

Microwave-assisted extraction optimization of Sabah snake grass (*Clinacanthus nutans* Lindau) herbal infusion and evaluation of antioxidant and acetylcholinesterase inhibition activity *in vitro*

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Article history:

Received: 25 October 2019
Received in revised form: 26 February 2020
Accepted: 28 February 2020
Available Online: 4 June 2020

Keywords:

Acetylcholinesterase inhibition,
Clinacanthus nutans,
Microwave-assisted extraction,
Optimization,
Response surface methodology

DOI:

[https://doi.org/10.26656/fr.2017.4\(5\).353](https://doi.org/10.26656/fr.2017.4(5).353)

Abstract

Clinacanthus nutans is a great medicinal herb with high polyphenols content and had been used in its infusion form to treat illness. In this paper, microwave-assisted extraction of *C. nutans* tea infusion was investigated by using central composite design combined with response surface methodology. The influence factors of microwave power (80 - 800 W); brewing time duration (0 - 240 s) and extraction time duration (60 s - 300 s) on total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (DPPH) and acetylcholinesterase inhibition activity (AChE) were investigated. The optimum extraction conditions were determined as follows: microwave power X_1 , 560 W; brewing duration, X_2 , 60 s and extraction time, X_3 , 240 s. Under this optimal extraction conditions, the responses were as followed: Total phenolic content (251.58 ± 10.98 mg GAE/L); Total flavonoid content (102.21 ± 5.16 mg RE/L); DPPH ($62.22 \pm 0.03\%$) and acetylcholinesterase inhibition activity (AChE) of $24.77 \pm 0.12\%$. Results demonstrated that MAE could be used to extract bioactive constituents having antioxidant and anti-acetylcholinesterase properties that may benefit in Alzheimer's disease.

1. Introduction

Clinacanthus nutans (Burm f.) Lindau from the Acanthaceae family is a native species to tropical Southeast Asian countries, and commonly known as "Belalai gajah" or "Sabah snake grass" in Malaysia. In Malaysia and Singapore, the fresh leaves of *C. nutans* were traditionally used as an infusion and served in tea form and have great health benefits to the consumers. *C. nutans* have been used to treat skin rashes, insects and snake bites, (Sakdarat *et al.*, 2009), mental stress, diabetes, rheumatoid arthritis, fever, dysentery, burns, scalds, diarrhea (Rahim *et al.*, 2016), skincare, cancer prevention, cold and flu and even goitre (Siew *et al.*, 2014). *C. nutans* extracts also were reported to exhibit antiviral activity against herpes simplex virus infection, varicella-zoster virus, derived from the family of Herpesviridae (Kongkaew and Chaiyakunapruk, 2011), antibacterial, anti-inflammatory, anticancer (Ng *et al.*, 2017), antioxidant, antiproliferative and antinociceptive activity (Rahim *et al.*, 2016). Apart from that, oral toxicity study suggested that *C. nutans* are safe for consumption in Dawley female rats (Lau *et al.*, 2014). Hexane fraction of *C. nutans* leaves revealed compounds

of stigmasterol, β -sitosterol and a triterpenoid lupeol (Le *et al.*, 2017). The phytochemical compounds of terpenoids, flavonoids, C-glycosyl flavones, sulfur-containing glucosides and glycerylcerolipids were identified in most of *C. nutans* plants research study. These phytochemical compounds were found to have pharmacological properties of antiviral, anticancer, antioxidant, antidiabetic, immunomodulatory, wound healing, anti-inflammatory and analgesic activities (Yahaya *et al.*, 2015).

Acetylcholine (ACh) is neurotransmitter located in the peripheral and central nervous system (CNS) that have a crucial role in memory, learning and behavioral activities. Reduction of ACh levels in the brain could lead to Alzheimer's disease (AD). One of the therapeutic approaches in treating AD via the inhibition of acetylcholinesterase (AChE). The AChE role is to terminate the cholinergic neurotransmission by hydrolysis of ACh (Grisaru *et al.*, 1999). The ACh restoration can be generated by using acetylcholinesterase inhibitors (AChEIs) that varies from natural (alkaloids, terpenoids, glycosides, coumarins), semisynthetic and synthetic derivatives. Tacrine,

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donepezil, rivastigmine and galanthamine are AChEIs that are approved by the US Food and Drug Administration and used to treat AD symptoms (Ouattara *et al.*, 2013). Studies have shown that phytochemicals can be great AChEI with little or no side effect and could be used as dietary supplements (Neagu *et al.*, 2015). Thus, free radical and antioxidant balanced production play a role in the prevention of AD and having AChEI that possessed antioxidant activities derived from the natural origin are a big concern.

Extraction techniques are one of the crucial analytical steps in determining extract compositions. Phenolic compounds are commonly extracted using conventional extraction such as simple maceration, infusion and decoction (Dai and Mumper, 2010). However, these conventional methods take up longer time and use a large amount of solvents. Thus, finding a convenient extraction method of shorter extraction time with lower solvent consumption with great yields is a great benefit. Microwave-assisted extraction (MAE) is a green technology that meets the requirements on finding a convenient extraction method compared to other extraction techniques (Mustapa, Martin, Galego *et al.*, 2015; Krishnan and Rajan, 2016; Panja, 2018).

MAE use the microwave to enhance the heating process of the solvent-sample system with shorter extraction time and lower solvent volume compared to conventional extraction. Mechanisms involved in microwave heating are dipole rotation and ionic conduction (Faridah *et al.*, 2018). Dipole rotation occurs as solvent and sample molecules having dipole moments align on electric field rotate and produce thermal energy (Veggi *et al.*, 2013). The ionic conduction occurs in solvent containing ions when the electromagnetic field is applied, where heat is generated due to friction between ions flow and the solvent itself. A study showed that brewing green tea for 30 s in freshly boiled water before irradiation for 60 s in microwave oven improved extract yield of catechins and caffeine compared to used microwave oven alone (Vuong *et al.*, 2012). Thus, brewing time outside the microwave oven is added in this study to assist in the microwave-assisted extraction.

Apart from one-factor-one-time approach (single factor experiment), response surface methodology (RSM) could also be used in the optimization process. RSM is a statistical technique to optimize and evaluate multiple factors effects and the influence on one or more response variables (Kormin *et al.*, 2010; Simic *et al.*, 2016; Caleja *et al.*, 2017). In this project, we investigated the RSM technique of central composite design (CCD) to optimize microwave-assisted extraction infusion parameter such as microwave power, brewing time and

extraction time to maximize the total phenolic content, total flavonoid content, DPPH antioxidant activity and AChE inhibition activity of *C. nutans* in its tea form.

2. Materials and methods

2.1 Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH); 5,5-dithio-bis-(2-nitrobenzoic acid) DTNB; Rutin hydrate; galanthamine hydrobromide were purchased from Sigma-Aldrich, USA. acetylcholinesterase from electrophorus eel, aluminium chloride anhydrous; ascorbic acid; di-Sodium hydrogen phosphate di-hydrate; folin-ciocalteu; gallic acid; hydrochloric acid magnesium chloride hexahydrate; methanol; sodium dihydrogen phosphate monohydrate; sodium hydroxide pellet; sodium nitrite; sodium chloride and Tris-HCL were purchased from Merck, Germany. 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) was purchased from Sigma, USA. All reagents used were of analytical grade.

2.2 Sample preparation

The herbal plant was obtained from a local supplier in Batu Pahat, Johor, Malaysia. The plant leaves were first cut and washed several times with running tap water to remove dirt. Next, the leaves were rinsed with distilled water to remove any impurities followed by drying in hot air oven (Memmert, Germany) for 2 days at 45°C (Abdullah *et al.*, 2012). Then, the leaves were ground into a fine powder using an electric grinder. The plant powder obtained was stored in an airtight container at room temperature until further use.

2.3 Microwave infusion preparation

A commercial microwave oven (nns215mf, Panasonic, Japan, 800 W) was used. A total of 2 g of dried leaves were infused with 200 mL boiling deionized water at 100°C under magnetic stirrer (brewing time) before placed in microwave oven under the following parameter as shown in Table 1 (microwave power, irradiate time). The infusion then was filtered with Whatman filter paper no 1 before used in the assay. The infusion was prepared freshly before the assay was carried out.

2.4 Experimental design

Response surface methodology (RSM) of central composite design for MAE of *Clinacanthus nutans* phytochemical content, antioxidant capacities and enzymatic activity was determined to optimize the process as in Table 1. Variables that were used in the MAE optimization were microwave power (80 – 800 W); brewing time (0 – 240 s) and extraction time (60 – 300 s). A full factorial central composite design with 20

Table 1. Experimental design layout and responses for Central Composite Design (CCD) based on response surface methodology (RSM) of *C. nutans* infusions phytochemical content, antioxidant activities and enzymatic activity using microwave assisted extraction (MAE) (in triplicate).

Run	A	B	C	Y ₁	Y ₂	Y ₃	Y ₄
8	560	180	240	301.35±2.09	117.67±4.58	65.43±0.74	28.27±2.51
3	240	180	120	250.21±2.37	93.42±3.78	72.84±0.45	24.25±0.26
13	440	120	60	239.25±5.97	87.67±10.65	69.56±0.39	21.76±0.67
7	240	180	240	247.47±6.85	84.64±3.28	70.73±0.63	20.13±1.68
6	560	60	240	322.81±15.8	124.33±10.14	63.85±0.88	28.28±0.44
18	440	120	180	250.66±0.79	105.24±3.78	62.59±1.03	26.99±0.36
10	800	120	180	338.33±39.92	133.12±7.35	60.64±0.82	31.12±2.01
1	240	60	120	247.01±17.13	99.18±1.57	71.22±0.45	23.68±1.47
2	560	60	120	263.9±11.86	116.15±6.05	65.04±0.68	27.42±0.71
20	440	120	180	250.21±6.28	106.45±4.17	62.72±1.03	26.46±0.23
15	440	120	180	247.47±15.44	93.42±10.14	68.37±0.93	21.20±0.95
11	440	0	180	283.54±17.4	74.64±1.39	71.62±3.08	18.99±0.69
12	440	240	180	273.95±10.37	91.61±2.62	69.08±0.31	22.58±1.26
4	560	180	120	250.66±9.12	109.18±2.73	66.30±1.13	22.52±0.48
14	440	120	300	289.02±10.1	118.88±3.44	65.29±1.45	26.66±0.92
5	240	60	240	248.38±11.16	93.73±5.96	71.44±1.01	20.14±1.14
19	440	120	180	260.25±4.81	96.15±8.20	65.05±1.27	26.90±0.21
16	440	120	180	256.14±5.54	106.45±3.28	63.54±0.30	25.74±0.14
9	80	120	180	259.79±14.5	81.91±1.82	71.75±0.73	21.70±0.76
17	440	120	180	257.51±3.45	99.18±7.10	64.50±0.27	30.16±3.67

A = Power (W); B = Brewing time (s); C = Irradiate time (s); Y₁= Total phenolic content (TPC; mg GAE/L); Y₂= Total flavonoid content (TFC, mg RE/L); Y₃= DPPH (% Inhibition); Y₄= AChE (% Inhibition).

experiments run was completed. The experiments were done according to the run numbers as arranged by the software to avoid errors. Responses of the experimental design were phytochemical content (Total phenolic content; Total flavonoid content), antioxidant activities (DPPH, FRAP, ABTS) and enzymatic activity (AChE inhibition activity). The obtained data of the responses were analyzed by using multiple regressions to fit the second-order polynomial model.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where Y= response function; β_0 = regression coefficient for intercept; β_i = regression coefficient for linear; β_{ii} = regression coefficient for quadratic; β_{ij} = regression coefficient for interaction; and x_i and x_j = independent variables

2.5 Total phenolic content determination (TPC)

Determination of TPC assay used Folin - Ciocalteu colorimetric method (Maisarah *et al.*, 2013). 200 μ L herbs infusion was mixed with 1.5 mL of Folin-Ciocalteu's reagent (10 folds), left for 5 mins before 1.5 mL of 6% (w/v) sodium bicarbonate solution was added and left for another 90 mins and measured at 725 nm by using a spectrophotometer. The polyphenols concentration was calculated by using gallic acid as a

standard reference with a concentration between 0 to 120 μ g/mL. The sample was analyzed in triplicates and expressed as milligram gallic acid equivalents per liter (mg GAE/L). The standard equation curve of gallic acid was $y = 0.0073x + 0.0635$, ($R^2 = 0.9985$).

2.6 Total flavonoid content determination

This assay was determined by aluminium chloride colorimetric method (Lusia *et al.*, 2015). Sample infusion (1mL) was mixed with 4 mL deionized water, 5% sodium nitrate solution (0.3mL), 10% aluminium chloride solution (0.6 mL) and 1 M sodium hydroxide solution (2 mL). The mixture was vortex before absorbance was taken at 510 nm spectrophotometrically. The flavonoid concentration was calculated by using rutin as a standard antioxidant reference with concentration ranging from 0 to 120 μ g/mL. Samples were analysed in triplicate and expressed as milligram rutin equivalents per litre (mg RE/L). The standard equation curve of rutin was $y = 0.007x + 0.002$, ($R^2 = 0.9918$).

2.7 DPPH free radical scavenging assay

The infusion scavenging activity was determined by measure decolorization of the stable free radical violet colour of DPPH (Lusia *et al.*, 2015). A solution of 0.1

mM DDPH in methanol was prepared. Next, infusion and methanolic DPPH free radical reagent was mixed together in ratio of 1:1. The reaction mixture was kept in dark at room temperature for 30 mins before absorbance was taken at 517 nm. Blank of deionized water and methanol in ratio of 1:1 was used in the assay. Ascorbic acid with concentration ranging from 1 to 10 µg/mL was used as a reference antioxidant compound. The infusion ability to scavenge the DPPH free radical was calculated as follow:

$$\text{Inhibition(\%)} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \right] \times 100\% \quad (2)$$

Where A_{sample} = Test sample absorbance (DPPH solutions with infusion) and A_{control} = Control absorbance (DPPH solution exclude sample)

2.8 In vitro acetylcholinesterase inhibition assay (AChE)

Acetylcholinesterase inhibition activity of infusion was determined spectrophotometrically by using Ellman's colorimetric method (Ahmed and Gilani, 2009). 745 µL of 50 mM Tris-HCL buffer (pH 8.0), 100 µL of infusion, 15 µL of 2U/mL acetylcholinesterase enzyme was added and left to incubate for 10 mins at room temperature. Next, 20 µL 10 mM DTNB in sodium phosphate buffer (pH 8.0) and 20 µL 14 mM acetylthiocholine iodide (ATCI) was added into the reaction mixture, vortex and incubated at room temperature for another 12 mins. A total of 10 µL of 1 mg/mL galanthamine was added into the reaction mixture to stop the enzymatic reaction. Absorbance was taken at 412 nm for every 30 s for three times with blank of Tris-HCL buffer and sample infusion. Galanthamine hydrobromide, a standard for AChE inhibitor was dissolved in deionized water and used as positive control and percentage of acetylcholinesterase inhibition was calculated by using the following formula:

$$\text{Inhibition(\%)} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \right] \times 100\% \quad (3)$$

Where A_{sample} = Infusion absorbance and A_{control} = Negative control absorbance (enzymatic reaction without sample)

2.9 Statistical analysis

All assays results were determined in triplicate with three occasions (n = 3). Results were expressed as mean ± standard deviation (S.D). Response surface methodology experimental design was generated and analysed by Design Expert 6.0.4.

3. Results and discussion

3.1 Optimization of MAE parameter

Central composite experimental design was shown in

Table 1. By using analysis of variance (ANOVA) of the response surface quadratic model, the relationship between the response variable and the independent variable can be identified and explained briefly as shown in Table 2. Significant model of responses variables with insignificant lack of fit TPC (0.2088), TFC (0.2822), DPPH (0.4331), and AChE (0.7920) at $p > 0.05$ indicated that model used is fit with the response variable and able to represent the relationship between responses and independent variables.

The correlation coefficient (R^2) reveals the total variation present in a model (Hayat *et al.*, 2009). As the correlation coefficients of the responses TPC (0.9718), TFC (0.8793), DPPH (0.8280) and AChE (0.7401) nearly to 1, these correlation coefficients are acceptable as it explained that 74.01% to 97.18% variability of the data can be explained by the model. Apart from that, adequate precision of response variables, TPC (22.329), TFC (10.711), DPPH (8.320) and AChE (6.609) with a ratio greater than 4 is assumed to be desirable and indicated that the model can be used to navigate the signal (Alara *et al.*, 2017).

In optimization coefficient estimation, linear, quadratic and interaction effect of each and between independent variable towards the responses can be examined. A value of $P < 0.05$ indicated that the model terms are significant. Based on Table 2, the total phenolic content was affected most significantly by power (A), followed by Irradiate time (B), quadratic terms power² (A^2), brewing² (B^2), and interaction between power and irradiate time (AC). Total flavonoid content response was affected by two linear parameters (Power (A) and Irradiate time(C) whereas, single factor of power (A), quadratic factor of brewing time (B^2) and irradiate time (C^2), would affect DPPH response. However, only power (A), and its quadratic relation would affect the effectiveness of AChE response. By neglecting non-significant parameter ($P > 0.1$), final predictive equation from each actual factors obtained for each responds were as follows:

$$Y_{\text{TPC}} = 252.35 + 18.92 A - 3.22 B + 12.99 C + 10.66 A^2 + 5.58 B^2 + 1.93 C^2 - 4.62 AB + 13.87 AC - 1.54 BC \quad (4)$$

$$Y_{\text{TFC}} = 101.65 + 12.42 A - 2.92 B + 4.05 C + 1.84 A^2 - 1.0 B^2 + 0.78 C^2 + 0.15 AB + 3.86 AC - 0.38 BC \quad (5)$$

$$Y_{\text{DPPH}} = 64.80 + 2.99 A + 0.55 B - 0.78 C + 0.60 A^2 - 1.01 B^2 + 0.91 C^2 + 0.24 AB - 0.02 AC - 0.25 BC \quad (6)$$

$$Y_{\text{AChE}} = 26.23 + 2.32 A + 0.18 B + 0.55 C + 0.035 A^2 - 1.37 B^2 - 0.51 C^2 - 0.68 AB + 1.78 AC + 0.54 BC \quad (7)$$

Table 2. Analysis of variance (ANOVA) response surface quadratic model total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picryl-hydrazyl free radical scavenging activity (DPPH), acetylcholinesterase inhibition (AChEI).

Response variable	TPC (mg GAE/L)		TFC (mgRE/L)		DPPH (% Inhibition)		AChE (% Inhibition)	
	Sum of Squares	p-value (Prob > F)	Sum of Squares	p-value (Prob > F)	Sum of Squares	p-value (Prob > F)	Sum of Squares	p-value (Prob > F)
Model	13490.78	<0.0001	3136.88	0.0015	199.03	0.0075	175.24	0.0435
Linear terms								
A	5728.21	<0.0001	2469.79	< 0.0001	143.06	0.0002	86.22	0.0038
B	166.4	0.0661	136.11	0.1058	4.77	0.3078	0.5	0.7822
C	2697.83	<0.0001	262.83	0.0331	9.77	0.1551	4.77	0.3991
Quadratic terms								
A ²	2858.81	<0.0001	85.34	0.1895	9.16	0.1674	0.031	0.9446
B ²	783.76	0.0012	25.07	0.463	25.85	0.0314	47.26	0.0197
C ²	93.68	0.1527	15.36	0.5635	20.89	0.0483	6.66	0.3225
Interaction terms								
AB	171	0.063	0.18	0.9492	0.46	0.7458	3.74	0.4537
AC	1538.98	<0.0001	119.42	0.1268	3.25E-03	0.9782	25.45	0.0693
BC	19	0.5017	1.15	0.8735	0.51	0.7319	2.32	0.5528
Lack of fit	267.41	0.2088	268.95	0.2948	18.36	0.5941	19.43	0.792
Adeq precision	22.329		10.711		8.32		6.609	
R ²	0.9718		0.8793		0.828		0.7401	

A = microwave power, B = brewing time and C = irradiate time.

Where A = microwave power; B = brewing time (s); C = irradiate time (s).

3.2 Microwave parameter effects on total phenolic content (TPC)

Total phenolic content (TPC) was between 239.25 and 338.33 mg gallic acid per litre of infusion. The analysis of data indicated that linear terms of microwave power and irradiate time shows significant effects on the TPC. While interaction term of AC (microwave power and irradiate time) and quadratic terms of A² and B² shows significant influenced towards total phenolic content. Equation 4 shows microwave power and irradiate time gave positive effect while brewing time gave negative effects on total phenolic content. All quadratic shows positive effect but only interaction between microwave power and irradiate time shows positive effect towards total phenolic content.

The response surface plot was generated to visualize the effect of the parameters on total phenolic content as shown in Figure 1 to Figure 3. Figure 1 shows the influence of microwave power and irradiates time, Figure 2 shows the influence of microwave power and brewing time while Figure 3 shows brewing time and irradiate time influence on total phenolic content. Higher microwave power with longer extraction time increased the total phenolic content of the infusion.

3.3 Microwave parameter effect on total flavonoid content

The total flavonoid content was found in ranged of 81.91 to 133.12 mg rutin per litre of infusion. From the analysis of variance (ANOVA), the linear term of microwave power and irradiate time shows a significant effect on total flavonoid content. However, the interaction and quadratic term between microwave power, brewing time and irradiate time show no

DESIGN-EXPERT Plot

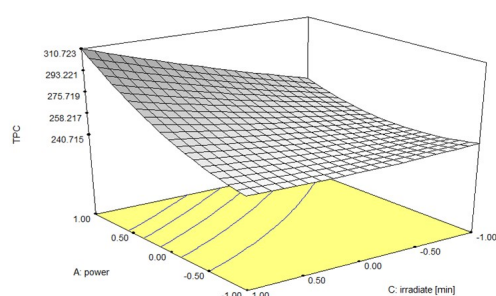
TPC
X = A: power
Y = C: irradiate [min]Actual Factor
B: brewing [min] = 0.00

Figure 1. Surface plot effect of microwave power and irradiate time on total phenolic content when brewing time was kept at zero level.

DESIGN-EXPERT Plot

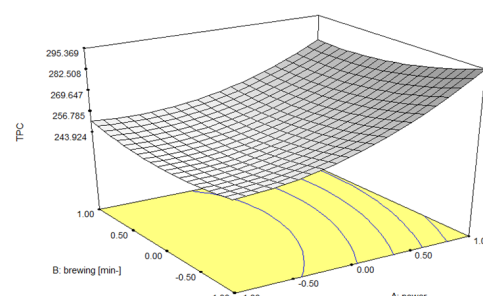
TPC
X = A: power
Y = B: brewing [min]Actual Factor
C: irradiate [min] = 0.00

Figure 2. Surface plot effect of microwave power and brewing time on total phenolic content when irradiate time was kept at zero level.

DESIGN-EXPERT Plot

TPC
X = B: brewing [min.]
Y = C: irradiate [min.]
Actual Factor
A: power = 0.00

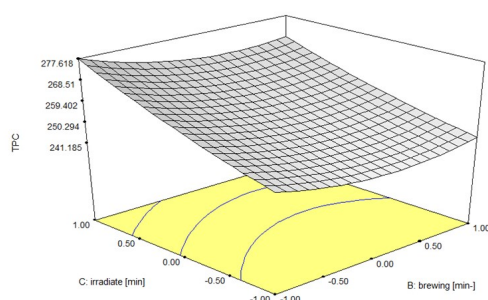


Figure 3. Surface plot effect of irradiate and brewing time on total phenolic content when microwave power was kept at zero level.

DESIGN-EXPERT Plot

TFC
X = A: power
Y = B: brewing [min.]
Actual Factor
C: irradiate [min.] = 0.00

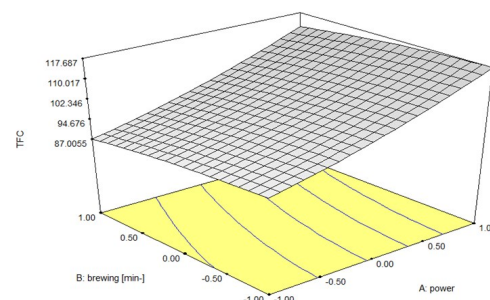


Figure 4. Surface plot effect of brewing time and microwave power on total flavonoid content when irradiate time was kept at zero level.

DESIGN-EXPERT Plot

TFC
X = A: power
Y = C: irradiate [min.]
Actual Factor
B: brewing [min.] = 0.00

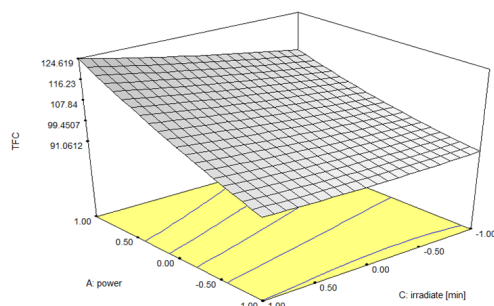


Figure 5. Surface plot effect of microwave power and irradiate time on total flavonoid content when brewing time was kept at zero level.

DESIGN-EXPERT Plot

TFC
X = B: brewing [min.]
Y = C: irradiate [min.]
Actual Factor
A: power = 0.00

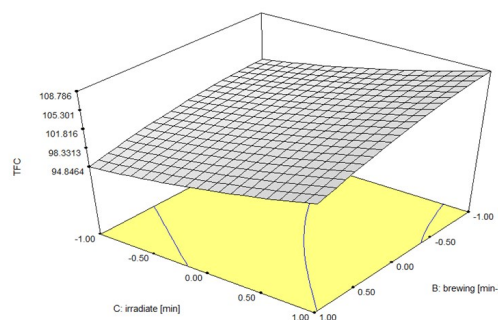


Figure 6. Surface plot effect of brewing time and irradiate time on total flavonoid content when microwave power was kept at zero level.

DESIGN-EXPERT Plot

DPPH
X = A: power
Y = B: brewing [min.]
Actual Factor
C: irradiate [min.] = 0.00

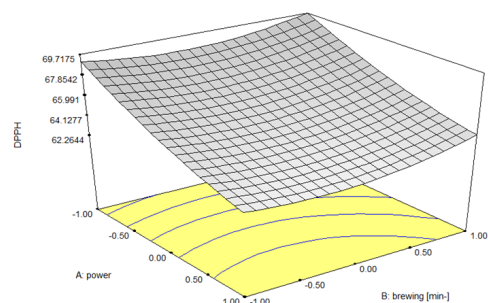


Figure 7. Surface plot effect of microwave power and brewing time on antioxidant DPPH free radical inhibition when irradiate time was kept at zero level.

DESIGN-EXPERT Plot

DPPH
X = A: power
Y = C: irradiate [min.]
Actual Factor
B: brewing [min.] = 0.00

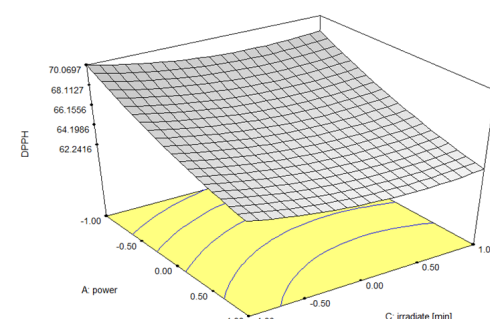


Figure 8. Surface plot effect of microwave power and irradiate time on antioxidant DPPH free radical inhibition when brewing time was kept at zero level.

DESIGN-EXPERT Plot

DPPH
X = B: brewing [min.]
Y = C: irradiate [min.]
Actual Factor
A: power = 0.00

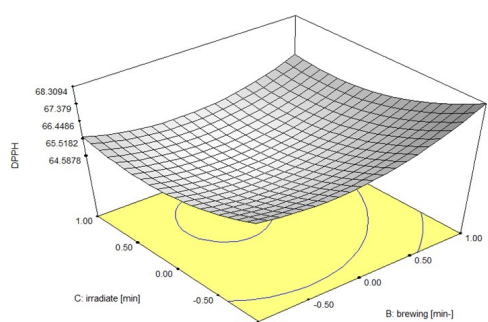


Figure 9. Surface plot effect of irradiate time and brewing time on antioxidant DPPH free radical inhibition when microwave power was kept at zero level.

DESIGN-EXPERT Plot

ACHE
X = A: power
Y = B: brewing [min.]
Actual Factor
C: irradiate [min.] = 0.00

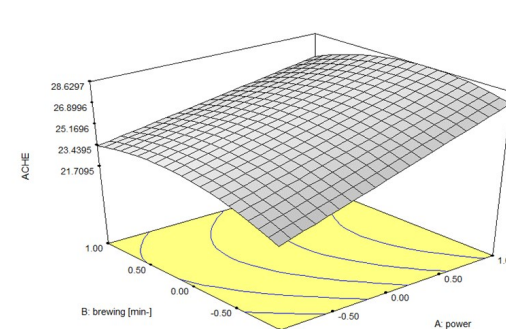
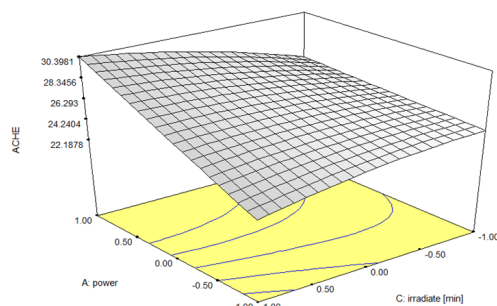


Figure 10. Surface plot effect of microwave power and brewing time on acetylcholinesterase inhibition when irradiate time was kept at zero level.

DESIGN-EXPERT Plot

ACHE
X = A: power
Y = C: irradiate [min]
Actual Factor
B: brewing [min] = 0.00



DESIGN-EXPERT Plot

ACHE
X = B: brewing [min]
Y = C: irradiate [min]
Actual Factor
A: power = 0.00

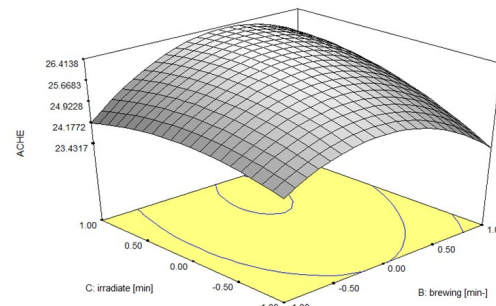


Figure 11. Surface plot effect of microwave power and irradiate time on acetylcholinesterase inhibition when brewing time was kept at zero level.

significant effect on total flavonoid content of the infusion. From equation 5, linear and quadratic of microwave power and irradiate time showed a positive effect on total flavonoid content. Whereas the interaction of microwave power with brewing time (AB) and microwave power and irradiate time (AC) gave a positive effect. While the interaction of brewing time and irradiate time gave negative effect to total flavonoid content. Figures 4 to 6 visualize the effect of the parameters on the total flavonoid content extracted from the *C. nutans* microwave-assisted infusion. In Figure 4 to Figure 6, the total flavonoid content increased as the parameters of microwave power, brewing time and irradiate time increased.

3.4 Microwave parameter effect on free radical scavenging DPPH

Antioxidant inhibition of DPPH radical scavenging under different microwave parameter was between 60.64 to 72.84%. Linear term of microwave power gave significant effect while brewing time and irradiate time gave a non-significant effect on antioxidant inhibition activity. The quadratic term of microwave power was not significant to antioxidant inhibition activity. Whereas quadratic term of brewing time and irradiate time gave significant effect to the response activity. However, no significant interaction term was present in the response. Based on equation 6, the positive effect was found in the linear term of microwave power and brewing time on antioxidant inhibition activity. Whereas quadratic of microwave power and irradiate time along with the interaction between microwave power and brewing time gave a positive effect to the response activity. Response surface was generated and illustrated as shown in Figure 7 to Figure 9. In Figure 7 to Figure 9, the antioxidant DPPH scavenging activity shown an increasing pattern when the microwave power, brewing time and irradiate time increase.

3.5 Microwave parameter effect on acetylcholinesterase inhibition activity

The acetylcholinesterase inhibition activity under

Figure 12. Surface plot effect of brewing time and irradiate time on acetylcholinesterase inhibition when microwave power was kept at zero level.

different microwave parameters was found to be between 18.99 to 31.12%. Linear term of microwave power gave significant effect while linear term of brewing time and irradiate time gave a non-significant effect on response activity. Quadratic term of brewing time gave significant effect while quadratic term of microwave power and irradiate time gave non-significant effect on acetylcholinesterase inhibition activity. Moreover, interaction terms between microwave power with brewing time (AB), microwave power with irradiate time (AC) and brewing time and irradiate time (BC) gave a non-significant effect on acetylcholinesterase inhibition activity. Equation 7 indicated that linear of microwave power, brewing time and irradiate time gave positive effect on acetylcholinesterase inhibition activity. While other positive effect was found only on quadratic of microwave power and interaction between microwave power with irradiate time (AC) and brewing time with irradiate time (BC). The response surface activity of acetylcholinesterase inhibition under the influence of the parameters was illustrated as in Figure 10 to Figure 12. An increasing acetylcholinesterase inhibition pattern was observed in Figures 10 and 11. While in Figure 12, as the irradiate time and brewing time increased, the acetylcholinesterase inhibition activity increased and decreased afterwards.

3.6 Validation of optimize MAE parameter

Table 3. Predicted and actual value under optimal condition, (560W, 60 s brewing time, 240 s irradiate time).

Responses	TPC (mg GAE/L)	TFC (mg RE/L)	DPPH (% Inhibition)	ACHE (% Inhibition)
Predicted value	252.35	101.65	64.8	26.22
Actual value	251.58	102.21	62.23	24.77

The optimal condition that was obtained by using the mathematical model were as follow: microwave power (560 W); brewing time (60 s) and irradiate time (240 s). Thus, optimal verification was performed. Under this optimal condition, the predicted value and actual value

of each response were shown in Table 3. As the actual values were closed to predictive value, indicating that the model was reliable.

4. Conclusion

From this study, the influence of MAE variables towards response phytochemical activities (TPC, TFC), antioxidant activities (DPPH) and enzymatic activity AChE of *C. nutans* tea infusions was studied using central composite design (CCD) of response surface methodology (RSM). The findings show that brewing time, microwave power and irradiate time significantly affected the responses, With the optimal condition of 560 W, 60 s brewing time and 240 s irradiate time, the experimental data is in agreement with the predicted data of the mathematical model (TPC 251.58 mg GAE/L; TFC 102.21 mg RE/L; 62.23% DPPH inhibition; 24.77% AChE inhibition). In general, this study suggested this model can be used to enhance extractions of phytochemical, antioxidant and acetylcholinesterase inhibition properties.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

Financial support from research fund MDR H506 and E15501 provided by Research Management Centre, Universiti Tun Hussein Onn Malaysia is gratefully acknowledged.

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