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PLANT VEGETATIVE STAGES AND DRYING METHODS AFFECT FLAVONOID CONTENT OF CLINACANTHUS NUTANS EXTRACTS

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Abstract

Background: *Clinacanthus nutans*, also known as 'Sabah snake grass' or 'Belalai gajah', is a herb well known locally for its medicinal values. The primary chemical constituents of the leaves are schaftoside, vitexin, isovitexin, orientin and isoorientin, and antiviral activity is shown by two glycoglycerolipids. Despite the importance of *C. nutans*, complete information with respect to commercial production and postharvest handling of the herb in the local herbal industry is still lacking. Thus, the objective of this study was to determine the optimum postharvest handling processes that could retain the quality of phytochemicals of *C. nutans.*
Materials and Methods: The flavonoid compounds of *C. nutans* were analysed by using ultra fast liquid chromatography (UFLC). Total phenolic content and antioxidant activity were determined using a spectrophotometer.
Results: The total phenolic compounds and antioxidant activity in *C. nutans* were found to be higher in the young vegetative stage than in the mature vegetative stage. Flavonoid compounds (schaftoside, isovitexin, vitexin and orientin) were also found to be highest in the young vegetative plant compared to the mature vegetative plant. All of the assayed phytochemicals and flavonoid compounds levels were found to be highest in oven dried samples compared to the sun, air and solar dried samples.
Conslusion: This study suggests that oven-drying young vegetative *C. nutans* plant material is the optimum method to retain postharvest quality.

Keywords: Flavonoid, Sabah snake grass, schaftoside, vitexin, drying.

Abbreviations: varicella-zoster virus (VZV), herpes simplex virus (HSV), total phenolic content (TPC) 2,2-diphenyl-1-picrylhydrazyl (DPPH), ultra fast liquid chromatography (UFLC), solid phase extraction (SPE), acetonitrile (ACN), completely randomized design (CRD) and analysis of variances (ANOVA).

Introduction

Clinacanthus nutans Lindau, from the family Acanthaceae, is commonly known in Malaysia as Sabah snake grass. It is widely grown in the tropical regions such as in Thailand, Malaysia and Indonesia. Many studies have reported antimicrobial, anti-inflammatory and anti-viral properties of *C. nutans* extracts such as against varicella-zoster virus (VZV) and herpes simplex virus (HSV) (Charuwichitratana et al., 1996; Sakdarat et al., 2009). In addition, it has been used as medicinal herbs for snake and insect bite allergic reactions (Tuntiwachwuttikul et al. 2004). Recently, researchers have

been interested in *C. nutans* because of its potential cancer fighting properties and have been conducting studies on it (Putwatana et al., 2009; Shiuan et al., 2012).

The quantity and stability of herbal phytochemicals can be affected by several factors, for example, genotype, plant parts and plant maturity/growth vegetative stage at the harvesting stage, in addition to the preharvest conditions and postharvest handling of the product (Böttcher et al., 2003). Variations in phytochemical content are also affected by endogenous changes taking place at different plant growth stages, as the photosynthetic competence of the plant may vary throughout its growth. Studies on other herbs, such as *Ilex paraguariensis* (Blum-Silva et al., 2015), *Ficus deltoidea* (Said et al., 2015), *Leucas aspera* (Chew et al. 2012), and *Portulaca olerecea* (Uddin et al., 2012), showed that their bioactive compounds are affected by their vegetative stages. Zaidi et al. (2006) found that the total polyphenol content of *F. deltoidea* leaves harvested from the top portion of the plant was significantly higher than the middle and bottom portions. This is to be expected since Hartmann, (1996) stated that most young plant tissues have higher rates of metabolite biosynthesis. However, although the influence of age has been discussed in some plant species, there is no general conclusion that is applicable to all plant species.

Lower moisture content makes the material easier to handle and less susceptible to microbial degradation. Drying is a very common preservation method used in foodstuffs and the quality of the final products is strongly dependent on the techniques and the process variables used (Doymaz, 2004). Thermal drying is usually used to reduce the moisture content in materials. Drying of herbs inhibits microbial growth and forestalls certain biochemical changes, but at the same time, it can give rise to other alterations that affect herb quality.

The growing interest in obtaining sufficient levels of natural phytochemicals from plants, especially *C. nutans,* has lead us to search for the optimal combination of the plant vegetative stage at harvest and an efficient drying method to produce a consistent source of plant materials with maximum benefits and desired qualities. This study represents the first systematic analysis of the effects of different plant vegetative stage (young and mature) and drying methods (sun, air, solar and oven) on the phytochemical properties and flavonoid compounds of the *C. nutans* plant.

Materials and Methods

Plant materials

The experiment was carried out at You Dun Chao Herbs Farm, Sendayan, Negeri Sembilan (2.6457° N, 101.8607° E) and the voucher specimen is SK 2883/15. The plants were planted under 40% of shade to produce bigger leaves compared to those planted without shade. Watering was done twice a day by using a sprinkler irrigation system in the morning and late evening. Nitrogen rate of 200 kg/ha was applied after 5 days transplanting.

Harvesting

C. nutans plants were harvested using sharp secateurs after the second ratoon crop (4.5 months). For the young vegetative stage, harvesting was done at 1.5 months of 2nd ratoon crop and 3 months after harvest for the mature vegetative stage. The samples were brought to the Postharvest Laboratory at the Department of Crop Science, Universiti Putra Malaysia, for postharvest handling processes. The samples were first chopped into 1-2 cm fragments. Then, the *C. nutans* samples were proportioned into four treatment groups corresponding to four drying methods: oven at 50 ^{o}C (Memmert ULM 500, Inc. Germany), solar (Green Energy Technology Innovation Park, UKM Malaysia), air (Postharvest Laboratory, Department of Crop Science, UPM) and sun (Drying House, Taman Pertanian Universiti, UPM). Each drying method comprised of five replicates of *C. nutans* samples weighing 500 g each. After the drying process, the samples were analyzed for phytochemical properties and flavonoid compounds.

Determination of phytochemical properties

Sample extraction

One gram of dried sample was taken and weighed using an electronic beam balance. Then, the dried samples were put into vials and extracted with 25 mL of 80% ethanol (HPLC grade). The sample was then ultrasonicated (FB 15055, Fischer Scientific, USA) for 30 minutes at 40 ºC. The mixture was then centrifuged at 10, 755 RCF (PK110 ALC, Virginia, USA) for 30 minutes. The ethanol extract was kept at -80 ^{o}C until used for the following chemical analysis (Raya et al., 2015).

Total phenolic content (TPC)

TPC of the herb extracts were determined according to Singleton et al. (1999) with some modifications. A 200 µL aliquot of the ethanol extract obtained above was mixed with 1.5 mL Follin-Ciocalteau reagent (Sigma-Aldrich, Germany). Then, the mixture was allowed to stand at 22 ºC (air-conditioned room) for 10 minutes in the dark before adding 375 µL of 20% sodium carbonate (Na_2CO_3) solution. After 2 h of incubation at 22 ºC in the dark, the absorbance was measured at the 725 nm wavelength using a spectrophotometer (Fisher Thermo Scientific, Multiskan Go, UK).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

This DPPH radical scavenging activity assay was performed according to the method described by Maizura et al. (2011). An aliquot of 1 mL of the ethanol extract was mixed with 2 mL of 0.1 mM DPPH (Sigma-Aldrich, Germany) and incubated in the dark for 1 hour. Then, the absorbance was measured by using a spectrophotometer at 517 nm.

Identification and quantification of flavonoid compound contents in *C. nutans* by ultra fast liquid chromatography (UFLC)

Standard, reagents and samples

Standard flavonoids, namely, orientin, vitexin, isovitexin and schaftoside were purchased from Fluka Sigma-Aldrich (Sigma-Aldrich and Co., USA). HPLC grade methanol was purchased from Merck, while the HPLC grade acetic acid was purchased from Nacalai Tesque (Japan). The other chemicals used for the extraction and standard preparation were of analytical grade. Water, used as the eluent, was purified by a distillation unit and was filtered using a nylon filter membrane (0.20 µm, 47 mm, Phenomenex, USA).

Solid phase extraction (SPE)

A 1 mL aliquot of ethanol extract was purified using solid phase extraction (SPE). The SPE cartridge was first conditioned with 2 mL of 100% acetonitrile (ACN) and equilibrated with 10% ACN/H_2O. After sample loading, the column was washed with 2 mL of 10% ACN/H_2O and the analytes were eluted with 2 mL of 20% ACN/H_2O. The eluate was membrane-filtered through a nylon syringe filter (0.22 µm, Thermo Scientific, USA), and 20 µl of the filtrate was subjected to UFLC analysis as described below.

UFLC separations

The composition of the flavonoid compounds was determined by using a modified UFLC method as described by Globinmed (2015). The UFLC system consisted of a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) equipped with a binary solvent manager, an auto-sampler (SIL-20A) and a Shimadzu diode array detector (SPD-M20A). The standards and extract solutions which had been filtered, were injected separately into a reversed phase Phenomenex Luna C18 (2) 100A column (250 mm × 4.6 mm, 5 µm). A gradient elution system was used and achieved using methanol (A) and 0.05% formic acid/deionized water (B) as shown in Table 1. The flow rate was 1.0 mL/min and scanning was performed between 200-400 nm.

Table 1: Gradient composition of mobile phase for flavonoid separation by UFLC

Time (min)	A%	B%
0	76.5	23.5
1	75.5	24.5
2	75	25
5	73.5	26.5
10	72.5	27.5
20	72	28
35	70	30
39	68	32
40	67	33
55	67	33

Experimental design and data analysis

The study was conducted using completely randomized design (CRD) in a factorial arrangement of treatments (two plant harvesting stages × four drying methods), with five replicates. Fifty plants were harvested for each stage. Data on phytochemical properties and flavonoid compounds of the *C. nutans* samples were subjected to analysis of variances (ANOVA) by GLM procedure of SAS (Statistical Analysis System Version 9.4 SAS Institute, Cary, NC).

Results

Total phytochemical content and activity

TPC for dried *C. nutans* were found to be between 12-16 mg GAE/g. There was a significant interaction effect between plant vegetative stage and drying methods for TPC ($P \leq 0.05$). As shown in Figure 1, the TPC for the young vegetative stage was significantly higher in both oven and solar drying methods compared to air drying method by 13.9% and 9.9%, respectively. Meanwhile, for the mature vegetative stage, oven drying gave the highest TPC compared to other drying methods, thus indicating highest retention of TPC content after oven drying compared to other drying methods. The TPC in sun dried *C. nutants* was significantly lower than oven drying by 20.3%.

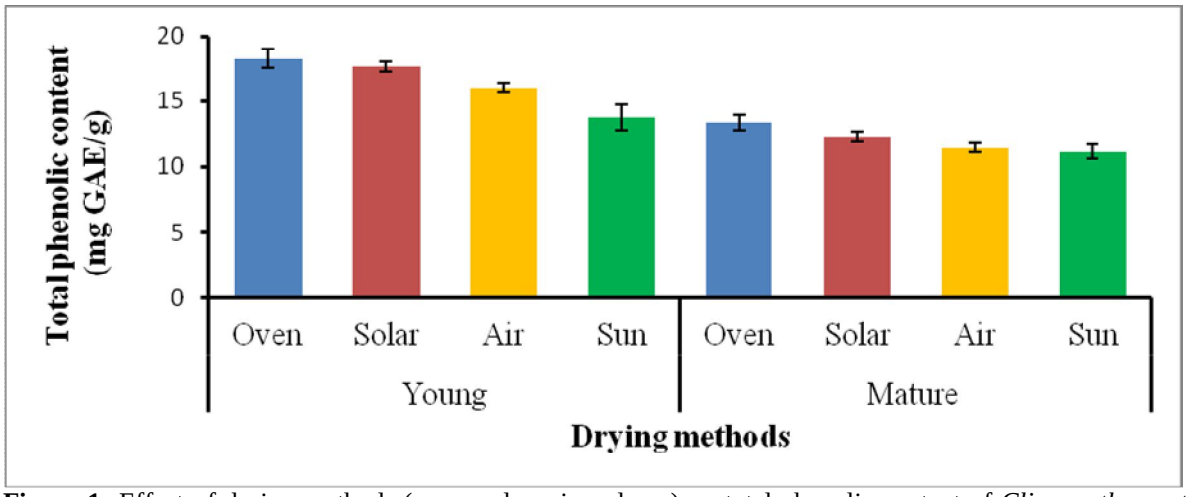

Figure 1: Effect of drying methods (oven, solar, air and sun) on total phenolic content of *Clinacanthus nutans* plants at young and mature vegetative stages.
Data was shown as mean ± SD for young and mature plant vegetative stages, significance using LSD test was considered at $P \leq 0.05$.

Phytochemical activities were measured by DPPH radical scavenging activity assay. In this study, the results revealed that there were significant interaction effects of plant vegetative stage and drying methods for DPPH ($P \leq 0.05$). The radical scavenging activities of dried sample extracts are presented in Figure 2 and expressed as percentage reduction of the initial DPPH absorption by the tested compound. For the young vegetative stage, the highest DPPH radical scavenging activity was observed in oven dried samples while the lowest DPPH activity was found in air and sun dried samples. The DPPH activity of oven dried samples was significantly higher by 17.6% as compared to sun dried samples for the mature vegetative stage.

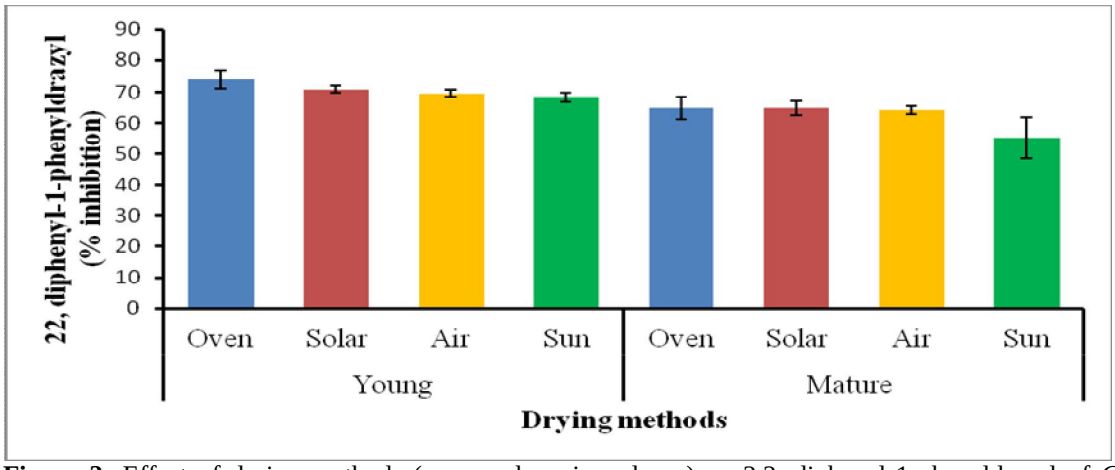

Figure 2: Effect of drying methods (oven, solar, air and sun) on 2,2, diphenyl-1-phenyldrazyl of *Clinacanthus nutans* plants at young and mature vegetative stages.
Data was shown as mean ± SD for young and mature plant vegetative stages, significance using LSD test was considered at $P \leq 0.05$.

The UFLC chromatogram data regarding the bioactive compounds available in *C. nutans* are shown in Figure 3. Vitexin, isovitexin, schaftoside, orientin and isoorientin were identified according to their retention time and the spectral characteristics of their peaks as compared to the respective standards. These bioactive compounds are grouped under the flavonoids and are present in plants in the form of glycosides. Four of the flavonoid compounds detected in the *C. nutans* extracts include orientin, schaftoside, vitexin and isovitexin, with retention times of 28.5, 32.0, 43.5 and 48.3 min, respectively (Figure 3). Among the bioactive compounds detected, schaftoside was the major flavonoid compound, while vitexin was present in the lowest amount. A research conducted by Chelyn et al. (2014) also validated the detection of schaftoside, orientin, isovitexin and vitexin in the current study.

There were no significant interaction effects between plant vegetative stages and drying methods on vitexin and orientin contents (Table 2). However, quantitative UFLC analysis of *C. nutans* showed a higher content of vitexin and orientin in the young vegetative stage compared to mature vegetative stage by 2.0 and 2.2 fold, respectively. In the case of drying method, the highest vitexin compound was observed in oven dried *C. nutans* extracts. The extracts contained 38.8% higher quantities of vitexin compared to the sun dried extracts. In contrast, the lowest vitexin compound was observed in sun and air dried extracts. The schaftoside and isovitexin contents in *C. nutans* samples that were dried at different vegetative stages and drying methods are shown in Figure 3.

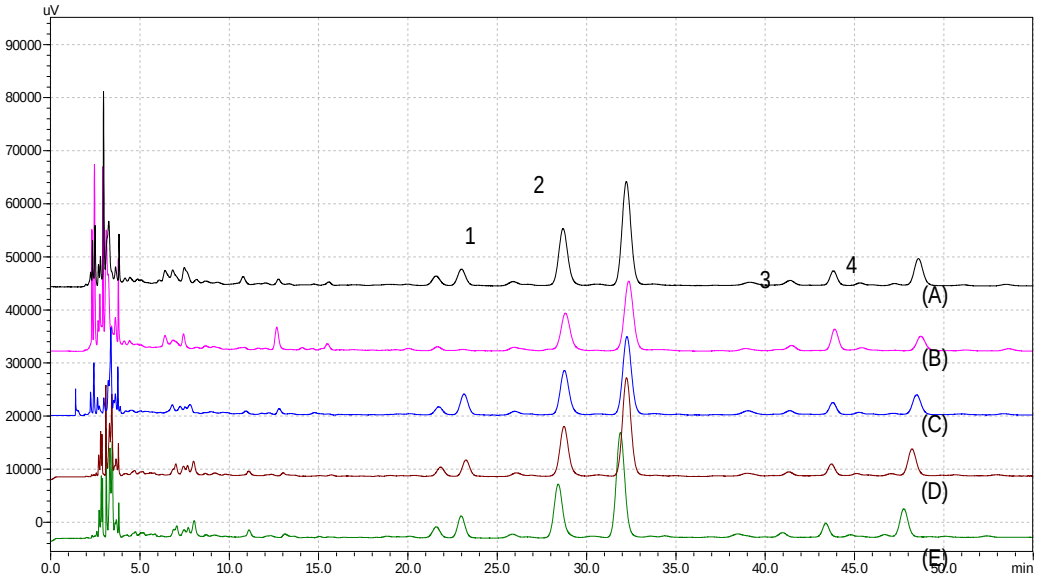

Figure 3: Ultra fast liquid chromatography profile of young *Clinacanthus* dried using oven (A), solar (B), air (C) sun (D) and mature vegetative stages (E), orientin (1), schaftoside (2), vitexin (3) and isovitexin (4) peaks.

Table 2: Main and interaction effects of plant vegetative stages and drying methods on vitexin orientin, schaftoside and isovitexin content of *Clinacanthus nutans*.

Factor	Vitexin	Orientin	Schaftoside	Isovitexin
	(µg/g DW)			
Plant vegetative stage (PVS)				
Young	139.42 ± 5.77 a	393.57 ±10.1 a	4726.72 ± 200 a	259.82 ± 11.5 a
Mature	68.43 ± 4.28 b	172.06 ±10.2 b	1620.87 ± 55 b	95.11 ± 10.1 b
Drying method (DM)				
Oven	125.92 ± 13.0 a	341.03 ± 40.1 a	3311.48 ± 653 a	216.53 ± 22.2 a
Solar	108.78 ± 18.3 ab	276.94 ± 46.4 b	3569.09 ± 745 a	193.43 ± 42.2 ab
Sun	90.71 ± 11.2 b	248.16 ± 38.2 c	3232.42 ± 412 a	131.94 ± 24.5 c
Air	90.34 ± 13.7 b	265.01 ± 44.2 bc	2582.11 ± 579 b	168.29 ± 38.1 b
PVS x DM	ns	ns	**	**

Means within a factor and column followed by the same letter are not significantly different at $P \leq 0.05$ by using LSD test. Mean ± standard deviation. [ns,] **= not significant at $P \geq 0.05$ and highly significant at $P \leq 0.01$, n = 5.

There were significant interaction effects between plant vegetative stages and drying methods on schaftoside and isovitexin contents. Quantitative data for the young vegetative stage showed that oven drying gave 38.3% higher amount of schaftoside than sun dried extracts. However, at the mature vegetative stage, the amounts of schaftoside were not significantly different between the drying methods applied (Figure 4A). Isovitexin of sun dried samples at the young vegetative stage decreased by 31.8%, relative to oven dried samples (Figure 4B). Furthermore, at the mature vegetative stage, oven-dried *C. nutans* showed highest isovitexin compared to solar, air and sun dried samples.

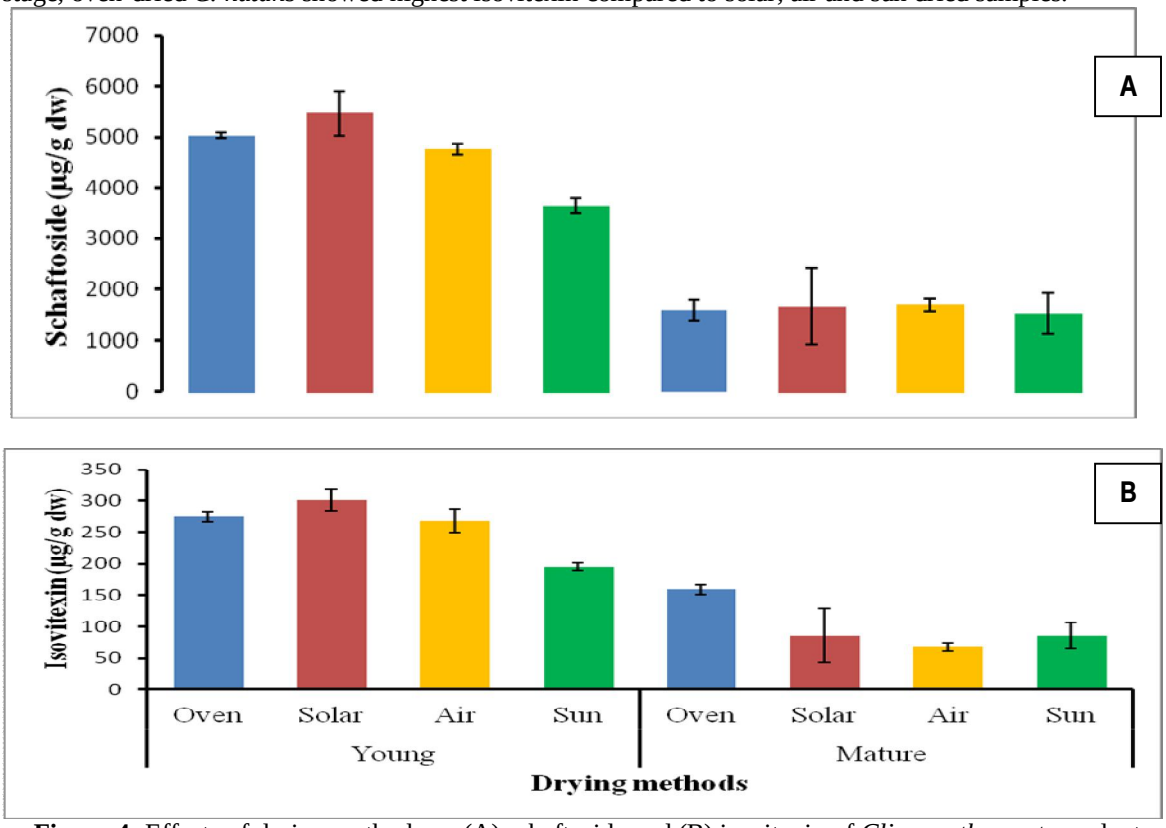

Figure 4: Effects of drying methods on (A) schaftoside and (B) isovitexin of *Clinacanthus nutans* plants at young and mature vegetative stages.

Data was shown as mean±SD for young and mature plant vegetative stages, significance using LSD test is considered at $P \leq 0.05$.

Discussion

Total phenolic content

It is well known that plant maturity significantly influences the quality of herbs, especially in terms of their chemical composition. The current study showed that the young vegetative stage of *C. nutans* contained higher TPC compared to the mature vegetative stage (Figure 1). Research by Blum-Sillva et al., (2015) similarly found a decrease in the production of TPC compounds over time in *Ilex paraguariensis*. They found that the TPC is highest in one month leaves, intermediate at two months and lowest at sixth months. Similar findings were also observed on *Moringa oleifera* where tender leaves had higher TPC and TFC than in mature leaves by 7.6% and 35.4%, respectively (Jahan et al., 2015). However, Dartora et al. (2011) did not find any significant differences in TPC levels between 1 and 6 months old *I. paraguariensis*, while only a slight increase in TPC content was observed with the age for sweet pepper (Serrano-Martínez et al., 2014). It is clear that the concentration of antioxidant compounds in relation to the maturity process is not uniform in all plants, though the present study suggests that *C. nutans* harvested at a young age could potentially provide the greatest concentrations of antioxidants.

It has been demonstrated that in plants, antioxidants are part of a complex defense mechanism against a wide range of stresses and thus, accumulate in response to these stresses (Viacava et al., 2014). In fact, secondary metabolites, particularly low molecular weight phenolic compounds, are at the highest levels in the early stage of growth or only synthesized at young plant vegetative stages when leaves are most in need of defense from herbivores (Parr and Bolwell, 2000). Many plants secrete stored TPC resins over newly expanding foliage and the inactive meristem cells may store previously formed secondary metabolites in small vesicles that later fuse with the vacuole as they develop during cell growth (Herms and Mattson, 1992). According to Raya et al. (2015), *C. nutans* leaves contain more TPC than the stems. In this experiment, the results showed that the young vegetative stage contained more TPC and TFC than the mature vegetative stage since the latter stage has more stem and branches than leaves. Khoo et al. (2015) also stated that *C. nutans* leaf extract contains higher TPC than stem extracts by 67%.

Our study showed that different drying methods applied on *C. nutans* had significant effects on the composition of phytochemical contents in the samples. Evidently, the loss of TPC during drying was due to the drying conditions, in particular the temperatures and duration used (Michalczyk et al., 2009). According to Davey et al. (2000), the drying process can affect the phytochemical contents by thermal breakdown. This can affect the cell structure integrity and results in the migration of components, leading to losses by leakage or breakdown by various chemical reactions involving enzymes, light and oxygen. However, the loss of TPC, as expected, was found to be lower with oven drying at 50 ^{o}C followed by solar, air and sun drying. The best drying method that could retain the TPC compound in *C. nutans* was the oven drying method. Youssef and Mokhtar (2014) found a similar result for *Postulaca oleracea* leaves in which oven drying at 50^{o}C produced the highest TPC and TFC concentrations. This could be due to the fact that oven drying rapidly removes water and inactivates oxidative enzymes, thus allowing better retention of phytochemicals in the extracts (Ji et al., 2012). Furthermore, drying stabilizes the plant materials and conserves not only the plant but also enhance the antioxidant properties and the contents of bioactive components (Doan et al., 2004).

Degradative enzymes, such as polyphenol oxidase, are not neutralised instantly by sun drying. Instead, phenolic compounds are degraded before the plant materials dry out. During drying, the binding of polyphenols with other compounds or changes in the chemical structures of polyphenols which are incalculable using existing methods are among the reasons for the reduction in total phenolic contents. Since sun drying did not immediately deactivate degradative enzymes such as polyphenol oxidase, therefore they degrade phenolic compounds before the plant materials reached constant weight. During drying, the decrease in total phenolic contents could be attributed to the binding of polyphenols with other compounds or to alterations in the chemical structures of polyphenols which cannot be extracted or determined by available methods (Mrad et al., 2012). Furthermore, sun dried samples are exposed to adverse UV radiation, which has been reported to degrade TPC (Kade et al., 2008).

Antioxidant activity

Similar findings were reported by Rafat et al. (2010) who showed that young leaves of *Andrographis paniculata* had higher antioxidant activity compared to those of matured leaves. However, Wongsen et al., (2013) found that the top leaves of lemon basil had the lowest antioxidant radical scavenging activity than the middle and bottom leaves. Interestingly, their findings were completely different from that of sweet basil, in which the top leaves had the highest antioxidant activity compared to the middle and bottom leaves. Ghasemzadeh et al. (2014) claimed that 1-year-old buds of *C. nutans* had higher antioxidant activity than in 6-month-old buds. Therefore, it was suggested that the level of antioxidant activity was related to the factors of leaf position and harvesting stages. The drying methods were shown to have variable effects on the phytochemical activities of *C. nutans*. Antioxidant activity was lowest after using the air drying method. The

antioxidant activity might be associated with the accumulation and generation of different phytochemical compounds having varying antioxidant activity, resulting in the synergistic or antagonistic effects with other antioxidants or with the extract compounds (Zielinski et al., 2000).

Flavonoid compounds

The amount of vitexin, isovitexin, schaftoside and orientin, tested in *C. nutans* varied in relation to plant vegetative stages. Higher contents of bioactive compounds were detected at the young stage compared to the mature stage. Findings by Siddiqui et al. (2013) showed that the rate of synthesis of bioactive molecules increased progressively with advanced maturity in *Capsicum chinense.* Blum-Silva et al. (2015) also reported that for Yerba-mate (*I. paraguariensis*), leaf age has a significant influence on caffeine, theobromine and total methylxanthine contents of the extracts.

A similar result reported by Do Thi and Hwang (2014) on *Aronia melanocarpa* showed that young leaves contained more *p*-coumaric acid, chlorogenic acid and rutin than older leaves. These trends are similar to those displayed in the present study. Our findings suggest that younger *C. nutans* could be more favorable to be processed into higher functioning antioxidative ingredients because they have larger amounts of flavonoids. During maturation, changes in ascorbic acid, α-tocopherol and total carotenoids gradually increase (Lepeduš et al., 2011). The export and accumulation of phytochemicals also change during the vegetative stage. Several authors have discussed the effects of plant age and vegetative stage on secondary metabolites content, and the relative proportions of these chemical components in *Tanacetum parthenium* (Hendricks et al., 1997) and *Tarbernaemontana pachysiphon* (Höft et al., 1998). Hartmann (1996) reported that young tissues generally have higher rates of metabolites biosynthesis. According to Jiménez and García-Carmona, (1998), during maturation, the loss of flavonoids may reflect the metabolic conversion to secondary phenolic compounds or degradation via enzymatic actions.

As shown in the results of the drying methods, oven drying gave the highest values for all bioactive compounds, while the lowest values were detected in the sun dried samples. This might be due to the oven temperature used, which was at 50 ^{o}C. As mentioned by Guan et al. (2005) dehydration of *Hippophae rhamnoides* leaves at temperatures ranging from 50 to 100 ^{o}C resulted in decreased concentrations of carotenoid. Heating may degrade L-ascorbic acid which affects cell wall integrity and causes leakage of some flavonoid compounds (Davey et al., 2000). In addition, the loss of flavonoids could be due to the breakdown or migration by chemical reactions including oxygen, enzymes and light (Madrau et al., 2009). According to Sukrasno et al. (2011), increasing the temperature causes decreased activity of polyphenoloxidase enzyme to degrade flavonoids, resulting in increased flavonoid content.

Extended drying times could be the reason for the loss of orientin, schaftoside and isovitexin compounds in *C. nutans.* According to Sun et al. (2011), drying processes causes loss of naturally occurring synephrine present in *Diospyros kaki* fresh sample and longer drying times causes a greater decline in product quality. Farmers commonly use sun drying to dry their herbs, vegetables and fruits. However, long drying time is a disadvantage as the produce degrades in quality (Laguerre et al., 1999). During drying, metabolically active plants lose moisture slowly and could sense moisture loss as stress (Hossain et al., 2010).

Conclusion

In conclusion, plant vegetative stages and drying methods affected the physicochemical properties and flavonoid compounds of *C. nutans.* Harvesting *C. nutants* at the young plant vegetative stage produced the optimum phytochemical properties and flavonoid compounds. All of the tested drying methods (sun, air, solar and oven) had adverse effects on TPC, antioxidant activity and also flavonoid compounds of *C. nutans.* However, oven drying had the lowest negative effects. The flavonoid compounds were highest in the young plant vegetative stage and oven dried samples. Thus, due to its high phytochemicals content and antioxidant activity, *C. nutans* is considered to be a potential dried herb. Overall, harvesting at the young plant vegetative stage and oven drying at 50 ^{o}C can be applied as the recommended method to retain postharvest quality.

Conflict of Interest statement
We declare that we have no conflict of interest.

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