Total phenol content of \textit{Clinacanthus nutans Lindau (C.nutans)} medicinal plant extracted using vacuum solvent-free microwave extraction (SFME)

Siti N S Othman\textsuperscript{1}, Ana N Mustapa\textsuperscript{1}, Ku Halim Ku Hamid\textsuperscript{1}

\textsuperscript{1} Faculty of Chemical Engineering, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.

Abstract. \textit{Clinacanthus nutans Lindau (C.nutans)} is a popular medicinal herb plant in Asia regions such as Malaysia, Indonesia, Thailand and China for its high polyphenols content. Various conventional extraction techniques have been used to extract bioactive compounds in \textit{C.nutans}, however, the methods have limitations such as usage of organic solvent, time-consuming and low extraction yield. In this study, polyphenols content from \textit{C.nutans} leaves extracted by solvent-free microwave extraction (SFME) with addition of water, to enhance absorption of energy from microwaves. Energy density or irradiation power per unit of sample volume is used instead of applied microwave power to determine actual energy absorbed by the microwave extraction. It is determined from energy absorbed by microwave power of 200, 300 and 400 W. The effect of process parameter on the polyphenol contents: energy density of 0.073, 0.078 and 0.108 W/ml in 5, 10 and 15 min with 6 ml/g of solvent-to-feed (S/F) ratio was studied. The highest total phenol content (TPC) was obtained at 0.073 W/ml, 6 ml/g of S/F ratio and 5 min of extraction time with TPC of 24.08 ± 0.51 mg GAE/g DW at temperature of 79 °C.

Results demonstrated that the TPC of \textit{C.nutans} was higher when extracted under vacuum pressure compared to TPC at ambient pressure. The results suggest that SFME method using water under vacuum state appears as the most efficient and green technique for the extraction of polyphenols from \textit{C.nutans} extract in shorter time with no use any of organic solvent to produce reasonably high TPC.

1. Introduction

\textit{Clinacanthus nutans Lindau} or \textit{C.nutans} is one of medicinal herbs popularly known in Asia regions as Sabah Snake Grass [1], [2], [3]. This plant is rich with phytochemicals which used to treat skin rashes, snake bites and lesions caused by herpes simplex virus, fever and diabetes [4]. People had long practiced the medicinal uses of \textit{C.nutans} throughout centuries because of its popularity as a traditional herbal tea in Asian countries such as Malaysia, Indonesia, Thailand and China [5]. While Chinese medical practitioner uses \textit{C.nutans} for controlling menstrual pain, pain relief, anemia, jaundice and setting of fractured bones. A fresh leaves of \textit{C.nutans} is boiled to treat diabetes in Indonesia [4]. \textit{C.nutans} is rich with bioactive compounds mainly phenolic and flavonoids [6]. Polyphenols is one of phenolic compounds in \textit{C.nutans} that has powerful antioxidant properties as well as their valuable effect in the hindrance of oxidative stresses has attracted interest among researchers [3], [7], [8]. Phenolic compounds are major phytochemicals found in all plants and many researches have focused on the extraction conditions and analysis for its medicinal properties [6].
The laboratory scale of inquisition of *C. nutans* oil is done through a method called extraction. Firstly, conventional extraction such as Soxhlet extraction [9], maceration extraction [10], advanced extraction technology such as supercritical carbon dioxide (SFE-\text{CO}_2) [11] and microwave-assisted extraction (MAE) [11] are methods to extract the valuable compounds from the plants. However, all these methods have lacks in several aspects such as usage of large amount of solvent, long extraction time and thermally unsafe that would be unfavorable commercially and required purification of products in later process [12], [13]. In addition, high temperature used in most of these extraction methods may cause degradation to thermolabile compounds [14], [15], [6].

Recently, microwave extraction without any use of organic solvent has received increasing attention [13], [16], [17] due to its advantages for thermosensitive compounds. This extraction method called as solvent-free microwave extraction (SFME) [15]. SFME is a new technology approach towards green extraction method. In this technique, moisture content plays important roles absorbing heat from microwave irradiation, expand the plant cells and lead to the extraction of solutes. Another way is by using water as solvent in the microwave extraction. Water has been regarded as universal solvent because it can extract various polarities of organic compounds by increasing the temperature of water during extraction process [18], [19], [20]. In this study, polyphenols compound is extracted from *C. nutans* plant by SFME method. Despite the technique is solvent-free, an amount of water is added accordingly by solvent-to-feed ratio (S/F) to maintain the moisture content of the sample matrix to absorb the microwave energy efficiently [21]. The high value of dielectric constant of water remain the sample at lower temperature which benefits the extraction of thermosensitive compounds [15].

Besides, dried plant sample can burn during certain stage of heating when there is no more water to evaporate from the sample and producing residue that could contaminate the oil yield. Therefore, vacuum condition is introduced to SFME method to overcome this problem as well as to enhance the amount of extraction yield. The application of vacuum condition allows boiling point of the water becoming lower than at ambient pressure. Thus, the water can continuously boil and refluxing at lower temperature. This would give advantages for the sample to mix thoroughly with solvent and extracting the thermolabile compounds while preventing it from degrade [22], [23].

Moreover, microwave technique is always associated with the problem of hot spot [24]. The hot spot could affect the heating efficiency of the sample as the heat supply is not uniform throughout the sample. This limitation of microwave also leads to irregular temperature profile during extraction of *C. nutans* leaves. Stirring method with designed glass stirrer rod was embedded in the flask containing sample to provide uniform heating during the microwave irradiation. In this work, effect of SFME parameters i.e. microwave power (200 to 400 W), time of extraction (5 to 15 min) and temperature in vacuum condition on TPC from *C. nutans* fresh leaves were investigated. A Folin-Ciocalteu colorimetric method was used to quantify the extracted TPC.

2. Experimental

*Chemicals.*

Reagents used for determination of total phenol content were Folin-Ciocalteu and gallic acid purchased from Merck (Malaysia). A sodium carbonate anhydrous was purchased from R&M Chemicals (Malaysia). A 10.6 wt% solution of sodium carbonate (Na$_2$CO$_3$) was prepared for TPC analysis.

*Sample.*

Fresh plant of Clinacanthus nutans Lindau was obtained from Forest Research Institute Malaysia (FRIM) in Kepong, Kuala Lumpur. The moisture content of fresh leaves *C. nutans* was determined gravimetrically prior to the extraction.
Procedures.
A modified inverter microwave oven (Panasonic, 27L, 1000W maximum power) connected with condenser system and vacuum pump outside the microwave oven with operating wave frequency of 2.45 MHz, using small portion of water as solvent to extract the plant sample. The microwave oven was equipped with CENTER305 K Type digital thermometer and data logger to measures the temperature profile during the extraction process. The parameters studied were power at 200, 300 and 400 at extraction time of 5, 10 and 15 minutes. The solvent-to-feed (S/F) ratio of 5/1, 15/3, 25/5 and 30/5 (ml/g) of sample were determined in preliminary study to find a suitable S/F ratio on extraction temperature, under mentioned extraction time. It was found that S/F ratio of 30/5 gave relatively suitable temperature of below 80 °C for the extraction of polyphenols.

Fresh leaves of *C.nutans* with moisture content of 85 wt% was cut to size approximately 0.5 x 0.2 ± 0.5 cm, placed in 250 ml flask and 30 ml of distilled water was added into the flask for microwave extraction. The system is put under vacuum condition at 70 kPa. During microwave heating, the sample was constantly stirred with glass rod stirrer to avoid the occurrence of hot spots thus, temperature profile of the sample is uniform. After microwaves irradiation, the sample was immediately cooled down in an ice bath to dropped the temperature to 40 °C, to stop the heating process that could further degrade the polyphenols compound in *C.nutans* extract [10]. Then, addition of 20 ml of distilled water was added into the flask to reach the same ratio used for conventional extraction process. Then, the flask was placed in warm water bath (40 °C) for normal infusion. First liquid sample was extracted after microwave irradiation of 5, 10 and 15 min (t = 0) and the next sample was extracted for every 20 min for total of 500 min. Extracts were filtered through 0.20 µm PTFE membrane to filtrate solid residue before storage in amber vials and stored under -8˚C. The experiments were replicates three times for each operating condition and the extracts were collected for TPC analysis.

Statistical analysis
All analyzes were done in triplicate and results are reported as mean values and standard deviations. ANOVA tests with two factor with replication analysis of variance were performed in Microsoft Excel 2013 to analyzed the effect of microwave power and extraction time, where difference between individual means was statistically significant (*p* < 0.05).

Total Phenol Content Analysis.
The total phenol contents were determined by Folin-Ciocalteau colorimetric method. For this analysis, 40 µL of sample extracts were added in test tubes containing 3 mL of distilled water. Then, 200 µL of Folin-Ciocalteau reagent was added and shaken gently. After 5 min, 600 µL of 10.6 wt% Na₂CO₃ was added and the mixture was gently shaken and left incubated in a warm water at 40 °C for 30 min. The solution was covered with aluminium foil and placed in dark area to avoid contact to light. Then, the solution was measured for absorbance at 765 nm (Lambda 750 UV/Vis spectrophotometer). Calibration curve of standard solution of gallic acid were use at concentration 50-900 ppm. The phenol yield of samples was calculated with equation (1) below and standard curve equation was \( y = 0.002415x + 0.016893 \) where \( R^2 = 0.9999 \). The analysis was conducted three times and reported as average with standard deviation.

\[
\text{Phenol yield} = \frac{\text{Concentration (mg/L) x Volume of extract (mL)}}{\text{Dry weight (g) x 100}} = \text{mg GAE/g DW}
\]  

\[
(1)
\]

3. Result and discussion

Total phenols content (TPC)
The total phenols content of sample extracts is tabulated in Table 1. The highest TPC was obtained at 200 W in 5 min of extraction time with value of 24.08 ± 0.51 mg GAE/g DW was obtained at energy
density of 0.073 W/ml in 5 min and 79 °C of extraction time and temperature (significantly difference with \( p < 0.05 \)). The TPC was decreased as the extraction time and energy density increased from 5 to 15 min and 0.073 to 0.108 W/ml, respectively. At fixed energy density of 0.073 W/ml with different extraction time (5, 10, and 15 min), the TPC obtained was decreased from 24.08 ± 0.51 to 23.04 ± 1.29 mg GAE/g DW. Results indicated that as time increased at fixed power, the TPC is reduced due to longer extraction increased the temperature of the plant sample and lead to the degradation of phenol compound. The rise in temperature resulted from the longer period of extraction had destroyed the phenol content in \( C.\) nutans. A similar finding on the effect of temperature on TPC also noted in \( P.\) sajor-caju methanolic extracts, where the TPC content was decreased from 0.080 ± 0.05 to 0.074 ± 0.03 mg GA/g DW at temperature of 100 °C in 15 min and 30 min of extraction time respectively [25].

Furthermore, at different energy density which are 0.073, 0.078 and 0.108 W/ml with fixed extraction time, the TPC obtained was also decreased from 24.08 ± 0.51, 19.61 ± 1.11 and 15.58 ± 1.83 mg GAE/g DW, respectively, however the difference is relatively high. The heat generated by higher microwave irradiation can be too powerful that may cause thermal degradation of phenol compound hence resulted in low TPC value [26], [27], [28].

**Table 1.** Total phenol content (TPC) at different conditions

| Power (W) | Average Energy Density (W/ml) | Average Temperature (°C) | TPC values (mg GAE/g DW) |
|-----------|-------------------------------|--------------------------|--------------------------|
| 200       | 0.073                         | 79.0 ± 1.56              | 24.08 ± 0.51             |
|           |                               | 85.5 ± 0.62              | 19.61 ± 1.11             |
| 300       | 0.078                         | 98.9 ± 1.13              | 24.04 ± 0.61             |
|           |                               | 100.8 ± 0.57             | 23.04 ± 1.30             |
|           |                               | 100.0 ± 0.56             | 17.42 ± 0.72             |
|           |                               | 102.8 ± 0.85             | 13.73 ± 0.70             |
| 400       | 0.108                         | 99.7 ± 0.45              | 15.58 ± 1.83             |
|           |                               | N/A                      | 15.44 ± 0.45             |

N/A Sample found burnt during microwave heating.

Phenol compounds are sensitive to several environmental conditions such as pH, oxygen, light, storage temperature and time [29]. Degradation of phenol compounds may occur under those conditions. In this study, it can be observed that the longer the exposure of sample (5 min to 15 min) to higher energy density (0.073 to 0.108 W/ml), the lower the phenol content. This is due to the increasing temperature of the sample during microwave irradiation period from 79 °C to 102.3 °C. \( C.\) nutans have thermosensitive of phenol compounds and can deteriorate under high temperature [15]. This is hypothesized that longer extraction time caused high temperature of the sample that lead to the degradation of target compound.

According to Mustapa et al., (2015), they found that the total phenol content of \( C.\) nutans extract using pressurized microwave-assisted extraction (P-MAE) is at highest value of 14.56 ± 0.77 mg GAE/g DM with pressure of 2.7 bar [11]. However, this method was using solvent ethanol at concentration of 50% v/v. Use of solvent is unfavorable economically and commercially as there is trace of solvent in the product that must be treated in purification process [30], [31]. Substantially high TPC obtained in
this work at 0.073 W/ml and 5 min (24.08 ± 0.51 mg GAE/g DW), in comparison to the TPC extracted by P-MAE at 2.7 bar (14.56 ± 0.77 mg GAE/g DM) reported by Mustapa et al. (2015), indicating that vacuum pressure and low energy density is adequate to extract high polyphenols from C. nutans. Furthermore, SFME method is more environmentally friendly method with considerable high phenol content. Moreover, during the extraction at energy density of 0.108 W/ml and extraction time of 15 min, the sample was found burnt. This is due to the increment of irradiation power from 200 to 400 W with extraction time beyond 10 min caused high temperature to the plant sample significantly ($p < 0.05$) degrade the polyphenols. Sharma et al. (2015) have studied the heating effect of six different varieties of onion on the total phenolic and flavonoids. It was found that the percentage weight loss of each type of onions increased for temperature from 80 °C to 150 °C in 30 min duration of heating [32]. As for the C. nutans leaves, at 0.108 W/ml and 15 min of extraction time, the temperature was higher than 102 °C lead to lost a lot of moisture during extraction process and caused destruction (burnt) of plant sample. Therefore, the extraction time longer than 10 min is disregarded and considered as inefficient in SFME of C. nutans.

**Effect of vacuum and ambient state on TPC**

Water used as solvent in SFME of C. nutans can be heated up at temperature lower than its boiling point (100 °C) when using vacuum pressure [15]. Vacuum pressure is used to employ the powerful penetration of microwave power and at the same time elevate the internal and external temperatures of plant sample thus resulting in uniform heating. Besides, water is evaporated swiftly under vacuum state, enable the sample to be heated at low temperature. Thus, hindering the occurrence of oxidation and maintaining the valuable compounds in plant sample [33].

Extraction curve of TPC of C. nutans extract when subjected under conditions of vacuum pressure and ambient pressure is illustrated in Figure 1. Vacuum pressure used in the study was 70 kPa. During 500 min of water-bath infusion of C. nutans extract after microwave extraction for 5 min, the TPC values for conditions at vacuum pressure was two times higher the TPC extracted at ambient pressure. The use of vacuum condition is purposely to avoid rise in temperature hence, conserve polyphenols content in C. nutans. It was clearly shown under vacuum condition at energy density of 0.073 W/ml and 5 min extraction time, the temperature was 79 °C. However, at ambient condition the temperature was 101 °C under higher energy density of 0.091 W/ml and 5 min extraction. This indicates that the difference in state of pressure used in this study is crucial factor to the rise of temperature hence, degrade the polyphenols content in C. nutans.

![Figure 1](image-url) **Figure 1.** Extraction curve of C. nutans with solvent-free microwave extraction at \( \eta = 0.073 \) W/ml and \( t = 5 \) min. Symbols represent: (■) vacuum pressure, (▲) ambient pressure
4. Conclusion
The TPC was found to be dependent on energy density, extraction time and temperature with $p < 0.05$. Extraction by SFME at 0.073 W/ml and 5 min was found as the best condition to obtain the highest TPC i.e. 24.08 ± 0.51 mg GAE/g sample. Lowest TPC of 15.44 ± 0.45 mg GAE/g DW at high energy density and extraction time (0.108 W/ml and 10 min respectively), was due to degradation of TPC at high temperature of 102.3 °C. Low energy density at 0.073 W/ml is observed to produce high TPC of C. nutans extract. The extraction at vacuum condition resulted to high TPC in comparison to the extraction at ambient pressure, indicating SFME with vacuum condition essentially maintain the low temperature of water to control the degradation of target compound. This work demonstrated that SFME as one of promising extraction technique to recover polyphenols from medicinal plant C. nutans in cleaner, safer and faster way.

Acknowledgement
Authors thanked the Faculty of Chemical Engineering, UiTM Shah Alam, Malaysia with their fully support, resources and facilities. This research is funded with grant BESTARI (P) (050/2018).

References
[1] P'ng X W, Akowuah G A and Chin J H 2012 Acute oral toxicity study of clinacanthus nutans in mice Jipsr. 3 4202–05
[2] Sakdarat S, Shuyprom A, Pientong C, Ekalaksananan T and Thongchai S 2009 Bioactive constituents from the leaves of clinacanthus nutans lindau Bioorg. Med. Chem. 17 1857–60
[3] Che Sulaiman I S, Basri M, Masoumi H R F, Chee W J, Ashari S E and Ismail M 2017 Effects of temperature, time, and solvent ratio on the extraction of phenolic compounds and the anti-radical activity of clinacanthus nutans lindau leaves by response surface methodology Chem. Cent. J. 11 1–11
[4] Sakdarat S, Shuyprom A, Na A T D, Waterman P G and Karagianis G 2006 Chemical composition investigation of the clinacanthus nutans lindau leaves Thai J. Phytopharm. 13 13–24
[5] Alam A, Ferdosh S, Ghafoor K, Hakim A, Juraimi A S, Khatib A and Sarker Z I 2016 Clinacanthus nutans: a review of the medicinal uses, pharmacology and phytochemistry Asian Pac. J. Trop. Med 9 402-09
[6] Khoddami A, Wilkes M A and Roberts T H 2013 Techniques for analysis of plant phenolic compounds Molecules 18 2328–75
[7] Dai J and Mumper R J 2010 Plant phenolics: extraction, analysis and their antioxidant and anticancer properties Molecules 15 7313–52
[8] Maestri D M, Nepote V, Lamarque A L and Zyngadlo J A 2006 Natural products as antioxidants Phytochemistry Advances in Research vol 37/661 ed Imperato F (Kerala: Reseach Signpost)
[9] Seidel V Initial and bulk extraction of natural products isolation Natural Products Isolation: Methods and Protocols vol 864 ed Sarker S D and Nahar L (New York: Springer) chapter 2 pp 27-42
[10] Nn A 2015 A review on the extraction methods use in medicinal plants, principle, strength and limitation Med. Aromat. Plants 4 3–8
[11] Mustapa A N, Martin Á, Mato R B and Cocero M J 2015 Extraction of phytocompounds from the medicinal plant clinacanthus nutans lindau by microwave-assisted extraction and supercritical carbon dioxide extraction Ind. Crops Prod. 74 83–94
[12] Mandal V, Mohan Y and Hemalatha S 2007 Microwave assisted extraction - An innovative and promising extraction tool for medicinal plant research Pharmcogn. Rev. 1 7–18
[13] Filly A, Fabiano-Tixier A S, Louis C and Fernandez X 2016 Water as a green solvent combined with different techniques for extraction of essential oil from lavender flowers Comptes Rendus Chim. 19 707–17
[14] Alupului A, Călinescu I and Lavric V 2012 Microwave extraction of active principles from medicinal plants UPB Sci. Bull. Ser. B Chem. Mater. Sci. 74 130-42
[15] Destandau E, Michel T and Elfakir C 2013 Microwave-assisted extraction Natural Products Extraction: Principles and Applications vol 21 ed Rostagno M A and Prado J M (Cambridge: Royal Society of Chemistry) chapter 4 pp 113-56

[16] Kusuma H S, Alway A and Mahfud M 2018 Solvent-free microwave extraction of essential oil from dried patchouli (Pogostemon cablin Benth) leaves J. Ind. Eng. Chem. 58 pp 343–48

[17] Filly A, Fernandez X, Minuti M, Visinoni F, Cravotto G and Chemat F 2014 Solvent-free microwave extraction of essential oil from aromatic herbs: from laboratory to pilot and industrial scale Food Chem. 150 pp 193–98

[18] Muhamad I I, Hassan N D, Mamat S N H, Nawi N M, Rashid W A and Tan N A 2017 Extraction technologies and solvents of phytocompounds from plant materials: physicochemical characterization and identification of ingredients and bioactive compounds from plant extract using various instrumentations Ingredients Extraction by Physicochemical Methods in Food vol 4 ed Grumezescu AM and Holban A M (Amsterdam: Elsevier) chapter 14 pp 524-60

[19] Mustafa A and Turner C 2011 Pressurized liquid extraction as a green approach in food and herbal plants extraction: a review Analyt. chimica acta 703 pp 8-18

[20] Azmir J, Zaidul I S M, Rahman M M, Sharif K M, Mohamed A, Sahena F, Jahurul M H A, Ghafoor K, Norulaini N A N and Omar A K M 2013, Techniques for extraction of bioactive compounds from plant materials: a review J. Food Eng. 117 pp 426–36

[21] Álvarez A, Poejo J, Matias A A, Duarte C M M, Cocero M J and Mato R B 2017 Microwave pretreatment to improve extraction efficiency and polyphenol extract richness from grape pomace. effect on antioxidant bioactivity Food Bioprod. Process 106 pp 162–170

[22] Wang J X, Xiao X H and Li G K 2008 Study of vacuum microwave-assisted extraction of polyphenolic compounds and pigment from Chinese herbs J. Chromatogr. A 1198–1199 pp 45–53

[23] Xiao X H, Wang J X, Wang G, Wang J Y and Li G K 2009 Evaluation of vacuum microwave-assisted extraction technique for the extraction of antioxidants from plant samples J. Chromatogr. A 1216 pp 8867–73

[24] Kok L P, Boon M E and Smid H M 1993 The problem of hot-spots in microwave equipment used for preparatory techniques - theory and practice Scanning 15 pp 100–09

[25] Saad, W.Z., Hashim, M., Ahmad, S., & Abdullah, N. (2014). Effect of heat treatment on total phenolic contents, antioxidant and anti-inflammatory activities of Pleurotus Sajor Caju extract. International Journal of Food Properties, 17 (1), 219-225.

[26] Alara O R, Abdurahman N H, Mudalip S K A and Olalere O A 2018 Microwave-assisted extraction of Vernonia amygdalina leaf for optimal recovery of total phenolic content. J. Appli. Res. Med. Aromat. Plants 10 pp 16-24

[27] Dahmoune F, Nayak B, Moussi K, Remini H and Madani K 2015 Optimization of microwave-assisted extraction of polyphenols from Myrtus communis L. leaves Food Chem. 166 pp 585-95

[28] Spigno G and De Faveri D M 2009 Microwave-assisted extraction of tea phenols: A phenomenological study. J. Food Eng. 93 pp 210-17

[29] Yang L, Jiang J G, Li W F, Chen J, Wang D Y and Zhu L. Optimum extraction process of polyphenols from the bark of Phyllanthus emblica L. based on the response surface methodology J. Sep. Sci. 32 pp 1437–44

[30] Kumar S P J, Prasad S R, Banerjee R, Agarwal D K, Kulkarni K S and Ramesh K V 2017 Green solvents and technologies for oil extraction from oilseeds. Chem. Cent. J. 11 pp 1-7

[31] Castejoń N, Luna P and Señoráns F J 2018 Alternative oil extraction methods from Echium plantagineum L. seeds using advanced techniques and green solvents. Food Chem. 244 pp 75-82

[32] Sharma K, Ko E Y, Assefa A D, Ha S, Nile H S, Lee E T and Park S W 2015 Temperature-dependent studies on the total phenolics, flavanoids, antioxidant activities, and sugar content in six onion varieties. J. Food Drug Anal. 23 pp 243-52
[33] Song C, Wu T, Li Z, Li J and Chen H 2018 Analysis of heat transfer characteristics of blackberries during microwave vacuum heating *J. Food Eng.* 223 pp 70-78