Synergistic Effects of Polyherbal Formulations on Antioxidant Properties and Functional Group Analysis using FTIR

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Phyllanthus emblica, *Camellia sinensis,* and *Allium sativum L.* (black solo garlic), contain phytochemicals associated with antioxidant properties. However, most studies have focused on these plants individually, thus overlooking the investigation of synergistic effects. Hence, sixteen aqueous extract formulations with varying proportions were developed in this study using a simplex lattice mixture design generated by Design Expert software. Antioxidant properties were quantitatively assessed through colorimetric analyses, measuring total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometrically. Principal component analysis (PCA) was applied to differentiate between the extracts, and Fourier Transform Infrared Spectroscopy (FTIR) was utilised to analyse their functional groups and specific characteristic regions. The results indicated a synergistic effect in most combinations, with *P. emblica* and *C. sinensis* contributing the highest TPC and DPPH values across the formulations. The optimised formulation (36.4 % *P. emblica*, 17.5 % *C. sinensis*, and 46.1 % *A. sativum L.*) yielded 1205.1 ± 20.1 mg GAE/g for TPC and 96.2 ± 0.1 % for DPPH. FTIR spectra revealed the wavenumber ranges associated with functional groups of TPC, which strongly correlated with the chemometric results (R^2 = 1). PCA demonstrated a clear correlation between FTIR data and antioxidant properties. These findings suggest that polyherbal formulations may be useful in the development of natural antioxidant-rich products in the food industry.

Keywords: FTIR Analysis; polyherbal optimisation; principal component analysis; synergistic antioxidant effects

Received: January 2025; Accepted: April 2025

The development of herbal supplements from traditional medicinal plants is proliferating in today's fast-paced world. Medicinal plants are renowned for their unique bioactive compounds, which provide health benefits that surpass those of ordinary plants [1]. These plants are prominently acknowledged for their extensive array of phytochemical compounds, featuring phenolics, flavonoids, tannins, fatty acids, and other active substances believed to possess substantial antioxidant benefits. Additionally, they have been reported to potentially lower the likelihood of several health problems [2]. A defining feature of herbal medicine is its use of herbal combinations and the intricate interactions between their constituents, which can enhance the therapeutic effects [3].

In the present investigation, *Phyllanthus emblica* (PE), *Camellia sinensis* (CS), and *Allium sativum L.* (black solo garlic) (B-AS) were selected for formulation development based on their rich phytochemical profiles and antioxidant properties. However, research focusing on their combined effects remains limited. PE, extensively utilised in a variety of herbal and patented medicinal applications [4], is distinguished by its abundance of vital constituents, encompassing gallic acid, ellagic acid, quercetin, rutin, catechol, ethyl gallate, chebulagic acid, kaempferol, isocorilagin, chebulanin, and mallotusini [5]. Furthermore, PE is a preeminent source of vitamin C, exhibiting concentrations between 600 and 1300 mg per 100 g of the consumable portion, which exceeds the levels found in conventional citrus fruits [4]. However, research on the antioxidant properties of PE fruits in Malaysia remains limited due to a lack of awareness among the local populace regarding the fruit's edibility, its considerable vitamin C content, and its potential health benefits [6]. As a further

note, the CS tea variety provides one of the most broadly appreciated beverages internationally. Green tea (GT) is renowned for its health-promoting properties, largely attributed to its high polyphenol content, particularly catechins, and its potent antioxidant activity. Compared to black or oolong tea, GT contains significantly higher levels of catechins [7]. Studies have identified certain plants with high phenolics and flavonoids which, when used in a polyherbal formulation with GT, exhibited superior antioxidant activity compared to the individual extracts [8]. In contrast, B-AS which contains S-allyl-cysteine, demonstrates pharmacological properties as an antidiabetic, antioxidant, and anti-inflammatory agent, exhibiting enhanced bioactivity compared to standard garlic varieties [9]. B-AS possesses sixfold greater concentrations of S-allyl-cysteine than fresh garlic, leading to augmented antioxidant activity. Nevertheless, investigations specifically examining B-AS remain limited, particularly in terms of the efficacy of its bioactive compounds [10].

Despite their well-established individual benefits, the combined antioxidant interactions and potential synergistic effects of these herbs remain underexplored. Thus, this study aims to investigate the antioxidant properties and potential interactions when these three herbs are combined by optimising the formulation using a simplex lattice mixture design (SLMD). Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) spectroscopy was employed to identify the essential functional groups and to review spectral modifications in the PE, CS, and B-AS extract formulations. Additionally, chemometric analysis was conducted to determine correlations between antioxidant properties and infrared fingerprint profiles.

EXPERIMENTAL

Sample Collection and Chemicals

The PE fruits were gathered from the Merlimau region in Malacca, while CS and B-AS were procured from local sources. DPPH, gallic acid, methanol, and Folin-Ciocalteu's phenol reagent were purchased from Sigma Aldrich, USA. Sodium carbonate, methanol, and ethanol were obtained from Bendosen, Malaysia. All chemicals were of analytical grade.

Preparation and Extraction of Samples

PE fruit extraction was conducted using the cold-pressed method [6], while CS and B-AS were extracted using the water decoction method [11, 12] with a 1:10 ratio of plant-to-distilled water [13]. PE fruits (10 kg) went through a rigorous cleansing procedure, involving washing with tap water and subsequent rinsing with distilled water. Following this, the fruits were diced into small portions, and extracted using a multipurpose extractor machine (Arshia, Germany) to produce 1 litre of liquid extract. For CS and B-AS, 500 g of each sample was boiled in 5000 ml of distilled water until it reduced to half of the original volume. Then, the extracts (PE, CS, and B-AS) were filtered separately using Whatman No. 1 (Cytiva, UK) filter paper to ensure they were free of impurities. The extracts (PE, CS, and B-AS) obtained were subjected to a freeze-drying (LabGene Scanvac Coolsafe, UK) process, and then stored in a refrigeration unit (Haier, China) set at a temperature of 4 °C prior to further analysis.

Experimental Design of Polyherbal Formulation

The plant extracts were combined in various proportions to produce 16 different formulations, as shown in Table 1. These formulations were designed using the commercial statistical software package Design Expert version 13 (USA). The mixture design approach was employed to determine the optimum polyherbal formulation in terms of its antioxidant properties. The simplex lattice mixture design (SLMD) was applied using three components: *P. emblica*, *C. sinensis*, and *A. sativum L.*, where the sum of their proportions equalled 100 % (A + B + C = 100 %) [14]. Total phenolic content (TPC) and DPPH inhibition percentage were selected as response variables. This design was chosen because all components shared the same range (0–100), with no constraints imposed on the design space. Polynomial models for the response variables were generated through multiple regression analysis, with model suitability assessed based on model significance, lack of fit, and multiple correlation coefficient (R^2) [15]. The best model was selected based on R^2 values closest to 1 and an insignificant lack of fit [16]. An analysis of variance (ANOVA) was conducted to determine the statistical significance of each term at a 95 % confidence level [17]. Furthermore, an optimal formulation was identified using a multiple-response method known as the desirability value [15]. A single data point was selected for validation, and the response was predicted using the model. The predicted value was then compared against the actual experimental value, with the percentage error determined using the formula below [18].

$$\text{Percentage of error (\%)} = [(E_v - P_v) / P_v] \times 100 \quad (1)$$

Where E_v is the experimental value and P_v is the predicted value.

Table 1. Design formulation of the polyherbal extract by simplex lattice mixture design.

Run order	Independent variable proportion (%)		
	PE extract	CS extract	B-AS extract
1	16.67	16.67	66.67
2	16.67	66.67	16.67
3	100	0	0
4	0	0	100
5	66.67	33.33	0
6	0	33.33	66.67
7	100	0	0
8	33.33	66.67	0
9	0	100	0
10	66.67	16.67	16.67
11	0	0	100
12	66.67	0	33.33
13	0	66.67	33.33
14	33.33	0	66.67
15	0	100	0
16	33.33	33.33	33.33

Total Phenolic Content Analysis

The total phenolic content (TPC) of the extract was evaluated using a modified version of the Folin-Ciocalteu method, as described by Sari et al. (2023) [19]. A 20 µl sample of the extract was mixed with 20 µl of Folin-Ciocalteu reagent and agitated for 1 minute. After allowing the mixture to stand for 5 minutes, 200 µl of a 7 % sodium carbonate solution and 10 µl of distilled water were added. The mixture was then shaken for 1 minute at a medium-continuous speed. Following 120 min of incubation in the dark at room temperature (25 °C), the absorbance was measured at 750 nm using a microplate reader (VersaMax, USA). Each sample was analysed in triplicate. To establish a standard calibration curve, a standard solution of gallic acid was prepared with concentrations ranging from 10 to 60 µg/ml. The TPC value was determined using the calibration curve and expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of the sample extract.

2,2´-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Analysis

The free radical scavenging activity of the sample extract was conducted using the procedure outlined by Mamat et al. (2021) [20], with certain variations. The DPPH solution was prepared by dissolving 3.8 mg of DPPH in 95 mL of methanol, resulting in a concentration of 0.1 mM. A 0.1 mM DPPH working solution (150 µL) was added to the sample (50 µL) and mixed in a 96-well microliter plate. The mixture was allowed to stand in the dark for 30 minutes. The change in absorbance was measured at 515 nm using a microplate reader. Methanol was used as a control and ascorbic acid was used as an antioxidant standard. Triplicate measurements were carried out, and the percentage of DPPH scavenging activity was calculated as follows.

$$\text{DPPH Scavenging Activity (\%)} = [(A_b - A_s)/A_b] \times 100 \quad (2)$$

where A_b is the absorbance of the control and A_s is the absorbance of the sample.

Synergistic Effect Analysis on Total Phenolic Content and DPPH Radical Scavenging Value

The predicted response values were calculated by combining the percentage of each single extract's response based on the proportions in the mixtures. These predicted inhibition values were then compared with the experimental percentages to assess interaction effects [16]. An interaction was considered synergistic if the experimental TPC and DPPH values exceeded the theoretical values [13]. Conversely, if the combined effect of two TPC or DPPH values was less than the sum of the individual components, it was interpreted

as an antagonistic effect [21]. In cases where the combination neither significantly improved nor deteriorated in terms of TPC or DPPH values, the result was considered indifferent, meaning the mixture had no noticeable effect on overall efficacy [22].

Fourier Transform Infrared (FTIR) Analysis of Polyherbal Formulation

The polyherbal formulation extracts, prepared in varying proportions, were scanned over a 4000-400 cm^{-1} spectral range with a resolution of 4 cm^{-1}. The FTIR spectra of these samples were recorded using an FTIR spectrometer (UATR-FTIR Two, PerkinElmer, USA).

Principal Component Analysis

The TPC, DPPH radical scavenging activity and infrared fingerprint profile data of the 16 formulations obtained were subjected to chemometric analysis to explore their correlations. The spectral peaks were recorded in the ranges of 3000 cm^{-1} to 3500 cm^{-1} (vibration of -OH), 2800 cm^{-1} to 3000 cm^{-1} (vibration of -CH), 1600 cm^{-1} to 1700 cm^{-1} (vibration of C=O), and 1000 cm^{-1} to 1400 cm^{-1} (vibration of C-O) [23][24]. The Metabo Analyst 6.0 software (University of Alberta, Canada) automatically normalised the data and generated the PCA score plot, as well as a biplot corresponding to PC loadings.

Statistical Analysis

Statistical analysis was performed using Design Expert Software® version 13 from State-Ease Inc., USA. A simplex lattice mixture design formulated the mixtures, with PE, CS, and B-AS as the factors. The responses measured were TPC and DPPH activity, expressed in values and percentages. Data analysis involved one-way analysis of variance (ANOVA), three-dimensional plots, and triangular contour diagrams. Statistical significance ($p < 0.05$) was assessed using Tukey-LSD in SPSS version 22.

RESULTS AND DISCUSSION

Synergistic Effect Towards TPC and DPPH Value

The TPC value and DPPH radical scavenging method are generally applied to determine the antioxidant characteristics of extracts from different plant species. Table 2 presents the antioxidant characteristics of the analysed extract. A linear standard curve of gallic acid was employed to determine TPC ($y=0.0141x + 0.0507$), with an R^2 value of 0.9936. Based on the results obtained, nine (9) synergistic and one (1) antagonistic interaction effects were observed for the TPC values. This observation is consistent with existing literature that highlights the rich phenolic profile of PE and CS extracts [25][26][27]. Similarly, the combination of PE and CS extracts in runs 5 and 8 resulted in comparable TPC values and showed a synergistic interaction. In contrast, the B-AS extract demonstrated a relatively diminished contribution to the phenolic content. However, the TPC values found in this study reached 163.9 ± 0.6 mg GAE/ g, which was notably higher than previous reports, which had values of 21.14 ± 1.06 to 115.64 ± 6.42 m g GAE/ g [28] and ranged between 6.73 ± 0.67 to 74.86 ± 1.13 mg GAE/ g [12]. This indicates that combining the three extracts may lead to an enhancement in the TPC. For instance, in run 16, where the extracts were evenly distributed (33.33 % PE, 33.33 % CS, 33.33 % B-AS), a synergistic interaction was observed, with the TPC value increasing from 1016.9 mg GAE/g to 1091.8 mg GAE/ g. Similarly, runs 2 and 10 showed synergistic interactions, producing higher than predicted TPC values. Conversely, run 6 exhibited an antagonistic interaction, where a high proportion of B-AS extract combined with a moderate CS extract negatively impacted the phenolic content. This may be due to inhibitory interactions between the compounds in these two extracts, possibly linked to covalent interactions between proteins in CS and organosulfur compounds in the B-AS extract [29]. Additionally, the presence of diverse constituents may lead to chemical incompatibility, resulting in instability [30]. Synergistic interactions in the DPPH assay were observed in experimental runs 2 and 10, aligning with the findings of Alexandru et al. (2015) [31], which demonstrated that combining herbal extracts could enhance antioxidant activity. Increasing the proportion of PE or CS in the formulation was likely to produce a synergistic effect, whereas an equal distribution of the extracts tended to result in indifferent interactions. This suggests that the DPPH activity of the B-AS extract played a significant role in the interaction effect when its proportion was increased. Run 16 exhibited the highest DPPH inhibition (94.0 ± 0.2 %), closely approaching ascorbic acid (95.1 ± 0.4 %) with no significant difference observed. These results indicate that combining the three extracts, particularly with higher amounts of PE or CS, enhanced phenolic content and antioxidant activity, suggesting a complementary interaction among their phytochemical components. Past studies have indicated that the chosen individual botanical species were rich in phenolic and flavonoid compounds. Their combination with CS in a polyherbal formulation exhibited superior antioxidant activity compared to the individual extracts [32]. Therefore, the synergistic interplay between the PE, CS, and B-AS extracts led to notably higher TPC and DPPH values in several formulations, highlighting the potential of combining these extracts to boost therapeutic efficacy.

Table 2. Experimental data obtained for TPC and DPPH values of the studied extracts.

Run order	TPC (mg GAE/ g)			DPPH (%)		
	Actual	Predicted	Interaction	Actual	Predicted	Interaction
1	1031.4 ± 26.9^{cd}	1014.7	Synergistic	90.7 ± 0.2^{c}	90.5	Indifferent
2	1062.6 ± 31.2^{bcd}	1045.9	Synergistic	90.8 ± 0.4^{c}	89.3	Synergistic
3	1085.1 ± 1.5^{bcd}	1085.2	Indifferent	91.7 ± 1.7^{bc}	91.7	Indifferent
4	169.3 ± 5.1^{f}	169.0	Indifferent	41.1 ± 3.4^{d}	40.9	Indifferent
5	1108.4 ± 7.8^{bc}	1091.8	Synergistic	91.3 ± 0.1^{bc}	91.7	Indifferent
6	704.1 ± 11.2^{e}	769.2	Antagonistic	89.7 ± 0.6^{c}	89.3	Indifferent
7	1085.2 ± 3.1^{bcd}	1085.2	Indifferent	90.4 ± 0.6^{c}	90.3	Indifferent
8	1102.1 ± 40.1^{bc}	1076.9	Synergistic	91.2 ± 0.0^{bc}	91.5	Indifferent
9	1213.1 ± 4.6^{ab}	1213.2	Indifferent	89.0 ± 0.4^{c}	89.2	Indifferent
10	1086.1 ± 9.6^{bcd}	1069.4	Synergistic	91.2 ± 0.1^{bc}	89.5	Synergistic
11	163.9 ± 0.6^{f}	163.5	Indifferent	39.8 ± 0.5^{d}	39.9	Indifferent
12	1102.6 ± 19.1^{bc}	1004.8	Synergistic	91.0 ± 0.2^{c}	91.6	Indifferent
13	1106.1 ± 56.6^{bc}	1074.7	Synergistic	90.3 ± 0.0^{c}	90.9	Indifferent
14	1003.0 ± 23.3^{d}	907.0	Synergistic	90.9 ± 0.2^{c}	90.5	Indifferent
15	1131.9 ± 46.2^{ab}	1131.7	Indifferent	89.3 ± 0.4^{c}	89.2	Indifferent
16	1091.8 ± 64.9^{bcd}	1016.9	Synergistic	94.0 ± 0.3^{ab}	94.6	Indifferent
Ascorbic acid				95.1 ± 0.4^{a}		

Values are expressed as the mean ± standard deviation (n=3). Different superscript letters within the same column indicate significant differences ($p < 0.05$).

Model Fitting

The regression coefficients corresponding to all variables within the optimised models are presented in Table 3. This study aimed to enhance plant extract blending to identify the optimal mixture in terms of TPC and DPPH radical scavenging activity. A statistical analysis of the lattice design batches was executed by employing a one-way ANOVA, with a significance threshold set at $p < 0.05$. TPC values exhibited statistical significance within the special quartic model, whereas DPPH demonstrated significance within the cubic model. The "Prob > F" values for TPC and DPPH were recorded as < 0.0001, confirming statistical significance below the 0.05 threshold. The lack of fit for the dependent variables was statistically insignificant, which was advantageous, as the objective was to achieve a well-fitting model. The R^2 values computed for TPC and DPPH were 0.9821 and 0.9989, respectively, which are reasonably proximate to 1, and thus deemed acceptable. The R^2 value served as the primary criterion for evaluating the model's suitability based on the determination coefficient. The optimal model for each assay was identified by selecting those characterized by a low standard deviation, diminished predicted sum of squares, and elevated predicted R^2 values [16]. In addition, the difference between the predicted R^2 and adjusted R^2 should be less than 0.2 for consistency, while an adequate precision ratio greater than 4 is desirable. This ensures the model's suitability for navigating the design space effectively.

Table 4 presents the definitive empirical models for the coded factors of TPC and DPPH radical scavenging activity, wherein A, B and C denote PE, CS, and B-AS, respectively. The antioxidant characteristics exhibited substantial variability across the different extracts. Analysis of the polyherbal formulation's antioxidant properties revealed TPC values ranging from 163.9 ± 0.6 to 1213.1 ± 4.6 mg GAE/g. The extract derived from CS exhibited the highest TPC value, followed by the PE extract, while the B-AS extract had the lowest value when assessed independently. The TPC values for CS and PE did not differ significantly ($P < 0.05$), as indicated in Table 2. This suggests that both extracts were particularly rich in phenolic compounds, contributing significantly to the overall TPC in the polyherbal formulation. Similarly, when the three extracts were combined in various ratios, no significant differences ($P < 0.05$) were observed, reinforcing the notion that combining

extracts enhanced phenolic yield. In terms of DPPH, a cubic model was employed to facilitate analysis of the interaction effects among the three extract types under investigation, revealing that the combination of PE, CS, and B-AS extracts yielded the highest percentage of inhibition when utilised together, where the proportion of PE or CS was higher than B-AS, as shown in Table 2. The results indicate a strong association between antioxidative activities and phenolic compounds, suggesting that phenolic compounds are responsible for the antioxidative activities of the PE, CS, and B-AS extracts. A comparable observation has been documented, wherein phenolic compounds were recognized as pivotal contributors to the antioxidant effectiveness of polyherbal formulations, as their proficient hydrogen-donating capacity renders them excellent antioxidants [33].

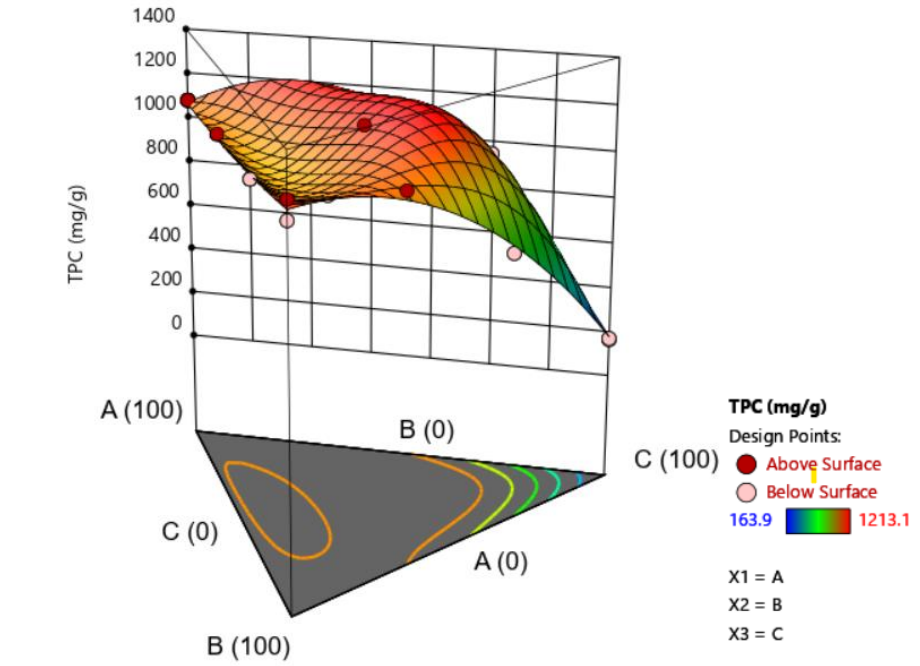
Table 3. Analysis of variance (ANOVA) of the regression equation for optimisation of total phenolic content and DPPH radical scavenging activity in the polyherbal formulations.

Response Variable	Sum of square	Degree of freedom	Mean Square	F – value	Probability	
TPC- Special quartic						
Regression	1555000	8	194400	47.97	< 0.0001	Significant
Residual	28361.8	7	4051.69			
Lack of fit	25050.5	4	6262.62	5.67	0.0927	Not significant
Pure error	3311.3	3	1103.77			
Cor total	1583000	15				
R^2						0.9821
Adjusted R^2						0.9616
Predicted R^2						0.9103
DPPH – Cubic						
Regression	4455.25	9	495.03	582.68	<0.0001	Significant
Residual	5.10	6	2.03			
Lack of fit	3.39	3	1.13	1.98	0.2949	Not significant
Pure error	1.71	3	0.5707			
Cor total	4412.47	15				
R^2						0.9989
Adjusted R^2						0.9971
Predicted R^2						0.9863

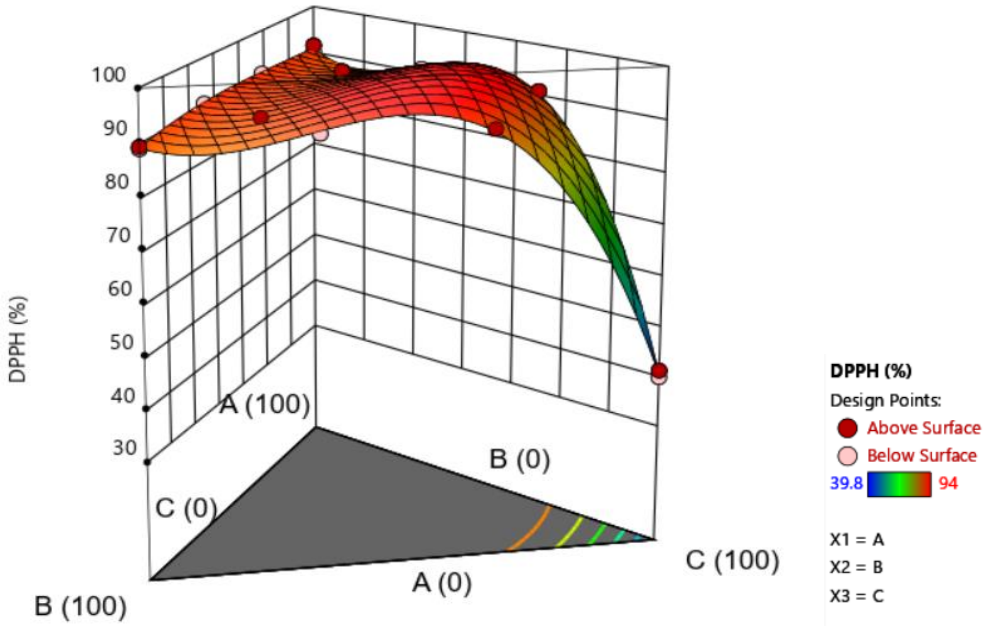
Table 4. Final empirical model equations for total phenolic content and DPPH radical scavenging activity value of the polyherbal formulations.

Parameter	Model	Final equation
Total phenolic content (mg GAE / g)	Special quartic	$1073.02A + 1175.94B + 178.39C - 72.58AB + 1935.88AC + 1039.65BC - 11194.12A^2BC - 11223.93AB^2C + 21197.88ABC^2$
DPPH radical scavenging activity (%)	Cubic	$91.08A + 89.15B + 40.50C + 6.29AB + 113.10AC + 113.03BC – 130.05ABC - 3.26AB(A-B) - 105.75AC(A-C) - 98.52BC (B-C)$

A is *Phyllanthus emblica*, B is *Camellia sinensis* and C is *Allium sativum var.* black solo garlic.



(a)



(b)

Figure 1. Three-dimensional surface plots of (a) TPC and (b) DPPH.

Optimisation of the Polyherbal Formulation

The optimised formulation was determined using Design Expert software to enhance the TPC and DPPH values. Empirical studies have demonstrated that extracts abundant in polyphenols frequently exhibit pronounced antioxidant characteristics [34]. The impact of various formulations on TPC and DPPH is illustrated by the three-dimensional surface plots, as displayed in Figure 1 (a) and (b). The graphical representations depict three distinct interaction variations among the responses, wherein the dark red region signifies the highest acceptance value. Conversely, the green and blue regions denote medium and low acceptance values among the responses relative to the influencing factors. A desirability score close to one was chosen, reflecting the highest satisfaction with the parameters. For this formulation, the desirability was 0.990, as shown in Figure 2, giving a polyherbal formulation consisting of 36.4 % PE, 17.5 % CS, and 46.1 % B-AS. In this study, TPC values were lower, but DPPH radical scavenging activity was more favourable in ternary polyherbal

formulations. Each extract possesses unique antioxidant properties that contribute to the overall therapeutic effect. Previous phytochemical studies indicated that phenolic acids such as gallic acid and ascorbic acid were the predominant bioactive compounds contributing to TPC in PE [35][36]. In CS, catechins were the key contributors [37], while the B-AS extract was characterized by organo-sulfur compounds, such as S-allyl-cysteine, which played a significant role in its antioxidant activity [38]. It has been noted that combinations of herbal substances frequently yield a more favourable therapeutic effect in treating diseases compared to administering a singular pharmaceutical agent [39].

Validation of the Optimised Polyherbal Formulation

The efficacy of the model equation in forecasting the optimal response value was evaluated through computation of the percentage error to the predicted value produced by Design Expert software. Table 5 presents a comparative analysis of the target response's predicted and experimental values resulting from the optimised polyherbal formulation. The percentage error remained below 10 %, confirming the model's reliability and accuracy in forecasting the response values [18].

Fourier Transform Infrared (FTIR) Analysis of the Polyherbal Formulation

The predominant functional groups present were identified using Fourier-Transform Infrared (FTIR) spectroscopy, allowing for the detection of spectral variations in the formulations detailed in Table 1. This rapid and non-invasive technique, often combined with chemometric analysis, is particularly well-suited for the characterisation of herbal pharmaceuticals [40]. The infrared spectra of individual botanical extracts and their combinations exhibited varying transmittance values corresponding to specific chemical constituents. It was previously explained that the extracts of PE and CS are distinguished by their substantial total TPC levels. Phenolic compounds, a major class of plant-derived phytochemicals, are characterised by the presence of one or more phenolic (-OH) groups attached to an aromatic ring [23]. These naturally occurring compounds exhibit strong antioxidant properties, effectively scavenging free radicals and reactive oxygen species (ROS) [41]. Figure 3 (a), (b), and (c) illustrate the characteristics of the FTIR absorption spectra of PE, CS, and B-AS individually, while (d) and (e) present FTIR peaks for binary and ternary combinations of the plant extracts, spanning the mid-infrared region (4000 to 400 cm^{-1}). The FTIR spectra for PE, CS, and B-AS revealed the existence of multiple peaks indicative of various bioactive functional groups. Upon investigation, the major peaks were noted to vary between 758.58 cm^{-1} and 3261.57 cm^{-1} for PE, 761.47 cm^{-1} and 3229.22 cm^{-1} for CS, as well as 776.80 cm^{-1} and 3280.65 cm^{-1} for B-AS, as pointed out by upgraded annotation. The findings indicated that the significant peaks for PE were situated at 3261.57, 2941.23, 1766.33, 1701.77, 1610.30, 1534.93, 1443.99, 1320.01, 1201.65, 1023.58, 919.40, 867.84, 813.03, and 758.58 cm^{-1}. For CS, they were at 3229.22, 2933.53, 2360.17, 1691.26, 1602.94, 1552.27, 1514.37, 1448.65, 1342.04, 1236.05, 1142.19, 1031.08, 917.50, 818.15, and 761.47 cm^{-1}; while for B-AS, the peaks were located at 3280.65, 2934.63, 1629.88, 1406.73, 1341.24, 1240.32, 976.29, 918.78, 864.93, 815.74, and 776.80 cm^{-1}. All these peaks were recorded within the fingerprint section of the FTIR spectrum.

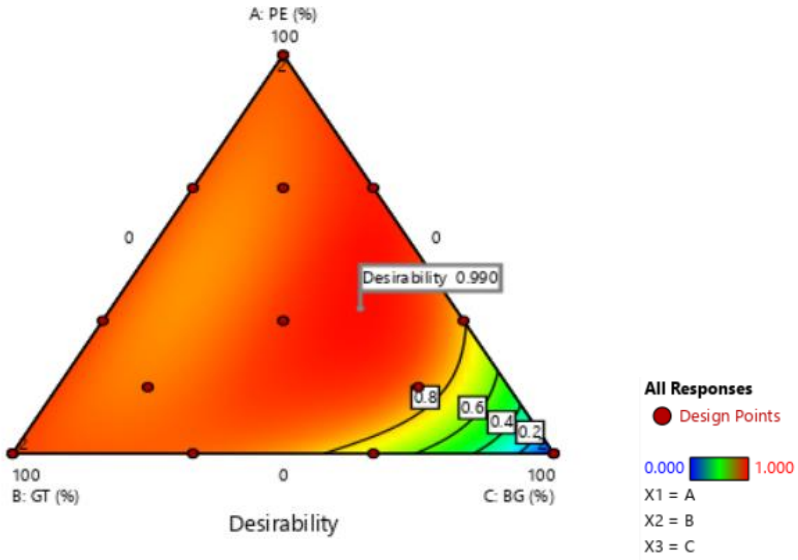


Figure 2. Triangular contour diagram optimised at a desirability of 0.990.

Table 5. Values of predicted and experimental responses for TPC and DPPH in the polyherbal formulation.

Parameter	Predicted value	Experimental value	Percentage error (%)
TPC (mg GAE / g)	1192.3	1205.1 ± 20.1	1.07
DPPH (%)	96.1	96.2 ± 0.1	0.06

Values are presented as the mean ± standard deviation (n=3).

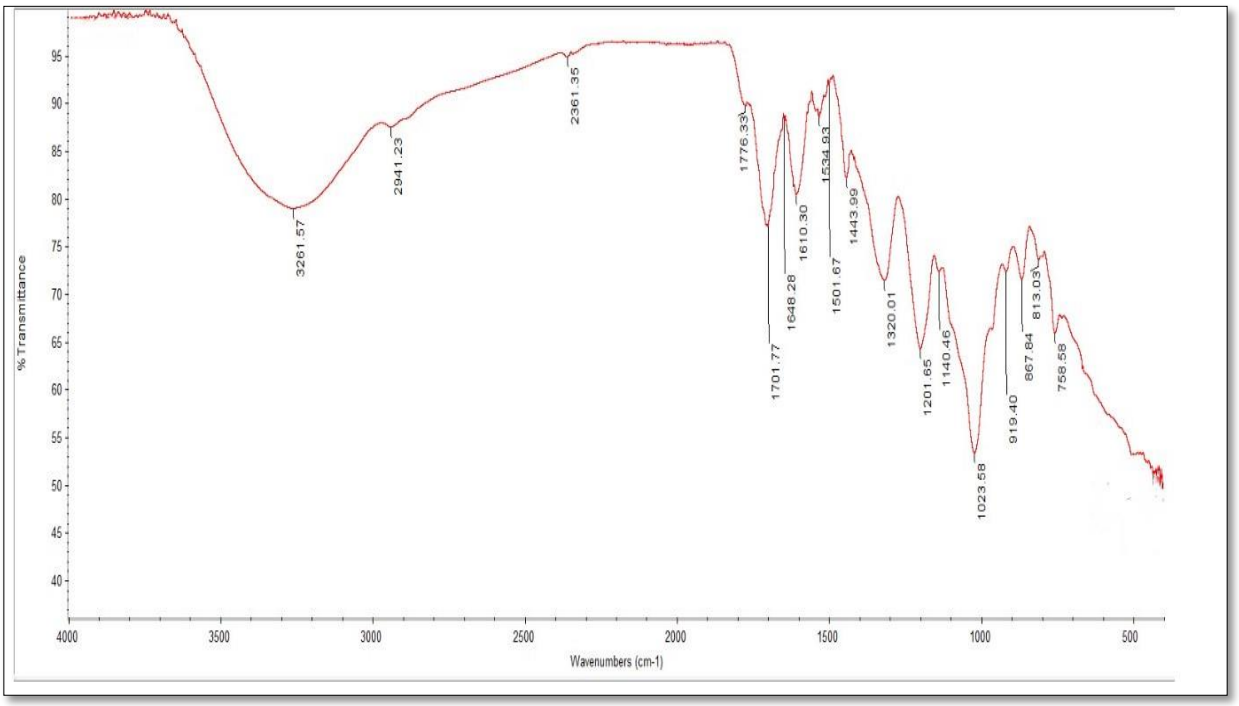
In the context of PE, the peak at 3261.57 cm^{-1} signified a pronounced and extensive hydroxyl group (-OH) stretching vibration, associated with both the phenolic and alcohol functionalities [42]. The C–H stretching vibrations were identified at 2941.23 cm^{-1}, potentially indicative of phenolic aromatic compounds [43]. The spectral data revealed a peak at 1766.33 cm^{-1} which was attributed to the C=O stretching vibration of the ester functional group [24]. In contrast, the peak at 1701.77 cm^{-1} suggests the presence of symmetric or asymmetric –CH aliphatic bonds [42]. The peaks observed at 1610.30, 1534.93, and 1443.99 cm^{-1} are likely associated with the presence of carbonyl groups or ketones, as well as C–C stretching vibrations within phenyl and aromatic rings [42], and the C=C-C stretching vibration of the aromatic ring [44]. The peak positioned at 1342.04 cm^{-1} predominantly corresponds to C–O stretching vibrations associated with ester functionalities [42]. The detection of C–O stretching within the spectral range of 1200–1000 cm^{-1} indicated phenolic aromatic ring vibrations [26]. The observed spectral wavelengths at 919.40, 867.84, 813.03, and 758.58 cm^{-1} may be attributed to HC–CH stretching vibrations characteristic of aromatic amides [42].

The TPC value is characterized by the presence of functional groups including –OH, C=O, and C-O [24]. Previous studies have confirmed the presence of phenolic compounds in PE fruit, including gallic acid [35], caffeic acid, quercetin [45], kaempferol [46], ellagic acid, and ascorbic acid [47] [36], in addition to alkaloids such as phyllantidine and phyllantine [48]. The findings from this spectral analysis align with the known chemical composition of PE fruit and are consistent with existing literature.

As for CS, the broad peak from 3000 to 3500 cm^{-1} may be assigned to O-H and N-H stretching modes, due to the presence of some polyphenols, polysaccharides, and amide groups of proteins [49]. The peak at 2933.53 cm^{-1} is associated with anti-symmetric and symmetric C–H stretching vibrations of methylene (CH_2) groups [50], while the O-H stretching of carboxylic acids appeared at 2360.17 cm^{-1} [51]. Another band at 1691.26 cm^{-1} was related to protein C=O stretching, and 1602.94 cm^{-1} was due to CH_2 symmetric stretching [37]. The 1600 – 1500 cm^{-1} peak represents flavonoids, polyphenols, and catechin [52]. The out-of-plane bending vibrations corresponding to the N-H amide II and III, in conjunction with the C-N stretching vibrations present in protein structures, were detected at 1514.37 cm^{-1} and 1342.04 cm^{-1}, respectively. Furthermore, the absorption band identified at 1448.65 cm^{-1} was ascribed to the bending vibrations of CH_2 alkane groups, the COO^- moiety of carboxylate functional groups in proteins and lipids, along with the rocking vibrations of C-H bonds in cis-disubstituted alkenes [49]. The peak at 1236.05 cm^{-1} and 1142.19 cm^{-1} was due to the stretching vibration of the C-O-C group of ether and could indicate the presence of alcohols, esters, and carboxylic acid groups in the sample, respectively [42]. The absorption peak at 1031.08 cm^{-1} represented C–O–C stretching, while the strong band at 917.50 cm^{-1} represented C=C bending [51]. All peaks below 1000 cm^{-1} corresponded to bending modes of sp2 C-H of alkenes and aromatic rings [49].

Phytochemical analysis of the CS aqueous extract indicated the presence of phenolic compounds such as flavonoids and phenolic acids [53]. Previous studies indicate that the major TPC in CS was catechin [37]. Therefore, this observation proves the involvement of (-)-epicatechin, (-)- epigallocatechin, (-)-epicatechin-3-gallate, and (-)-epigallocatechin-3-gallate as well as gallic acid [54]. On the other hand, evidence derived from previous research has found that the amino acid profile of CS, in both free and bound forms, enhanced its quality and nutritional value [55].

In the analysis of the B-AS extract, the prominent peak at 3280.65 cm^{-1}, corresponded to O-H stretching of hydroxyl groups, suggesting the presence of polyhydroxy compounds such as flavonoids, non-flavonoids, and saponins [56] or polyphenolic compounds [57]. The peak at 2934.63 cm^{-1} was attributed to C-H bonds' symmetric and asymmetric stretching vibrations [58]. The band at 1629.88 cm^{-1} indicated C=O bonding, associated with peptide linkages, carbonyl groups, and carboxylic functionalities [56, 59]. The signal at 1406.73 cm^{-1} was linked to the bending of the –O–H group in carboxylates [60]. The peaks at 1341.24 cm^{-1} and 1240.32 cm^{-1} indicated O-H bending related to carboxylic acids and carbonyl stretching vibrations, respectively. In the region between 1100 and 1000 cm^{-1}, the S=O stretching of sulfoxides suggested the presence of organosulfur compounds, such as alliin, allicin, and diallyl disulfide [58]. Additionally, the peaks at 918.78 cm^{-1}, 815.74 cm^{-1}, and 776.80 cm^{-1} may be attributed to C–H deformation of CH_2, N–H bending of primary amines, and C–H bending of alkynes, respectively [60].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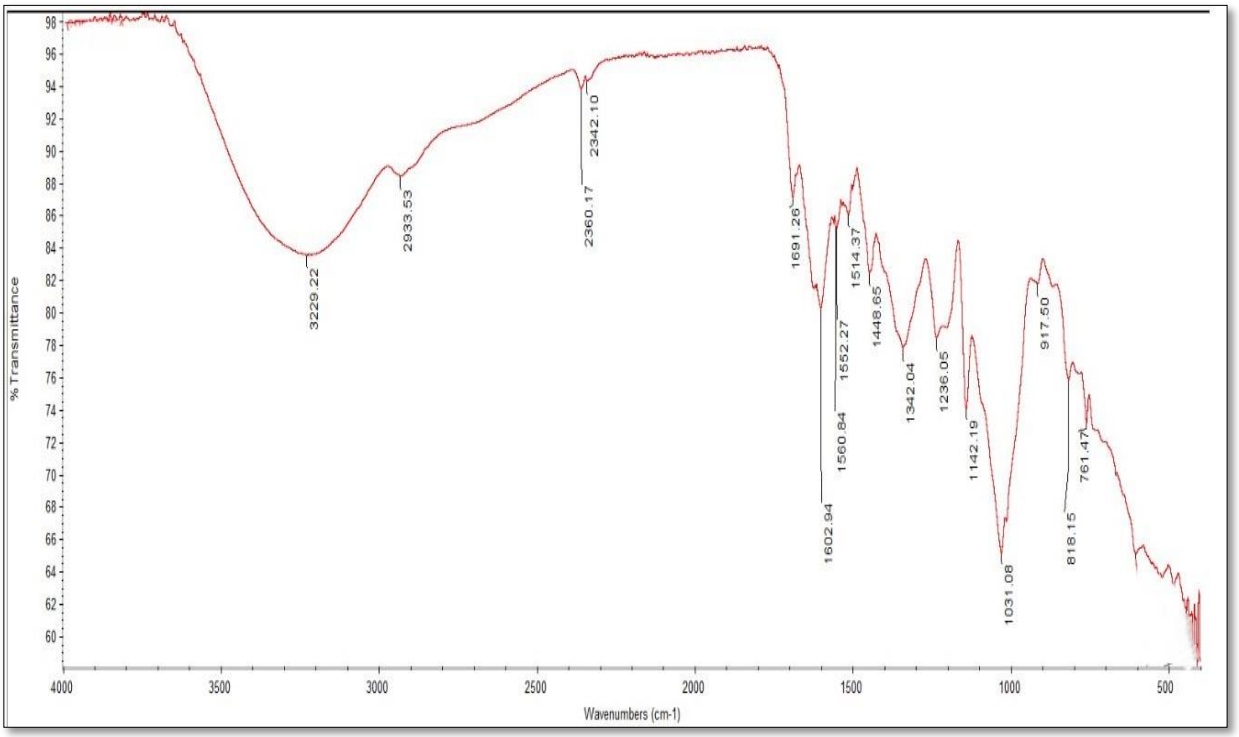
2941.23
2361.35
1776.33
1701.77
1648.28
1610.30
1534.93
1501.67
1443.99
1320.01
1201.65
1140.46
1023.58
919.40
867.84
813.03
758.58

(a)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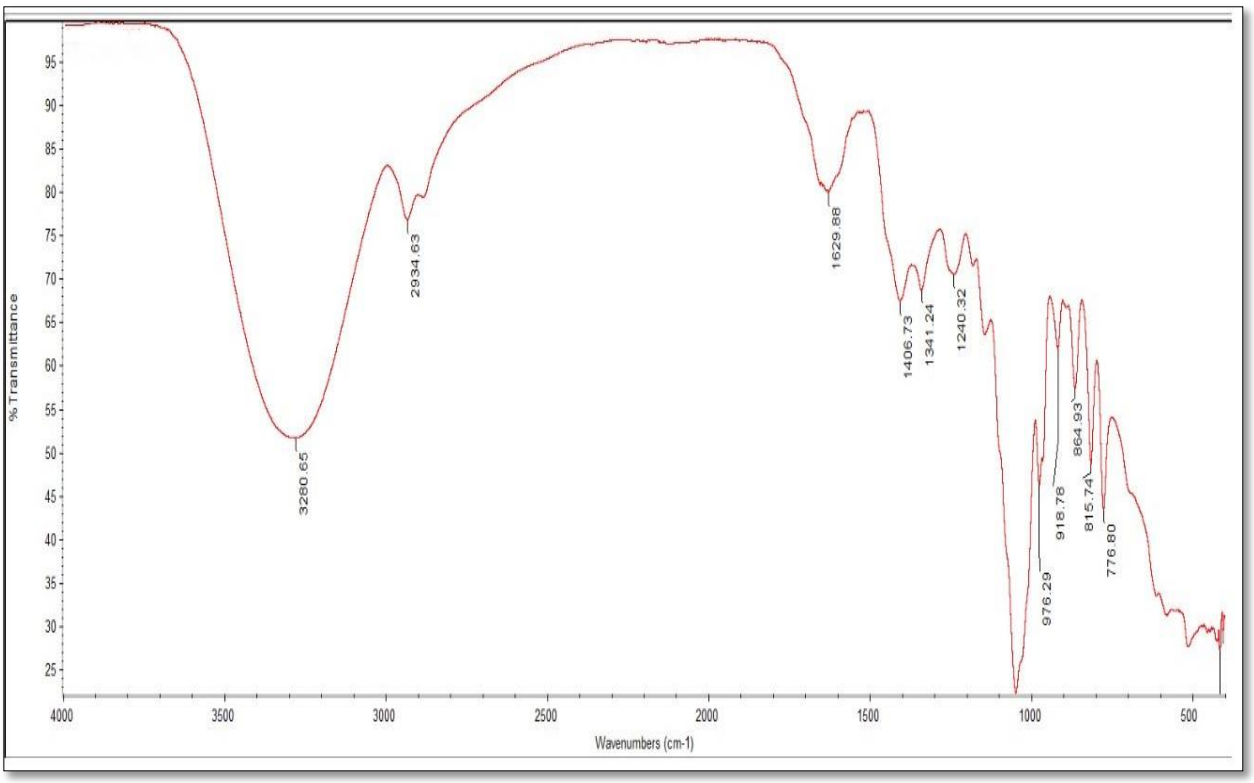
917.50
818.15
761.47

(b)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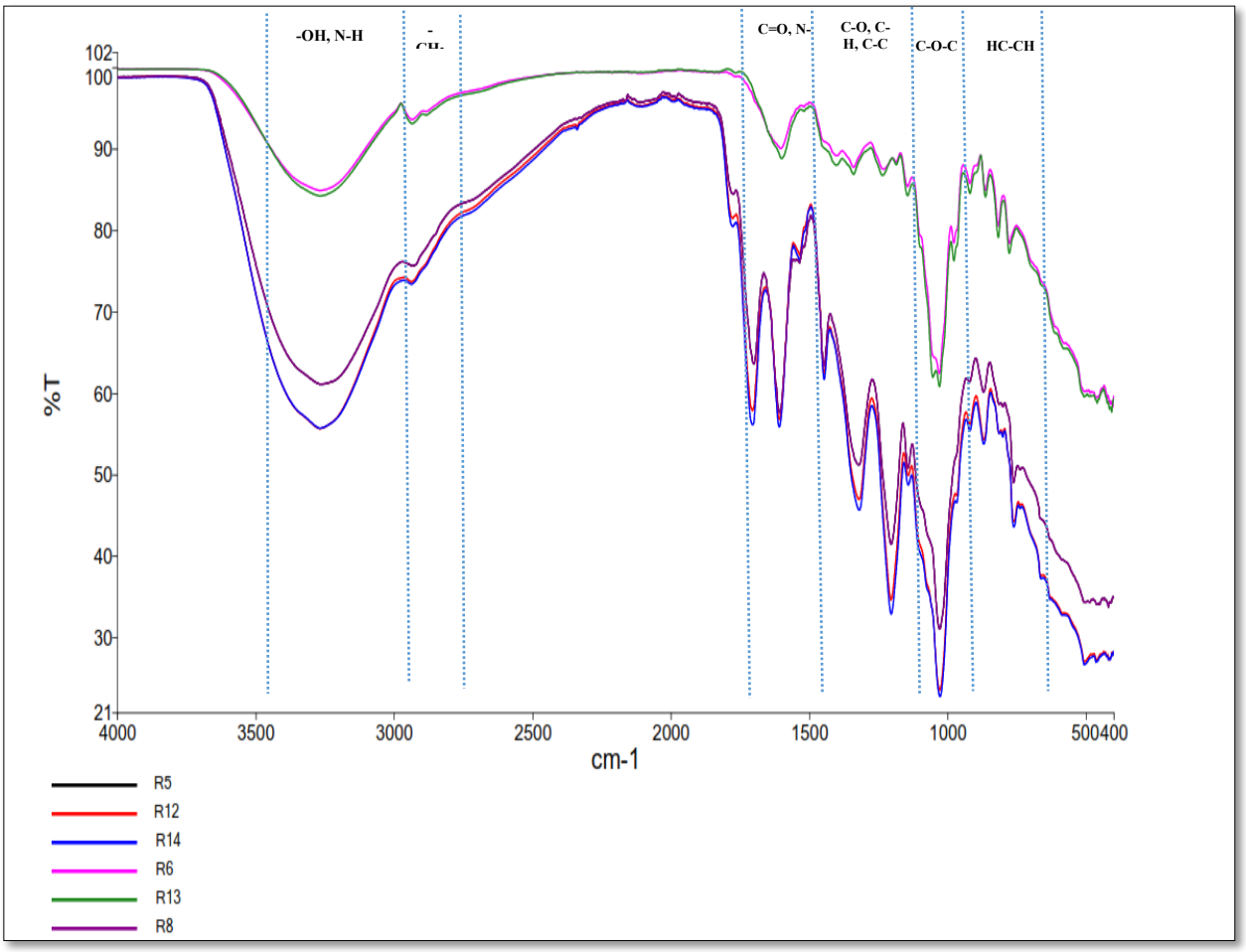
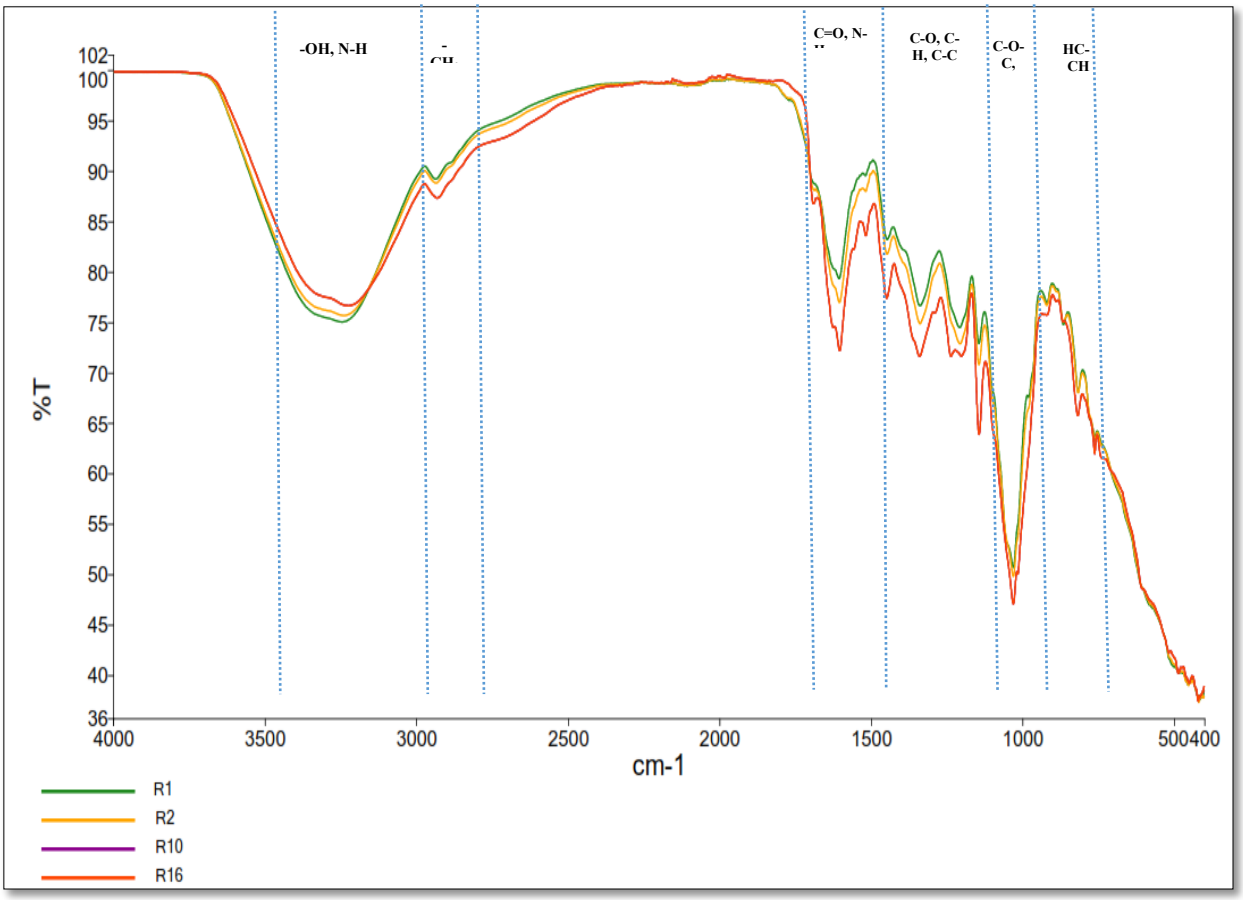
776.80

(c)


-OH, N-H
C=O, N-
C-O, C-H, C-C
C-O-C
HC-CH
%T
cm-1
R5
R12
R14
R6
R13
R8

(d)



(e)

Figure 3. FTIR spectra for (a) PE extract, (b) CS extract, (c) B-AS extract, (d) Binary combinations of extracts, and (e) Ternary combination of extracts.

From the graphical representation, it can be discerned that the absorption maxima of all spectra manifested at a nearly identical wavenumber within the specified region, albeit with minor intensity variations for each extract and the combined plant extracts (binary and ternary) as shown in Figure 3 (d) and (e). These variations may indicate molecular interactions among the functional groups of the various compounds present within the individual extracts. Three primary functional group regions (OH-stretching, CH- stretching, and C=O stretching) were evident across all run orders. In binary combinations, the spectral regions of R6 and R13 (CS + B-AS) were quite different from R5 and R8 (PE + CS) and R12 and R14 (PE + B-AS). This may be due to the covalent interactions between protein in the CS and organ sulphur compounds in the B-AS extract [29], resulting in subtle shifts and enhancements in certain spectral regions. For R5, R8, R12, and R14, strong O-H and C=O stretching peaks dominated the spectra due to the polyphenol groups present in PE extracts. Upon incorporating all three extracts, the spectrum evolved into a more intricate configuration as supplementary functional groups and interactions materialized. This resulted in more pronounced and overlapping peaks within the 3000–3500 cm^{-1} (O-H stretching) and 1600–1700 cm^{-1} (C=O stretching) regions, alongside the fingerprint region (1500–600 cm^{-1}) [44] where complex bending vibrations transpired. This observation suggests the incorporation of polyphenols [24], flavonoids [61], and other hydroxyl-containing compounds [44] from the three plant extracts. Similar absorption patterns have been documented in other studies, indicating the presence of phenolic compounds in crude extracts of *Emblica officinalis* [24], *Garcinia schomburgkiana* [62] and *Orthosipon stamineus* [63]. These extracts exhibited functional groups such as alcohols and phenols (H-bonded hydroxyl groups), carboxylic acids (C–O stretching), amino acids (COO^-), methyl and aldehyde groups (C–H bond stretching), aldehydes or ketone (C=O stretching), alkenes (C=C stretching), amines and amides (N–H bending), and aromatics (C–C and C=C-C stretching).

Principal Component Analysis

Chemometric analysis was performed based on the data obtained from antioxidant properties and infrared fingerprints. PCA and PLS-DA are the common chemometric methods applied to identify compounds in herbal plant and fruit varieties for quality assessment [64]. In the present study, principal component analysis

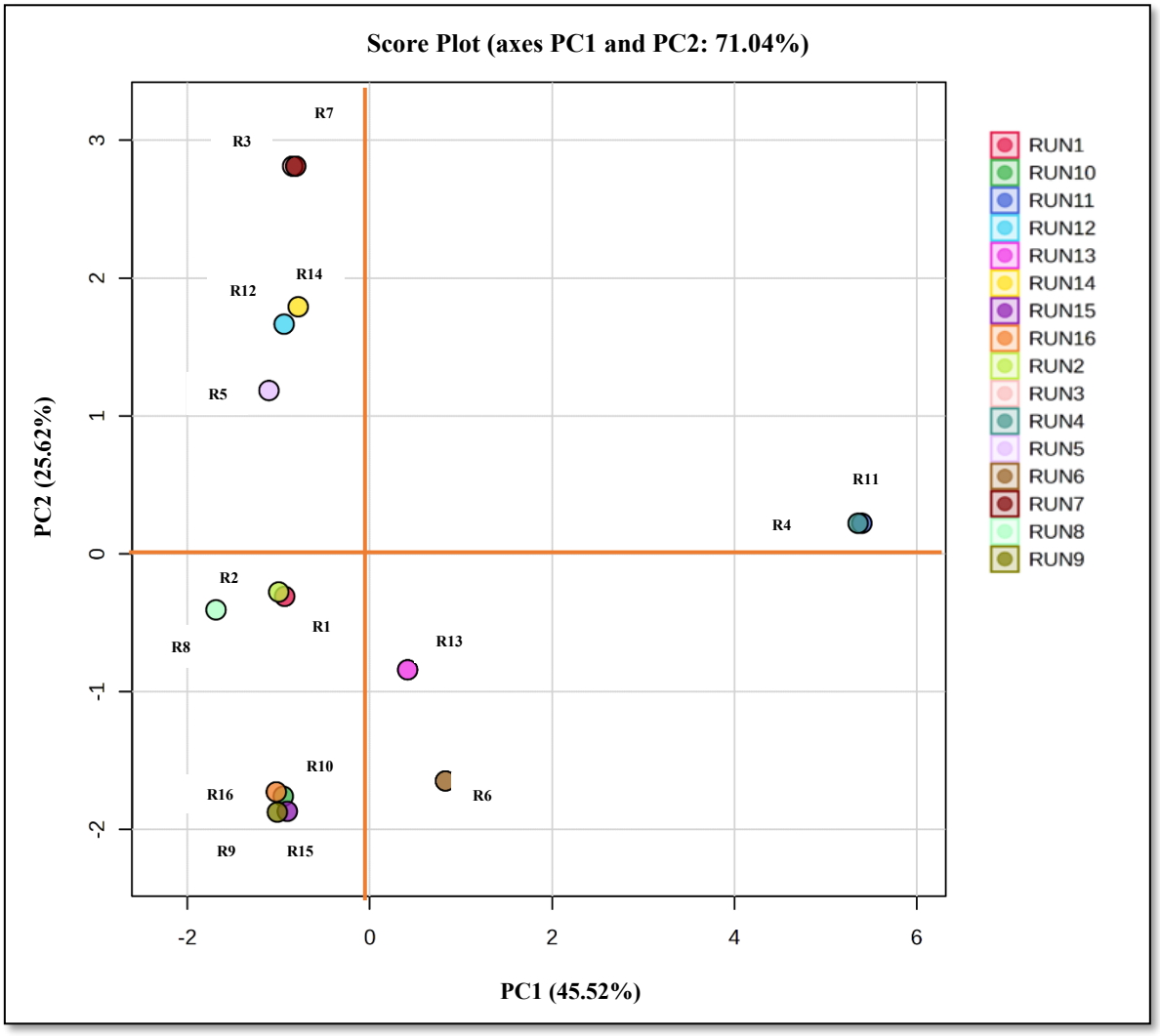
(PCA) was chosen to interpret the principal components that characterize each variable and to ascertain the parameters that elucidate the variance observed among the samples [65][66]. Previously, it has been highlighted that the TPCs are characterized by the presence of functional groups including –OH, C=O, and C-O [24] which correspond to the wavenumber ranges of 3000 cm^{-1} to 3500 cm^{-1} (vibration of -OH), 2800 cm^{-1} to 3000 cm^{-1} (vibration of -CH), 1600 cm^{-1} to 1700 cm^{-1} (vibration of C=O), and 1000 cm^{-1} to 1400 cm^{-1} (vibration of C-O). Table 6 lists the wavenumber ranges for the 16 formulations obtained in the experimental design.

The analysis shown in Figure 4(a) revealed the PCA score plot of the two components, wherein component PC 1 and PC 2 accounted for 45.52 % and 25.62 % of the variance, respectively, and R^2 was equal to 1. Meanwhile, Figure 4(b) illustrates the PCA biplot. The samples were grouped based on their spectral fingerprints and antioxidant activities (TPC and DPPH scavenging activity). Notably, R1, R2, R8, R9, R10, R15, and R16 were positioned on the negative side of PC1, strongly associated with TPC and DPPH values, as well as spectral absorption ranges of 1400 cm^{-1} to 1500 cm^{-1} and 1000 cm^{-1} to 1100 cm^{-1}. This suggests that these samples had higher phenolic content and antioxidant activity, potentially due to the presence of functional groups like –OH, C=O, and C-O, which contribute to radical scavenging ability [24]. Conversely, samples R4 and R11, which were positioned further away from TPC and DPPH, showed a weak correlation with these antioxidant properties. Their association with absorption bands at 3000 cm^{-1} to 3500 cm^{-1} (O-H stretch), 2800 cm^{-1} to 3000 cm^{-1} (C-H stretch), and 1600 cm^{-1} to 1700 cm^{-1} (C=O stretch) suggests that these formulations may contain more aliphatic and carbonyl compounds rather than polyphenols, which could explain their lower antioxidant effectiveness. The PCA results showed that samples R3, R5, R7, R12, and R14 along the positive side of PC2 indicated a different chemical profile. Their correlation with the absorption region of 1600 cm^{-1} to 1700 cm^{-1} suggests they might contain a significant amount of conjugated carbonyl compounds and aromatic functionalities.

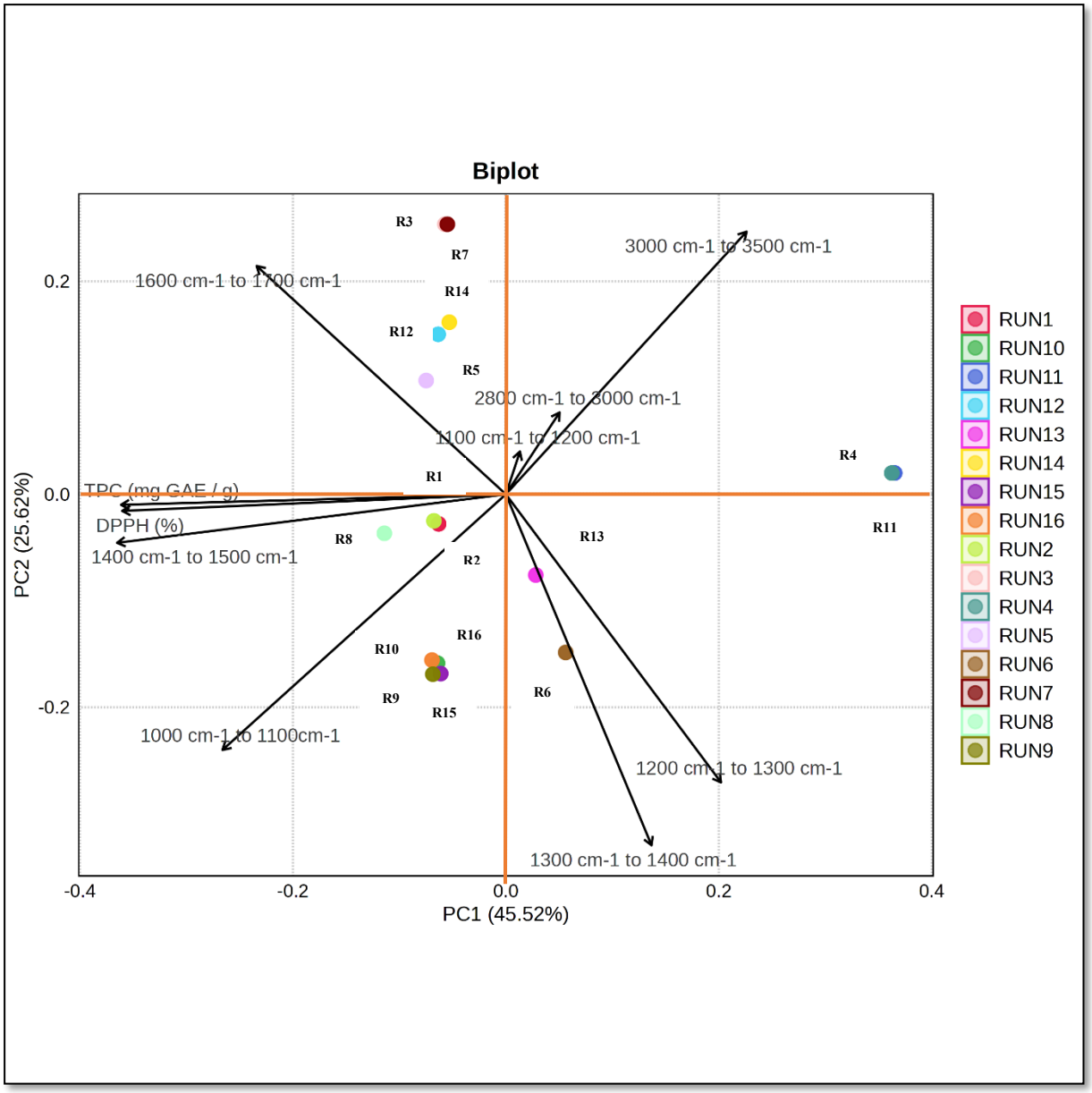
Table 6. Wavenumber ranges of samples.

Run order	Wavenumber ranges (cm^{-1})							
	3000 - 3500	2800 - 3000	1600 - 1700	1400 - 1500	1300 - 1400	1200 - 1300	1100 - 1200	1000 – 1100
1	3247.71	2933.82	1692.95	1448.02	1338.23	1207.44	1144.17	1030.43
2	3242.28	2940.12	1693.14	1447.04	1338.07	1206.48	1143.97	1030.66
3	3261.57	2941.23	1610.30	1443.99	1320.01	1201.65	1140.46	1023.58
4	3280.65	2934.63	1629.88	1406.73	1341.24	1240.32	1150.00	1020.00
5	3263.46	2929.46	1699.52	1446.97	1321.88	1203.63	1142.68	1028.44
6	3245.57	2937.22	1603.18	1441.23	1339.47	1232.28	1145.60	1029.87
7	3261.57	2941.23	1610.30	1443.99	1320.01	1201.65	1140.46	1023.58
8	3225.61	2928.35	1698.82	1451.14	1333.23	1202.17	1147.63	1029.37
9	3229.22	2933.53	1691.26	1448.65	1342.04	1236.05	1142.19	1031.08
10	3227.99	2931.23	1692.92	1448.71	1339.57	1236.55	1143.55	1030.52
11	3280.65	2934.63	1629.88	1406.73	1341.24	1240.32	1150.00	1020.00
12	3271.04	2930.91	1625.16	1446.41	1320.19	1204.11	1178.27	1028.05
13	3269.82	2936.22	1623.15	1447.24	1339.35	1235.64	1235.77	1029.18
14	3270.67	2928.34	1625.32	1446.57	1319.33	1203.53	1203.01	1027.32
15	3229.22	2933.53	1691.26	1448.65	1342.04	1236.05	1142.19	1031.08
16	3225.93	2933.67	1693.22	1448.71	1339.57	1236.55	1143.50	1030.53

The PCA results also indicated that the absorption peaks within the ranges of 3000 cm^{-1} to 3500 cm^{-1}, 1600 cm^{-1} to 1700 cm^{-1}, 1400 cm^{-1} to 1500 cm^{-1}, 1300 cm^{-1} to 1400 cm^{-1} and 1000 cm^{-1} to 1200 cm^{-1} could be associated with TPC functional groups (-OH and NH stretch, C=O bending vibrations, aromaticity, primary or secondary -OH in-plane bending, phenol or tertiary alcohol -OH bend and C-O stretching vibrations) and may lead to radical scavenging activity [67][62]. This suggests that these functional groups might be co-occurring in the polyherbal formulation, possibly contributing synergistically to the chemical structure of polyphenols, as evidenced in R1, R2, R5, R8, R10, R12, R13, R14, and R16. Additionally, sample runs that had major contributions to the TPC and DPPH values were identified at negative PC1 values along the y-axis. Consequently, minor variations in the chemical composition across different plant varieties may be effectively discerned by applying this chemometric analysis of infrared spectral data [63]. In this study, PCA provided a clear visual relationship between TPC, DPPH, and infrared spectral fingerprints in the plant extracts.



(a)



(b)

Figure 4. PCA analysis of the first two components: (a) score plot and (b) biplot.

CONCLUSION

Optimisation of three plant extracts was successfully achieved using a simplex lattice mixture design aimed at TPC values and the DPPH radical scavenging activity. The optimised polyherbal formulation composed of PE (36.4 %), CS (17.5 %), and B-AS (46.1 %), demonstrated a TPC of 1205.1 ± 20.1 mg GAE/g and a DPPH inhibition of 96.2 ± 0.1 %. FT-IR spectra of the formulation were recorded to assess the presence of functional groups such as -OH, C=O, and C-O that were related to antioxidant potential. Interestingly, principal component analysis indicated a strong correlation between TPC, DPPH, and FT-IR spectra, where R^2 was equal to 1. These findings pave the way for the creation of innovative nutraceutical products with optimized antioxidant properties in the food industry. Future research should incorporate toxicity assessments through *in-vitro* studies using cell cultures to evaluate the formulation's protective effects against oxidative stress.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude for the fiscal assistance rendered by the *'Hadiah Latihan Persekutuan'* initiative, facilitated by the Ministry of Education Malaysia. A special thanks to Universiti Tun Hussein Onn Malaysia, Politeknik Merlimau Melaka, and Universiti Teknologi

Malaysia for providing the laboratory facilities and apparatus.

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