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Optimization and Interaction Effects of a Traditional Antioxidant Polyherbal Drink using Design Expert

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ABSTRACT
A polyherbal drink consisting of ginger, aromatic ginger, turmeric and rice starch is utilized by the people of Baturraden, Central Java, to treat masuk angin. This study contributed to optimize the ratio of the component crude drugs for obtaining a formulation with most favorable antioxidant properties. Twenty formulations were prepared by mixing powdered ginger, aromatic ginger, turmeric, and rice starch in different weight ratios. Each formulation was extracted using the decoction method for 30 minutes, with a crude drug-to-water ratio of 1:20. The extracts were subjected to the standard total flavonoid content (TFC), total phenolic content (TPC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assays. The TFC, TPC, and DPPH scavenging activity was used as the response for predicting the interaction effect and optimal composition of the formulation by simplex lattice design (LSD). The results showed that the highest TFC, TPC, and DPPH scavenging activity was observed in Formula 8 (turmeric only, 10.0 ± 0.01 mg QE/g), Formula 3 (a combination of equal ratios of ginger and turmeric, 9.01 ± 0.01 mg GAE/g), and Formula 10 (rice starch only, 333.76 ± 0.04 mmol TE/g), respectively. The antagonistic interaction effect on the DPPH scavenging activity was observed in most formulations. A synergistic effect was only predicted to occur in a combination of ginger, aromatic ginger, and turmeric. The incorporation of rice starch into the formulations resulted in an antagonistic interaction. These findings support the refinement of traditional polyherbal drinks by optimizing crude drug ratios to enhance antioxidant potential.

KEYWORDS
Antioxidant; Ginger; Optimization; Polyherbal formulation; Turmeric

1. INTRODUCTION

Polyherbal formulations are composed of multiple medicinal plant components, and have long been utilized in Indonesian traditional medicine. The formulations are preferable over single-constituent ones for their synergistic therapeutic effects. Combining various components is believed to broaden the spectrum of bioactive compounds and enhance overall biological activity, including antioxidant potential [1], [2]. Such antioxidant properties are primarily attributed to phenolic compounds and flavonoids. These compounds exert their activity by donating hydrogen atoms or electrons to neutralize free radicals or mediate the reduction-oxidation process [3]. The interactions between active constituents of the components in the formulation may result in synergistic, additive, or antagonistic effects, which significantly affect the safety and efficacy during use [4]. Hence, the optimization of the components is required to design a polyherbal formulation with synergistic effects that satisfies the safety and efficacy profile [5].

People in Baturraden, Central Java, utilized a traditional beverage obtained from a decoction of the mixture of ginger (*Zingiber officinale* Roscoe), aromatic ginger (*Kaempferia galanga* L.), turmeric

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(*Curcuma longa* L.), and rice (*Oryza sativa* L.) starch for alleviating *masuk angin* [2]. *Masuk angin* refers to a general state of malaise accompanied by symptoms such as fatigue, chills, and bloating resulting from bodily imbalance [6]. In *masuk angin* treatment, the antioxidant effects of the agent act as a supporting activity [7]. Ginger, aromatic ginger, and turmeric are *Zingiberaceae* plants, which contain antioxidants such as gingerols, shogaols, curcuminoids, and phenolic acids [8], [9], [10]. Those compounds contribute to the antioxidant properties of the polyherbal drink. Rice starch, although typically used as an excipient to maintain viscosity and increase the sensorial aspects of the drink, can also exert antioxidant activity due to the generation of reducing sugars during the extraction process of the drink [11]. There is a wide variation in the composition and ratio of each component of this *masuk angin* polyherbal drink, as every household in Baturraden has its signature concoction recipe. On the other hand, there is limited scientific evidence on whether component interactions in the formulation lead to the expected synergistic effects.

Previous studies on herbal mixtures have reported varying relationships between total phenolic content (TPC), total flavonoid content (TFC), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. While some formulations exhibit strong positive correlations between TPC and antioxidant activity, others demonstrate weak or even absent correlations, highlighting the complex interplay between phytochemical composition and antioxidant properties [12], [13], [14]. Furthermore, simplex lattice design (SLD) offers a powerful way to optimize formulations and analyze ingredient interactions. Yet, it has not been applied to traditional Indonesian polyherbal drinks combining phenolic-rich rhizomes with carbohydrate-based components. This study aimed to optimize the proportions of ginger, aromatic ginger, turmeric, and rice starch in a traditional polyherbal drink using SLD, with the primary focus on maximizing antioxidant potential as assessed by DPPH radical scavenging activity. The interaction effects between components were evaluated to identify synergistic and antagonistic relationships.

2. MATERIALS AND METHODS

2.1. Materials

Crude drugs of ginger, aromatic ginger, and turmeric were obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT) Tawangmangu, Indonesia. Rice was purchased from the local market in Purwokerto. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), acetic acid, sodium acetate, sodium carbonate, aluminium chloride, Folin-Ciocalteau reagent, gallic acid, quercetin, Trolox, deionised water, ethanol, and methanol were purchased from Sigma-Aldrich (USA).

2.2. Formula Design of Polyherbal Formulation

The composition of the formulations was designed by the simplex lattice design of the Design Expert (Stat-Ease, USA) program using ratios of ginger, aromatic ginger, turmeric, and rice starch as the factors and the DPPH scavenging activity as the response [15].

2.3. Polyherbal Drink Preparation

The powdered crude drugs of ginger, aromatic ginger, turmeric, and rice starch were weighed in the specified ratios as designed by SLD (Table 1) and mixed homogenously with a total weight of 50 g for each formulation. The mixture of crude drugs was boiled in water for 30 minutes in a crude drug-to-solvent ratio of 1:20. Upon reaching room temperature, the concoctions were filtered, and the obtained extracts were freshly used for the assays [2].

2.4. Evaluation of TFC, TPC, and DPPH Scavenging Activity

The TFC, TPC, and DPPH scavenging activity of the polyherbal drinks was analysed according to their previous reports [12], [16]. Each appropriately diluted sample was mixed with 10% aluminium chloride solution, 1M sodium acetate solution, and water to determine the TFC. After being allowed to sit for 30 min, the absorbance was measured at 416 nm using a UV-Vis spectrophotometer (Shimadzu, Japan). Quercetin was used as the standard, and the TFC was reported in quercetin equivalent (QE)/g crude drugs. The appropriately diluted samples were mixed with the 7.5% Folin-Ciocalteau reagent to determine TPC.

The absorbance was measured at 751 nm after being allowed to sit for 60 minutes. Gallic acid was used as the standard, and TPC was reported in mg gallic acid equivalent (GAE/g). The appropriately diluted samples were incubated with 25 µg/ml DPPH solution for 30 min at room temperature in dark conditions. The absorbance was read at 517 nm. The inhibitory percentage was calculated from the relative comparison of the difference in DPPH absorbance with the sample to the absorbance of DPPH alone. The DPPH scavenging activity was expressed as mmol Trolox equivalent (TE/g).

2.5. Determination of The Interaction Effect and The Optimum Formulation

SLD was utilized to predict the interaction effect, optimum formulation and the suitability of the model based on the DPPH scavenging activity of 20 polyherbal formulations [15]. In addition, the interaction effect was also evaluated by the comparison and difference methods [4], [17], which were calculated by equations (1) and (2).

$$\text{Predicted value} = \frac{((Va \times fa) + (Vb \times fb) + \ldots(Vn \times fn))}{(fa + fb + \ldots fn)} \quad (1)$$

$$\text{Difference} = \frac{(Vmix \times 100)}{(Va + Vb + \ldots Vn)} - 100 \quad (2)$$

where V is actual DPPH scavenging activity, f is fraction of each component, mix is formulation the mixture of components, and a, b, n are components.

2.6. Data Analysis

The effects of formula composition on the TFC, TPC, and DPPH scavenging activity were individually evaluated by two-way ANOVA. The value of each variable between formulations was compared by Duncan's test. The actual and predicted DPPH scavenging activity were compared by a paired T-test. The effect and difference were significant at $p \leq 0.05$. The two-way ANOVA and post hoc test were performed using SPSS (IBM Statistics, USA).

3. RESULTS AND DISCUSSION

SLD suggested 20 formulation designs with eight single-component, seven two-component, and five four-component formulations. There were 15 different formulations in total, as five formulations were replicated; they were those containing a single component (ginger, aromatic ginger, turmeric, and rice starch only) and a combination of aromatic ginger and turmeric (Table 1).

The quercetin linear regression of $y=0.0179x–0.0307$ ($R^2=0.9908$) was used to calculate TFC in the polyherbal drink. The composition of the components in the polyherbal formulation significantly affected the TFC of the drink ($p=0.000$) (Figure 1, Table 2). Individually, turmeric showed the highest TFC, while rice starch did not contain flavonoids. Formula 3 (equal ratio of ginger and turmeric) and Formula 9 (a combination of turmeric and rice starch) were the drinks with the highest TFC. Formula 11, containing four components with ginger as the dominant one, contained a relatively high level of flavonoids. Nevertheless, flavonoids are minor compounds in ginger, aromatic ginger, and turmeric [9], [18], [19]. Flavonoids in turmeric are particularly in glycosidic forms of flavonol and isoflavone [18].

The gallic acid linear regression of $y=0.0057x+0.0258$ ($R^2=0.993$) was used to calculate TPC in the drinks. Similarly, the composition of the formulation affected the TPC of the polyherbal drink ($p=0.00$). Ginger showed the highest TPC and rice starch has a negligible level of phenolic compounds. Formula 3 showed the highest value, and the relatively high TPC was also observed in Formula 11, and Formula 15 (all four components in an equal ratio) (Figure 1, Table 2). Curcuminoids, the major compounds in turmeric, are phenolic compounds, and so are gingerols, shogaols, paradols, gingerdiols, and phenolic acids of ginger [20], [21]. Aromatic ginger contains phenolic acids [22].

Table 1. Polyherbal formulation design by simplex lattice design.

Formulation	Weight ratio (%)			
	Ginger	Aromatic ginger	Turmeric	Rice starch
Formula 1	100	0	0	0
Formula 2	50	50	0	0
Formula 3	50	0	50	0
Formula 4	50	0	0	50
Formula 5	0	100	0	0
Formula 6	0	50	50	0
Formula 7	0	50	0	50
Formula 8	0	0	100	0
Formula 9	0	0	50	50
Formula 10	0	0	0	100
Formula 11	62.5	12.5	12.5	12.5
Formula 12	12.5	62.5	12.5	12.5
Formula 13	12.5	12.5	62.5	12.5
Formula 14	12.5	12.5	12.5	62.5
Formula 15	25	25	25	25
Formula 16	0	100	0	0
Formula 17	0	0	100	0
Formula 18	100	0	0	0
Formula 19	0	0	0	100
Formula 20	0	50	50	0

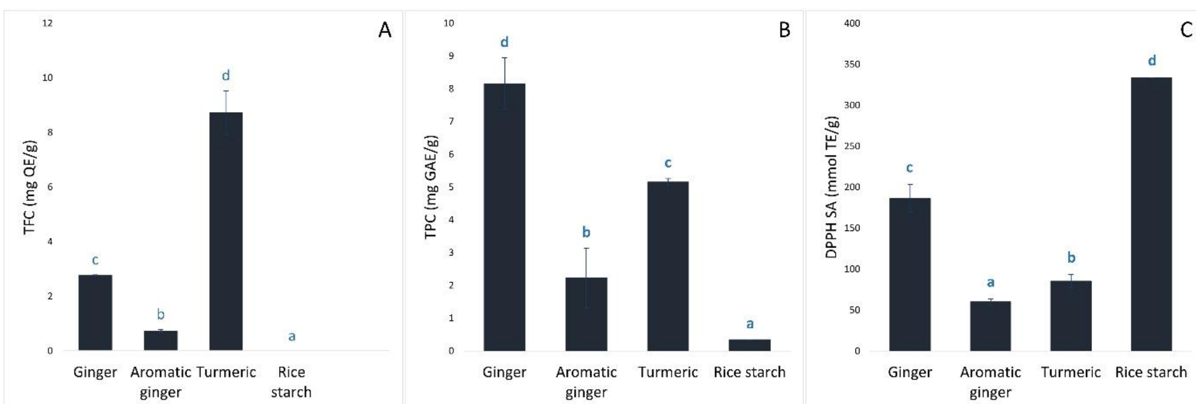

Figure 1. Profile of TFC (A), TPC (B), and DPPH scavenging activity (C) of the individual components of the polyherbal drink.

The Trolox linear regression of y=0.2438x–1.759 (R^2=0.991) was used to calculate the DPPH scavenging activity of the drinks. DPPH scavenging activity was also significantly affected by the formulation composition ($p = 0.014$). Rice starch showed the highest DPPH scavenging activity. The high DPPH scavenging activity of rice starch might relate to the generation of reducing sugars from starch during extraction. After being boiled for 30 minutes, powdered rice starch underwent gelatinization, releasing amylose. Amylose is prone to undergo hydrolysis to generate reducing sugars, including glucose. The free aldehyde or ketone group of glucose is capable of reducing DPPH [23]. Formula 3 and Formula 11 also showed high DPPH scavenging activity (Figure 1, Table 2). The free radical neutralizing activity of ginger, aromatic ginger, and turmeric has been reported elsewhere [22], [24], [25]. The higher DPPH scavenging activity of ginger than turmeric and aromatic ginger in this study was previously also reported in Indonesian studies [26], [27].

Table 2. TFC, TPC, and DPPH scavenging activity of the formulation containing multiple components.

Formulation	TFC (mg QE/g)	TPC (mg GAE/g)	DPPH (mmol TE/g)
Formula 2	1.17 ± 0.02^{ab}	5.51 ± 0.07^{f}	101.30 ± 0.17^{g}
Formula 3	8.46 ± 0.07^{h}	9.06 ± 0.09^{g}	103.73 ± 0.22^{gh}
Formula 4	4.27 ± 0.03^{d}	3.30 ± 0.04^{c}	99.07 ± 0.09^{f}
Formula 6	4.39 ± 0.20^{d}	4.34 ± 0.42^{e}	66.40 ± 9.15^{b}
Formula 7	0.94 ± 0.01^{a}	1.61 ± 0.02^{a}	55.08 ± 0.23^{a}
Formula 9	7.45 ± 0.07^{g}	3.36 ± 0.03^{c}	72.39 ± 0.27^{cd}
Formula 11	6.21 ± 0.08^{f}	5.35 ± 0.07^{f}	113.79 ± 0.25^{i}
Formula 12	3.90 ± 0.07^{c}	3.73 ± 0.00^{d}	65.88 ± 0.09^{bc}
Formula 13	4.21 ± 0.09^{d}	4.13 ± 0.04^{e}	73.68 ± 0.31^{cd}
Formula 14	2.76 ± 0.07^{b}	2.42 ± 0.03^{b}	69.19 ± 0.12^{bc}
Formula 15	5.14 ± 0.02^{e}	4.11 ± 0.07^{e}	80.76 ± 0.62^{e}

Note: Different subscripted alphabet in each column represented significantly different TFC, TPC, and DPPH scavenging activity (n=3).

Phenolic compounds donate hydrogen atoms or electrons to scavenge free radicals. This mechanism underlines the antioxidant activity of ginger, aromatic ginger, and turmeric [26], [28]. The moderate to strong correlations between TPC and DPPH scavenging activity were previously reported in all three *Zingiberaceae* plant extracts used to prepare the polyherbal drink in this study [29], [30]. However, the mixture of components in the polyherbal formulation showed a significant, negative, moderate correlation between TPC and DPPH scavenging activity (R=-0.675, p=0.001). Correlation between TPC and DPPH scavenging activity is widely varied according to the compositions and their ratios. For example, the correlation of those parameters in an Indonesian antidiabetic formulation, which consisted of the king of bitter, bitter vines, Java tea, papaya leaf, and pink-and-blue ginger, was weak [31]. A polyherbal drink from a mixture of turmeric, Java tea, and seed-under-leaf showed a very strong correlation between TPC and DPPH scavenging activity, while that of six commercial Indian polyherbal formulations indicated for diabetes treatment was none [14], [32].

A cubic model of the DPPH scavenging activity equation is suitable for capturing complex relationships in mixture designs (equation (3)). The model explained a significant portion of the variability in DPPH scavenging activity relative to the noise and demonstrated that the combination of ginger, aromatic ginger, turmeric, and rice starch, along with their interactions, significantly affects the free radical scavenging activity (F=84.94 and p<0.0001). On the other hand, the model fitted the experimental data well; hence, it accurately predicted the DPPH scavenging activity of the formulation based on different component proportions (R^2=0.9946). The model also fitted the data well, and there's no evidence of systematic error (Lack of fit=2.81, not significant). Other than the cubic model to predict its DPPH scavenging activity, the quadratic one was also reported in a Malaysian polyherbal formulation consisting of lemongrass, curry leaf, turmeric and ginger [13]. A quadratic model was also fitted to predict the radical neutralising activity of a polyherbal drink, which consisted of Java tea, turmeric, seed-under-leaf, cinnamon, and ginger [12].

$$\begin{aligned} DPPH = {} & (185.96 \times A) + (59.81 \times B) + (85.01 \times C) + (333.42 \times D) - (91.69 \times A \times B) - \\ & (132.37 \times A \times C) - (647.82 \times A \times D) - (26.81 \times B \times C) - (571.88 \times B \times D) - \\ & (553.01 \times C \times D) + (6569.69 \times A \times B \times C) - (1418.56 \times A \times B \times D) - \\ & (1857.76 \times A \times C \times D) - (1369.44 \times B \times C \times D) \end{aligned} \quad (3)$$

where A is ginger, B is aromatic ginger, C is turmeric, and D is rice starch.

Rice starch was predicted to make the most significant contribution to the DPPH scavenging activity of the formulations, followed by ginger, turmeric, and aromatic ginger. The reducing sugars in boiled rice starch and phenolic compounds in the rhizomes were likely responsible for the radical scavenging effects.

Hydroxyl groups, especially the ortho-hydroxy, along with conjugated double bonds and aromatic rings of phenolic compounds, donate hydrogen atoms to neutralize free radicals [33]. The free radical scavenging ability of reducing sugars comes mainly from their hydroxyl groups to donate hydrogen and their aldehyde/enediol to transfer electrons and exert reducing ability [34].

Table 3. Prediction of the interaction effect between components.

Formulation	The interaction effect predicted by				
	LSD equation	Comparison method		Difference method	
		Predicted/actual value (mmol TE/g)	Interpretation	Difference (%)	Interpretation
Formula 2	Antagonistic	123.23/101.30	Antagonistic	-58.90	Antagonistic
Formula 3	Antagonistic	135.83/103.73	Antagonistic	-80.95	Antagonistic
Formula 4	Antagonistic	260.04/99.07	Antagonistic	-74.85	Antagonistic
Formula 6	Antagonistic	72.76/66.40	Antagonistic	-54.37	Antagonistic
Formula 7	Antagonistic	196.96/55.08	Antagonistic	-86.02	Antagonistic
Formula 9	Antagonistic	209.56/72.34	Antagonistic	-82.74	Antagonistic
Formula 11	-	176.35/113.84	Antagonistic	-82.90	Antagonistic
Formula 12	-	113.28/65.93	Antagonistic	-90.09	Antagonistic
Formula 13	-	125.88/73.73	Antagonistic	-88.92	Antagonistic
Formula 14	-	250.08/69.24	Antagonistic	-87.87	Antagonistic
Formula 15	-	166.40/80.71	Antagonistic	-83.16	Antagonistic

The higher predicted DPPH scavenging activity compared to the actual one in the comparison method represented antagonistic effects, and was observed in all multi-component formulations. The antagonistic effects in the formulations were also confirmed by the negative value obtained in the difference methods. Prediction by SLD demonstrated that the least activity reduction in two-component formulations was observed in the combination of aromatic ginger and turmeric, while the highest reduction was in that of ginger and rice starch (Table 3). Phenolic compounds and reducing sugars act as antioxidants of the rhizomes and rice starches through different mechanisms, leading to antagonistic interaction effects among the components [33], [34]. Our results were different from the Malaysian study, reporting that the combination of ginger and turmeric resulted in a synergistic interaction effect toward DPPH scavenging activity [35].

Only a combination of ginger, aromatic ginger, and turmeric exerted the synergistic interaction effect, with the other three-component combinations resulting in reduced radical scavenging activity due to their antagonistic effects. The least negative effect on DPPH scavenging activity was observed in the combination of aromatic ginger, turmeric, and rice starch. Individual ginger, aromatic ginger, and turmeric moderately contributed to their DPPH scavenging activity; however, when they were combined, they created a powerful synergistic effect toward the activity. Although rice starch is the most potent radical scavenging agent on its own, its combination with two or three other components tends to generate negative interactions. The predicted formulation with the optimum DPPH scavenging activity consisted of 0.53% ginger, 0.2% aromatic ginger, 0.22% turmeric, and 0.05% rice starch. This combination was predicted to show TFC, TPC, and DPPH scavenging activity of 6.72 mg QE/g, 4.52 mg GAE/g, and 223.28 mmol TE/g, respectively. The desirability of this predicted optimum formulation was 0.58, which indicated that the predicted formulation partially meets the desired DPPH scavenging activity. It likely suggested the presence of antioxidants in each component significantly interacted with one another, which eventually generated the antagonistic effects.

4. CONCLUSION

The mixture of ginger, aromatic ginger, turmeric, and rice starch in a traditional polyherbal formulation generated beverages with different TFC, TPC, and DPPH scavenging activity. Antagonistic interaction effects were observed in most formulations, but a synergistic effect was predicted in the formulation of ginger, aromatic ginger, and turmeric. The optimum formulation was expected to be the mixture of 0.53% ginger, 0.2% aromatic ginger, 0.22% turmeric, and 0.05% rice starch.

AUTHOR CONTRIBUTION

Dwi Hartanti: Writing (original draft), writing (review & editing), formal analysis, supervision, conceptualization, funding acquisition. **Alwani Hamad**: Writing (review & editing), formal analysis, supervision, conceptualization, funding acquisition. **Satriyo Krido Wahono**: Writing (review & editing), formal analysis, conceptualization, funding acquisition. **Addien Anugerah Insani**: Investigation, writing (review & editing). **Firman Wicaksana**: Investigation, writing (review & editing).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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