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Determination of Phenolic and Flavonoid Contents, Antioxidant Activities and GC-MS Analysis of *Clinacanthus nutans* (Acanthaceae) in Different Locations

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ABSTRACT

Clinacanthus nutans is an essential medicinal plant that had been used in various local remedies to treat many illnesses. A study had been conducted to determine the phenolic, flavonoid, antioxidant activities and phytochemical compounds of *C. nutans* in different locations. *C. nutans* were harvested from eight locations and the leaves were extracted with 80 % methanol by maceration process. Then, the phytochemical screening using Gas Chromatography-Mass Spectrometry (GC-MS), 2,2 diphenyl-2-picrylhydrazyl hydrate (DPPH) assay method, total phenolic content by Folin-Ciocalteu's assay method and total flavonoid content by aluminium chloride ($AlCl_3$) were carried out. The *C. nutans* extracts showed higher antioxidant activities than phenolic and flavonoid content. The neutral pH sandy clay soil from location KKK (Kuala Ketil, Kedah, Malaysia) had higher antioxidant activities (58.0 %), phenolic (44.1 mg GA.100 g^{-1}) and flavonoid content (30.8 mg QE.100 g^{-1}) compared to other locations. The GC-MS analysis showed the presence of phytochemicals constituents of 20 compounds. The results revealed that environmental factors (light intensity, temperature and soil characteristics) of eight locations were responsible for variations of phenolic, flavonoids, antioxidants and GC-MS analysis in *C. nutans.* The findings of this study will provide baseline data for future breeding programs for commercial cultivation.

Keywords: antioxidant; *C. nutans*; environment; flavonoid; phenolic; phytochemistry

INTRODUCTION

Around 85 % to 90 % of the world's population consumes medicinal plants (Krishnamoorthy & Subramaniam, 2014). Plant secondary metabolites have their own roles in the human and also other living things same goes to *Clinacanthus nutans* that have its own role in pharmaceutical remedies in South-East Asia such as Malaysia, Thailand, Indonesia, Vietnam and China (Ismail, Arsad, Samian, Ab. Majid, & Hamdan, 2016). *C. nutans* belongs to family Acanthaceae that can be described as herbs or perennial shrubs that can grow up to 1 m tall. In Malaysia, it is well-known as 'Belalai Gajah' because of the curve stems appearances that resemble the curve of elephant's trunk that means Belalai Gajah in Malay word. Confirming to the people who used leaves of *C. nutans*, they used it as raw vegetable and sometimes mixed with apple juice, sugarcane juice, orange juice, green tea, and served as a fresh drink or a refreshing beverage. They have been practiced drinking *C. nutans* extracts due to their history of the disease in cancers, diabetes, fever, jaundice, pneumonia, and hepatitis. They also claimed that they have recovered from the illness especially cancer disease (Shim, Aziana, & Khoo, 2013). The scientific information on benefits of *C. nutans* had been proved to treat many diseases such as varicella-zoster virus and herpes-simplex virus, anti-snake venom, analgesic and anti-inflammatory and anti-proliferative in cancer cells (Ismail, Arsad, Samian, Ab. Majid, & Hamdan, 2016).

Plant of the same species may differ significantly for their secondary metabolite contents within different environments. This is because the quality and quantity of secondary metabolites in medicinal plants are largely influenced by the environmental conditions. In some cases, secondary metabolites accumulation has been affected by geographical altitude and local ecological conditions such as rainfall, humidity, temperature, water variability, exposure to soil microorganisms and variations in soil pH and nutrients. Furthermore, environmental factors also interact with the genetic of the plants resulting in a variable of gene

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expression that ultimately leads to the alteration of secondary metabolites concentration and thus affects the quality of medicinal plants (Sampaio, Edrada-ebel, & Batista Da Costa, 2016). Plant secondary metabolites have been divided into many classes, one of the general classes are phenolic that can be divided into two which are flavonoids and non-flavonoids. Non-flavonoids consist of secoiridoids, stilbenes, simple phenols, benzoic acids, lignans, hydrolysable tannins, acetophenones, cinnamic acids, phenylacetic acids, benzophenones, coumarins, and xanthones. While flavonoid compounds assemble from a large group of polyphenolic *compounds* which consist of benzo-γ-pyrone structure that present all over the secondary metabolite of the plant. The structure of flavonoids is categorised by a 15-carbon (C6-C3-C6) backbone comprising of two aromatic and one oxygenated ring. Both of them are grouped together into subclasses based on their basic chemical structures which are flavanones, flavones, chalcones, flavanols, flavonols, isoflavones and anthocyanins (Kumar & Pandey, 2013).

The research was conducted to study the amount of phenolic, flavonoid, antioxidant activities as well as GC-MS analysis of *C. nutans* in different location sites. Most of the researchers had been using colorimetric methods to obtain the total contents of phenolic, flavonoid and antioxidant assay from Folin-Ciocalteu assay, $AlCl_3$ assay, and DPPH assay respectively. To investigate the phytochemical compounds of *C. nutans* in different locations, GC–MS had been successfully applied. The GC-MS analysis had been used for plants such as *Ziziphus spina-christi* (Moustafa, Hesham, Quraishi, & Alrumman, 2016), *Cannabis sativa* (Tayyab & Shahwar, 2015), and *Justicia adhatoda* (Gilani, Fujii, Kikuchi, Shinwari, & Watanabe, 2011) to determined variations of chemical compounds found in different locations. In recent years, the screening of phytochemical compounds of *C. nutans* has already been studied such as stigmasterol, belutin, C-glycosyl flavones, vitexin, isovitexin, orientin, isoorientin, sulfur-containing glycosides, monoacylmonogalatosylglycerol, lupeol, β-sitosterol, shaftoside, isomollupentin-7-O-β-glucopyranoside, glycoglycerolipids and cerebrosides (Sakdarat, Shuyprom, Pientong, Ekalaksananan, & Thongchai, 2009). Until the scientific research have informed, the diversity of phenolic, flavonoid and antioxidant activities of *C. nutans* are still unclear whether the quantity of phenolic, flavonoid, antioxidant activities and secondary metabolites from GC-MS analysis were varied in different locations. Therefore, it is important to understand that the chemical properties of the plant in different locations as they might contain powerful compounds in certain locations that can benefit to human health and also other living things. Moreover, the selected *C. nutans* locations can be used as breeding programs and commercial cultivation for medicinal purposes.

MATERIALS AND METHODS

In this study, eight locations had been selected in the northern region of Peninsular Malaysia. The code names for locations of *C. nutans* were shown in Table 1 and the map was shown in Fig. 1. At Penang, four locations of *C. nutans* cultivation had been identified which were Pongsu Seribu (PBF) and Tasek Gelugor (PTG) (situated at north Seberang Perai), Batu Maung (PBM) (situated at the south-eastern part of Penang Island) and Batu Ferringhi (PBF) (situated at the hilly northern side of Penang Island). In Kedah, three locations of *C. nutans* cultivation had been identified which were Jeniang (KJN) (a small town that situated between Gurun and Sik), Sungai Petani (KSP) and Kuala Ketil (KKK). At Perlis, it had been identified one location site which was at Taman Herba Perlis at Sungai Batu Pahat (SBP). It lies at the northern part of the west coast of Peninsular Malaysia. *C. nutans* and are distributed at the elevation between 8 m to 44 m above sea level. Temperature among eight locations showed averaged of 32 ^{o}C between 30 ^{o}C to 34 ^{o}C. The soil characteristics and light intensity were determined using soil and light tester meter (soil moisture, soil pH and light) while the amount of nitrogen, potassium and phosphorus level in the soil were determined using advanced soil test kit. The KSP and KKK showed the highest value of pH of 7.0 (neutrality) while the lowest value came from location KJN with a pH value of 5.0 (acidity). The mean rainfall in different location sites was collected from the nearest Malaysian Meteorological Department. This research was conducted for three months (February 2015 to April 2015) in Integrative Medicine Cluster Laboratory, Advanced Medical, and Dental Institute, Universiti Sains Malaysia. The identification of plant was confirmed by the herbarium of School of Biological Sciences, Universiti Sains Malaysia with voucher collection (No: 11536).

Table 1. Location site, geographical and soil characteristics at different locations of *C. nutans*

Location code	State	Region	Coordinate		Environmental conditions				Soil characteristics					
			Latitude	Longitude	Elv (m)	T (°C)	R (mm)	LI (lux)	Soil texture	pH	N	P	K	Soil moisture
PBF	Penang	Batu Feringgi	5.46913	100.24655	33	33	221	500	Sand	5.5	0	4	2	6.0
PTG	Penang	Tasek Gelugor	5.48783	100.50535	14	30	202	650	Sandy loam	6.5	1	4	3	7.5
SBP	Perlis	Sungai Batu Pahat	6.53414	100.16906	44	30	384	500	Sand	6.0	0	3	3	9.5
KSP	Kedah	Sungai Petani	5.70244	100.51216	13	34	211	800	Sandy clay	7.0	0	3	2	6.5
KKK	Kedah	Kuala Ketil	5.60518	100.65115	21	33	246	850	Sandy clay	7.0	1	4	2	7.5
PPS	Penang	Pongsu Seribu	5.49663	100.44532	11	33	202	700	Sandy loam	6.0	1	4	3	7.5
KJN	Kedah	Jeniang	5.81501	100.62629	34	33	146	500	Silt loam	5.0	0	3	3	3.5
PBM	Penang	Batu Maung	5.28496	100.28013	8	32	225	550	Sandy loam	6.5	2	4	3	6.0

Remarks: Elv (m) Elevation, T (°C) Temperature, R (mm) Rainfall mean (February 2015 to April 2015), LI (lux) Light intensity, Soil moisture (1-3 dry, 4-7 intermediate, 8-10 wet), N Nitrogen, P Phosphorus, K Potassium, 0=Depleted (0.0-7.5 ppm), 1=Deficient (~7.5-15.0 ppm), 2=Adequate (~ 15.0-22.5 ppm), 3=Sufficient (~22.4-30.0 ppm), 4=Surplus (~30+ ppm)

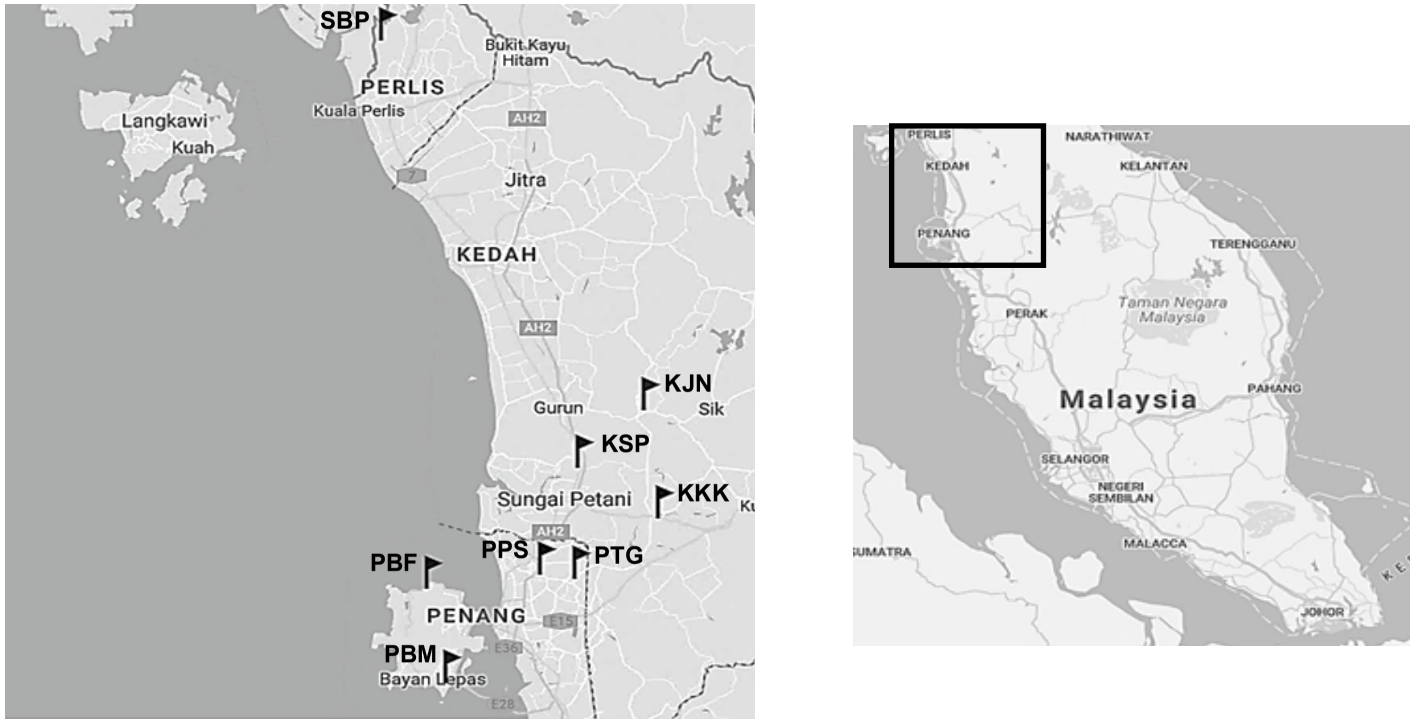


Fig. 1. Distribution of *C. nutans* in northern region of Peninsular Malaysia. The code names for location sites above were stated in Table 1

Preparation of Leaves Extract

The leaves of *C. nutans* from each location were separated and bagged independently. The leaves were cleaned with distilled water and cut into small slices. The extraction method used was maceration with methanol as solvent. The extraction begins by grinding the 200 g leaves into small particles. The leaves were soaked in the ratio of distilled water: methanol (1:4). The *C. nutans* extracts were kept under 24 hours agitation by using a mechanical stirrer set at 200 rpm. After that, the extracts were filtered. The extraction processes were repeated three times. The rotary evaporator was used to remove solvents under reduced pressure and leave only green coloured pastes extracts. The extracts obtained were freeze dried for three days to remove all solvents completely. The crude extracts were stored at -20 °C before used.

Total Flavonoid Content

The 0.001 g of crude extracts were diluted with distilled water to obtained 1 mg mL^{-1} of sample concentration. Then, the 100 *µL of C. nutans* extracts were mixed with 100 µL of 2 % $AlCl_3$ solution dissolved using distilled water. The samples were shaken and the absorbance was read at absorption of 430 nm after 15 minutes of incubation. The same method was repeated for the quercetin standard solution and the calibration line was interpreted. The concentration of flavonoids was read mg mL^{-1} on the calibration line. After that, the content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of quercetin per g).

Total Phenolic Content

The total phenolic content of *C. nutans* was obtained from Folin-Ciocalteu Assay. Concentrations of 1 mg mL^{-1} of crude extracts were prepared in distilled water. An amount of 15 µL of extract was dissolved with 75 µL of a 10 fold dilute of Folin-Ciocalteu's phenol reagent. Then, 60 *µL* saturated 35 % sodium carbonate (Na_2CO_3) solution was added to the mixture after 3 minutes incubation. The reaction mixtures were incubate for 30 minutes. After that, the absorbance was determined at 765 nm with a spectrophotometer. The standard that had been used was gallic acid and the total phenolic was expressed as mg g^{-1} gallic acid equivalents (GAE). Samples were analyzed triplicates.

Antioxidant Activities

An amount 24 mg DPPH in 25 mL methanol of stock solution was prepared to obtained 0.96 mg mL^{-1} (2.4 mM). The dilution of 1.8 mL of DPPH stock solution to 45 mL methanol was prepared as working solution. The 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox) working standards (0.5–3.0 mM) was diluted with the same solvent. In 96 well plates, 10 uL of extracts (1 mg mL^{-1}) was mixed thoroughly with 200 uL of DPPH working solution and incubated in the dark for 30 minutes. Then, the absorbance was measured at 517 nm against blank methanol with no DPPH solution, control methanol with DPPH solution and DPPH solution. The amount of antioxidant activity was characterised by the inhibitory concentration (IC_{50}), which was a concentration of the sample solution indicated a potential antioxidant that was needed to reduce DPPH by 50 % (Salusu et al., 2017). The percentage of inhibition of the DPPH radical was determined using the following equation:

$$\% \text{ inhibition of DPPH} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \text{ X } 100$$

Where: Abs control is the absorbance of DPPH solution without extracts.

GC-MS

The concentration of 1 mg mL^{-1} of crude extracts were used for GC-MS analysis The secondary metabolites from each of the extracts were determined using Agilent gas chromatograph model 6890 equipped with an Agilent 19091S-433 capillary column, HP-5MS (0.25 mm x 30 m x 0.25 um). Helium gas was used as a carrier gas at 1.0 mL per minute with splitless mode injection. The oven temperature was set as follows: 70 °C held for 2 minutes, then increased to 200 °C at the rate of 5 °C per minute, then increased to 325 °C at the rate of 10 °C per minute and finally maintained at 250 °C for 10 minutes.

Data Analysis

The data obtained were subjected to analysis of variance (ANOVA) and tested for normality by IBM SPSS Statistics version 19. Canonical correspondence analysis (CCA) with 1000 permutation was performed to determine which environmental variables (as the independent factor) influenced the productivity of phenolic, flavonoid and antioxidant activity (dependent factor) of *C. nutans* in different locations. The CCA was conducted using XLSTAT version 2017. The phytochemical compounds from GC-MS were compared by at least

80 % of the similarity index from the mass spectral library data from National Institute of Standards and Technology (NIST).

RESULTS AND DISCUSSION

Percentage Yield Crude Extracts

The choice of an extraction method and suitable solvent are an important step in drug discovery process. The 80 % methanol is known to be the best solvent to use for extraction because methanol and water give more polar organic properties and preferable to determine flavonoid, phenolic and antioxidant activities (Tasioula-Margari & Tsabolatidou, 2015). The leaves were soaked in methanol three times to extract out the polar compounds which constitute the majority compounds present in the samples. The percentage of average crude methanol extract yield was 4.5 % as shown in Table 2. The yield of *C. nutans* extract from location PBM (5.3 %) was the highest among eight locations whereas the yield of *C. nutans* extract from location KSP (3.5 %) was the lowest compared to the other locations. From the observation, *C. nutans* had been regularly being collected at the juvenile stage and the purpose of cultivation was for medicinal and research purposes. Thus, we had collected *C. nutans* at the juvenile stage (3 to 4 months) since a previous study had been conducted by Raya et al. (2015) that showed younger leaves of *C. nutans* produced high DPPH radical scavenging activities and total flavonoid as well as high phytochemical contents than older leaves of *C. nutans*.

Table 2. Percentage yield of crude extracts from *C. nutans* leaves in different locations

Location code	Weight of crude sample (g)	Percentage Yield (w/w) (%)
PBF	9.08	3.6
PTG	12.22	4.9
SBP	11.65	4.7
KSP	1.74	3.5
KKK	2.22	4.5
PPS	11.57	4.7
KJN	4.98	5.0
PBM	5.25	5.3
	Average	4.5

Antioxidant Activities, Total Phenolic and Flavonoid Contents of *C. nutans*

In this study, eight locations of *C. nutans* (PBF, PTG, SBP, KSP, KKK, PPS, KJN and PBM) were being investigated as potential locations to harvest *C. nutans* for commercial cultivation. Total phenolic content varied from 17.12 mg GAE.100 g^{-1} to 44.13 mg GAE.100 g^{-1}, location KJN being the lowest and the highest was in location KKK (Table 3). The descending order of phenolic contents in the studied locations was KKK>PBF>PTG>PPS>KSP>PBM>SBP>KJN. Total flavonoid content ranged from 14.08 QE.100 g^{-1} to 30.80 QE.100 g^{-1}. It was the highest in location KKK and the lowest in locations PTG. The descending order of flavonoid contents in the studied locations was KKK>KSP>PBM>PPS>SBP>KJN>PBF>PTG. For the antioxidant activities analysis, Table 3 showed a range of DPPH inhibition of 38.1 % to 58.0 % with descending order of KKK>PTG>PPS>SBP>PBF>PBM>KSP>KJN.

Table 3. The amount of phenolic, flavonoid and antioxidant activity of *C. nutans* extracts (1 mg/mL) in different locations

Location code	Total phenol (mg GAE.100 g^{-1})	Total flavonoid (mg QE.100 g^{-1})	DPPH inhibition (%)
PBF	34.28±0.02	17.56±0.01	45.2±0.3
PTG	32.55±0.04	14.08±0.01	55.0±0.6
SBP	18.25±0.00	21.39±0.13	45.3±0.6
KSP	25.26±0.03	25.13±0.03	38.1±0.1
KKK	44.13±0.00	30.80±0.18	58.0±0.7
PPS	30.22±0.01	22.41±0.15	54.9±0.4
KJN	17.12±0.00	20.15±0.08	35.1±0.3
PBM	19.25±0.00	24.13±0.05	40.7±0.5
ANOVA			
MS	167.04	114.83	123.01
SS	1169.28	803.82	861.03
F Value	425716.11	11362.36	588.15
p value	$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$

Remarks: MS Mean Squares, SS Sum of Square, Each value was expressed as mean ± standard deviation (SD) (n = 3)

The findings showed that antioxidant activities had the largest value than total phenolic followed by flavonoid content. This finding is in line with Sulaiman et al. (2015), who reported that higher antioxidant activities in *C. nutans* followed by total phenolic content and total flavonoid content. This is because many antioxidant properties such as terpenes, sterols and saponin may have contributed to these activities and not only phenolic compounds (Odchimar, Nuñeza, Uy, & Senarath, 2016). The result by IBM SPSS Statistics version 19 showed that there were significant values of ANOVA of phenolic, flavonoid and antioxidant activities among tested extracts in different locations ($P \leq 0.05$).

GC-MS analysis

Through many years, many people have been trying to treat their illnesses from different plant extracts and formulations. In this study, phytochemical constituents of *C. nutans* were identified by GC-MS. The GC-MS is the preferable tool in this research as it is easy to use, less expensive and detects universal analyses which are suitable to screen and compare the phytochemical compounds of this species in different locations. Phytochemical compounds of *C. nutans* were compared with chemical compounds from NIST data library by at least 80 % of the similarity index. The GC-MS analysis of 80 % methanol of *C. nutans* extracts revealed the presence of phytonutrients that beneficial in pharmacological activities. There were twenty compounds that matched at least 80 % similarity index from the library in Table 4. The peaks abundance area indicated that the abundance of phytochemical compounds was different in each *C. nutans* extracts which means that the quantity of each constituent present in the plant is different from each other. The information of variability of phytochemical compounds may be very useful for quality control and selection of commercial cultivation of *C. nutans*.

Table 4. Main phytochemical compounds found in *C. nutans* crude extracts at different locations

No.	Phytochemical compounds	Similarity Index (%)	Peak Abundance Area (%)							
			KJN	KKK	KSP	PBF	PTG	PPS	PBM	SBP
1.	2,3-dihydro-3,5-dihydroxy-6-methyl 4H-Pyran-4-one	87	-	5.34	-	2.33	3.55	5.05	2.45	1.87
2.	Phytol	90	5.19	5.18	5.17	5.18	3.48	3.47	5.17	5.17
3.	Phenol, 2,4-bis(1,1-dimethylethyl)	94	15.40	15.35	15.36	15.30	7.56	7.58	15.35	15.36
4.	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester	93	1.46	1.45	1.55	1.50	2.54	2.35	1.45	1.56
5.	Pentadecanoic acid, 14-methyl-, methyl ester	98	4.89	4.78	4.90	4.89	4.79	4.89	4.75	4.85
6.	Hexadecanoic acid, methyl ester	98	-	1.05	-	2.35	1.79	1.14	-	-
7.	n-Hexadecanoic acid	97	-	0.86	-	-	-	-	-	2.35
8.	9,12,15-Octadecatrienoic acid, methyl ester	99	-	-	2.59	5.36	5.45	5.30	7.37	1.54
9.	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	91	-	10.23	-	-	-	-	-	0.57
10.	2-Methoxy-4-vinylphenol	85	4.95	5.34	2.56	6.79	5.48	7.25	-	-
11.	10,13-Octadecadienoic acid, methyl ester	83	-	1.36	-	-	-	-	-	-
12.	.alpha.-D-Galactopyranoside, methyl	91	-	-	-	-	-	-	8.25	-
13.	Phthalic acid, isobutyl octyl ester	89	3.56	2.87	3.86	3.68	3.69	3.74	tr	3.74
14.	Oleic Acid	92	-	-	5.69	-	-	-	-	-
15.	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	86	-	-	-	-	-	-	3.47	tr
16.	Octadecanoic acid	96	-	-	6.12	-	-	-	-	-
17.	Benzofuran, 2,3-dihydro-	88	13.47	-	-	-	-	-	-	-
18.	Heptadecanoic acid, 16-methyl-, methyl ester	96	-	-	-	-	-	-	-	1.65
19.	Octadecanoic acid, methyl ester	99	2.05	2.36	-	-	-	-	2.65	-
20.	Vitamin E	96	-	2.85	-	-	-	-	2.90	-

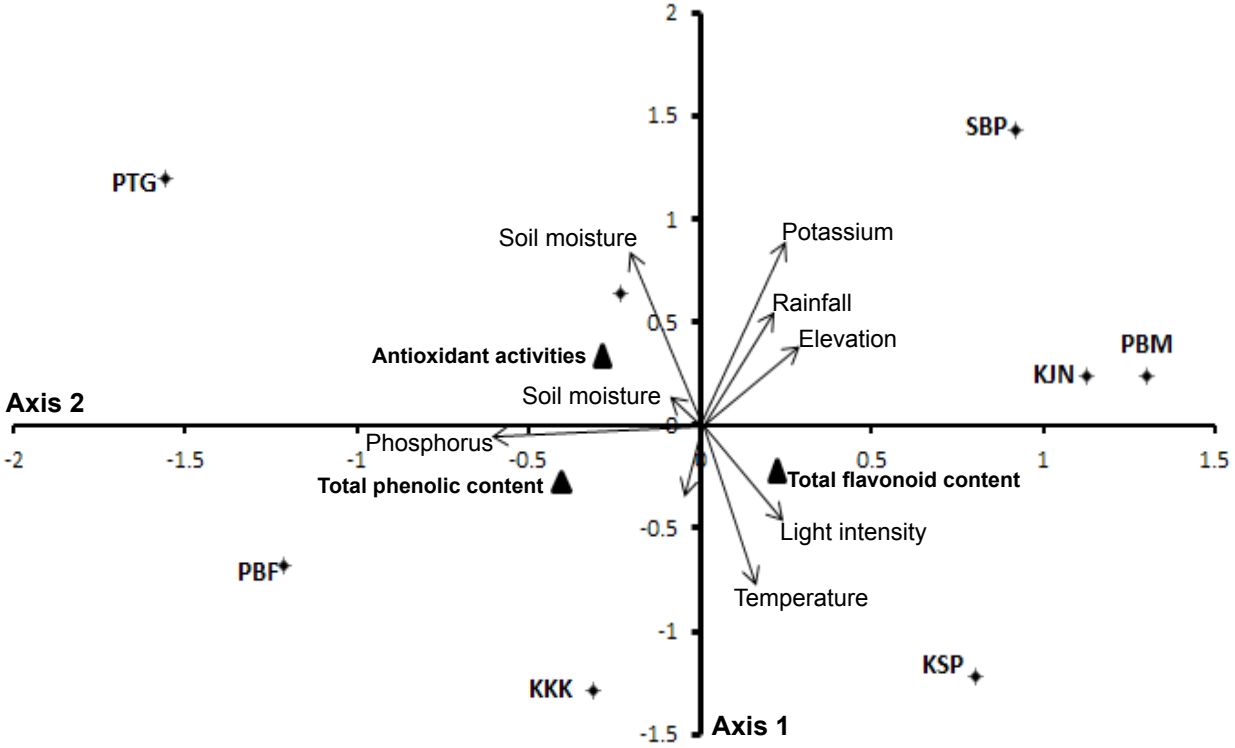
Remarks: tr: trace (<0.01%)

The findings showed that *C. nutans* extract was higher in location KKK with 13 chemical compounds and lowest in location KJN with 8 chemical compounds. The location PTG, PBF, and PPS had 9 similar compounds but different in peak abundance area. Phytol, phenol, 2,4-bis(1,1-dimethylethyl), 2-propenoic acid, 3-(4-hydroxyphenyl)-,methyl ester, pentadecanoic acid, 14-methyl-,methyl ester and phthalic acid, isobutyl octyl ester were present in all locations of *C. nutans* but in different peak abundance area. The result indicated that there was a variation of phytochemical compounds in the tested *C. nutans* extracts in different locations. This may be due to extraction process and also environmental condition at location sites. In this study, we used solvent methanol to extract *C. nutans* leaves. In the most study, methanol has been commonly used for phytochemical screening of several medicinal plants and is considered a volatile organic compound that is suitable for GC-MS analysis. Moreover, the use of 80 % methanol was reported as an efficient extraction solvent to detect bio phenol compounds (Tasioula-Margari & Tsabolatidou, 2015). Based on the GC-MS analysis, there was a wide range of biologically active compounds not only phenolic and flavonoid compounds only. Studies show that *C. nutans* have been a potential medicinal plant to treat cancer (Shim, Aziana, & Khoo, 2013). In this case, many compounds found in *C. nutans* by GC-MS analysis point towards bioactive compounds that can treat cancer such as 2,3-dihydro-3,5-dihydroxy-6-methyl 4H-pyran-4-one, phytol, phenol, 2,4-bis(1,1-dimethylethyl), 10,13-octadecadienoic acid, methyl ester, oleic acid, octadecanoic acid and vitamin E in Table 4. Such findings of phytochemicals compounds in the medicinal plant were significant in the contribution of new drugs development from the plant, especially in *C. nutans*. The understanding of the chemicals compound found in the plant can lead to systematic examination for economically valued of chemical compounds in the market.

Factors Affecting Phytochemical Composition of *C. nutans*

CCA was used to visualize and correlate between the environmental conditions of different location sites with response to phytochemical variables (total phenolic content, total flavonoids and DPPH) (Fig. 2) (Yudiyanto, Rizali, Munif, Setiadi, & Qayim, 2014). The results showed the association between the different locations and environmental factor (elevation, temperature, rainfall, light intensity and soil characteristics) to phytochemical variables (phenolic, flavonoids, and antioxidant activity). The CCA axes 1 and 2 explained 71.8 % and 28.3 % variation respectively. Fig. 2 showed that total phenolic content response to phosphorus and pH level in the soil. *C. nutans* in location PBF and KKK showed a high amount of total phenolic content with acidic and neutral pH level in the soil respectively. Both location sites showed the surplus amount of phosphorus level in the soil. The antioxidant activity can be associated with a deficient amount of nitrogen level of the soil and intermediate level of soil moisture at location sites. Among all locations that had been studied, *C. nutans* at the location PPS, PTG and KKK had the largest amount of antioxidant activities with a deficient amount of nitrogen level in the soils and intermediate level of soil moisture. The soil moisture at locations PPS, KKK and PTG showed intermediate level (7.5) according to the soil and light tester meter. Thus, the result showed that *C. nutans* was suitable being cultivated in intermediate (normal) soil moisture which was not too dry or too wet.

In Fig. 2, the result showed that high temperature and high light intensity were responsible for total flavonoid content in the *C. nutans*. Location KSP exhibited the highest temperature and high light intensity compared to other locations with high total flavonoid content after location KKK (Table 1). This finding concurs with the report by Ghasemzadeh, Jaafar, & Rahmat (2015) that high light intensity will increase the net of photosynthesis and exceeds carbon which leads to increase in production of flavonoid and phenol content in the plant. In this study, all environmental conditions measured play an important role in phytochemical variables of *C. nutans*. However, elevation, rainfall, and potassium level in the soil showed in location KJN, PBM and SBP could not correlate to the phenolic, flavonoid and antioxidant activities in *C. nutans* (Table 1, Table 3 and Fig. 2). Hence, the findings make it clear that more location sites need to be identified outside the northern region of Peninsular Malaysia since larger sample sizes may be needed to analyze total phenolic, flavonoid and antioxidant activity in different environmental conditions.



Remarks: PBF, PTG, SBP, KSP, KKK, PPS, KJN and PBM (location code based on Table 1)

Fig 2. CCA plot showing *C. nutans* locations with the relationship of phytochemical variables with different environmental conditions

Most of the location sites (PBF, PTG, SBP, KSP, KKK, PPS and PBM) in this study possessed sandy soils followed by sand clay to sandy loam except location KJN that showed silt loam (Table 1). The location KKK sampled in clay soils increased in antioxidant properties and phenolic content compared to the sandy soil as sandy soil tends to retain fewer nutrients (Akbarian, Rahimmalek, & Sabzalian, 2017). The high macro nutrient of potassium in soil stimulates the photosynthesis activity and increasing the translocation of carbohydrate to plants that enhanced biosynthesis of total phenolic and flavonoid content of plants (Ibrahim, Jaafar, Karimi, & Ghasemzadeh, 2012). Results revealed that most of the locations studied had a moderate amount of potassium content in the soil. Comparing to the nitrogen level in soils, most of the soils in the locations studied were in low nitrogen level. This is concurrent with the results by Munene, Changamu, Korir, & Joseph (2017), reported that accumulation polyphenol and flavonoid contents found in plant tissue will enhance under restricted nitrogen nutrition. Soil pH also played an important role for medicinal plants. The results showed that *C. nutans* that have been cultivated from saline soil (KJN) had the lowest activity in phenolic, flavonoid, antioxidant and also phytochemical compounds from the GC-MS analysis. According to Valifard, Mohsenzadeh, Kholdebarin, & Rowshan (2014), saline soil can affect phenolic compounds, the growth, yield and secondary metabolites of plants. However, different plant species have their own favorable environmental conditions.

On the other hand, the extraction method can also affect the phytochemical compounds of *C. nutans*. The extraction method that had been used was maceration with methanol to water (4:1) at room temperature. Extraction at room temperature is a preferable technique to avoid degradation of compounds and etraction under lower temperature

resulted in many production of polyphenols extracts compared to the higher temperature (Blicharski & Oniszczuk, 2017). Such variation that had been showed in phenolic and flavonoid content as well as antioxidant activities in different locations also depends on the genetic variation of the traits (Ismail, Arsad, Samian, Ab. Majid, & Hamdan, 2016) and the impact of the environmental conditions (soil contents and climatic conditions) (Gilani, Fujii, Kikuchi, Shinwari, & Watanabe, 2011). The impact of the environmental condition can affect the genetic diversity of plants (Ismail, Arsad, Samian, Ab. Majid, & Hamdan, 2016) which leads to the variable of gene expressions that can cause modification of secondary metabolites in the plant.

Relationship between GC-MS Analysis with Phenolic, Flavonoid and Antioxidant Activities of *C. nutans*

The phytochemical variables of antioxidant activities, phenolic and flavonoid contents of *C. nutans* in the different locations were significant between each other. However, the results of the GC-MS analysis cannot be compared directly with the results of those phytochemical variables. For phenolic, flavonoid and antioxidant activities, the standards used were not performed in the GC-MS analysis. Moreover, the data expressed from those phytochemical variables were based on absorbance from spectrophotometry and were expressed differently from the data obtained from the GC-MS analysis. In addition, phytochemical compounds found from GC-MS were not only phenolic and flavonoid contents but also consist of many other compounds such as diterpene, aromatic methyl esters, unsaturated fatty acid, ester, fatty acid, vitamin and so on which was compared by NIST mass spectral library.

CONCLUSION AND SUGGESTION

In conclusion, *C. nutans* extract in location KKK exhibited the highest in antioxidant (58.0 %), phenol (44.13 mg GAE.100 g^{-1}) and flavonoid (30.80 mg QE.100 g^{-1}) and possessed the most chemical constituents detected by GC-MS analysis. It is suggested that *C. nutans* needs to be cultivated in high light intensity, neutral pH sandy clay soil, intermediate soil moisture with deficient nitrogen level, surplus phosphorus level and adequate potassium level in the soil to obtain higher phenolic, flavonoid, and antioxidant activity.

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