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Optimization of Polyphenol Extraction Conditions from Rhizomes of *Curcuma zedoaria* with Antioxidant, Anti-Inflammatory and Anti-Diabetic Activities *In Vitro*

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Abstract

The recovery of polyphenol compounds is seen as an arduous task because polyphenol compounds are available as free aglycones, as sugar or ester conjugates, or as polymers with several monomeric components. The response surface method (RSM) as a tool to optimize the factors that affect extraction efficacy as well as to obtain maximum recovery of the compounds of interest. This study optimizes ultrasound-assisted polyphenol extraction from *Curcuma zedoaria* rhizomes using response surface methodology (RSM). Three variables were considered: Ethanol concentration (70 - 90 %, v/v), temperature (60 - 80 °C), and ultrasound time (15 - 25 min). Data were analyzed by ANOVA, yielding an R^2 of 0.9993, a significant interaction effect ($p < 0.0001$), and an insignificant lack-of-fit test ($p = 0.6684$). Optimal conditions for maximum polyphenol content (TPC = 31.05 ± 0.53 mg GAE/g powder) were 80.02 % ethanol, 68 °C, 20.47 min ultrasound time, and 1/10 (w/v) raw material/solvent ratio. Experimental values matched RSM predictions, confirming successful extraction optimization from *Curcuma zedoaria* rhizomes. The polyphenol-rich optimum extract from *Curcuma zedoaria* rhizomes has been studied for its antioxidant, anti-inflammatory and anti-inflammatory properties *in vitro*. The results were found, the optimum extract of *Curcuma zedoaria* rhizomes could perform effective neutralization activities of free radicals performed in DPPH test (IC_{50} = 5.89 ± 0.23 µg/mL), $NO^{\bullet}$ (IC_{50} = 5.89 ± 0.23 µg/mL) and $ABTS^{\bullet+}$ (IC_{50} = 8.28 ± 0.12 µg/mL). Besides, this optimum extract had the ability to protect red blood cell membranes and inhibit protein denaturation due to heat with IC_{50} times values such as 29.06 ± 0.35 and 30.24 ± 0.32 µg/mL. In addition, it also significantly inhibited α-amylase, α-glucosidase enzyme activities with IC_{50} values of 5.89 ± 0.23 and 9.62 ± 0.11 µg/mL, respectively. This investigation showed that the polyphenol-rich optimum extract from *Curcuma zedoaria* rhizomes was a promising antioxidant, anti-inflammatory and anti-diabetic agent.

Keywords: Anti-diabetic, Anti-inflammatory, Antioxidant, *Curcuma zedoaria* rhizomes, Extract, Response surface methodology, Polyphenol

Introduction

Curcuma zedoaria is widely known as a medicinal plant and could be notably used in traditional medicine to reduce stomachache, indigestion, bloating and blood clotting in periods. Recent research demonstrated that this species could also resist cancer, remedy cardiovascular diseases, cerebrovascular, fibrosis, and inflammation, control blood lipids and reduce blood sugar [1-3]. *C. zedoaria* has been proven to contain highly bioactive compounds such as curcumin, curcumol and beta-elemene. Especially, polyphenol-group compounds in which curcumin is a prime example [4,5].

Research on animals, humans and epidemiology indicates that polyphenol-group compounds demonstrate antioxidant, and anti-inflammatory properties that could prevent and cure cardiovascular diseases, degenerative mental disease, cancer, diabetes, liver diseases and overweight [6,7]. Therefore, the research on optimal conditions for separating polyphenol-group compounds has interested scientists. Factors such as solvents, temperature, time and ingredients/solvents ratio are focused on during the process of extracting compounds from medicinal plants. Currently, to optimize the conditions for the process, scientists usually use Response Surface Methodology (RSM). This method, proposed by Box and Wilson [8], is an optimization for designing complete experiments and mathematical models and has been applied widely in the extraction process of plants because of its high accuracy and good prediction capabilities [9,10].

Materials and methods

Sample collection

Rhizomes of *C. zedoaria* (**Figure 1**) were purchased at Thanh Phu District, Ben Tre Province, Vietnam on 1st January 2020. They are shape-categorized by Mr. Chi Linh Tran (Biology Department, College of Natural Sciences, Can Tho University) and stored in Biochem-Clinical lab room (room C11.105), Department of Biochemistry, Faculty of Medicine, Tra Vinh University under storage code BT_Cze01010010.

Figure 1 Leaves, rhizomes and herbal powder of *C. zedoaria*.

Prepare herbal powder from plants

After acquirement, the rhizomes (6.25 kg) are washed and removed from rotten parts, then divided into 0.5 cm slices. After that, *C. zedoaria* is then dried under shades and then finely grounded (1.85 kg). The powder is filtered over a 60 mesh-fine tray and has its humidity determined according to the description from Vietnam Pharmacopoeia V. The sample, which has 7.15 ± 0.25 % humidity, is preserved in a PE plastic bag, placed in a plexiglass box and stored at 4 °C.

Determination of polyphenol contents

The polyphenol content of *C. zedoaria* rhizomes's powder is determined following the methods by Singleton and Rossi [11], albeit with minor changes. The extract of *C. zedoaria* (100 nL) is mixed with 100 nL of Folin-Ciocalteu (25 %) reagent. After 4 min, add 100 nL of sodium carbonate 7.5 % (w/v) solution into the mixture and let incubate in darkness and at room temperature for 30 min. Electromagnetic spectrum absorbance for the sample is measured by the Multiskan GO UV/Vis microplate spectrophotometer from Thermo Scientific (United States) at 765-nanometer waves. Control gallic acid with different concentrations (10, 20, 40, 60, 80 and 100 µg/mL) is prepared to form a control line. Samples are calculated based on the control line and measured by milligrams of gallic acid over grams of powder (mg GAE/g powder).

Determining effects of singular factors and optimization of the polyphenol extraction process

A heated ultrasonic machine (Derui DR-MH30, made in China) set to a frequency of 40 kHz has been used as an ultrasonic generator. Polyphenol in the tuber is extracted with ethanol solvents of different concentrations, from 40, 50, 60, 70, 80, 90 to 99.5 % (v/v). Temperature is gradually increased from 30, 40, 50, 60, 70, 80 to 90 °C. The duration of the ultrasonic process is increased from 5, 10, 15, 20, 25, 30 to 35 min. The ratio between ingredient/solvent is adjusted from 1/10, 1/15, 1/20, 1/25, 1/30, 1/35 to 1/40 (w/v). While studying the influence of single factors, 90 % alcohol at 30 °C, 10-minute ultrasonic time and 1/10 ratio between ingredients/solvents were fixed factors. Filter extracts and determine the amount of polyphenol as previously described. After determining single factors' influence, the researchers chose 3 factors that had the most impact on the polyphenol content in powdered *C. zedoaria* rhizomes to develop the optimized extraction process. The RSM was designed by Box-Behnken's experimental design with 3 factors and 3 levels in Design Expert 11.0 software during the development and evaluation phase.

Surveying antioxidant properties *in vitro* of optimized balm from *C. zedoaria* rhizomes

Optimized balm from *C. zedoaria* rhizomes had its free radical neutralization properties with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) evaluated according to descriptions by Khorasani *et al.* [12] with adjustments. Free radical neutralization with $ABTS^{\bullet+}$ was evaluated by reacting 990 µL $ABTS^{\bullet+}$ with 10 µL of optimized balm for 6 min at room temperature. The electromagnetic spectrum absorbance of the reaction blend was identified by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 734 nm waves.

Optimized balm from *C. zedoaria* rhizomes had its free radical neutralization properties with 2,2-diphenyl-1-picrylrylhydrazyl (DPPH) evaluated according to descriptions by Mensor *et al.* [13] with adjustments. The 960 µL of optimized balm at various concentrations was reacted with 40 µL DPPH (1,000 µg/mL). The reaction blend was left in the dark for 30 min at room temperature and had its electromagnetic spectrum absorbance measured by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 517 nm waves.

Optimized balm from *C. zedoaria* rhizomes had free radical neutralization properties with nitric oxide ($NO^{\bullet}$) according to descriptions by Ebrahimzadeh *et al.* [14] with adjustments. One mL of optimized balm from *C. zedoaria* rhizomes at various concentrations was reacted with 1 mL of sodium nitroprusside (5 mmol/L) for 150 min at room temperature. Afterward, 0.5 mL of Griess reagent was added, and the electromagnetic spectrum absorbance was measured by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 546 nm waves.

Ascorbic acid was used as a positive reference substance for all 3 methods. Antioxidation efficiency and the half maximal inhibitory concentration (IC_{50}) were determined according to descriptions by Khorasani *et al.* [12].

Surveying anti-diabetes properties *in vitro* of optimized balm from *C. zedoaria* rhizomes

α-Amylase enzyme inhibitory activity of optimized balm from *C. zedoaria* rhizomes was carried out based on Mohamed *et al.* [15] description with adjustments. The reaction blend contained 100 µL of optimized balm at various concentrations and 100 µL of phosphate buffer pH = 7 incubated with 100 µL of starch (2 mg/mL) at 37 °C for 15 min. The reaction was then halted by adding 400 µL HCl 1 M. The electromagnetic spectrum absorbance of the reaction blend was determined by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 660 nm waves before adding 600 µL of iodine reagent.

α-Glucosidase enzyme inhibitory activity of optimized balm from *C. zedoaria* rhizomes was determined according to descriptions by Chipiti *et al.* [16] with adjustments. Optimized balm (250 µL) at various concentrations was incubated with 500 µL enzyme α-glucosidase 1 U/mL (mixed in 100 mM phosphate buffer; pH = 6.8) at 37 °C for 15 min. Two hundred fifty µL of 4-nitrophenyl-D-glucopyranoside 5 mM (mixed in 100 mM phosphate buffer; pH = 6.8) was then added. The blend continued to incubate at 37 °C for 20 min. The electromagnetic spectrum absorbance of the released *p*-nitrophenol post-reaction was measured by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 405 nm waves.

α-Amylase, α-glucosidase enzyme inhibitory activity was evaluated based on inhibitory efficiency (%) and concentration (µg/ML) to inhibit 50 % enzyme activity (IC_{50}) based on descriptions by Mohamed *et al.* [15]. In addition, α-amylase and α-glucosidase enzyme inhibitory activity of optimized balm is also compared with acarbose.

Surveying anti-inflammatory properties *in vitro* of optimized balm from *C. zedoaria* rhizomes

Protection of red blood cells (RBCs) from heat-induced thalassemia was carried out based on the description by Banani *et al.* [17] with adjustments. Mice blood was centrifuged at 3,000 rpm for 10 min to remove inner fluids and rinsed with saline solution 3 times. Then, the blood was diluted with saline until a 10 % RBCs solution was obtained. Then, 1 mL of optimized balm from *C. zedoaria* rhizomes at various concentrations was reacted with 1 mL 10 % RBCs. The mixture was incubated at 56 °C for 30 min to cool down. It was then centrifuged at 2,500 rpm for 5 min. The electromagnetic spectrum absorbance was measured by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 560 nm wavelength.

Inhibition of protein denaturation: Albumin denaturation inhibition activity (Bovine Serum Albumin, BSA) was carried out according to descriptions by Sakat *et al.* [18] with adjustments. The reaction blend contained a 100 µL solution of BSA 0.5 %. It was then left to incubate at 37 °C for 15 min. Protein denaturation was caused by maintaining the blend's temperature at 70 °C for 10 min. After cooling down,

the electromagnetic spectrum absorbance was measured by Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (United States) at 660 nm wavelength.

Protection of RBCs and inhibition of BSA denaturation was evaluated based on inhibitory efficiency (%) and concentration (μg/ML) 50 % effective (IC_{50}) based on descriptions by Banani *et al.* [17]. In addition, the ability to protect RBCs from heat-induced thalassemia and inhibit BSA denaturation of optimized balm from *C. zedoaria* rhizomes was also compared with diclofenac.

In the experiments above, optimized balm from *C. zedoaria* rhizomes was also compared with its unoptimized counterpart regarding antioxidant, anti-diabetes and anti-inflammatory properties. Unoptimized balm from *C. zedoaria* rhizomes is extracted under unoptimized conditions using ultrasonic waves, 99.5 % ethanol (v/v), 30 °C ultrasonic temperature, 5 min of ultrasonic time, and has a ratio between ingredient and solvent of 1/10. In this article, we will call it "unoptimized balm".

Processing and analyzing statistics

Statistics in the single-factor study were presented as Mean ± Standard deviation (SD) and processed with ANOVA-Tukey test in Minitab 16.0. The graph is designed in Microsoft Excel 2013. Statistics in the optimization model were interpreted with Design Expert 11.0.

Results and discussion

Impacts of single-factors on the polyphenol extraction process

The solvent is considered the main factor that heavily affects the number of natural compounds during extraction. Most polyphenols are polar compounds; therefore, selecting a solvent with a suitable polarity to extract polyphenols is necessary. Previous research has shown that carbonic solvents mixed with water form an environment with suitable polarity for polyphenol extraction [19]. Therefore, the research team mixed 99.5 % ethanol with water to form different ethanol concentrations from 40 to 99.5 % (v/v) to extract polyphenol from powdered *C. zedoaria* rhizomes. As presented in **Figure 2**, the largest polyphenol extract from powdered *C. zedoaria* rhizomes was 10.45 ± 0.11 mg GAE/g powder using 80 % ethanol. Therefore, ethanol mixes with 70 - 90 % concentration were chosen for the next experiment.

Aside from solvents, temperature also impacts the number of natural compounds during extraction. Research indicated that the molecular speed, diffusibility and solubility of compounds rapidly increase when ultrasonic temperature rises [20]. Appropriate temperature rise can increase the extraction efficiency as it reduces viscosity, increases solvent osmosis into cells and increases solubility with the extraction coefficient of extricated compounds [21]. However, polyphenol is prone to destruction if the extraction temperature is unsuitable. Temperature can increase or decrease the number of substances during extraction.While experimenting with temperatures ranging from 30, 40, 50, 60, 70, 80 to 90 °C, the most polyphenol extracted was 12.93 ± 0.04 mg GAE/g powder. Therefore, it can be concluded that temperature has a noticeable impact on the polyphenol content. The research team chose the 60 - 80 °C range for the following experiments.

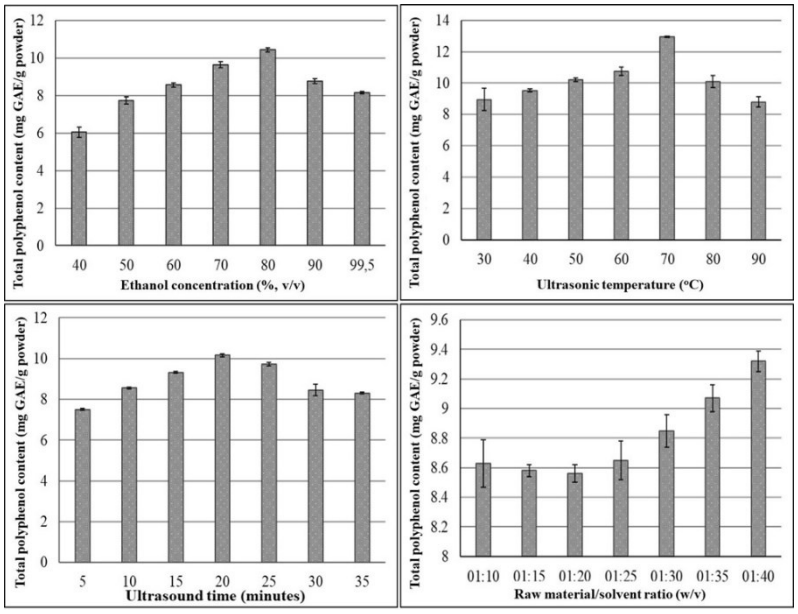

Figure 2 Polyphenol content of *C. zedoaria* powder.
Note: The mean ± standard deviation (min-max) was compared using Tukey test.

The ultrasonic time is an essential factor directly impacting the polyphenol content in the extracted plant sample. Therefore, researchers evaluated the polyphenol content with various ultrasonic times, ranging from 5 to 35 min. The results demonstrated that the maximum polyphenol extraction is at 20 min (10.16 ± 0.07 mg GAE/g powder) and decreases with time. They align with what Tomaz *et al.* [22] researched, as polyphenol-group compounds dissolved and formed emulsions as time increased. Therefore, it was concluded that ultrasonic time is around 15 - 20 min for the following optimization experiments.

The ratio between ingredient and solvent is another relevant factor to their contact area, affecting the natural compound's extraction process. The research group experimented with 1/10, 1/15, 1/20, 1/25, 1/30, 1/35 and 1/40 ingredient/solvent ratio (w/v). According to the results presented in **Figure 2**, it is visible that the polyphenol content extracted from *C. zedoaria* rhizomes increased with an increase in the ingredient/solvent ratio. The polyphenol content rose from 8.63 ± 0.16 mg GAE/g powder (1/10, w/v) to 9.32 ± 0.07 mg GAE/g powder (1/40, w/v). The researchers concluded that there are no significant changes to polyphenol contents as the ratio between ingredient and solvent increases [23]. Therefore, to conserve solvents, the ratio is fixed at 1/10 (w/v) compared with acarbose.

Optimization of polyphenol extraction process

Based on the evaluations of factors that impact the condition for ultrasonic polyphenol extraction, ethanol concentration, temperature and ultrasonic time are 3 factors that influence the experiment process most. The Response Surface Methodology following Box-Behnken with 3 variables, and 3 levels were used to build an optimized strategy for polyphenol extraction from *C. zedoaria*'s powder. Results from **Table 1** indicate that central methods (Methods 13 - 17) yield higher polyphenol content than others. Method 14 (30.93 ± 0.61 mg GAE/g powder) yield the highest polyphenol content. In addition, this research demonstrates a similarity in the actual and predicted model of polyphenol content.

Table 1 Box-Behnken experiment matrix with variables of powdered *C. zedoaria* rhizomes.

Method(s)	Independent variables			Polyphenol content (mg GAE/g powder)	
	Ethanol (%, v/v)	Temperature (°C)	Time (min)	Actual yield	Predicted yield
1	70	60	20	$16.89^{e} \pm 0.32$	17.00
2	90	60	20	$17.77^{e} \pm 0.30$	17.92
3	70	80	20	$10.53^{fg} \pm 0.57$	10.40
4	90	80	20	$9.16^{g} \pm 0.52$	9.07
5	70	70	15	$21.64^{bc} \pm 0.13$	21.56
6	90	70	15	$20.54^{cd} \pm 0.65$	20.43
7	70	70	25	$21.89^{bc} \pm 0,23$	22.02
8	90	70	25	$22.63^{b} \pm 0.57$	22.73
9	80	60	15	$17.99^{e} \pm 0.23$	17.98
10	80	80	15	$10.17^{fg} \pm 0.24$	10.40
11	80	60	25	$19.72^{d} \pm 1.44$	19.50
12	80	80	25	$11.61^{f} \pm 0.23$	11.64
13	80	70	20	$30.07^{a} \pm 0.60$	30.54
14	80	70	20	$30.93^{a} \pm 0.61$	30.54
15	80	70	20	$30.33^{a} \pm 0.21$	30.54
16	80	70	20	$30,79^{a} \pm 0.61$	30.54
17	80	70	20	$30.55^{a} \pm 0.13$	30.54

Note: The suffixes following identical columns are not significant to the data ($p < 0.05$). The mean ± standard deviation (min-max) was compared using Tukey test.

The research group analyzed the data and retrieved the following model for polyphenol content calculations:

$$\text{Polyphenol} = -930.09 + 8.31\times A + 16.72\times B + 5.56\times C - 0.006\times A\times B + 0.009\times A\times C - 0.001\times B\times C - 0.05\times A^2 - 0.12\times B^2 - 0.15\times C^2.$$

where A is ethanol content, B is temperature and C is time.

Analysis of variance (ANOVA) was used to evaluate the model, which outputted results in **Table 2**. The regression function for polyphenol content is a polynomial and is in good accordance with meaningful data ($p < 0.05$). These results indicate the suitability of this model for precise prediction of the target function. The polyphenol content function also shows statistics explaining the variance of the target function with good accuracy of $R^2 = 0.9993$. Therefore, it is determined that there is an important correlation between predicted and actual polyphenol content. Considering R^2 correlation coefficiency, a multi-variable regression model is considered good with a minimum value of 0.8, much lower than the value of R^2 in this research, meaning that the polyphenol content function explains 99.93 % of actual data. Moreover, the variability (CV = 1.5 %) for the proposed model is smaller than 10 %, demonstrating the accuracy and

dependability of the experimental process. The coefficients in the regression model contain meaningful differences ($p < 0.05$), apart from A, B and C ($p > 0.05$).

Table 2 Analyzing coefficients of factors impacting polyphenol content.

Sources	Sum of squares	Df	Mean square	F-value	*p*-value
Model	950.84	9	105.65	1085.61	< 0.0001
A-ethanol content	0.0882	1	0.0882	0.9063	0.3728
B-temperature	119.35	1	119.35	1226.42	< 0.0001
C-time	3.81	1	3.81	39.14	0.0004
AB	1.27	1	1.27	13.01	0.0087
AC	0.8556	1	0.8556	8.79	0.0210
BC	0.0210	1	0.0210	0.2160	0.6562
A^2	108.26	1	108.26	1112.48	< 0.0001
B^2	593.83	1	593.83	6101.96	< 0.0001
C^2	60.34	1	60.34	620.08	< 0.0001
Residual	0.6812	7	0.0973		
Lack of fit	0.2017	3	0.0672	0.5608	0.6688
Pure error	0.4795	4	0.1199	N = 17	CV = 1.50 %
Cor total	951.52	16	$R^2 = 0.9993$	$R^2_{Adj} = 0.9984$	$R^2_{Pre} = 0.9958$

Note: Cor total is corrected total sum of squares.

In addition, 3D surface response figures are made by fixing a factor to 0 and altering the other 2 factors in the survey range. These 3D figures described the interactive effect of factors on the polyphenol content, as described in **Figure 3**. During optimization, ethanol concentration and temperature are the 2 most important numbers affecting polyphenol content. From actual experiment results, polyphenol content scales up with ethanol concentration and temperature, up to 80 % concentration and 70 °C before decreasing. Using a mixture of catalysts (ethanol and water) can increase the number of polyphenols or other compounds with the highest or low polarity [24]. Combining with ultrasonic temperature during the separation process, the catalyst's polarity may decrease and be suitable for the target compound, as the mixture of water and ethanol can improve separation effectiveness and increase surface area for the catalyst and contacting powder [25]. It can be seen from **Figure 3** that increasing separation time decreases the polyphenol content in powdered *C. zedoaria* rhizomes. Based on the resulting analysis, the best method to extract polyphenols from *C. zedoaria* rhizomes' powder is described in **Figure 4**, with the optimal polyphenol content of 30.88 mg GAE/g powder obtained at 68 °C ultrasonic temperature, 20.47 min of ultrasonic time, 80.02 % ethanol concentration and an ingredient/solvent ratio of 1/10 (w/v). Actual experimentation results in a yield of 31.05 ± 0.53 mg GAE/g, close to prediction results.

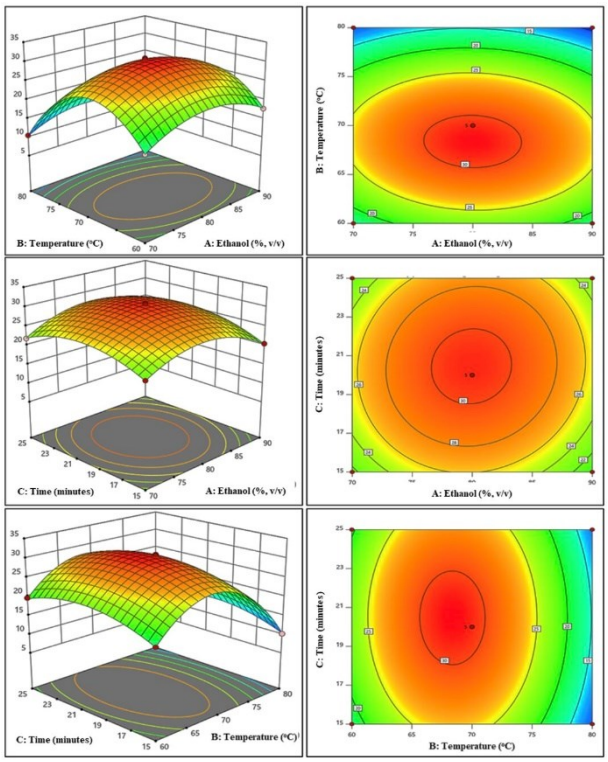

Figure 3 Response surface to polyphenol content of powdered *C. zedoaria* rhizomes.

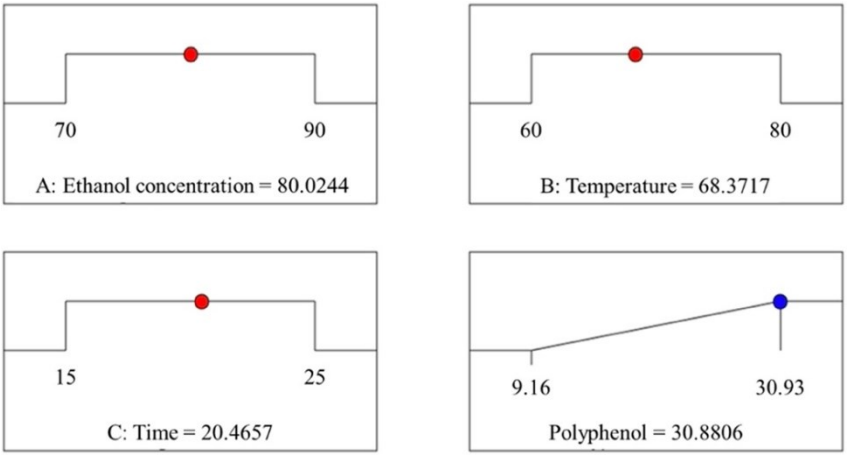

Figure 4 Expected model and optimized polyphenol extraction conditions.

Results of the bioactivity survey

In vitro antioxidant activity

Antioxidant ability of experiment models was evaluated by inhibiting free radicals belonging to active oxygen groups represented by $ABTS^{\bullet+}$ and inhibiting free radicals belonging to active nitrogen groups represented by DPPH and $NO^{\bullet}$. Results have indicated that both optimized and unoptimized balm from *C. zedoaria* rhizomes and ascorbic acid can neutralize all 3 free radicals $ABTS^{\bullet+}$, DPPH and $NO^{\bullet}$. Antioxidant activity of the optimized balm was surveyed at concentrations from 1 to 10 µg/mL with antioxidant efficiency ranging from 3.82 ± 0.82 to 89.84 ± 1.32 % (**Figure 5**). The optimized balm has better $ABTS^{\bullet+}$, DPPH and $NO^{\bullet}$ neutralization efficiency than the unoptimized balm. Research has indicated

that polyphenols can electrify free radicals, returning them to a neutral, stable state and harmless to human grand biomolecules [26].

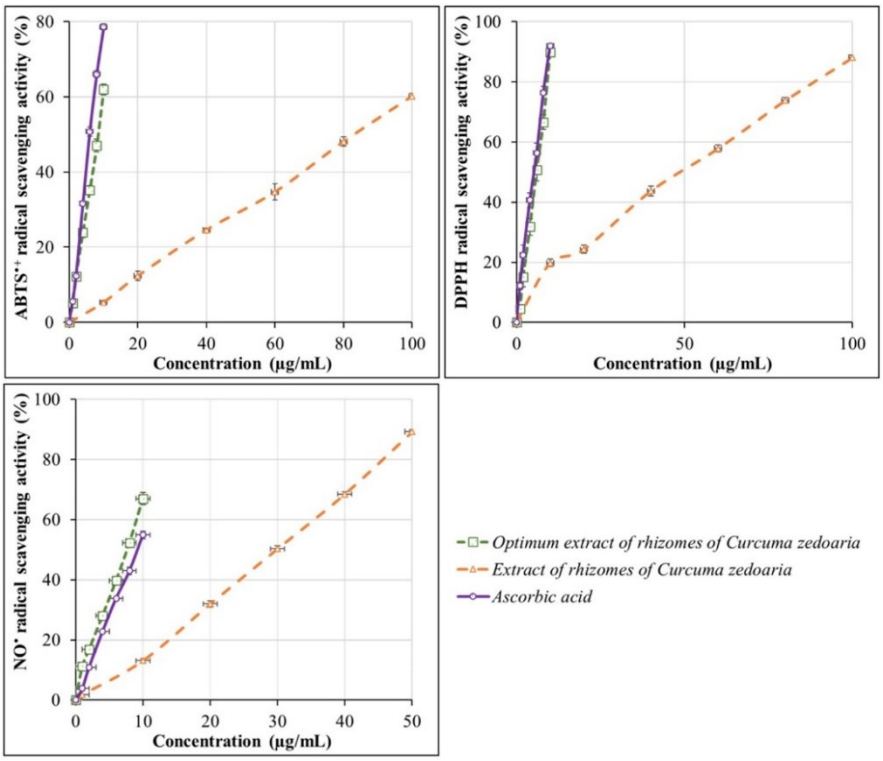

Figure 5 The antioxidant activities in 3 assays.
Note: The mean ± standard deviation (min-max) was compared using Tukey test.

In vitro anti-diabetes activity

Diabetes is increasing at an alarming rate and significantly affects human health. An important strategy in controlling blood sugar is inhibiting main carbohydrate-digesting enzymes, such as α-amylase and α-glucosidase, which also prevents complications from diabetes. Inhibitants to α-amylase and α-glucosidase enzymes slow down the carbohydrate digestion process, decreasing the glucose induction rate of the small intestine and decreasing blood sugar after eating. Therefore, inhibiting α-amylase and α-glucosidase enzymes is the key to managing and curing diabetes [27]. In this research, optimized balm from *C. zedoaria* has an inhibition efficiency against α-amylase enzyme from 3.13 ± 0.31 to 91.94 ± 1.84 % and against α-glucosidase enzyme from 6.13 ± 0.45 to 51.29 ± 1.25 %. A-amylaseand α-glucosidase enzyme inhibition of *C. zedoaria* rhizomes is tightly correlated with polyphenol content. Polyphenol is capable of inhibiting α-amylase and α-glucosidase enzymes depending on the content, structure, placement and number of hydroxyl groups. Polyphenol induces α-amylase and α-glucosidase enzymes inhibition by forming hydrogen bonds between hydroxyl groups of polyphenol and amino acids at active locations of α-amylase and α-glucosidase enzymes [28] (**Figure 6**).

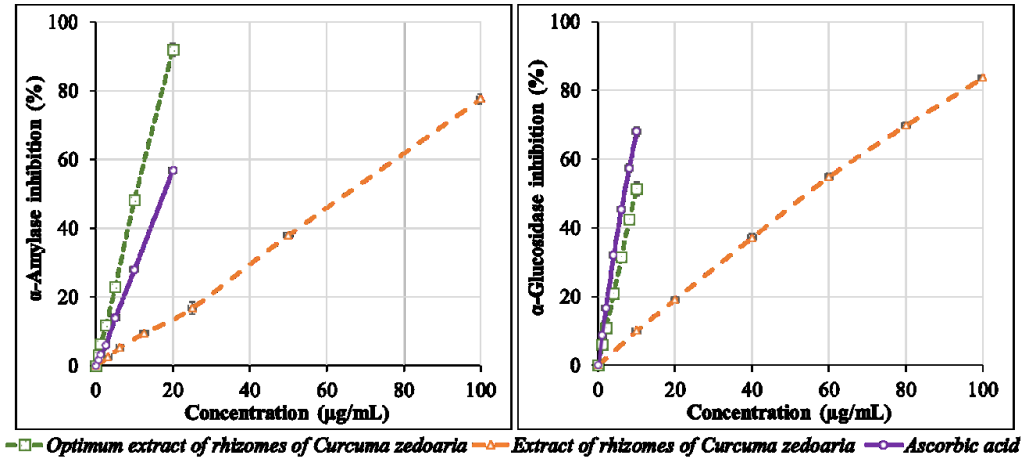

Figure 6 The percentage inhibition of α-amylase and α-glucosidase.
Note: The mean ± standard deviation (min-max) was compared using Tukey test.

In vitro anti-inflammatory activity

In this research, cow serum albumin was used as a denatured source of protein. Under high temperatures, cow serum albumin underwent the denaturation process and demonstrated antigens related to type 3 hypersensitivity linked to diseases such as renal corpuscle inflammation, rheumatoid arthritis and lupus rashes [29]. Therefore, inhibiting the denaturation of cow serum albumin partially demonstrated the potential to cure inflammatory diseases of plants. Optimized balm from *C. zedoaria* rhizomes can inhibit denaturation of cow serum albumin from 3.04 ± 1.15 % at 1 µg/mL concentration to 80.01 ± 0.55 % at 50 µg/mL concentration. Ullah *et al.* [29] research also showed that *C. zedoaria* can inhibit white egg yolk albumin denaturation under heat. Many projects have proven that polyphenol and protein interaction increases protein stability under heat [30]. Ali *et al.* [31] research confirmed that polyphenols improved protein stability under heat in soybean glycinin, cow serum albumin and cow *p*-lactoglobulin.

In inflammatory reactions, antibodies release lysosomal components, such as protease, to damage organism cells [32]. Therefore, plant extracts can prevent enzyme release from lysosomes and stabilize lysosome membranes, making them an essential anti-inflammatory base [33]. Due to the similarities between RBC membranes and lysosomal, protection of RBC membranes from heat-induced damage has been used to evaluate the anti-inflammatory potentials of polyphenols. In this research, optimized balm from *C. zedoaria* rhizomes protected RBC membranes well against heat-induced thalassemia better than their unoptimized counterparts (**Figure 7**). The optimized balm has better RBC cells protection efficiency with 66.13 ± 1.91 % at 40 µg/mL concentration, compared with the unoptimized balm's 22.30 ± 1.40 % at the same surveyed concentration. Polyphenol can manage interactions with hydrophile ends of cytoplasmic membranes, reducing maneuverability and increasing the rigidity of the membranes. These interactions support hydrogen bonds to protect the membranes against harmful molecules, such as pre-oxidants, ensuring membrane intactness and biophysical functions. Polyphenol plays an important role in preventing RBC membranes from lipid peroxidation and stabilizing these membranes against hypotonic lysis [34].

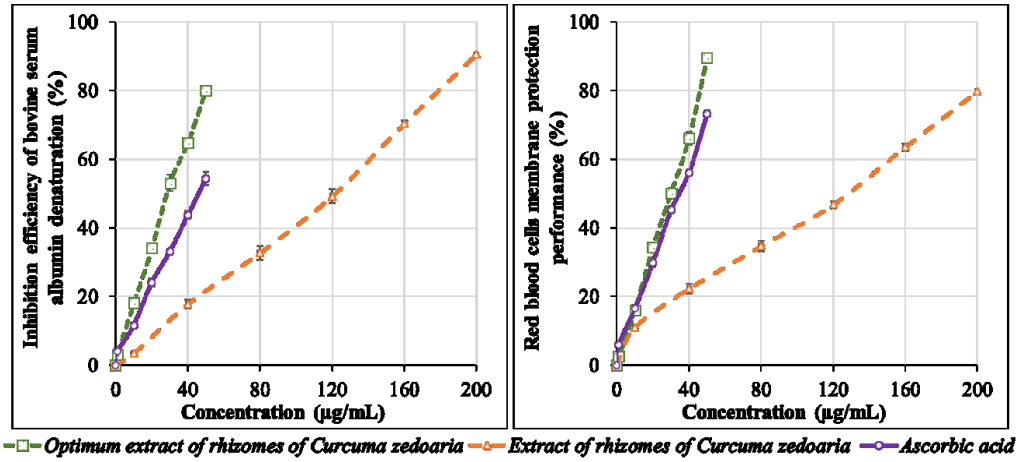

Figure 7 *In vitro* anti-inflammatory performance.

Note: The mean ± standard deviation (min-max) was compared using Tukey test.

Comparing bioactivity of optimized balm from *C. zedoaria* rhizomes, unoptimized balm from *C. zedoaria* rhizomes and standard substances based on 50 % effective concentration

In vitro antioxidant, anti-inflammatory and anti-diabetes abilities of optimized balm from *C. zedoaria* rhizomes and balm from *C. zedoaria* rhizomes are compared with ascorbic acid, acarbose and diclofenac through IC_{50} values presented in **Table 3**.

Optimized balm from *C. zedoaria* rhizomes can neutralize free radicals $ABTS^{\bullet+}$, DPPH and $NO^{\bullet}$ with IC_{50} values of 8.28 ± 0.12, 5.89 ± 0.23 and 7.47 ± 0.16 µg/mL, respectively. According to Blois [35], a test sample with IC_{50} values below 50 µg/mL is considered a powerful anti-oxidant. Therefore, optimized balm from *C. zedoaria* rhizomes is determined to possess powerful antioxidant properties. Optimized balm from *C. zedoaria* rhizomes is 10.13, 8.75 and 3.21 times stronger than unoptimized balm against $ABTS^{\bullet+}$, DPPH and $NO^{\bullet}$ free radicals, respectively. However, optimized balm is still weaker than ascorbic acid as an anti-oxidant. In Shehna *et al.* [36] research, unoptimized balm from *C. zedoaria* rhizomes can neutralize $ABTS^{\bullet+}$ and DPPH free radicals with IC_{50} of 33.9 and 82.32 µg/mL respectively, matching our research. Through optimized extraction, the optimized balm has an $ABTS^{\bullet+}$ and DPPH free radicals neutralization efficiency 4.09 and 13.08 times higher than that of the unoptimized balm in Shehna *et al.* [35] research. α-Amylase and α-glucosidase enzymes directly break down starch into simpler carbohydrates. α-Amylase and α-glucosidase inhabitants reduce glucose ingestion for diabetes patients. Optimized balm from *C. zedoaria* rhizomes indicated higher inhibitory activity for α-amylase (IC_{50} = 26.87 ± 0.60 µg/mL) and α-glucosidase (IC_{50} = 9.62 ± 0.11 µg/mL) than unoptimized balm ($IC_{50,\ \alpha\text{-amylase}}$ = 65.09 ± 0.99 µg/mL; $IC_{50,\ \alpha\text{-glucosidase}}$ = 56.77 ± 0.09 µg/mL), but still weaker than acarbose ($IC_{50,\ \alpha\text{-amylase}}$ = 17.66 ± 0.20 µg/mL; $IC_{50,\ \alpha\text{-glucosidase}}$ = 6.97 ± 0.05 µg/mL) by 1.52 and 1.38 times, respectively. Optimized balm from *C. zedoaria* rhizomes has higher BSA denaturation inhibition (IC_{50} = 30.24 ± 0.32 µg/mL) and RBCs protection (IC_{50} = 29.06 ± 0.35 µg/mL) than unoptimized balm ($IC_{50,\ BSA}$ = 114.63 ± 1.18 µg/mL; $IC_{50,\ RBCs}$ = 122.41 ± 1.93 µg/mL) by 3.79 and 4.21 times, respectively. In comparison with diclofenac ($IC_{50,\ BSA}$ = 45.74 ± 1.14 µg/mL; $IC_{50,\ RBCs}$ = 34.12 ± 0.39 µg/mL), optimized balm from *C. zedoaria* rhizomes possesses 1.51 times better BSA denaturation inhibition and 1.17 times better RBCs protection. The results indicated that polyphenol content is tightly related to the bioactivity of plants. The higher the polyphenol content, the more bioactivity the plant possesses and vice versa, which is similar to Göger *et al.* [37] research findings. Therefore, this research has established an optimized extraction process for polyphenols from *C. zedoaria* rhizomes, along

with proving the role of polyphenols as an *in vitro* antioxidant, anti-inflammatory and anti-diabetes substance.

Table 3 Regression model, 50 % effective concentration.

Method	Sample	Regression model	IC_{50} value (µg/mL)
$ABTS^{\bullet+}$	Optimum extract of RCz	$y = 6.1035x - 0.5266$ ($R^2 = 0.9983$)	$8.28^{b} \pm 0.12$
	Extract of RCz	$y = 0.601x - 0.1285$ ($R^2 = 0.9991$)	$83.85^{a} \pm 0.64$
	Ascorbic acid	$y = 8.2527x - 1.5305$ ($R^2 = 0.9954$)	$6.24^{c} \pm 0.05$
DPPH	Optimum extract of RCz	$y = 8.9863x - 2.8732$ ($R^2 = 0.996$)	$5.89^{b} \pm 0.23$
	Extract of RCz	$y = 0.8364x + 6.895$ ($R^2 = 0.9855$)	$51.54^{a} \pm 1.20$
	Ascorbic acid	$y = 9.0861x + 2.622$ ($R^2 = 0.9976$)	$5.21^{c} \pm 0.20$
$NO^{\bullet}$	Optimum extract of RCz	$y = 5.5302x - 0.3281$ ($R^2 = 0.9982$)	$9.10^{b} \pm 0.08$
	Extract of RCz	$y = 1.7765x - 1.8932$ ($R^2 = 0.9965$)	$29.21^{a} \pm 0.29$
	Ascorbic acid	$y = 6.3451x + 2.6123$ ($R^2 = 0.9949$)	$7.47^{c} \pm 0.16$
α-Amylase	Optimum extract of RCz	$y = 1.845x + 0.4071$ ($R^2 = 0.9994$)	$26.87^{b} \pm 0.60$
	Extract of RCz	$y = 0.7737x - 0.3539$ ($R^2 = 0.9987$)	$65.09^{a} \pm 0.99$
	Acarbose	$y = 2.8494x - 0.3395$ ($R^2 = 0.9997$)	$17.66^{c} \pm 0.20$
α-Glucosiadase	Optimum extract of RCz	$y = 5.1426x + 0.5608$ ($R^2 = 0.9992$)	$9.62^{b} \pm 0.11$
	Extract of RCz	$y = 0.8419x + 1.9596$ ($R^2 = 0.9969$)	$56.77^{a} \pm 0.09$
	Acarbose	$y = 6.826x + 2.3862$ ($R^2 = 0.9941$)	$6.97^{c} \pm 0.05$
BSA	Optimum extract of RCz	$y = 1.5997x + 1.6217$ ($R^2 = 0.9968$)	$30.24^{b} \pm 0.32$
	Extract of RCz	$y = 0.4469x - 1.2246$ ($R^2 = 0.9969$)	$114.63^{a} \pm 1.18$
	Diclofenac	$y = 1.0592x + 1.582$ ($R^2 = 0.9974$)	$45.74^{c} \pm 1.14$
RBCs	Optimum extract of RCz	$y = 1.7374x - 0.4876$ ($R^2 = 0.9965$)	$29.06^{b} \pm 0.35$
	Extract of RCz	$y = 0.3725x + 4.4091$ ($R^2 = 0.9912$)	$122.41^{a} \pm 1.93$
	Diclofenac	$y = 1.3996x + 2.2512$ ($R^2 = 0.9962$)	$34.12^{c} \pm 0.39$

Note: RCz = *Curcuma zedoaria* rhizomes. The suffixes following identical columns are not significant to the data ($p < 0.05$). The mean ± standard deviation (min-max) was compared using Tukey test.

Conclusions

Polyphenols were efficiently extracted from *C. zedoaria* rhizomes powder using ultrasonication, and optimization was carried out through Response Surface Methodology, employing the Box-Behnken model to assess the interplay of independent variables like ethanol concentration, temperature and ultrasonic time. The results revealed significant interactions within the model ($p < 0.001$). The optimized conditions included a 68 °C ultrasonic temperature, 20.47 min of ultrasonic time, 80.02 % ethanol concentration and a 1/10 (w/v) ingredient/solvent ratio. Notably, the predicted and actual results closely matched (30.88 mg GAE/g powder and 31.05 ± 0.53 mg GAE/g, respectively). The polyphenol extraction method from *C. zedoaria* rhizomes was effectively optimized in this study, and the optimized balm had better anti-inflammatory, anti-diabetic and antioxidant qualities than the unoptimized version.

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