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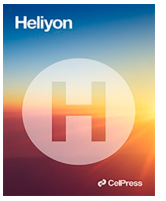

Research article

Optimizing ultrasound-assisted extraction of bioactive compounds from *Canthium horridum* blume leaves utilizing polyols: A study on skin-related activities

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A B S T R A C T

The focus on reducing organic solvent usage, owing to their negative environmental and health impacts, is driving a search for innovative green alternative solvents in academia and industry. *Canthium horridum* Blume (CH) exhibits many therapeutic activities, including antioxidant and anti-inflammatory efficacy. The objective of this study is to evaluate the optimal solvent concentration using a simplex-lattice design with an aqueous-polyols mixture and to optimize the parameters for extracting bioactive compounds and antioxidant activities from ultrasound-assisted extraction (UAE) of CH leaves through central composite design (CCD) in response surface methodology (RSM). This study examines the total phenolic content (TPC), antioxidant activities, comparison of different extraction conditions, identification of bioactive compounds, cell cytotoxicity, cellular antioxidant activity, and melanin content reduction efficacy of the extracts. According to the findings from the simplex-lattice model, the ideal solvent composition consisted of 32.57%w/w butylene glycol, 32.92%w/w glycerine, and 34.51%w/w water. Furthermore, based on the response model, optimal extraction conditions were identified as a 15-min extraction time and a solvent-to-sample ratio of 32.94:1. In comparison to alternative extraction methods, ultrasonic-assisted extraction using the aqueous-glycerine-butylene glycol (GB-UAE) extract resulted in notably elevated TPC and antioxidant responses ($p < 0.05$). Major antioxidant bioactive compounds included 4-(Butoxymethyl) phenol, 3-*O*-Caffeoyl-4-*O*-methyl-quinic acid, Quercetin 3-(2G-glucosylrutinoside), 2,4-Dihydroxybenzoic acid and other bioactive compounds. The GB-UAE extract revealed greater cell viability than UAE using ethanol (EtOH-UAE) extract in both cytotoxicity and cellular antioxidant assays at the same concentration. Additionally, it exhibited comparable melanin content reduction efficacy at a higher concentration compared to that of EtOH-UAE extract. The researcher anticipates that the current study will advance the utilization of an aqueous-polyols system for extracting bioactive compounds extending beyond CH leaves. Although the potential applications of CH leaves in cosmetics and pharmaceutical formulations have been identified, further comprehensive mechanistic and clinical studies are required to fully understand their effects.

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1. Introduction

Currently, there is a significant focus on minimizing the utilization of organic solvents, having adverse impacts on human well-being and the ecological systems. This emphasis aligns with a growing public awareness of environmental protection. Therefore, the search for innovative green solvents to replace organic solvents addressing environmental concerns and health hazards has become a significant challenge in academia and industry [1]. Green chemistry principles require solvents to be low toxicity, non-flammable, non-mutagenic, non-volatility, cheap, easy to handle, and recyclable [2]. Recently, water, ionic liquids, supercritical fluids ($scCO_2$), and perfluorinated solvents have emerged as promising approaches for solvent innovation. However, their utilization is constrained by factors such as high costs and insufficient data regarding toxicity and biocompatibility. Additionally, challenges arise with product separation in aqueous-based processes. Therefore, a universal green solvent is not yet available, prompting the scientific community to continuously search for sustainable solvents for catalytic and organic processes [2]. Currently, there is increasing interest in using polyols including glycerine, propylene glycol, and butylene glycol from the viewpoint of green chemistry as an alternative approach for bioactive compounds extraction from plant materials. Comparable to polar solvents, these polyols exhibit the capacity to facilitate the dissolution of diverse substances [2,3]. These polyols have been classified as safe chemicals, consequently, their application extends across a spectrum of industries, including but not limited to the pharmaceutical, cosmetic, and food sectors. Contrary to the conventional use of organic solvents in plant-based bioactive compound extraction, the employment of these polyols offers a unique advantage. Specifically, the need to eliminate residual solvents from the resulting extracts is mitigated, and the potential hazards associated with organic solvent residues are obviated [4,5]. As GRAS chemicals, polyols enable the direct incorporation of extracts obtained through polyol-based plant extraction into cosmetic and medicinal applications.

In the context of green chemistry, it is important to select the extraction method considerately, as it profoundly influences the environmental impact and sustainability of chemical processes [6]. UAE is a non-conventional extraction method that offers significant economic and environmental advantages. The utilization of ultrasonic waves in the UAE method significantly improves the capacity to extract bioactive compounds from plant materials. By operating at lower temperatures and shorter extraction periods than traditional techniques, the technology minimizes its impact on the environment and conserves energy. Furthermore, the UAE lessens the requirement for harmful chemical solvents, which helps to lessen the production of hazardous waste and promotes an extraction method that is more environmentally friendly [7]. Because ultrasonic-assisted extraction is environmentally safe, it fits nicely with modern green chemistry concepts and can be a useful method for producing plant extracts that are both ecologically friendly and sustainable [8].

The majority of the Akha community resides in Mae Sai, located in Chiang Rai District, utilize CH leaves for medicinal purposes, particularly for treating skin conditions through topical application to combat skin diseases and alleviate itching and skin rash [9]. CH belongs to the *Rubiaceae* plant family and contains various bioactive compounds, including quercetin, *p*-hydroxybenzoic acid, and vanillic acid, exhibiting significant antimicrobial properties [10]. Moreover, studies on the biological properties of the *Canthium* genus reveal antioxidant, anti-cancer, anti-inflammatory, wound healing, diuretic, hypoglycemic, and cholesterol-lowering effects [10,11]. Consequently, plants belonging to the *Canthium* genus are employed in traditional medicine to treat various ailments such as coughs, diabetes, and hypertension [11].

Recent studies encompassing gas chromatography-mass spectrometry (GC-MS) analysis of essential oils derived from CH leaves and a bioassay-guided investigation into the isolation of bioactive compounds from the CH stem have been conducted [10,12,13]. Nevertheless, the investigation of UAE utilizing polyols for extracting phenolic compounds and analyzing bioactive compound compositions in CH leaf extracts through LC-MS/MS is not extensively documented in existing literature. Additionally, previous studies in the field of aqueous-polyols systems have predominantly focused on the use of a single polyol [14,15], with limited exploration of systems incorporating two or more polyols for the extraction of bioactive compounds from plants. Consequently, this research aims to develop a novel solvent system for phenolic extraction using a simplex-lattice design, optimizing extraction conditions through response surface methodology (RSM) employing a central composite design (CCD). Additionally, this study aims to compare the efficacy of ultrasonic-assisted extraction with conventional maceration, undergo composition analysis of the extracts through ultra-high performance liquid chromatography coupled with electrospray ionization/quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS/MS), and evaluate cytotoxicity, cellular antioxidant activity, and melanin content reduction capacity of CH leaf extracts for potential cosmetic applications introducing an environmentally friendly extraction method.

2. Materials and methods

2.1. Samples and chemicals

In November 2019, CH leaf samples were collected from Ban Huai San, located in Chiang Rai District, Thailand. The botanical identification of the plant was conducted by botanists, and a voucher sample (MFU-00618) was filed at the herbarium of Mae Fah Luang University in Chiang Rai, Thailand. The study utilized cosmetic-grade glycerine and butylene glycol, obtained from Chanjao Longevity Company Limited, Bangkok, Thailand. All other chemical reagents utilized in the experiment adhered to a minimum analytical grade standard.

2.2. Sample preparation

The freshly harvested CH leaves were subjected to dry in a tray dryer set at 50 °C for a duration of 24 h. Following this, the dried

leaves were finely ground into powder and stored at room temperature for use in subsequent experiments.

2.3. Selection of the appropriate solvent concentration

Glycerine, butylene glycol, and water were selected to evaluate the optimal solvent concentration using a simplex-lattice mixture design (Table 1). This experimental design comprised 15 experiments. The extraction efficiency was assessed by measuring the TPC, and statistical analyses of variance (ANOVA) were conducted to identify solvent mixtures that significantly influenced the extraction process.

2.4. Ultrasonic-assisted extraction

The method for extracting CH leaf involved employing a 6 mm probe ultrasonicator (VCX 130, Vibra cell, Sonics & Materials, USA), operating consistently at a frequency of 20 kHz and at room temperature as outlined in a specified protocol [15].

2.5. Selection of extraction duration and solvent-sample ratio

The experimental setup was utilized to identify the relevant parameters, specifically the extraction duration (ranging from 5 to 30 min) and the solvent-sample ratio (varied at 10:1, 15:1, 20:1, 25:1, and 30:1). Each experimental iteration involved the systematic alteration of a single independent variable while maintaining the constancy of other parameters. Subsequently, an assessment of the independent factors exerting a statistically significant influence on the efficacy of the extraction procedure was conducted by measuring TPC.

2.6. Experiment design for optimization of extraction method

The optimization of parameters for bioactive compounds extraction from CH leaves was systematically undertaken through the application of RSM employing a CCD. The aim was to identify the extraction conditions that would result in optimal values for both the bioactive compounds and antioxidant properties of CH leaves. Two variables, namely time (A, in minutes) and solvent-sample ratio (B), were varied at five coded levels (Table 2). Fourteen experimental runs were systematically evaluated to develop models for total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, and ferric reducing antioxidant power (FRAP).

2.7. Determination of the total phenolic content and antioxidant activities

TPC and antioxidant activities (DPPH, ABTS, and FRAP assays) were evaluated according to the method stated [16].

2.8. Validation of expected value

The established response model was validated under the optimized extraction conditions determined by CCD to assess the precision and reliability of the model.

Table 1
The TPC with expected and observed responses using simplex-lattice design.

No.	Butylene glycol (A)	Glycerine (B)	Water (C)	TPC (GAE mg/g sample)	
				Expected value	Observed value
1	1	0	0	3.98	3.84
2	1/2	1/2	0	12.02	10.46
3	1/2	0	1/2	18.97	19.36
4	0	1	0	9.41	9.10
5	0	1/2	1/2	19.06	19.03
6	0	0	1	13.79	14.64
7	4/6	1/6	1/6	14.23	16.04
8	1/6	4/6	1/6	16.07	16.77
9	1/6	1/6	4/6	19.85	18.74
10	1/3	1/3	1/3	19.20	19.18
11	1	0	0	3.98	3.68
12	0	1	0	9.41	9.65
13	0	0	1	13.79	13.45
14	1/2	1/2	0	12.02	12.58
15	1/2	0	1/2	18.97	18.18

Table 2
Levels of independent variables for central composite design.

					Levels	
Factor	Label	-α	−1	0	1	+α
Time (min)	A	10.86	15	25	35	39.14
Solvent-sample ratio	B	8.79	15	30	45	51.21

2.9. Comparison of UAE with maceration using selected solvent or ethanol

A comparative investigation was conducted between the UAE and maceration technique, employing various solvents, specifically the selected solvent or ethanol following the protocol delineated [15]. Following extraction, the resultant mixtures underwent centrifugation, with subsequent collection of the supernatants for further analysis. The quantification of TPC and assessment of antioxidant activities using DPPH, ABTS, and FRAP assays were subsequently performed to compare the efficacy of these extraction conditions.

2.10. Determination of bioactive compounds by UHPLC-ESI-QTOF-MS/MS

Phenolic compounds in CH leaf extracts were assessed using an UHPLC system, specifically the Agilent 1290 Infinity II, coupled with the Agilent 6545 LC-QTOF/MS system, according to the method outlined in Ref. [17].

2.11. Cell culture

NIH/3T3 fibroblasts (ATCC®CRL-1658™) and B16F10 melanoma cells (ATCC®CRL-6475™) were procured from the American Type Culture Collection (ATCC), Manassas, VA, USA. All cell culture experiments were performed in triplicate.

2.11.1. Cytotoxicity assay

The cytotoxicity assessment of NIH/3T3 fibroblasts and B16F10 melanoma cells was conducted using the application of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, following the established protocol outlined by Ref. [14]. Cells were then seeded in 96-well plates at densities of 15,000 cells/well for NIH/3T3 fibroblasts and 10,000 cells/well for B16F10 melanoma cells.

Cell viability percentage was determined through the application of the following formula:

$$\text{Percent of cell viability} = (\text{Sample absorbance} \ / \ \text{Control absorbance}) \times 100\%$$

2.11.2. Cytoprotective effect of CH leaf extracts against oxidative stress induced by hydrogen peroxide

The assay methodology employed adhered to the procedures delineated in our previous investigation [14].

Assessment of cell viability percentage ensured through the application of the subsequent formula:

Percent of cell viability = (Sample absorbance (Hydrogen peroxide treated)/Control absorbance) × 100 %

2.11.3. Melanin content reduction assessment

Melanin content reduction assessment was conducted according to the protocol stated by Ref. [18] with minor modifications. B16F10 cells were inoculated into a 12-well plate at a density of 10,000 cells per well. Following a 24-h incubation period in a carbon dioxide (CO_2) environment, each well was supplemented with 1 mL of complete Dulbecco's modified Eagle's medium (cDMEM) containing both samples and kojic acid as a positive control. The plate underwent further incubation for 72 h. To determine the melanin content, the cell suspension underwent rinsing with Dulbecco's phosphate buffered saline (D-PBS), followed by centrifugation at 15,000 rpm for 5 min, and underwent two further rinses with D-PBS before being dissolved in a solution of 1 N sodium hydroxide (NaOH). The resulting cell pellets were incubated at 80 °C for 1 h. The solubilized melanin was subjected to absorbance measurements at 490 nm.

Melanin content was quantified as a percentage relative to control cells, using the formula:

$$\text{Melanin content (\% of Control)} = (\text{Sample absorbance}) \ / \ (\text{Control absorbance}) \text{ x } 100\%$$

2.12. Statistical analysis

The statistical analysis of the data acquired through the simplex-lattice mixture design and RSM with central composite experiments was executed using the R software (version 4.2.1) and the dedicated mixexp and rsm package, respectively. The extraction conditions were compared using SPSS software, with a one-sample *t*-test utilized during validation to assess differences between expected and observed values. One-way ANOVA was used to evaluate comparisons of bioactive compounds and antioxidant activities among extraction methods. Significant level was set at $p < 0.05$. The reported results are presented as mean ± standard deviation.

3. Result and discussion

3.1. Selection of the appropriate solvent concentration

Polyols and their aqueous mixtures are commonly used in formulating of cosmetic and pharmaceutical products as they are considered safe, stable, and effective for delivering various compounds to the skin or body [19]. In addition, the dielectric constant is linked to a solvent's polarity, with high dielectric constants being better at dissolving polar solutes and low dielectric constants being more effective with nonpolar solutes [20]. Among polyols, glycerine (Gly) (dielectric constant – 41.14 at 25 °C) [21] has a relatively high dielectric constant and butylene glycol (BG) (dielectric constant – 28.8 at 25 °C) [22] has a relatively low dielectric constant. Hence, in this study, an assessment was conducted on Gly, BG, and water utilizing a simplex-lattice mixture design to improve compatibility between bioactive constituents present in the plant and extraction solvent, resulting in a greater yield of bioactive components. The outcomes of the effect of solvent concentration utilizing a simplex-lattice mixture design are presented in Table 1. TPC values ranged from 3.68 mg GAE/g sample to 19.36 mg GAE/g sample. Regarding pure solvent, water can yield approximately twice higher the TPC than BG and Gly. This may be elucidated by the capability of the extracting solvent to infiltrate and interact with ultrasonic waves, influencing the extraction of bioactive compounds [23]. Regarding binary solvents, an aqueous mixture of BG and Gly yielded higher TPC than BG and Gly mixture. In terms of ternary mixtures, the symmetrically distributed volume of three solvents yielded higher TPC than that of other volume ratios. Overall, the aqueous mixture of BG and Gly and the symmetrically distributed volume of three solvents yielded comparable TPC content, and these results were higher than those of pure solvents and a mixture of BG and Gly (Fig. 1). Previous studies supported the findings that alcohol-water mixtures enhanced the extraction yield of bioactive compounds [14,15,24].

The TPC values obtained from the experimental mixture design (Table 1) underwent statistical analysis through ANOVA and multiple regression fitting and polynomial equation as in equation [1] to describe the quantitative relationship between the concentrations of the three solvents and the resultant TPC,

$$TPC = 3.98A + 9.41B + 13.79C + 21.29(A*B) + 40.36(A*C) + 29.84(B*C) \quad [1]$$

where A refers to butylene glycol, B refers to glycerine, and C refers to water.

The obtained results demonstrate model significance, as evidenced by a $p < 0.05$ and F values reaching 72. The coefficient of determination (R^2) for the fitted model is computed as 0.997, indicating an exceptional degree of data representation exceeding 90 % [25]. The adjusted R square (adj R^2) is determined to be 0.995, implying minimal significant differences between predicted and adjusted values, thereby affirming the model's reliability [26]. The statistical insignificance ($p > 0.05$) of the lack of fit terms further attests to the model's appropriateness (Table 4). All linear and combination terms of Gly, BG, and water significantly influenced the TPC (Table 5). Utilizing the simplex-lattice model, the optimal solvent composition is identified as 32.57 %w/w BG, 32.92 %w/w Gly, and 34.51 %w/w water. Validation experiments conducted using the optimal solvent mixture confirmed the accuracy of the model, demonstrating no statistically notable distinctions ($p > 0.05$) between expected and observed values (Table 6). Thus, these findings validate the reliability of the response model.

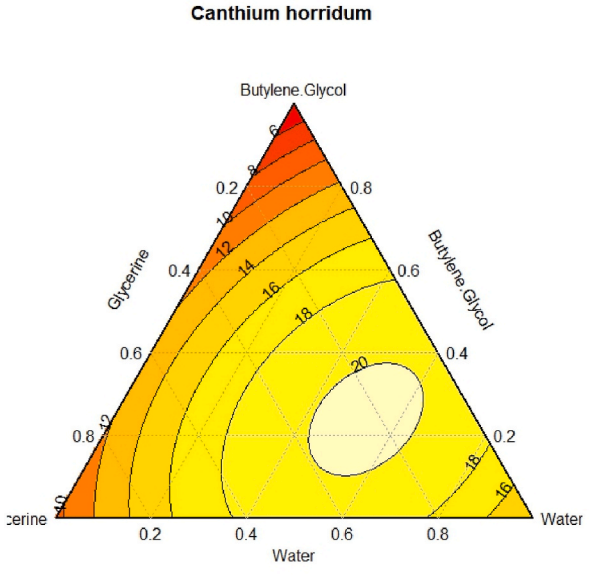

Fig. 1. Simplex-lattice mixture design for solvent concentration optimization.

Table 3
Central composite design with responses of variables.

Std run	Time (min)	Solvent-sample ratio	TPC (mg GAE/g sample)		DPPH (mg TEAC/g sample)		ABTS (mg TEAC/g sample)		FRAP (mg $FeSO_4$/g sample)	
			Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed
1	−1 (15)	−1 (15)	7.53	8.35	6.39	6.90	16.98	15.46	14.23	14.69
2	1 (35)	−1 (15)	8.10	8.36	6.87	7.22	14.12	15.83	14.67	14.71
3	−1 (15)	1 (45)	11.31	11.39	10.75	9.73	30.54	26.51	22.42	23.30
4	1 (35)	1 (45)	13.08	12.60	16.66	15.47	43.24	42.42	24.72	25.18
5	−1.68 (10.86)	0 (30)	10.63	10.69	8.52	9.38	24.78	29.60	22.97	21.38
6	1.68 (39.14)	0 (30)	12.59	13.12	13.89	14.13	33.04	32.01	25.27	23.92
7	0 (25)	−1.68 (8.79)	2.09	4.14	1.73	3.16	1.30	7.08	4.89	8.30
8	0 (25)	1.68 (51.21)	9.45	11.39	13.62	15.35	37.15	40.83	20.22	20.60
9	0 (25)	0 (30)	13.20	13.23	11.93	12.54	31.45	31.54	20.65	20.81
10	0 (25)	0 (30)	13.20	13.10	11.93	12.32	31.45	33.16	20.65	20.68
11	0 (25)	0 (30)	13.20	13.99	11.93	11.37	31.45	30.81	20.65	19.70
12	0 (25)	0 (30)	13.20	13.45	11.93	12.36	31.45	30.26	20.65	20.47
13	0 (25)	0 (30)	13.20	12.61	11.93	10.58	31.45	28.97	20.65	21.68
14	0 (25)	0 (30)	13.20	12.82	11.93	12.43	31.45	33.92	20.65	20.54

Note: GAE = Gallic acid equivalent, TEAC = Trolox equivalent antioxidant capacity, $FeSO_4$ = Ferrous sulfate equivalent.

Table 4
The analysis of variance of response in simplex-lattice design.

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)
Model	389.33	5	77.87	72.00	<0.0001*
Residual	9.73	9	1.08		
Lack of Fit	5.91	4	1.48	1.94	0.2430
Pure Error	3.82	5	0.76		
R square	0.997				
Adj R square	0.995				
Cor Total	399.0608	14			

Significance levels: '***' (<0.001), '**' (<0.01), '*' (<0.05), '.' (<0.1), and ' ' (≥0.1).

Table 5
Regression analysis of response in simplex-lattice design.

Factor	Estimated	Std. Error	t value	Pr (>|t|)
A	3.98	0.72	5.52	0.000372 ***
B	9.41	0.72	13.05	3.75e-07 ***
C	13.79	0.72	19.12	1.35e-08 ***
AB	21.29	3.39	6.27	0.000146 ***
AC	40.36	3.39	11.89	8.33e-07 ***
BC	29.84	4.04	7.39	4.15e-05 ***

Significance levels: '***' (<0.001), '**' (<0.01), '*' (<0.05), '.' (<0.1), and ' ' (≥0.1).

Table 6
The outcomes of validating expected values and observed value for extraction of TPC from CH leaves.

Conditions	Butylene glycol (%w/w)	Glycerine (%w/w)	Water (%w/w)	TPC (mg GAE/g sample)
Expected	32.566	32.923	34.511	19.36
Observed	32.57	32.92	34.51	19.18 ± 0.50^{ns}

"ns" refers that there is no significant difference ($p > 0.05$) within the same column.

3.2. Selection of extraction duration and solvent-sample ratio

Effect of duration of extraction.

The time dependent effect of extraction represents a pivotal aspect of plant extraction procedures, exerting control throughout solvent interaction with plant material and influencing the efficiency of chemical transfer from the plant matrix to the solvent [27,28]. The impact of varying extraction times on UAE of CH leaves (Fig. 2). Notably, a 25-min extraction duration resulted in the significantly highest TPC at 20.69 ± 2.36 mg GAE/g sample, while a 5-min duration yielded the lowest TPC at 15.57 ± 0.82 mg GAE/g sample ($p < 0.05$). This investigation reveals a positive correlation between extraction time and the yield of bioactive compounds, indicating that

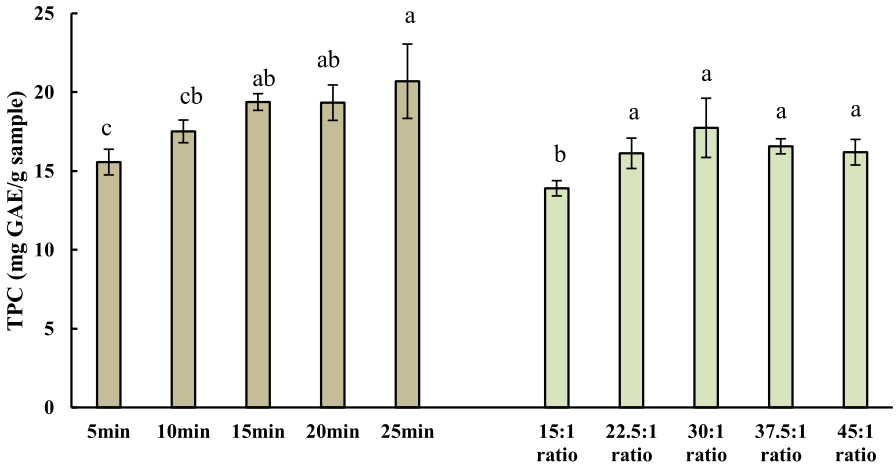

Fig. 2. The impact of extraction duration and solvent-sample ratio on the TPC during UAE of CH leaves. Distinct superscript letters refer statistically significant differences ($p < 0.05$) among values.

Table 7
The analysis of variance of the models through CCD.

Dependent variable	Source	Sum Sq	Df	Mean Sq	F value	Pr (>F)
	Model	93.63	5	18.73	47.84	<0.0001
	Linear	41.14	2	20.57	52.36	2.54E-05
	Interaction	0.36	1	0.36	0.92	0.3665
	Quadratic	52.14	2	26.07	66.36	1.05E-05
TPC	Residuals	3.13	8	0.39		
	Lack of fit	1.95	3	0.65	2.73	0.1535
	Pure error	1.19	5	0.24		
	R square	0.97				
	Adj R square	0.95				
	Total	96.76	13			
	Model	144.90	5	28.98	26.32	<0.0001
	Linear	120.65	2	60.33	54.81	2.14E-05
	Interaction	7.34	1	7.34	6.67	0.03245
	Quadratic	16.94	2	8.47	7.70	1.37E-02
DPPH	Residuals	8.81	8	1.10		
	Lak of fit	5.71	3	1.90	3.07	0.1292
	Pure error	3.10	5	0.62		
	R square	0.94				
	Adj R square	0.91				
	Total	153.71	13			
	Model	1160.63	5	232.13	30.32	<0.0001
	Linear	959.44	2	479.72	62.65	1.30E-05
	Interaction	60.37	1	60.37	7.89	0.022912
	Quadratic	140.57	2	70.28	9.18	8.48E-03
ABTS	Residuals	61.24	8	7.65		
	Lack of fit	44.25	3	14.75	4.34	0.0739
	Pure error	17.01	5	3.40		
	R square	0.95				
	Adj R square	0.92				
	Total	1221.87	13			
	Model	247.51	5	49.50	89.01	<0.0001
	Linear	170.07	2	85.04	153.19	4.19E-07
	Interaction	0.87	1	0.87	1.56	0.2472
	Quadratic	76.42	2	38.21	68.84	9.10E-06
FRAP	Residuals	4.45	8	0.56		
	Lack of fit	2.42	3	0.81	1.98	0.2352
	Pure error	2.03	5	0.41		
	R square	0.98				
	Adj R square	0.97				
	Total	251.96	13			

Table 8
Regression analysis of responses in central composite design.

	TPC				DPPH				ABTS				FRAP			
Factor	Estimated	Std. Error	t value	Pr (>|t|)	Estimated	Std. Error	t value	Pr (>|t|)	Estimated	Std. Error	t value	Pr (>|t|)	Estimated	Std. Error	t value	Pr (>|t|)
Intercept	13.20	0.25588	51.5872	2.209e-11 ***	11.93	0.42828	27.8633	2.971e-09 ***	31.45	1.12964	27.835	2.995e-09 ***	20.65	0.30417	67.879	2.470e-12 ***
A	0.58	0.22161	2.6266	0.03034 *	1.60	0.37093	4.3062	0.002594 **	2.46	0.97837	2.5157	0.036047 *	0.69	0.26344	2.6061	0.03132 *
B	2.19	0.22161	9.8901	9.218e-06 ***	3.54	0.37093	9.5438	1.202e-05 ***	10.67	0.97837	10.9078	4.421e-06 ***	4.56	0.26344	17.3086	1.264e-07 ***
A*B	0.30	0.31338	0.9573	0.36645	1.36	0.52454	2.5832	0.032453 *	3.89	1.38352	2.808	0.022912 *	0.47	0.37253	1.2482	0.24724
A^2	−0.56	0.2307	−2.4378	0.04070 *	−0.26	0.38614	−0.668	0.522962	−0.90	1.01847	−0.8842	0.402396	1.23	0.27423	4.4934	0.00202 **
B^2	−2.63	0.2307	−11.4134	3.138e-06 ***	−1.51	0.38614	−3.9061	0.004505 **	−4.33	1.01847	−4.2481	0.002807 **	−2.87	0.27423	−10.4619	6.053e-06 ***

Significance levels: '***' (<0.001), '**' (<0.01), '*' (<0.05), '.' (<0.1), and ' ' (≥0.1).

prolonged exposure enhances the transfer of these constituents from the plant into the extraction solvent [27]. Consequently, the extraction duration of 25 min was chosen for subsequent experiments because it yielded the highest TPC compared to other durations tested.

3.2.1. *Effect of solvent-sample ratio*

The quantity of polyphenols obtained is significantly influenced by the ratio of sample and liquid used in the extraction process [29]. Fig. 2 illustrates the impact of various solvent-sample ratios on the TPC of CH leaves when employing UAE. The TPC yield demonstrated a substantial and statistically significant increase ($p < 0.05$) as the solvent-sample ratio rose, peaking at a solvent-sample ratio of 30:1. Beyond this point, a further elevation of the solvent-sample ratio of 45:1 resulted in a decrease in TPC yield. This phenomenon is attributed to the dynamics of dissolution equilibrium in which during dissolution, wherein solute molecules transition from the solid phase to the liquid phase until reaching an equilibrium state where there is no net change in the amount of dissolved substance [30]. Typically, an increased ratio of solvent to sample promotes a more pronounced concentration difference, thereby facilitating enhanced mass transfer and dissolution of solutes. However, the efficiency of extraction plateaus after reaching equilibrium, indicating that a continued increase in the solvent-sample ratio does not enhance extraction efficiency [30,31].

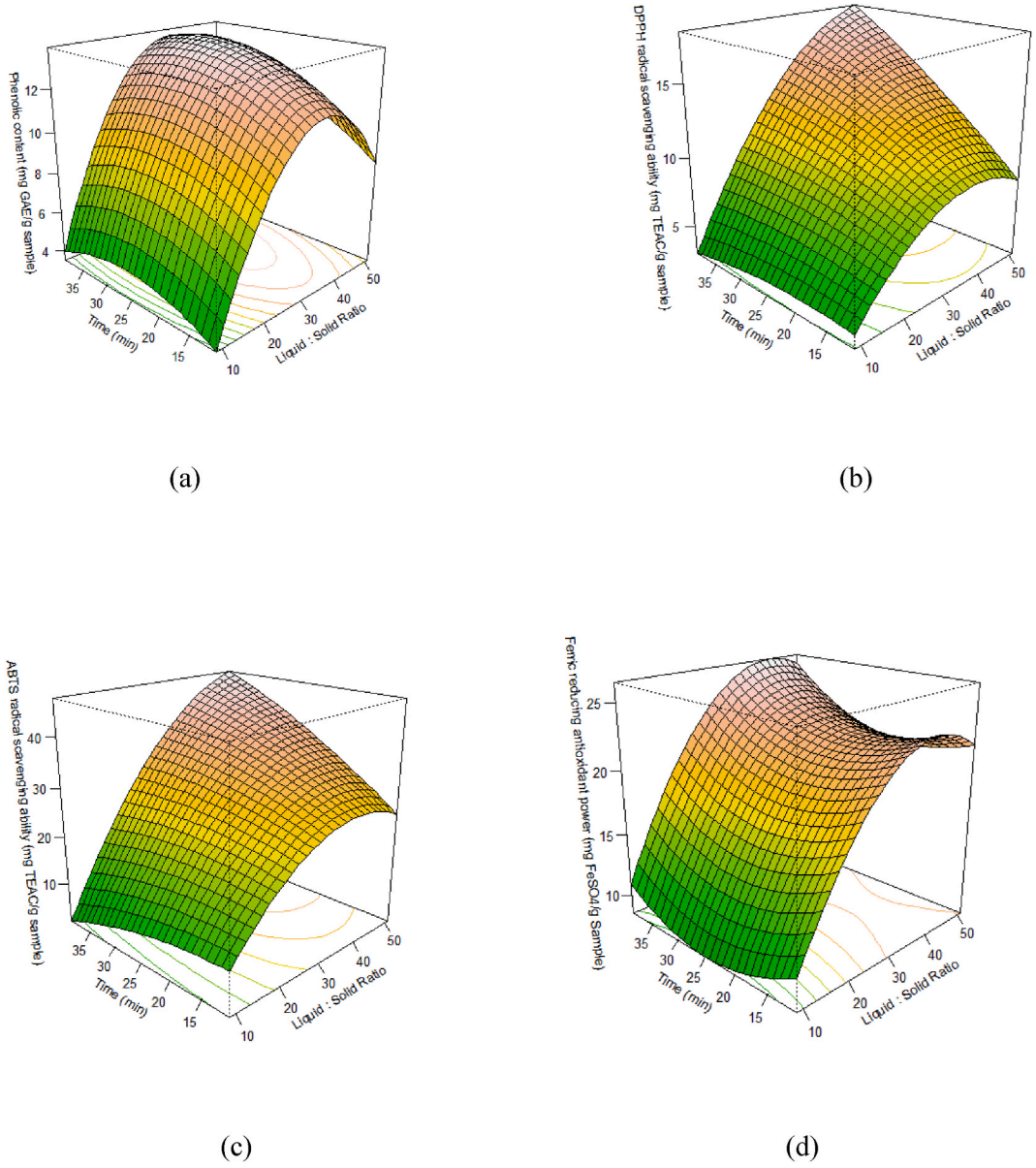

Fig. 3. Response surface plots displaying the influences of extraction duration, and solvent-sample ratio on TPC (a), DPPH (b), ABTS (c), and FRAP (d).

3.3. Extraction optimization by RSM

The process of optimizing conditions to extract bioactive constituents and evaluate antioxidant activities using RSM included varying extraction duration (A, in minutes) and solvent-sample ratio (B) across five different levels, as outlined in Table 2. The outcomes of various responses (TPC, DPPH, ABTS, and FRAP) of CH leaf extract were presented in Table 3.

3.3.1. Modeling the extraction conditions of CH leaves

The findings presented in Table 3 underwent analysis through multiple regression fitting and quadratic polynomial equations [2–5]. These equations were utilized to describe the correlations between the two variables and the measured parameters, including TPC, as well as the antioxidant activities (DPPH, ABTS, and FRAP),

$$TPC = 13.20 + 0.58A + 2.19B + 0.30AB\text{-}0.56A^2\text{-}2.63B^2 \quad [2]$$

$$DPPH = 11.93 + 1.60A + 3.54B + 1.36AB\text{-}0.26A^2\text{-}1.51B^2 \quad [3]$$

$$ABTS = 31.45 + 2.46A + 10.67B + 3.89AB\text{-}0.90A^2\text{-}4.33B^2 \quad [4]$$

$$FRAP = 20.65 + 0.69A + 4.56B + 0.47AB{+}1.23A^2\text{-}2.87B^2 \quad [5]$$

where A, and B represent the extraction duration and solvent-sample ratio.

The outcomes were assessed through R software (version 4.2.1), employing quadratic polynomial regression models with TPC, DPPH, ABTS, and FRAP as response variables. The findings revealed the statistical significance ($p < 0.05$) of all four models, as evidenced by F values of 47.86, 26.32, 30.32, and 89.01. The coefficient of determination (R^2) for TPC, DPPH, ABTS, and FRAP models exceeded 0.94, indicating a representation of over 90 % of the data by these models [25]. The adjusted R square (adj R^2) values for these models surpassed 0.90, implying minimal significant differences between predicted and adjusted values, affirming the model's reliability [32]. Additionally, the insignificance of the lack of fit terms ($p > 0.05$) underscored the appropriateness of the models (Table 7).

3.3.2. Influence of independent factors on TPC and antioxidant activities of CH extract

The influence of extraction duration (A) and solvent-sample ratio (B) on TPC, as well as the values of antioxidant assays of CH leaf extracts derived from 14 experimental trials, were analyzed using quadratic regression equations (equations [2–5]), with the corresponding coefficients and outcomes (Table 8). The interactions between time and solvent-sample ratio, along with their respective effects on TPC, DPPH, ABTS, and FRAP values, were graphically represented Fig. 3(a–d).

Regarding linear variables, both solvent-sample ratio and time demonstrated a significant positive impact on all responses ($p < 0.05$), indicating that increasing these factors could enhance the response values. In addition, the interaction of duration and solvent-sample ratio was positively significant ($p < 0.05$) in DPPH and ABTS assays, indicating that a rise in both variables could synergistically increase the values of DPPH and ABTS. In the quadratic terms, solvent-sample ratio was negatively significant ($p < 0.05$) in all responses, indicating the presence of curvature in their response surface and suggesting that their values rose as the solvent-sample ratio rose up to the optimal condition and after that, further rises in the solvent-sample ratio could not raise their values. This can be explained by the change in concentration gradient after increasing the solvent [27]. A higher solvent-sample ratio increases the quantities of components dispersed in solvents, which improves mass transfer. A further increase in solvent quantities did not cause an increase in bioactive compounds yield once the bioactive compounds concentration of the plant had been depleted [33]. In addition, extraction duration demonstrated a negatively significant quadratic term ($p < 0.05$) in the TPC assay, suggesting that TPC values had a downward curve-shaped trend with an increase in extraction duration, and positively significant ($p < 0.05$) in the FRAP assay, suggesting that FRAP values had an upward curve-shaped trend with an increase in extraction duration (Fig. 3). Time-dependent enhancements in extraction efficiency occur along with an increase in extraction duration. However, prolonged extraction time can generate heat that could degrade the bioactive compounds of the plant [34]. Earlier research on the UAE of coffee pulp with an aqueous-propylene glycol mixture and tea flower with an aqueous-BG mixture showed that TPC, as well as antioxidant activities, remained relatively unchanged with prolonged extraction duration. However, these values exhibited an increase with higher solvent-to-sample ratios until reaching a saturation point, beyond which additional solvent did not lead to further enhancement in response values [14,15]. In contrast, Pandey et al. (2018) found that the duration of extraction did not significantly affect response values such as TPC, ABTS, and DPPH. However, they observed that the ratio of solvent to sample had a negative linear impact on ABTS values and a positive linear impact on DPPH values [35]. The impact of the ratio of solvent to sample was observed to have a greater

Table 9
The outcomes of validating expected values at a 95 % confidence interval.

Conditions	Time (min)	Solvent-sample ratio	TPC	DPPH	ABTS	FRAP
			(mg GAE/g sample)	(mg TEAC/g sample)	(mg TEAC/g sample)	(mg $FeSO_4$/g sample)
Expected	15	32.94:1	12.3245	10.4473	29.2512	21.8845
Observed	15	32.94:1	13.34 ± 1.09^{ns}	11.81 ± 0.60^{ns}	30.45 ± 1.87^{ns}	22.17 ± 0.93^{ns}

"ns" refers that there is no significant difference ($p > 0.05$) within the same column.

influence compared to the duration of extraction across all measured responses.

3.4. Validation of expected value

The optimum parameters, as determined by the response model, were identified as an extraction duration of 15 min and a solvent-sample ratio of 32.94:1. Subsequently, a verification test was performed to assess the accuracy of the response model under these optimal conditions. The anticipated values were compared with the observed experimental outcomes (Table 9). Significantly, no notable distinctions were detected between the expected and observed outcomes, with p-values exceeding 0.05. Consequently, the findings affirm the reliability of the response model for the given extraction process.

3.5. Comparison of UAE with maceration using selected solvent or ethanol

Extracts from CH leaves were obtained using four different methods (GB-UAE, EtOH-UAE, GB-maceration, and EtOH-maceration) under optimal conditions determined by the response model, with the results summarized in Table 10. GB-UAE exhibited significantly greater efficiency across all measured response variables (TPC, DPPH, ABTS, and FRAP), surpassing outcomes achieved by other extraction methods. Notably, GB-maceration ranked second in efficiency for all parameters except DPPH, where EtOH-UAE showed the second-highest value. In contrast, EtOH-maceration consistently yielded the lowest values across all responses. These results suggest that employing an aqueous mixture of BG and Gly as an extraction solvent lead to higher yields of bioactive compounds and antioxidant activity in plant extracts, regardless of whether UAE or maceration is employed, compared to ethanol.

Moreover, when comparing UAE and maceration using the same extraction solvent, UAE consistently produced greater results across all parameters and necessitated considerably less time. A previous study of bioactive compounds extraction using an aqueous-ethanol system from Sacha inchi husk compared the efficacy of UAE and maceration in terms of the yield value and TPC value and reported that the values of each method were comparable, while UAE required a shorter time of extraction [36]. In addition, prior studies supported our findings that polyols, BG or propylene glycol, yielded larger quantities of bioactive compounds with antioxidant properties compared to ethanol, whether using UAE or maceration [14,15].

3.6. Determination of bioactive compounds by LC-QTOF-MS/MS

Different polyphenols demonstrate unique antioxidant properties contingent upon the arrangement of hydroxyl groups on the benzene ring [37]. Identifying phenolic components within an extract is crucial. Identifying phenolic components within an extract is crucial. Hence, the employment of UHPLC-ESI-QTOF-MS/MS for the characterization of phenolic constituents in CH leaves generated comprehensive MS total ion chromatograms (TIC) through the analysis of CH leaf extracts under negative ionization mode, accompanied by the corresponding MS/MS spectra elucidating specific bioactive compounds (Fig. 4(a and b) and 5 (a-c)). The assessment of compound identification quality is facilitated by the MFG score, preferring high MFG scores and low mass differences (ppm) [38]. Tentatively identified metabolites with MFG scores exceeding 75 and errors below 3 ppm are presented in Table 11, accompanied by key indicators substantiating their identification. In this study, it was observed that both extracts contained a total of seven phenolic compounds, namely 4-(butoxymethyl) phenol, methyl 3,4,5-trimethoxycinnamate, 3-*O*-caffeoyl-4-*O*-methylquinic acid, dihydrocaffeic acid 3-*O*-glucuronide, kaempferol 3-isorhamninoside, quercetin 3-(2G-glucosylrutinoside), and 2,4-dihydroxybenzoic acid. The comparison of these compounds in both extracts was based on their respective height in UHPLC-ESI-QTOF-MS/MS, presented as a percentage difference. Among the seven phenolic compounds analyzed, GB-UAE displayed a greater percentage difference in 4-(butoxymethyl)phenol, 3-*O*-caffeoyl-4-*O*-methylquinic acid, quercetin 3-(2G-glucosylrutinoside), and 2,4-dihydroxybenzoic acid, whereas the EtOH-UAE extract exhibited a higher percentage difference in the remaining phenolic compounds. Additionally, the GB-UAE extract revealed the presence of panzhihuacycaside, dihydroferulic acid 4-*O*-glucuronide, and phenyl glucuronide, while the EtOH-UAE extract contained luteolin 7-neohesperidoside-4′-sophoroside and kaempferol 3-rutinoside-4′-glucoside. Notably, no previous report exists on the LC-MS analysis of CH leaf extract. However, a previous study on the GC-MS analysis of leaves found 1,2-benzenedicarboxylic acid bis(2-methylpropyl) ester to be the main bioactive substance [12]. Additionally, a previous bioassays-guided isolation study involving thin-layer chromatography of ethanol extracts from the CH stem reported a total of 20 bioactive compounds. These included quercetin, *p*-hydroxybenzoic acid, and vanillic acid, as the compounds isolated from the CH stem [10].

Table 10
The results of comparison between UAE with maceration using polyols or ethanol.

Conditions	Time	Solvent-sample ratio	TPC	DPPH	ABTS	FRAP
			(mg GAE/g sample)	(mg TEAC/g sample)	(mg TEAC/g sample)	(mg $FeSO_4$/g sample)
GB-UAE	15 min	32.94:1	13.34 ± 1.09^{a}	11.81 ± 0.60^{a}	30.45 ± 1.87^{a}	22.17 ± 0.93^{a}
EtOH-UAE	15 min	32.94:1	11.93 ± 0.30^{b}	11.11 ± 1.53^{ab}	28.55 ± 0.53^{a}	18.74 ± 0.89^{b}
GB-maceration	24 h	32.94:1	12.78 ± 0.47^{ab}	9.96 ± 0.96^{ab}	30.30 ± 0.28^{a}	19.69 ± 1.07^{b}
EtOH-maceration	24 h	32.94:1	11.09 ± 0.41^{bc}	9.54 ± 0.98^{b}	25.22 ± 1.22^{b}	18.70 ± 0.70^{b}

Varying superscript letters within the same column indicates statistically significant differences ($p < 0.05$).

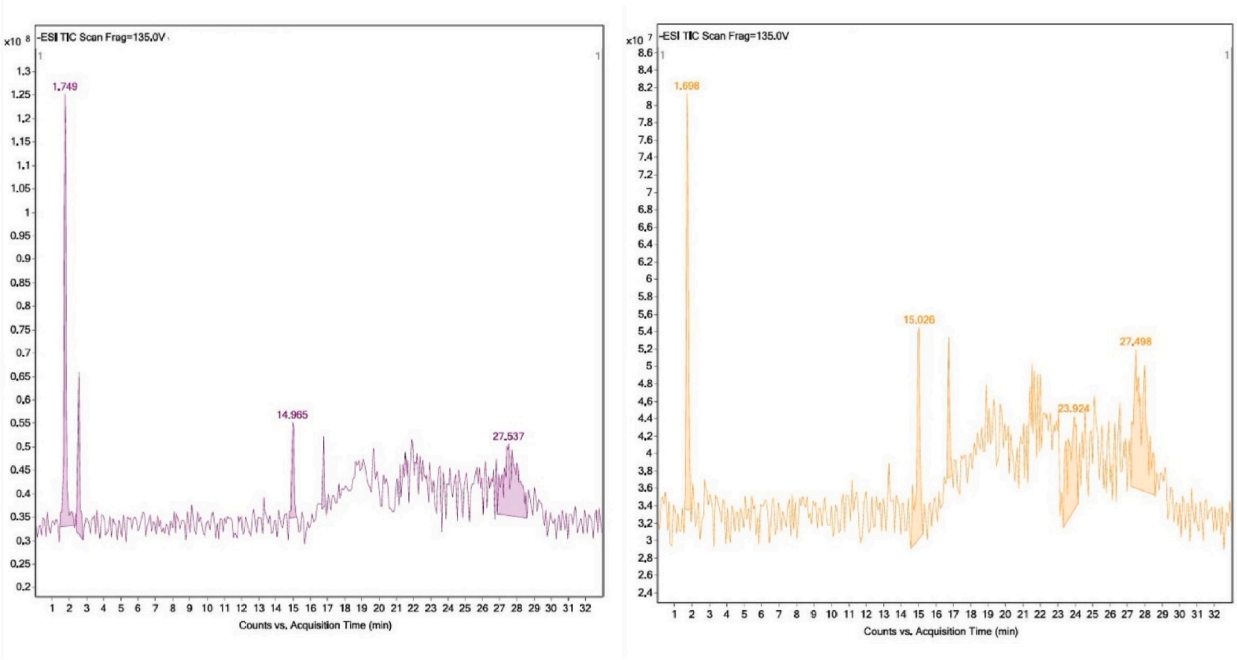

Fig. 4. Total ion chromatograms obtained through mass spectrometry in negative ionization mode of GB-UAE extract (a) and EtOH-UAE extract (b).

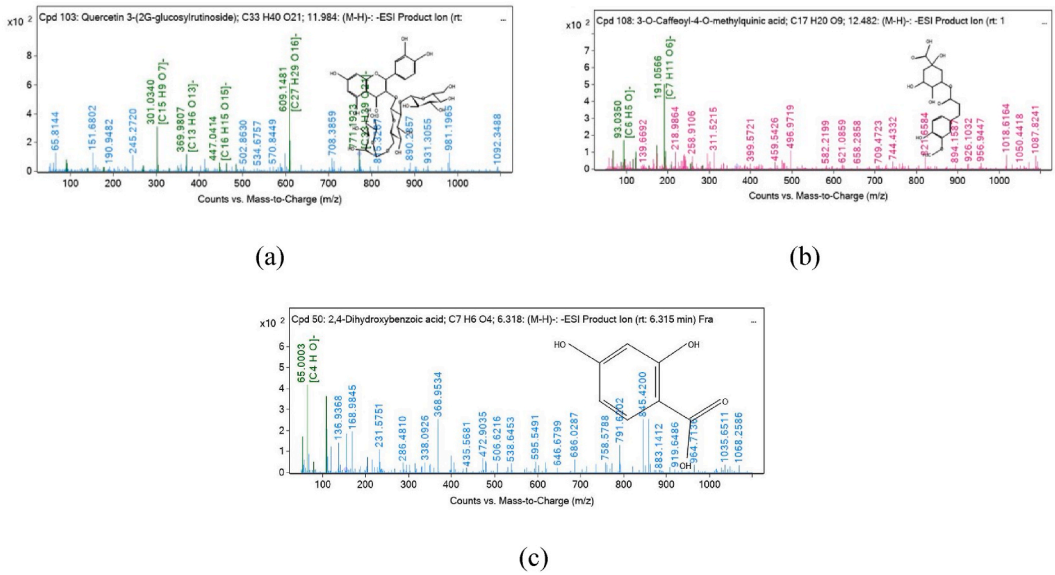

Fig. 5. MS/MS spectra of Quercetin 3-(2G-glucosylrutinoside) (a), 3-*O*-caffeoyl-4-*O*-methylquinic acid (b), and 2,4-dihydroxybenzoic acid (c).

3.7. Cell culture

3.7.1. Cytotoxicity assessment on NIH/3T3 mouse fibroblast and B16F10 melanoma cells

Dose-response experiments were carried out to evaluate the highest non-toxic concentration of extracts derived from CH leaves, as well as the influence of ascorbic acid on NIH/3T3 mouse fibroblast cells and kojic acid on B16F10 melanoma cells. Fig. 6 illustrates the outcomes of the cytotoxicity assay, depicting the effects of both ascorbic acid and CH leaf extracts on NIH/3T3 mouse fibroblast cells. Similarly, Fig. 7 presents the results of the cytotoxicity assay for kojic acid and CH leaf extracts on B16F10 melanoma cells.

Table 11
Phenolic constituent analysis of CH leaf extracts by LC-QTOF-MS/MS.

Phenolic compounds	Molecular Formula	Mass	Retention time	Ionization (ESI+/ESI-)	*m/z*	Error	Fragments	% Difference	Sample
4-(butoxymethyl)phenol	C11H16 O2	180.12	19.378	(M − H)-	179.108	−0.18	92.99, 136.98	11.115	GB-UAE, EtOH-UAE
Methyl 3,4,5-trimethoxycinnamate	C13H16 O5	252.10	19.337	(M − H)-	251.092	0.79	122.04, 179.11	−0.948	GB-UAE, EtOH-UAE
3-*O*-caffeoyl-4-*O*-methylquinic acid	C17H20 O9	368.11	12.453	(M − H)-	367.105	−2.4	93.04, 191.06	3.730	GB-UAE, EtOH-UAE
Dihydrocaffeic acid 3-*O*-glucuronide	C15H18 O10	358.09	2.367	(M − H)-	403.088	−0.32	135.05, 223.02	−2.752	GB-UAE, EtOH-UAE
Kaempferol 3-isorhamninoside	C33H40 O19	740.22	13.28	(M − H)-	739.209	0.8	284.03, 593.15	−96.272	GB-UAE, EtOH-UAE
Quercetin 3-(2G-glucosylrutinoside)	C33H40 O21	772.20	12.004	(M − H)-	771.197	2.59	301.03, 609.15	4.684	GB-UAE, EtOH-UAE
2,4-dihydroxybenzoic acid	C7 H6 O4	154.03	6.332	(M − H)-	153.019	1.66	65.00, 108.02	15.805	GB-UAE, EtOH-UAE
Luteolin 7-neohesperidoside-4′-sophoroside	C39H50 O25	918.26	11.413	(M − H)-	917.256	0.47	285.04, 755.20	–	EtOH-UAE
Kaempferol 3-rutinoside-4′-glucoside	C33H40 O20	756.21	12.249	(M − H)-	755.203	1.45	151.00, 285.04	–	EtOH-UAE
Panzhihuacycaside	C39H50 O24	902.27	12.752	(M − H)-	901.260	1.64	284.03, 755.20	–	GB-UAE
Dihydroferulic acid 4-*O*-glucuronide	C16H20 O10	372.11	9.538	(M − H)-	417.104	−1.25	167.04, 267.07	–	GB-UAE
Phenyl glucuronide	C12H14 O7	270.07	5.195	(M − H)-	315.072	0.86	53.04, 108.02	–	GB-UAE

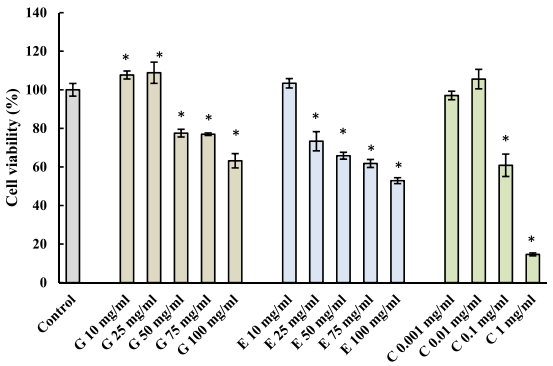

Fig. 6. Cytotoxicity assessment of GB-UAE (G), EtOH-UAE (E), and ascorbic acid (C) at various concentrations in NIH/3T3 fibroblast cells. An asterisk (*) denotes statistically significant variation in comparison with control ($p < 0.05$).

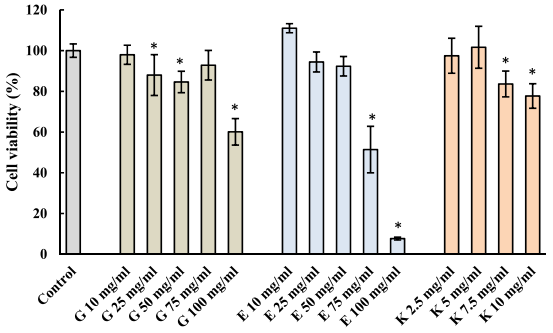

Fig. 7. Cytotoxicity assessment of kojic acid (K), GB-UAE (G), and EtOH-UAE (E) of different concentrations in B16F10 melanoma cells. An asterisk (*) indicates statistically significant variation in comparison with control, ($p < 0.05$).

In the context of NIH/3T3 mouse fibroblasts, ascorbic acid at concentrations of 0.001 mg/ml and 0.01 mg/ml were identified as non-cytotoxic, exhibiting cell viabilities of 97.05 ± 2.20 % and 105.56 ± 5.06 %, respectively. However, the concentration of 0.1 mg/ml demonstrated a significant suppressive effect on cell survival, resulting in a cell viability of 60.85 ± 5.81 % ($p < 0.05$). Notably, increased doses of ascorbic acid are recognized to induce cytotoxicity by inducing metabolic stress, potentially leading to cell apoptosis [39]. Additionally, concentrations of 25 mg/ml and 10 mg/ml for GB-UAE, as well as 10 mg/ml for EtOH-UAE extract samples, were found to be non-cytotoxic, as their respective cell viabilities exceeded 80 % [40].

Three concentrations of kojic acid, namely 2.5 mg/ml, 5 mg/ml, and 7.5 mg/ml, were identified as non-cytotoxic to B16F10 melanoma cells, exhibiting cell viability exceeding 80 %. However, a concentration of 10 mg/ml of kojic acid was observed to significantly suppress cell survival, resulting in a cell viability of 71.39 ± 7.70 % ($p < 0.05$). Suthiram et al. (2023) reported a significant dose-dependent decrease in cell viability with kojic acid, particularly at higher concentrations, attributed to the induction of oxidative stress [41]. With respect to GB-UAE and EtOH-UAE extracts, concentrations of 100 mg/ml of GB-UAE, and 75 mg/ml and 100 mg/ml of EtOH-UAE were found to suppress cell viability, while the remaining concentrations were regarded as non-cytotoxic doses due to their cell viability exceeding 80 % [40].

In the assessment of cell viability, GB extract exhibited superior results in comparison with ethanol extract in both cell lines. This observed difference in cell viability aligns with prior research suggesting that polyols are less cytotoxic than ethanol in cell culture [42]. Additionally, these findings are consistent with an earlier study that investigated UAE, wherein the BG extract of tea flower demonstrated heightened cell viability in comparison to ethanol extract under similar conditions [15].

3.7.2. Cytoprotective effect of CH leaf extracts against oxidative stress induced by hydrogen peroxide

The investigation evaluated the cytoprotective properties of CH leaf extracts by exposing cells to an oxidative stress assay induced by H_2O_2, a pro-oxidant agent known to induce cell death via DNA damage [43]. CH leaf extracts significantly mitigated oxidative stress induced by 400 μM H_2O_2 in NIH/3T3 fibroblasts (Fig. 8). The viability of cells treated with H_2O_2 markedly decreased to 58.64 ± 1.66 % compared to the control but significantly increased to 79.64 ± 4.09 % when pre-treated with ascorbic acid of 0.01 mg/mL before oxidative stress induced by hydrogen peroxide ($p < 0.05$). Remarkably, both extracts obtained through ultrasonic-assisted extraction (GB-UAE and EtOH-UAE) demonstrated a noteworthy increase in cell viability, statistically significant at $p < 0.05$, in comparison to treatment with hydrogen peroxide (H_2O_2). Additionally, GB-UAE extract exhibited greater cell viability than ethanol extract at equivalent concentrations, consistent with results from in vitro antioxidant assays and bioactive composition analyses. This phenomenon could potentially be triggered by the elevated levels of potent polyphenols, such as 3-*O*-caffeoyl-4-*O*-methylquinic acid and quercetin 3-(2G-glucosylrutinoside), in GB-UAE extract than EtOH-UAE extract [44,45]. Similarly, a previous study on cellular antioxidant activity in NIH/3T3 fibroblasts using BG and ethanol extract of tea flower reported that BG extract demonstrated superior cell protection and increased viability compared to ethanol extract [15]. There is limited literature regarding the cellular antioxidant activity of CH leaf extracts. However, in the previous study of the same genus, it was found that the hydroalcoholic extracts derived from the *Canthium coromandelicum* (Burm.f.) Alston leaves exhibited in vivo antioxidant activities by reducing oxidative stress markers in streptomycin-induced diabetic rats. Furthermore, it was reported that hydroalcoholic extracts also increased enzymatic antioxidant activities, such as superoxide dismutase and catalase, in streptomycin-induced diabetic rats [46].

3.7.3. Melanin content reduction assessment

GB-UAE and EtOH-UAE extracts were evaluated for their efficacy in reducing melanin content in B16F10 melanoma cells compared to kojic acid, which served as the positive control. Both kojic acid and extracts significantly decreased melanin content in dose-dependent manner ($p < 0.05$) (Fig. 9). When comparing GB-UAE and EtOH-UAE extracts at the same concentration, EtOH-UAE

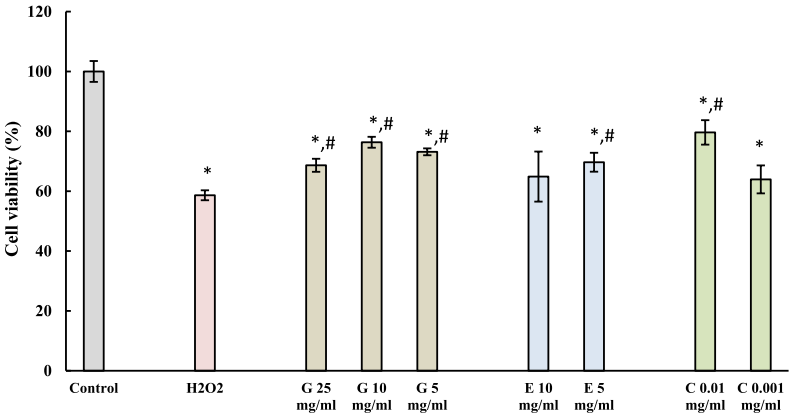

Fig. 8. Assessment of the cytoprotective effect of GB-UAE (G), and EtOH-UAE (E), and ascorbic acid (C). An asterisk (*) denotes statistically significant variation in comparison with the control ($p < 0.05$). The symbol "#" denotes a statistically significant difference in comparison with the H_2O_2 group ($p < 0.05$).

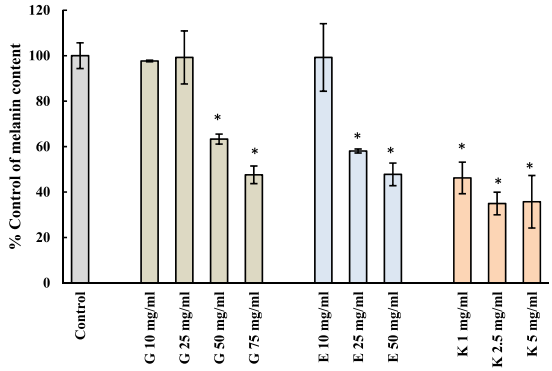

Fig. 9. Melanin content reduction activity assessment of kojic acid (K), GB-UAE (G), and EtOH-UAE (E). An asterisk (*) denotes statistically significant variation in comparison with the control ($p < 0.05$).

extract demonstrated a greater decrease in melanin content compared to GB-UAE extracts. However, GB-UAE extracts could obtain comparable efficacy in melanin content reduction within the non-cytotoxic concentration range at 75 mg/mL compared to EtOH-UAE extract at 50 mg/mL. The difference in the effectiveness of melanin suppression between EtOH-UAE and GB-UAE extracts may be attributed to variations in the solubility of the bioactive molecules responsible for melanin inhibition. Ethanol may be more efficient than the GB mixture at extracting or stabilizing specific active ingredients that suppress melanin. Based on the results of the bioactive composition analysis of the extracts by LC-Q-TOF-MS, a possible explanation might be that the EtOH-UAE extract exhibited a higher amount of phenolic compounds such as luteolin 7-neohesperidoside-4′-sophoroside and kaempferol 3-rutinoside-4′-glucoside than the GB-UAE extract, as these compounds (luteolin and kaempferol) have been reported to have potent melanin synthesis inhibition efficacy [47]. In a previous study of the same genus, hydroalcoholic extract of *Canthium coromandelicum* leaves exhibited potent skin whitening activity by inhibiting tyrosinase enzyme, which was comparable to the kojic acid at equivalent concentrations [48].

4. Conclusion and future perspectives

This investigation employed simplex-lattice and central composite designs within RSM to optimize the parameters for the UAE of CH leaves. The simplex-lattice model identified an optimal solvent composition consisting of 32.57%w/w butylene glycol, 32.92%w/w glycerine, and 34.51%w/w water. The response model pinpointed two optimal extraction conditions: a 15-min extraction time and a solvent-sample ratio of 32.94:1 mL/g, with the latter identified as the predominant factor influencing CH leaf extraction across all responses. Validation procedures substantiated the reliability of the response model. Comparative analysis revealed that GB-UAE outperformed EtOH-UAE in all responses. UHPLC-ESI-QTOF-MS/MS analysis exhibited 12 bioactive compounds, representing the first LC-MS exploration of CH leaf extracts. This is the first investigation for determining phenolic compounds in CH leaves using UHPLC-ESI-QTOF-MS/MS. GB-UAE exhibited greater cell viability and hydrogen peroxide-induced cellular antioxidant activity in cell cytotoxicity and antioxidant assays compared to EtOH-UAE. Notably, GB-UAE exhibited comparable melanin content reduction at higher non-cytotoxic concentrations, suggesting its potential utility in cosmetic (anti-aging and skin whitening products) and pharmaceutical applications (skin disorder treatment). While acknowledging the limitations of this study in the comprehensive exploration of CH leaves bioactive compounds and their mechanisms, further research is encouraged to explore the mechanisms of bioactive compounds on various biological activities through advanced analytical and molecular biology methods, alongside clinical trials to confirm the safety and effectiveness of these bioactive compounds and to scale up the extraction process for industrial applications. The researchers expect that this research will enhance the utilization of an aqueous-polyols system for extracting bioactive compounds from the plants, not limited to CH leaves, and uncover potential applications of CH leaves extracts in cosmetics, including anti-aging and skin whitening products, as well as in pharmaceutical formulations.

Data availability

The data will be available on request.

CRediT authorship contribution statement

Hla Myo: Writing – original draft, Methodology, Investigation. **Nuntawat Khat-udomkiri:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing

interests:

Nuntawat Khat-udomkiri reports financial support was provided by National Science, Research and Innovation Fund (NSRF). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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