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Original Article

POLYHERBAL FORMULATION OPTIMIZATION FROM *CLITORIA TERNATEA*, *ROSMARINUS OFFICINALIS* AND *AQUILARIA MALACCENSIS* USING SIMPLEX LATTICE DESIGN

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ABSTRACT

Objective: This study aimed to optimize the composition of *Clitoria ternatea* flowers, *Rosmarinus officinalis* herbs, and *Aquilaria malaccensis* leaves as a polyherbal formulation.

Methods: The polyherbal formulation (PHF) was systematically optimized using a simplex lattice design generated by Design Expert software. The selected independent variables were the percent of *C. ternatea* flowers extract (X1), the percent of *R. s officinalis* herbs extract (X2), and the percent of *A. malaccensis* leaves extract (X3). The dependent variables were total phenolic contents (Y1) and 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical scavenging activity (Y2).

Results: The results showed that the optimum composition of PHF was *C. ternatea* flowers extract (10%), *R. officinalis* herbs extract (80%), and *A. malaccensis* leaves extract (10%) to obtain 135.794 mg GAE/g dried extract for total phenolic contents and 22.879 µg/ml (IC_{50}) for DPPH radical scavenging activity.

Conclusion: The findings suggest that the polyherbal formulation consisting of *C. ternatea* flowers (CTF), *R. officinalis* herbs (ROH), and *A. malaccensis* leaves (AML), when formulated with the optimal composition has the potential to enhance the total phenolic content and antioxidant activity.

Keywords: Polyherbal formulation, Antioxidants, Optimization, Simplex lattice design

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INTRODUCTION

The impact of air pollution on human health is significant and widely recognized as the foremost environmental health concern globally. The combination of accelerated urban expansion and rapid population growth has impacted air quality deterioration substantially. As a consequence, poorer nations tend to experience the most significant impact. Nevertheless, the correlation between air pollution and mortality remains apparent in nations whose pollution levels are significantly lower than the established goal requirements [1]. There is a growing consensus within the scientific community regarding the ability of breathed pollutant gases to induce oxidative stress, which is a fundamental mechanism contributing to the development of many diseases [2].

Oxidative stress refers to an imbalance between reactive oxygen species' generation and removal mechanisms [3]. Reactive oxygen species (ROS) are typically synthesized within cells at minimal concentrations and play a crucial role in preserving cellular homeostasis and functionality [4]. ROS, including singlet oxygen and free radicals, are typically produced within the extracellular and intracellular milieus due to routine metabolic activities and electron transfer reactions. Nevertheless, in instances where the quantity of ROS surpasses the typical state of equilibrium, these ROS will initiate the process of extracting electrons from the lipid membrane, which is the most susceptible component of the cell. Oxidative stress is a physiological state characterized by a sudden elevation in the concentration of ROS [5]. The phenomenon of oxidative stress harms the functional and structural integrity of healthy cells inside the body due to its targeted assault. So far, free radicals have been involved in the pathophysiology of over 50 illnesses [6]. Examples of serious ailments include cancer, diabetes, hepatotoxicity, nephrotoxicity, and osteoarthritis, among others [5].

Antioxidants are chemical compounds that can impede the process of oxidation and the generation of free radicals. Antioxidant molecules can safeguard metabolic processes against the detrimental impacts of ROS and oxidative stressors. Antioxidants derived from natural sources such as vegetables, fruits, and plants have been widely recognized for effectively neutralizing ROS or free radicals. Hence, instead of synthetic antioxidants with deleterious consequences, there is a preference for naturally derived antioxidants with proven botanical provenance [7]. Historically, natural chemicals have been employed to prevent and treat a wide range of ailments on a global scale. Consequently, there has been an increasing inclination towards evaluating the significance of plant and food-derived bioactive compounds, such as polyphenols, which have been reported to possess antioxidant, anti-inflammatory, antimicrobial, anti-angiogenic, and regenerative properties for cancer, cardiovascular diseases, neurological disorders, and skin rejuvenation. Moreover, it has been observed that polyphenols possess the ability to stimulate activation [8-10]

Polyphenols are a class of secondary metabolites found in plants, characterized by their chemical structure consisting of aromatic phenolic rings [11]. The primary sources of their dietary intake consist of vegetables, fruits, legumes, red wine, tea, and coffee [12]. Polyphenols can be categorized into subgroups based on the number of phenol rings they possess and the structural components that connect them [13].

ROS and reactive nitrogen species (RNS) both cause damage to macromolecules, so polyphenolic compounds are crucial in protecting them. These compounds also enhance antioxidant status, lipid profile, and vascular and endothelial function [14]. The antioxidant attributes of polyphenols, which are comparable to or surpass those of vitamin E, are attributed to the existence of hydroxyl groups that are easily susceptible to oxidation. Therefore, these molecules exhibit efficacy in scavenging ROS [15]. Consequently, polyphenols are widely acknowledged as powerful antioxidants [16].

Numerous polyphenolic compounds, over 8,000 in number, have been successfully identified across a diverse range of plant species [17]. Several plants containing polyphenols have been proven to have antioxidant activity, such as *Clitoria ternatea*, [18], *Rosmarinus officinalis* [19], and *Aquilaria malaccensis* [20]. Previous studies have reported that the total phenolic content of CTF was measured to be

57.51 mg GAE/g DE [21], while its antioxidant activity, as determined by the DPPH assay, was found to be (IC_{50}) 26.10µg/ml [22]. These results indicate that CTF exhibits a high level of antioxidant activity, falling within the very strong antioxidant activity category.

CTF comprises phenolic chemicals, specifically the anthocyanins referred to as ternatins and the flavonols kaempferol, quercetin, and mirecithin. Research conducted on several extracts of CTF has revealed its *in vitro* and *in vivo* characteristics, which include antioxidant, antihyperglycemic, antihyperlipidemic, antibacterial, and hepatoprotective capabilities [23]. The primary basis for the therapeutic properties of anthocyanins is predominantly ascribed to their antioxidative capacities. The structural composition of anthocyanins enables them to exhibit direct antioxidant activity towards radicals through two distinct processes known as hydrogen atom transfer (HAT) and single electron transfer (SET) [24].

ROH is a widely utilized botanical species within the realm of spices, owing to its longstanding historical usage for its therapeutic attributes [7]. According to Jakubczyk *et al.*'s investigation, the total phenolic content of ROH was 44.23 mg GAE/l with a 33.53% inhibitory activity (IC_{50}) against oxidative compounds [25]. The ROH extracts, which were found to include rosmarinic acid, demonstrated a high level of efficacy in safeguarding both bacterial and human cells from the genotoxic effects caused by direct and indirect-acting mutagens, as well as a ROS-inducing agent [26].

AML (*Aquilaria malaccensis* Lam.) is utilized in several civilizations, mostly for its therapeutic properties associated with anti-inflammatory and related actions. The plant treats several ailments, such as rheumatism, arthritis, bodily discomfort, asthma, and gout, as a laxative, aphrodisiac, and stimulant. Additionally, it has been employed as a therapeutic measure for rheumatism, asthma, and liver illness [27]. In addition to its palatable flavour, agarwood tea is known for its potential benefits when consumed during cold or inclement weather since it may enhance our immune system. In addition, there are several other advantages associated with this practice, including the alleviation of headaches, enhancement of male endurance, prevention of common colds, and soothing of stomach discomfort [28].

Prior research has documented that the overall phenolic content of AML was quantified at 18.62 mg GAE/g DE [29]. Additionally, the antioxidant activity of AML, as assessed using the DPPH assay, was observed to have an IC_{50} value of 77.21µg/ml. The findings of this study suggest that CTF demonstrates a notable degree of antioxidant activity, placing it in the group of compounds having strong antioxidant properties [30].

The use of a polyherbal formulation, which combines two or more plants with various phytoconstituents that have either similar or dissimilar medicinal potential, has resulted in the therapy of human illnesses [31]. The therapeutic system's concept of PHF is impressive. PHF comprises different plant's active ingredients that are highly effective at treating disease, have low adverse effects, and are affordable, convenient, and environmentally friendly [32-33]. Compared to the separate extracts, mixtures of plants containing these elements may demonstrate greater activity. On the other hand, the existence of numerous ingredients may cause chemical incompatibility, which may result in instability [33]. The primary objective of this study is to ascertain the most favorable combination of *C. ternatea* flowers, *R. officinalis* herbs, and *A. malaccensis* leaves in PHF to enhance the overall phenolic content and antioxidant activity of these formulations.

MATERIALS AND METHODS

Materials

The dried flowers of *C. ternatea*, dried herbs of *R. officinalis*, and dried leaves of *A. malaccensis* were obtained from local markets in Bandung and North Jakarta. These specimens were authenticated by the Herbarium Jatinangor, Padjadjaran University, Indonesia. All other reagents and compounds employed in the investigation were of analytical grade.

Extract preparation

The aqueous extracts of all-natural ingredients were made using the slightly modified ultrasound-assisted extraction (UAE) method following Nurrahmah *et al.*, [34]. The solid-solvent ratio was maintained at 1:20, and the extraction process was conducted at 50 °C for 60 min with a power setting of 120 watts. The extracts obtained were concentrated in a water bath.

Phytochemical screenings

Phytochemical screens were undertaken to ascertain the presence of several chemical components in the extract, such as alkaloids, flavonoids, phenolics, tannins, saponins, quinones, triterpenoids, monoterpenes, and sesquiterpenes.

Polyherbal formulation (PHF) preparation

CTF, ROH, and AML aqueous extracts were separately dissolved in methanol (1000µg/ml). Design Expert Software® ver. 13.0.5 used Simplex Lattice Design (SLD) to design the polyherbal formulation. Various ratios of percent *C. ternatea* flowers aqueous extract (X1), percent *R. officinalis* herbs aqueous extract (X2), and percent *A. malaccensis* leaves aqueous extract (X3) and dependent variables were total phenolic contents (Y1) and 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical scavenging activity (Y2). The investigation examined a total of 14 different PHF combinations [35]. The design layout of the corresponding sample is displayed in table 1.

Table 1: PHF combination layout

Run	*C. ternatea* (%)	*R. officinalis* (%)	*A. malaccensis* (%)
1	10	80	10
2	21.667	56.667	21.667
3	10	10	80
4	45	10	45
5	45	45	10
6	33.333	33.333	33.333
7	80	10	10
8	45	45	10
9	56.667	21.667	21.667
10	10	45	45
11	21.667	21.667	56.667
12	10	10	80
13	10	80	10
14	80	10	10

Total phenolic content (TPC)

Total phenolics were determined in each PHF combination using the Folin-Ciocalteu (FC) assay, following previous studies by Molole *et al.*, 2022; Lohvina *et al.*, 2022; and Ridwan *et al.*, 2023, with modifications [37–39]. TPC was measured by reacting 1 ml of PHF (1000 µg/ml) with 5 ml of FC reagent (1:10 in water for injection) and incubating for 5 min. Then, 4 ml of 7.5% Na2CO3 aqueous solution was added. After 45 min of incubation in the dark at room temperature, absorbance was measured at 782 nm using a UV-VIS

spectrophotometer (Shimadzu UV1780, Kyoto, Japan). As a standard, gallic acid was used. Gallic acid was used to create the calibration curve, and the concentration ranged from 10 to 50 µg/ml. Each PHF's gallic acid content was determined using the regression equation and its absorbance. These values were then converted to milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g) according to the TPC.

Antioxidant activity

A DPPH (2′2-diphenyl-l-picrylhydrazyl) free radical assay was used to find out how well PHF scavenged free radicals. The present study employed a methodology similar to previous approaches, although with some modifications [40–42]. One ml of PHF with concentrations of 12.5, 25, 50, 100, and 200 g/ml was mixed with a new two ml methanolic solution of DPPH (40 g/ml). The final mixture was thoroughly mixed and left at room temperature in the dark for 45 min. Then, a UV-Vis spectrophotometer was used to capture this mixture at 516 nm. As a blank, methanol was used. The scavenging capacity of PHF was measured, and the IC_{50} was determined.

RESULTS AND DISCUSSION

Extraction and yield extract

All extracts were prepared with ultrasound-assisted extraction by mixing 100 g of dry powder of natural ingredients with 2 liters of distilled water (1:20). The yield value is shown in table 2. Until it was utilized, the dried extract was kept in a refrigerator at 4 °C.

Table 2: Yield value of extract

Extract	Yield value (%)
C. ternatea	61.55
R. officinalis	38.73
A. malaccensis	12.30

Phytochemical screenings

Secondary metabolites were discovered during the phytochemical screening of the aqueous extracts of *C. ternatea*, *R. officinalis*, and *A. malaccensis*. Phytochemical screenings are shown in table 3. The phytoconstituents of a medicinal plant, either alone or in combination, determine its therapeutic value. Finding the phytochemicals in a plant can help predict its pharmacological activity, which in this present study is antioxidant activity [40].

Total phenolic content (TPC)

Gallic acid equivalents were used to represent the total phenolic content of PHF as determined by the Folin-Ciocalteu assay (the equation of the standard curve: y = 0.0084x+0.0002, r2 = 0.9957). All of the extracts used in this present study have phenolic content due to phytochemical screenings, and these findings are related to previous studies [40–42]. The total phenolic content (table 4) ranged between 41.677+0.113 and 121.85+0.056 mg GAE/g dried extract.

Table 3: Phytochemical screening of aqueous extract

Secondary metabolites	*C. ternatea*	*R. officinalis*	*A. malaccensis*
Alkaloids	-	+	-
Flavonoids	-	-	+
Phenolics	+	+	+
Tannis	+	+	-
Saponins	+	-	+
Triterpenoids	+	+	+
Monoterpenes and sesquiterpenes	+	-	+
Quinones	+	+	+

+: Presence,-: Absence

Fig. 1 shows the relationship between independent factors of the percent of *C. ternatea, R. officinalis*, and *A. malaccensis* on total phenolic content. However, the large TPC could be seen in the PHF with a large ratio of ROH and AML (orange area of the figure), and ROH had a larger TPC than AML. Using SLD, the equation for total phenolic content was found, and it is given in Eq. 1. The simplex's interior and edges were investigated using SLD. It is simple to compute the model equation's coefficients [43].

$$Y = 49.11A+110.13B+108.88C \text{ ..... (Eq. 1)}$$

Y was the response of total phenolic content (mg GAE/g), A was *C. ternatea*'s percent, B was *R. officinalis*'s, and C was *A. malaccensis*. From Eq.1, it is clear that *C. ternatea*, *R. officinalis*, and *A. malaccensis* all impacted the total phenolic content, with ROH having a more decisive influence than the other two.

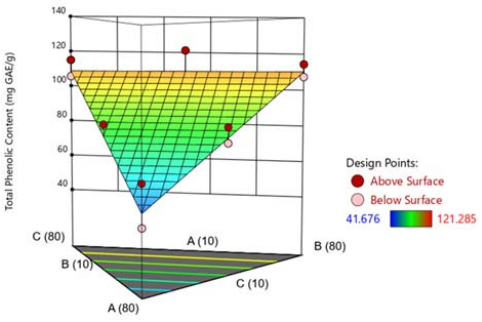

Fig. 1: Response surface of PHF for TPC

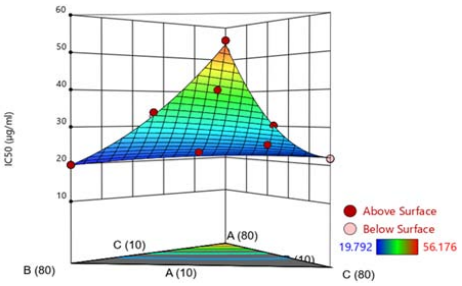

Fig. 2: Response surface of PHF for IC_{50}

Antioxidant activity

By employing a UV-Vis spectrophotometer at a wavelength of 516 nm, the antioxidant impact of the PHF was assessed using the DPPH method. On the PHF at varied extract combinations, the measurement of the IC_{50} value was done. Due to their concentrations being between 12.5 and 200 µg/ml, the study findings indicated that the PHF had a very strong antioxidant effect. Better antioxidant activity might be seen in the decreased IC_{50} value (table 4) [44]. The antioxidant activity of PHF proved that the higher the ROH, the IC_{50} decreased (fig. 2). ANOVA statistics showed that there was a significant difference ($P<0.05$) among the five formulas evaluated. Using SLD, the equation to describe IC_{50} was as in Eq. 2.

$$Y = 54.89A+19.74B+22.04C-17.27AB-33.48AC+5.76BC \text{ ..... (Eq. 2)}$$

In which Y was the response of IC_{50} (µg/ml), A was the percent of *C. ternatea*, B was the percent of *R. officinalis*, and C was *A. malaccensis*, AB was an interaction between *C. ternatea* and *R. officinalis*, AC was an

interaction between *C. ternatea* and *A. malaccensis*, and BC was an interaction between *C. ternatea* and *A. malaccensis*. Eq. 2 indicated that ROH and AML affected IC_{50} larger than CTF. From the equation findings, that CTF had a negative effect on IC_{50} if it was combined with ROH or AML, which is an indication of an increased composition of CTF that, when combined with ROH or AML, increased the IC_{50} value.

Table 4: Observed values of the polyherbal response

RUN	Total phenolic content (mg GAE/g DE)		DPPH radical scavenging activity (µg/ml)	
	Adjusted	Predicted	Adjusted	Predicted
1	110.110	107.279	19.180	19.792
2	99.738	95.427	22.871	23.268
3	108.850	115.140	21.700	21.700
4	78.995	85.132	29.435	30.128
5	79.625	76.752	31.850	32.806
6	89.367	82.977	26.289	23.293
7	49.140	41.676	54.320	52.747
8	79.625	85.211	31.850	33.920
9	69.253	59.194	37.174	40.498
10	109.480	121.285	21.665	23.341
11	99.108	87.646	22.906	25.138
12	108.850	105.842	21.700	21.810
13	110.110	114.342	19.180	19.816
14	49.140	63.583	54.320	56.176

Numerical optimization using the desirability function and verification

The purpose of this study was to create a PHF of three plants that matched the requirements outlined in the material and methods section. The value of the function with the highest desirability out of the two solutions estimated by the program was selected as the ideal solution, which corresponds to the ratios shown in table 1.

The desirability function was optimized to maximize the mixtures' total phenolics and antioxidant capacity. The results are presented in table 5. The simultaneous optimization, including all responses, suggested that the ternary mixture consisting of 10% X1, 80% X2, and 10% X3 was the most appropriate to obtain the best combination of variables. The combination of aqueous *Clitoria ternatea* flowers, *Rosmarinus officinalis* herbs, and *Aquilaria malaccensis* leaves extract with a ratio of 10:80:10 can decrease the IC_{50} value if compared with previous studies [20, 42, 45].

Table 5: Verification experiments under optimal conditions

Optimal condition: X_1 = 0.1, X_2 = 0.8, X_3 = 0.1 **Desirability: 0.690**			
Response	**Adjusted**	**Predicted**	**Error**
TPC	135.794	110.133	25.661 (23.3%)*
IC_{50}	22.879	19.745	3.134 (15.87%)*

*percent error

The experiment results were higher than the prediction to verify the optimal condition. For the IC_{50}, the experimental was fit with a range of 95% population (low = 15.558, high = 23.932); however, the TPC was unsuitable (low = 94.453, high =125.813). This condition can occur due to the extraction method using an ultrasonic bath. Ultrasonic baths are more economical and easy to handle, but their low reproducibility restricts their use in the extraction [46].

CONCLUSION

The three plants' PHF of phenolic antioxidants was successfully optimized using the response surface methodology. The research findings indicate that the PHF, consisting of *C. ternatea*, *R. officinalis*, and *A. malaccensis* extract at a ratio of 10:80:10, exhibited a total phenolic content of 135.794 mg GAE/g DE. The findings of this study suggest that the utilization of polyherbal formulation can significantly enhance the total phenolic content (TPC) value when compared to individual extracts. The obtained result is consistent with the antioxidant activity exhibited by the polyherbal mixture, which surpasses the antioxidant activity value of each extract, measuring (IC_{50}) 22.879 µg/ml and categorizing it as a very strong antioxidant.

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AUTHORS CONTRIBUTIONS

All the authors contributed equally.

CONFLICT OF INTERESTS

Declared none

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