxidant activity have also been reported. The ratio of plant components defines the final interaction effect (Mapeka et al., 2022; Rahim et al., 2020; Yap et al., 2023). Optimization of the composition of a polyherbal formulation is essential for obtaining a compound with a synergistic antioxidant effect. However, the interaction effect cannot be estimated and can only be proven after a specific response is measured. It is necessary to establish formulation compositions with optimum antioxidant activity.

This study aimed to standardize crude drugs and to optimize the ratio of java tea, turmeric, seed-under-leaf, cinnamon, and ginger crude drugs in a novel polyherbal formulation with 2,2-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity and ferric reducing antioxidant power (FRAP) as the responses, and to elucidate the interaction effects of the crude drugs in the selected formulations.

2. METHODS

2.1. Materials

Crude drugs of java tea, turmeric, cinnamon, and ginger were obtained from Wisata Kesehatan Jamu (WKJ) Kalibakung, Tegal, Indonesia, whereas seed-under-leaf drugs were purchased from Indoplant, Yogyakarta, Indonesia. Analytical grade chemicals and reagents, that is, 2,4,6-tripyridyl-s-triazine (TPTZ), $CHCl_3$, CH_3COONa, CH_3COOH, chloral hydrate, DPPH, and Trolox, solvents (acetone, chloroform, deionized water, ethanol, ethyl acetate, formic acid, methanol, n-hexane, and toluene), and silica gel F_{254} plates were purchased from Sigma-Aldrich (St. Louis, MO, USA). The instruments used were a light microscope (Olympus, Tokyo, Japan) connected to a digital camera (Optilab, Anand, India), analytical balance (Shimadzu, Kyoto, Japan), and a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The software used was Design Expert ver. 6.0.4 (Stat-Ease, Minneapolis, USA) and SPSS ver. 26.0 (IBM, New York, USA).

2.2. Standardization of Crude Drugs

The identity of java tea, turmeric, seed-under-leaf, cinnamon, and ginger crude drugs was determined using thin-layer chromatography (TLC) profiles and macroscopic and microscopic morphology. Separation was conducted on silica gel F_{254} plates, with the other chromatographic conditions presented in **Table 1**.

The loss of drying, total ash, acid-insoluble ash, ethanol extractable, water extractable, and chemical content of the crude drugs were evaluated using methods and compared to standard values according to the Indonesian Herbal Pharmacopeia (IHP) (Indonesian Ministry of Health, 2017).

Table 1. Thin layer chromatographic conditions for the separation of crude drug extracts

Crude drugs	Separated sample	Reference compound	Mobile phase	TLC plate visualization
Java tea	Ethanolic solution, 10%	Sinensetin	Chloroform – ethyl acetate (60:40)	UV_{366}
Turmeric	Ethanolic solution, 5%	Curcuminoids	Chloroform – methanol (95:5)	UV_{366}
Seed-under-leaf	Ethanolic solution, 2%	Quercetin	Chloroform – acetone – formic acid (14:6:0.8)	UV_{366}
Cinnamon	Ethanolic solution, 5%	Cinnamaldehyde	n-Hexane – ethyl acetate (90:10)	Visible light, after derivatization with anisaldehyde -sulphuric acid
Ginger	Ethanolic solution, 10%	Eugenol	Toluene – ethyl acetate (97:3)	UV_{254}

2.3. Polyherbal Formulation Preparation

A simplex lattice design (SLD) was used to determine the composition of the crude drug mixtures. The mass fractions of the crude drugs in java tea (X_1), turmeric (X_2), seed-under-leaf (X_3), cinnamon (X_4), and ginger (X_5) ranged from 0 to 100, with a total weight fraction of 100. A total of 26 formulations of the crude drug mixture were prepared (**Table 2**).

Table 2. Composition of crude drug mixtures for each formulation

Formulation	Ratio (%)				
	Java tea (X_1)	Turmeric (X_2)	Seed-under-leaf (X_3)	Cinnamon (X_4)	Ginger (X_5)
1	10	10	10	10	60
2	10	10	10	60	10
3	0	0	0	100	0
4	0	0	100	0	0
5	0	0	0	0	100
6	0	50	0	50	0
7	0	0	0	50	50
8	60	10	10	10	10
9	100	0	0	0	0
10	20	20	20	20	20
11	50	0	0	0	50
12	0	100	0	0	0
13	0	0	50	0	50
14	100	0	0	0	0
15	0	0	50	50	0
16	0	50	50	0	0
17	0	0	0	0	100
18	0	0	100	0	0
19	0	0	0	100	0
20	50	50	0	0	0
21	50	0	50	0	0
22	10	60	10	10	10
23	50	0	0	50	0
24	10	10	60	10	10
25	0	50	0	0	50
26	0	100	0	0	0

2.4. Preparation of Water Extract

Crude drugs from java tea, turmeric, seed-under-leaf, cinnamon, and ginger were weighed in ratios according to **Table 2** and homogeneously mixed. A total of 1 g of each crude drug mixture was added to 20 ml of water and indirectly heated in a 100˚C water bath for 30 min. The water extract was filtered, allowed to reach room temperature, and used for the antioxidant assays.

2.5. DPPH Scavenging Activity Assay

Five hundred microliters of each properly diluted formulation of water extract or Trolox standard was mixed with 5000 µL of 25 µg/ml DPPH solution. The reaction mixture was allowed to sit for 45 min, protected from light, and absorbance was measured at 518 nm. The same DPPH solution was used as a blank, and the percentage of DPPH inhibition (% inhibition) was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{A0 - A1}{A1} \times 100$$

Where A0 and A1 are the absorbances of the blank and reaction mixture, respectively, and the obtained % inhibition was plotted on the standard curve equation ($y = 0.1687x + 15.336$, $R^2 = 0.97$), which was prepared from Trolox solution concentrations (50-300 µM) and their respective % inhibition. The DPPH scavenging activity of the crude drugs was reported as µmol Trolox equivalent (TE)/g) (Hartanti et al., 2023).

2.6. FRAP Assay

A total of 210 µL of each properly diluted formulation of water extract or Trolox standard was mixed with 4000 µL of FRAP reagent, which was freshly prepared from ten parts of 300 mM acetate buffer, 10 mM TPTZ solution in HCl, and 20 mM $FeCl_3$ mixture at a final pH of 3.6. The reaction mixture was allowed to stand for 40 min, and the absorbance was measured at 595 nm. The obtained absorbance was plotted onto the standard curve equation ($y = 0.0021x + 0.1092$, $R^2 = 0.96$) prepared from Trolox solution concentrations (50-300 µM) and their respective absorbances. The FRAP of crude drugs was reported as µmol TE/g (Hartanti et al., 2023).

2.7. Prediction of Optimum Formulation

The optimum formulation and suitability of the model were predicted by SLD using DPPH scavenging activity and FRAP as responses (Monton et al., 2020).

2.8. Interaction Effect of the Polyherbal Mixture

The interaction effect of the crude drug mixture on antioxidant activity was analyzed by statistical comparison of the predicted and obtained DPPH scavenging activity and FRAP values. The obtained values were measured during the experiments, whereas the predicted values were calculated using the equation suggested by SLD (Yap et al., 2023).

2.9. Statistical Analysis

The data are reported as the mean ± standard deviation (SD). The effect and mean separation of the formulation on antioxidant activities were evaluated using one-way ANOVA and Duncan's test. The predicted and experimental values of DPPH scavenging activity and FRAP during the optimization stage were compared using a t-test. The effect and difference were significant at $p \leq 0.05$, and calculated using the general procedure of SPSS.

3. RESULTS AND DISCUSSION

The macroscopic characteristics of all crude drugs were similar to those described for the IHP. Similarly, most identity fragments of each crude drug were observed during microscopic evaluation. For example, covering trichomes and spiral vessels were observed in java tea leaves, while the parenchymal cortex and spiral vessels were found in turmeric crude drugs. Epidermis with calcium oxalate crystals and epicarps was observed in seed-under-leaf crude drugs. Cinnamon crude drugs produced sclereid and sclerenchyma, whereas crude ginger drugs produced amylum starch and spiral vessels. The TLC profiles of the crude drugs were also similar to their respective standard chromatograms (**Figure 1**). Based on these three parameters, the identity of all crude drugs was confirmed to be correct. In addition, the reference standards for TLC of java tea, turmeric, and cinnamon crude drugs were their respective identity markers, that is, sinensetin, curcumin, and cinnamaldehyde. Spots with Rf values or colors identical to those markers were observed in the chromatogram of the respective crude drugs. Hence, the identities of java tea, turmeric, and cinnamon crude drugs were confirmed by the presence of their identity markers.

Java tea, turmeric, cinnamon, and ginger crude drugs were of good quality, whereas the seed-under-leaf drugs were not (**Table 3**). The values of all quality parameters of java tea, turmeric, cinnamon, and ginger crude drugs were within the specified values in the IHP. Good quality ginger crude drugs were also observed in our previous study, which was subjected to one of the different batches purchased from WKJ Kalibakung (Hartanti & Hamad, 2024; Puspitasari et al., 2024). Loss on drying, total ash, and acid-insoluble ash represent the purity aspect of quality, which directly affects safety during crude drug use. In contrast, ethanol extractable, water extractable, and chemical content represent the content aspect of quality that defines the efficacy of crude drugs (Al-Harrasi et al., 2022). Hence, all four crude drugs are predicted to be safe and effective for medicinal use. However, the opposite was observed with seed-under-leaf crude drugs, which only met the loss on drying requirements and were not within the IHP specifications for other parameters.

The antioxidant activities and potential therapeutic uses of various extracts of java tea, turmeric, and seed-under-leaf crude drugs have been well-characterized (Ashraf et al., 2020; Navarro et al., 2017; Quirós-Fallas et al., 2022). The mixture of these crude drugs in a ratio of 2-1-7 as a polyherbal formulation generated a water extract with a considerably high DPPH scavenging activity and FRAP (Hamad & Hartanti, 2023). Hence, these three crude drugs were the main components with antioxidant activity. On the other hand, the role of cinnamon and ginger crude drugs in the formulation is for the supporting components.

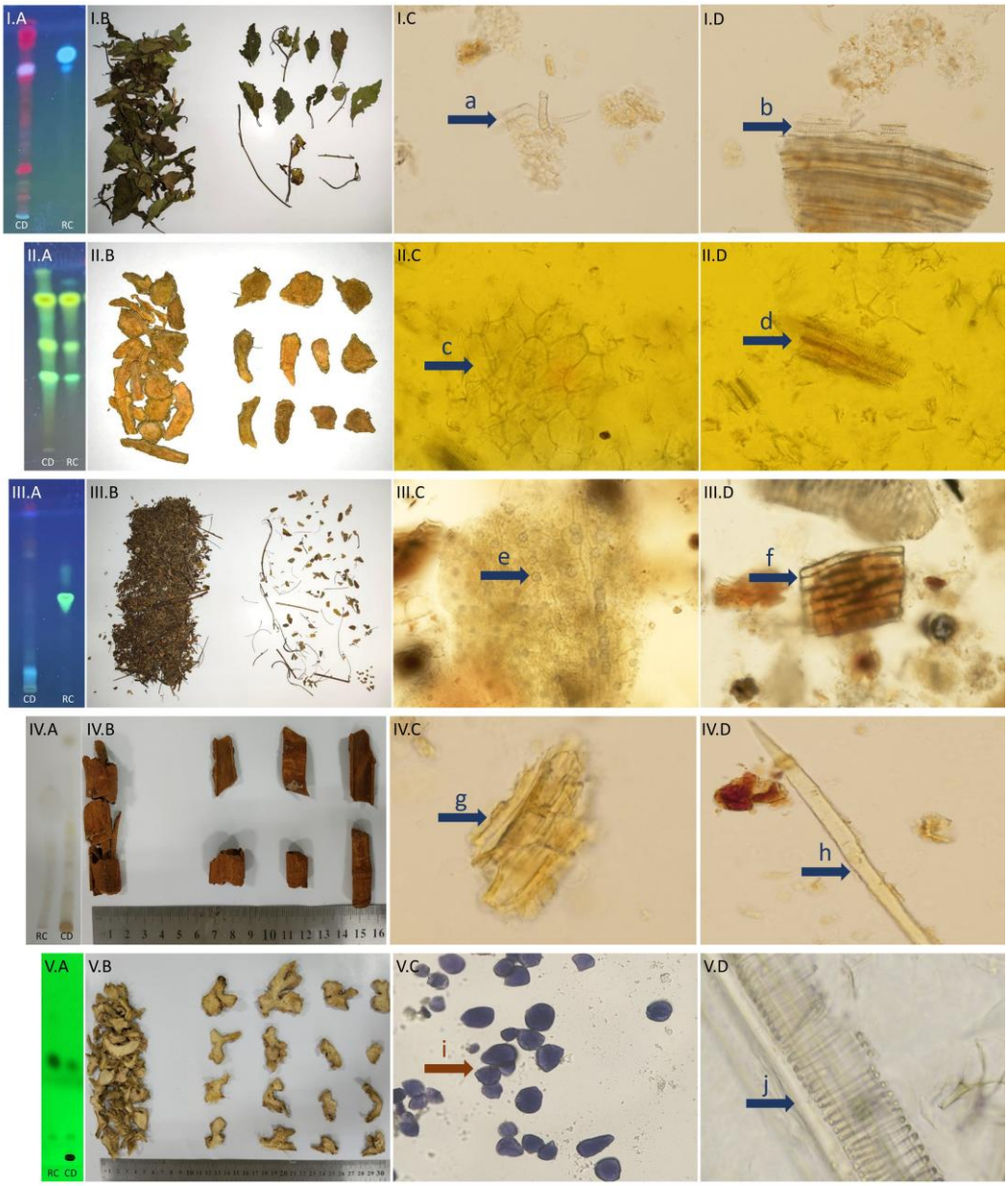

Figure 1. The identity aspects of java tea (I), turmeric (II), seed-under-leaf (III), cinnamon (IV), and ginger (V) crude drugs showing TLC profiles (A), macroscopic characters (B), and selected microscopic features (C-D), i.e., covering trichome (a), vessels (b, d, j), parenchymatous cells with yellow pigment (c), upper epidermis with rosette-shape calcium oxalate chrystals (e), exocarps (f), sclereids (g), sclerenchyma (h), and starch granules (i); CD = crude drugs, RC = reference compound

Table 3. Profile of purity and content aspects of crude drug quality

Parameters	Crude drugs									
	Java tea		Turmeric		Seed-under-leaf		Cinnamon		Ginger	
	Value (%)	Standard	Value (%)	Standard	Value (%)	Standard	Value (%)	Standard	Value (%)	Standard
LoD	9.37 ± 0.45	≯10%	7.65 ± 0.52	≯ 10%	9.61 ± 0.13	≯ 10%	8.46 ± 0.71	≯ 10%	9.86 ± 0.73	≯ 10%
TA	9.03 ± 0.36	≯ 10.2%	6.68 ± 0.85	≯ 8.2%	12.94 ± 0.30 *	≯ 7.2%	1.40 ± 0.10	≯ 10.5%	2.45 ± 0.49	≯ 4.2%
A-IA	1.49 ± 0.36	≯ 3.4%	0.63 ± 0.24	≯ 0.9%	2.89 ± 0.27*	≯ 1.2%	0.28 ± 0.06	≯ 0.3%	2.05 ± 0.52	≯ 3.2%
EE	10.99 ± 0.78	≮ 7.2%	11.96 ± 0.21	≮ 11.4%	7.26 ± 0.49*	≮ 10.5%	19.41 ± 3.16	≮ 16.0%	6.39 ± 0.75	≮ 5.7%
WE	20.43 ± 0.93	≮ 10.2%	15.89 ± 0.36	≮ 11.5%	14.16 ± 0.25 *	≮ 20.3%	7.92 ± 1.78	≮ 4.0%	18.79 ± 0.37	≮ 15.8%
CC	0.93 ± 0.07	≮ 0.10%	11.37 ± 3.72 ; 1,85	≮ 3.82%; ≮ 1.85%	0.36 ± 0.0*	≮ 0.9%	0.50	≮ 0.42%	0.98	≮ 0.8%

The asterisk (*) represents the parameter that did not meet the official specifications in the IHP. The chemical content of java tea, turmeric, seed-under-leaf, cinnamon, and ginger crude drugs were sinensetin, curcumin total and volatile oil, flavonoid total, volatile oil, and volatile oil, respectively. LoD = loss on drying, TA = total ash, A-IA = acid-insoluble ash, EE = ethanol extractable, WE = water extractable, CC = chemical content, ≯ = not more than, ≮ = not less than

Both materials contain pleasant aroma essential oils with spicy-pungent tastes and are commonly used in various food and beverage products (dos Santos et al., 2015; Vitalini et al., 2023). These properties enable their use to increase the palatability of polyherbal formulation extracts by improving their aroma and taste. Cinnamon and ginger have shown prominent antioxidant properties (Antasionasti & Jayanto, 2021; Haroen et al., 2024). Hence, both crude drugs may have contributed to the antioxidant activity of the formulation.

The antioxidant activities of the water extracts for formulation optimization are presented in **Table 4**. The single crude drugs generally showed a higher DPPH scavenging activity than their mixture counterparts, as shown for turmeric, seed-under-leaf, and cinnamon crude drugs. The highest DPPH scavenging activity was shown by Formulas 3 and 4, which were 100% cinnamon and seed-under-leaf crude drugs, respectively. The superior free radical scavenging activity of turmeric over ginger has been reported in an Italian study on the valorisation of both plants (Tinello & Lante, 2019). The same case was also applied to the higher DPPH scavenging activity of cinnamon over java tea, as reported in a Malaysian study (Ismail et al., 2017). Similarly, single plant materials tended to produce higher FRAP, particularly that of turmeric and seed-under-leaf. As for DPPH scavenging activity, higher FRAP of turmeric than that of ginger was also previously reported in Italy (Tinello & Lante, 2019). The formula with the best FRAP was Formula 13, which consisted of an equal ratio of seed-under-leaf and cinnamon crude drugs.

The different antioxidant activities observed in the DPPH scavenging activity and FRAP of a given formula extract represent the different antioxidant mechanisms evaluated by both methods. DPPH scavenging activity was used to measure the ability of the antioxidant compounds in the extract to donate hydrogen atoms and electrons to neutralize free radicals. Hence, the extracts showed higher DPPH scavenging activity, likely containing compounds that easily transfer their hydrogen atoms or electrons to free radicals available in the system. This reflected the direct free radical scavenging potential that mimicked the quenching of reactive oxygen species (ROS) in the body, which is commonly associated with the etiology of various degenerative diseases. On the other hand, FRAP measures the ability of antioxidant compounds to reduce ferric ions to ferrous ions under acidic conditions. This relies on the capacity of the compound to transfer electrons for redox reactions. Extracts with higher FRAP showed better reducing potential, which is essential for regenerating oxidized antioxidants and maintaining redox homeostasis in biological systems (Munteanu & Apetrei, 2021).

A quadratic model was chosen for formula optimization to fit the evaluation of antioxidant activity. Prob >F was significant, the value of R^2 was nearly 1, and the lack of fit was insignificant (**Tables 5** and **6**). Hence, the selected SLD model can be used to optimize the formula. The optimum formulation was modeled based on the assumption that turmeric, java tea, and seed-under-leaf crude drugs were the main antioxidant components of the formulation, with a maximum fraction of 0.8, and cinnamon and ginger crude drugs were the supportive components. The optimum ratio for java tea - turmeric - seed-under-leaf - cinnamon - ginger crude drugs was 20-20-40-10-10. A model desirability value of 0.93 indicates that they are the optimum formulations and meet the objectives based on the expected response criteria. The percentage error of the optimum formulation for DPPH scavenging activity was 4.26%, which was within the ideal range (Crespo et al., 2019).

Table 4. Antioxidant activity response result of formulation optimization

Formulations	Antioxidant activity (μmol TE/g)	
	DPPH	FRAP
1	4.72 ± 0.31^{E}	21.49 ± 0.60^{lm}
2	6.46 ± 1.63^{GHI}	19.19 ± 1.04^{hi}
3	8.16 ± 0.16^{NO}	14.58 ± 2.10^{d}
4	8.59 ± 0.34^{O}	$20.79 \pm 0.51k^{lm}$
5	0.44 ± 0.46^{A}	10.16 ± 0.30^{b}
6	7.01 ± 0.31^{HIJK}	18.73 ± 0.04^{gh}
7	4.36 ± 0.05^{DE}	21.19 ± 0.34^{klm}
8	4.35 ± 0.90^{E}	21.17 ± 0.34^{lm}
9	3.75 ± 0.65^{D}	7.52 ± 0.42^{a}
10	6.85 ± 0.48^{HIJ}	20.70 ± 0^{jkl}
11	2.37 ± 0.07^{C}	9.60 ± 2.50^{b}
12	7.07 ± 0.72^{IJK}	17.33 ± 0.92^{ef}
13	6.06 ± 0.58^{FG}	23.74 ± 0.03^{n}
14	4.22 ± 0.42^{DE}	11.78 ± 0.69^{c}
15	8.07 ± 0.18^{MN}	19.86 ± 1.21^{ij}
16	7.60 ± 0.32^{KLM}	20.58 ± 0.05^{jk}
17	1.27 ± 0.41^{B}	12.15 ± 0.31^{c}
18	8.00 ± 0.02^{MN}	21.85 ± 0.26^{lm}
19	7.70 ± 0.09^{LMN}	14.29 ± 0.06^{d}
20	6.03 ± 0.06^{FG}	20.85 ± 0.03^{klm}
21	6.39 ± 0.16^{GH}	21.89 ± 0.03^{m}
22	5.87 ± 0.67^{FG}	17.81 ± 0.93^{fg}
23	5.49 ± 0.63^{F}	19.81 ± 0.32^{ij}
24	7.07 ± 0^{JKL}	19.31 ± 0.02^{ij}
25	5.98 ± 0.08^{FG}	19.63 ± 0.59^{ij}
26	7.39 ± 0.10^{JKLM}	16.64 ± 0.98^{e}

Each column's capital and lowercase letters represent significantly different DPPH scavenging activity and FRAP, respectively.

In contrast, the percentage error for FRAP was -13.14%. Previous studies have reported the use of SLD to obtain polyherbal formulations with optimal antioxidant effects. A study from Malaysia reported a combination of lemongrass (53.7%), curry leaves (43.4%), and ginger (2.9%), in which turmeric had to be excluded from the formulation (Rahim et al., 2020). On the other hand, the optimum antioxidant activity of the classical Triphala formulation was achieved from an equal ratio of myrobalan, belleric myrobalan, and Indian gooseberry (Monton et al., 2020).

The interaction effects between the components in a given mixture are classified as synergistic, additive, or antagonistic. Synergistic effects were observed when the predicted values were statistically lower than the measured counterparts, while antagonistic effects

were observed when the opposite was observed. Additive effects were identified when there was no statistical difference between predicted and measured values (Yap et al. 2023).

The mixture of the two crude drugs generated various interaction effects on the DPPH scavenging activity. The interaction effect depended on the crude drugs constituting the mixture. The additive, in which no interaction was observed, was detected in all four formulations. Synergistic and antagonistic effects were observed in two and three crude drug mixtures, respectively. The mixture of all five crude drugs produced an additive effect on the DPPH scavenging activity, as observed in the six formulations. Formulations with cinnamon and java tea crude drugs as the major components showed additive effects, such as those with equal ratios of crude drugs and optimum formulation. However, the synergistic effect was only demonstrated if the formulation had ginger as a major component.

In contrast, an antagonistic effect was shown in the polyherbal formula, which mainly consisted of turmeric and seed-under-leaf crude drugs. The interaction effect pattern of FRAP differed from that of DPPH scavenging activity. Synergistic, additive, and antagonistic effects were observed in three-, four-, and three-component mixtures, respectively (**Table 7**).

Table 5. Evaluation of the SLD model

Response	Model	F-value	Prob>F	SD	R^2	Adjusted R^2	Lack of fit	Probability
DPPH	Quadratic	27.17	<0.0001(sig)	0.54	0.971	0.936	2.50 (not sig)	0.166
FRAP	Quadratic	6.31	0.0020 (sig)	2.20	0.889	0.748	2.89 (not sig)	0.132

Table 6. Quadratic equation of the SLD model

Response	Final equation
DPPH	DPPH (μmol TE/g) = $(3.94*X_1)+(7.14*X_2)+(8.27*X_3)+(7.91*X_4)+(0.92*X_5)+(1.12*X_1*X_2)+(0.77*X_1*X_3)$-$(2.02*X_1*X_4)+(0.12*X_1*X_5)$-$(1.10*X_2*X_3)$-$(2.65*X_2*X_4)+(7.86*X_2*X_5)$-$(0.16*X_3*X_4)+(6.38*X_3*X_5)+(0.37*X_4*X_5)$
FRAP	FRAP (μmol TE/g) = $(10.07*X_1)+(16.85*X_2)+(21.13*X_3)+(14.46*X_4)+(11.54*X_5)+(28.73*X_1*X_2)+(23.87*X_1*X_3)+(30.66*X_1*X_4)$-$(1.48*X_1*X_5)+(0.63*X_2*X_3)+(8.30*X_2*X_4)+(20.64*X_2*X_5)+(3.82*X_3*X_4)+(28.09*X_3*X_5)+(32.96*X_4*X_5)$

X_1 = java tea, X_2 = turmenic, X_3 = seed-under-leaf, X_4 = cinnamon, X_5 = ginger

Table 7. The interaction effects of the polyherbal mixture

Formulation	DPPH scavenging activity			FRAP		
	Predicted (μmol TE/g)	Obtained (μmol TE/g)	Interaction	Predicted (μmol TE/g)	Obtained (μmol TE/g)	Interaction
1	4.29*	4.72 ± 0.31*	Synergistic	19.64*	21.49 ± 0.60*	Synergistic
2	6.79	6.46 ± 1.63	Additive	20.87*	19.13 ± 1.04*	Antagonistic
6	6.93	7.01 ± 0.31	Additive	17.95*	18.73 ± 0.04*	Synergistic
7	4.53*	4.35 ± 0.05*	Antagonistic	21.39	21.19 ± 0.34	Additive
8	5.14	4.35 ± 0.90	Additive	19.50*	21.17 ± 0.34*	Synergistic
10	6.18	6.85 ± 0.48	Additive	26.71*	20.70 ± 0*	Antagonistic
11	2.47*	2.37 ± 0.07*	Antagonistic	10.51	9.60 ± 2.50	Additive
13	6.23	6.06 ± 0.58	Additive	23.50*	23.74 ± 0.03*	Synergistic
15	8.11	8.08 ± 0.18	Additive	18.89	19.86 ± 1.21	Additive
16	7.52	7.60 ± 0.32	Additive	19.38*	20.58 ± 0.04*	Synergistic
20	5.94*	6.03 ± 0.06*	Synergistic	21.19*	20.85 ± 0.03*	Antagonistic
21	6.37*	6.39 ± 0.16*	Synergistic	21.93*	21.88 ± 0.03*	Antagonistic
22	7.35*	5.87 ± 0.67*	Antagonistic	21.49*	17.81 ± 0.93*	Antagonistic
23	5.50	5.50 ± 0.63	Additive	20.37*	19.81 ± 0.32*	Antagonistic
24	7.31*	7.07 ± 0*	Antagonistic	23.81*	19.31 ± 0.02*	Antagonistic
25	6.15*	5.98 ± 0.08*	Antagonistic	19.88	19.63 ± 0.59	Additive
Optimum	6.75	7.05 ± 0.94	Additive	22.30*	19.37 ± 0.94*	Antagonistic

Asterisk (*) indicated the significantly different values between the prediction and the experimental result

The interaction effects of the plant components in a polyherbal mixture vary widely. As we observed in the mixtures of turmeric and ginger, as well as cinnamon and ginger, a combination of an equal ratio of lemongrass and curry leaf, lemongrass and ginger, or lemongrass and turmeric generated antagonistic interactions with DPPH scavenging activity (Rahim et al., 2020).

The synergistic effects observed in some formulations in this study were also reported for the combination of turmeric, ginger, black pepper, calamansi, and bee honey in a ratio of 2-2-2-1-2 (Yap et al., 2023). In addition, the DPPH scavenging activity that was observed in most formulations evaluated in this study, including the optimum formulation, was recorded between cinnamon and lemon balm essential oils, as well as in the mixture of bitter, bitter

vine, turmeric, *wan chak motluk* curcuma, and seed-under-leaf crude drugs in a ratio of 5-5-3-3-3 (Hartanti & Hamad, 2023; Mapeka et al., 2022). Our results on the antagonistic FRAP in the optimum formulation were similar to those of turmeric, ginger, black pepper, calamansi, and bee honey mixture (Yap et al., 2023).

CONCLUSION

Java tea, turmeric, cinnamon, and crude ginger drugs were of good quality. The five-component formulation with optimum antioxidant activity consisted of 20% java tea, 20% turmeric, 40% seed-under-leaf, 10% cinnamon, and 10% crude ginger drugs. Formula 21 (a mixture of java tea and seed-under-leaf crude drugs) showed the highest synergistic effect toward DPPH scavenging activity, while Formula 13 (a combination of equal seed-under-leaf and ginger crude drugs) showed one toward FRAP.

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