TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITYBY DIFFERENT DRYINGAND EXTRACTION METHODS OF *Clinacanthus nutans* LEAVES

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ABSTRACT. *This report presents a study on the total flavonoid content and antioxidant activity of Clinacanthus nutans leaves from different drying and extraction methods. The C. nutans leaves were subjected through three different drying methods: oven (40°C, 60°C and 80°C), vacuum oven and air drying. Afterwards, extraction on the dried leaves was performed using three different extraction techniques: soxhlet, maceration and ultrasound-assisted solvent extraction. Finally, total flavonoid and antioxidantactivity was determined spectrophotometrically by aluminium chloride colorimetric assay and 2-Diphenyl-1-picryl hydrazyl (DPPH) method respectively. The results showed that the choice of drying and extraction methods had a significant influence on total flavonoid and antioxidant content of C. nutans extract. The combination of drying C. nutans leaves using laboratory oven at 60°C and soxhlet extraction obtained the highest amount of total flavonoid and antioxidant content at 24.53 ± 0.95 mg RU/g and 89.73 ± 4.39mg TE/ g respectively. The highest antioxidant activity for C. nutansfromair-dried samples and vacuum oven-dried samples were obtained through maceration extraction at 85.46 mg TE/ g and 83.96 mg TE/ g respectively.*

KEYWORDS. *Clinacanthus nutans*; Drying; Extraction method; Flavonoid; Antioxidant

INTRODUCTION

Plants are natural sources of antioxidant and they synthesize antioxidant compounds as part of their defense mechanism to counteract destructive effects of free radicals for survival (Kasote *et al*., 2015). Antioxidant compounds from natural sources have received much attention from researchers due to their potential applications in different life science fields. As such, research on local herbs with antioxidant properties is important to gather scientific data on potential natural product high in natural source of antioxidant. This study will focus on an increasingly popular medicinal herb known locally as Sabah Snake Grass or its scientific name *Clinacanthus nutans* (Burm. f.) Lindau. The herb is used in the preparation of traditional remedies to treat snake bites, skin rashes, fever, inflammation, heal burns and

kidney problems (Zulkipli *et al*., 2017). Due to the increasing public awareness of its potential medicinal value, many studies were conducted to investigate the phytochemical and antioxidant properties of *C. nutans*. Therefore, it is important to maximize the extraction yield of the bioactive compounds associated with the plant. Factors such as herb dehydration, extraction solvent and techniques were reported to have significant influence on the bioactive compounds yield from crude extract of plants (Khoo et al., 2015, Kumar, 2013).Thus, the aim of this study was to determine the effect of combining different drying methods and extraction techniques on the total flavonoid content (TFC) and antioxidant activity of *C. nutans* leaves.

LITERATURE REVIEW

C. nutans belongs to the family of Acanthaceae and is a perennial herb widely distributed in Malaysia, Thailand and Indonesia (Arullappan *et al*., 2014).It is a shrub with pubescent branches that can grow 1 - 3 meters in height. The plant grows well at the elevation ranging from 500 to 620 meters, particularly at low altitude forest areas (Chen *et al*., 2015). The leaves are pale green, with simple, narrowly elliptic, oblong or lanceolate-ovate shapes, that ranges between 2.5 - 13 cms long and 0.5 - 1.5 cms wide. The stems of the plant are terete, striate and glabrescent, while the petioles are sulcate, bifariously pubescent and between 0.3 - 2 cms long. *C. nutans* formed flowers in compact cymes that are dull red in color with green base and yellow stripes on the lower lip. The flower's upper lip is located in the throat and is triangular in shape with two stamens. The plant's ovary is compacted into two cells in which each cell haves two ovules. Its capsule is oblong in shape, and basally wrapped into a 4-seeded short, solid stalk. *C. nutans* produce seeds that are around 2mm in diameter. (Alam *et al*., 2016; Hu and Daniel, 2011; Kunsorn *et al*., 2013, Panyakom, 2006; Zulkipli *et al*., 2017). *C. nutans* has been used in the preparation of traditional remedies in Malaysia and other Asian countries for treating nettle rash, blisters, oral inflammatory symptoms, burns, dysentery, insect stings, gout, fever, urinates neuropathies, kidney problems, nasal cavity cancer, liver cancer and uterine fibroid (Arullappan *et al*., 2014; Tiew *et al*., 2014; Zulkipli *et al*., 2017). The herb is also one of the medicinal plants used traditionally to treat poisonous snake bites, herpes simplex virus (HSV) and varicella-zoster virus (VZV) in Thailand (Yahaya *et al*., 2015; Watson and Preedy, 2008).

Due to its many medicinal properties, researches were conducted to investigate the phytochemical and antioxidant properties of *C. nutans*. A study by Pannangpetch *et al*. (2007) concluded that ethanolic extract of *C. nutans* exhibited free radicals scavenging activity, ferric reducing antioxidant power (FRAP) and exerted intracellular inhibition effect on phorbol myristate acetate (PMA)-induced free radical production. Ghasemzadeh *et al*. (2014) demonstrated that *C. nutans* buds had higher antioxidant activity as compared to leaves extracts. However, leaves extract was reported to contain higher DPPH scavenging activity when compared to stem extract (Khoo *et al*., 2015). The value of DPPH scavenging activity of *C. nutans* that had been reported in several studies were 67.65% (Pannangpetch *et al*., 2007), 82.0% (Arullappan *et al*., 2014), 66.2% (Ghasemzadeh *et al*., 2014), 44.31%

(Khoo *et al*., 2015) and 55.12% (Sarega *et al*., 2016). A study conducted by Mai *et al*. (2016) concluded that leaves extracts of *C. nutans* shows anti-inflammatory effect by inhibiting all LPS induced TLR – 4 inflammatory proteins in a dose-dependent manner. Also, *C. nutans* extract displayed antibacterial properties and exhibited inhibitory effect towards *Propionibacterium acnes, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Bacillus cereus, Salmonella enterica Typhimurium* and *Candida albicans* (Yang *et al*., 2013; Arullappan *et al*., 2014). Besides that, *C. nutans* extract also shows inhibitory effect towards herpes simplex virus (HSV) and vavicella- zostervirus (VSZ) (Kunsorn *et al*., 2013; Thawaranantha *et al*., 1992).

The bioactive compounds of *C. nutans* had been investigated in previous studies, and the chemical constituents were reported and characterized. Extraction by using different solvent had identified chemical compounds such as glycosides, glycoglycerolipids, cerebrosides, belutin, shaftoside, lupeol, b–sitosterol, stigmasterol, orientin, isoorientin, isovitexin, vitexin, steroids, triterpenoids monoacylmonogalactosylglycerol, clinamides A, B and C, 2-cis-entadamide A, 6,8-apigenin-C-α-l-pyranarabinoside, isomollupentin 7-O-β-glucopyranoside, pupurin-18-phytyl ester, phaeophorbide and chlorophyll derivatives (Aslam *et al*., 2015; Zulkipli *et al*., 2017). A study by Sakdarat *et al*. (2006) had successfully isolated different varieties of chlorophyll A and chlorophyll B compounds from chloroform extract of *C. nutans* leaves. The chlorophyll A related compounds were 13^2-hydroxy-(13^2-S)-phaeophytin A, 13^2-hydroxy-(13^2-R)-phaeophytin A, purpurin-18-phytyl ester and phaeophorbide A, whereas the chlorophyll B related compounds isolated in the study were 13^2-hydroxy-(13^2-S)-chlorophyll B, 13^2-hydroxy-(13^2-R)-chlorophyll B, 13^2-hydroxy-(13^2-S)-phaeophytin B and 13^2-hydroxy-(13^2-R)-phaeophytin B. In 2009, the same group isolated three more structures related to chlorophyll a and chlorophyll b, 13^2-hydroxy-(13^2-R)-phaeophytin b, 13^2-hydroxy-(13^2-S)-phaeophytin a and 13^2-hydroxy-(13^2-R)-phaeophytin a (Sakdarat *et al*., 2009).

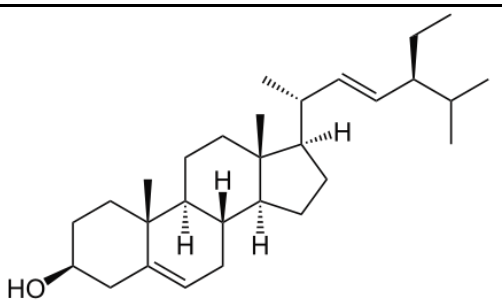
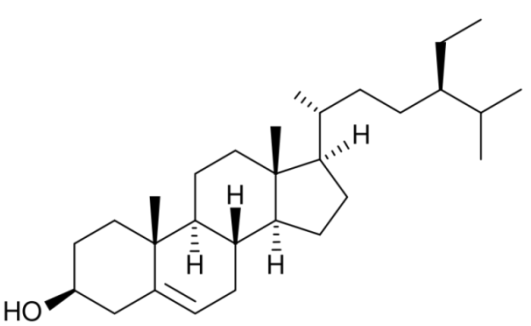
Fresh raw material generally needs to undergo proper drying before storage to prolong their shell life. Although drying inhibits the microbial growth, it can also cause colour and aroma changes to the harvested plant due to degradation of bioactive compounds or the formation of new volatiles as a result of oxidation reactions or esterification reactions (Hossain *et al*., 2010). Different drying methods have been proven to significantly effect in the yield of bioactive compounds and antioxidant activity of medicinal plants (Pham *et al*., 2015). Research on proper drying method is important to achieve the highest quality product in the shortest amount of time, avoiding a waste of time and energy.

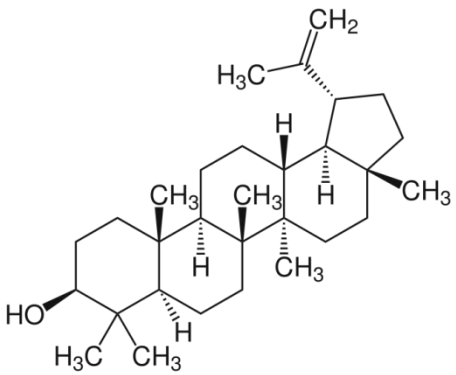
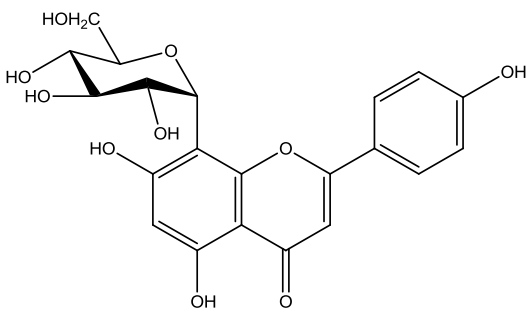
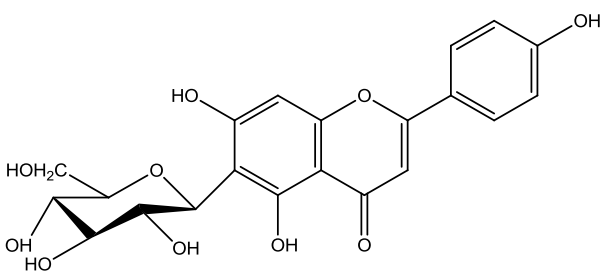
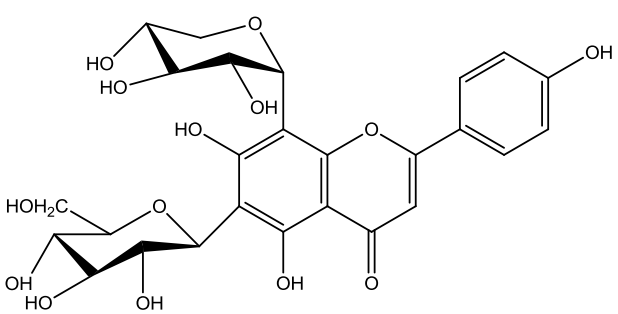
To date, literature reviews of *C. nutans* showed that the plant had been extracted through both conventional and modern extraction techniques. The conventional extraction methods, such as Soxhlet extraction, hydrodistillation and maceration combined solvent of choice with the use of heat or agitation to extract nutraceuticals from plant matrices (Wang and Weller, 2006). Modern extraction technique such as ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and microwave assisted extraction (MAE) showed promising potential in the extraction of

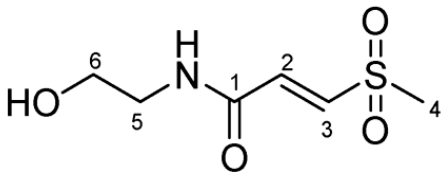
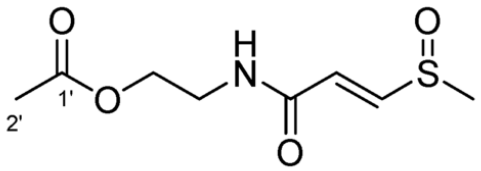
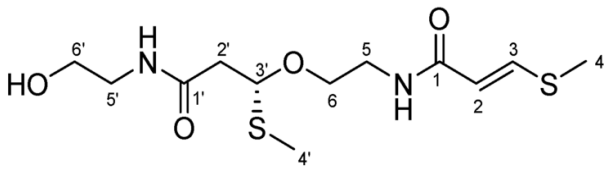
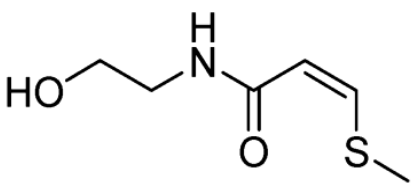
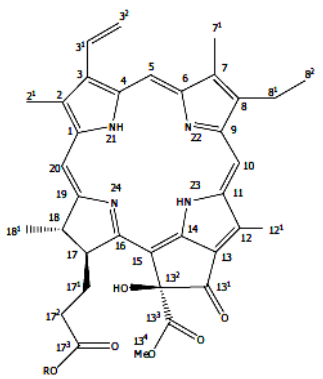
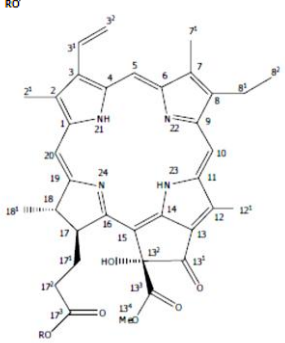
nutraceuticals from plants at laboratory or bench-scale (Wang and Weller, 2006). Conventional extraction method using soxhlet apparatus and maceration were generally used by researchers for *C. nutans* extraction in several scientific studies (Sriwanthana *et al*., 1996; Direkbusarakom *et al*., 1998; Sakdarat *et al*., 2006; Kunsorn *et al*., 2013; Peng, *et al*., 2014; Aslam *et al*., 2016).Conventional methods are usually time consuming, tedious and require the use of large amount of organic solvent, but they are still widely used due to its simplicity (Okoduwa *et al*., 2016). Modern extraction methods have demonstrated in various studies that smaller volume of organic solvent can be used, while allowing an increase bioactive compound yield at lower cost (Azwanida, 2015). These different extraction techniques offered different advantages with respect to extraction time, solvent consumption, extraction yields and reproducibility.

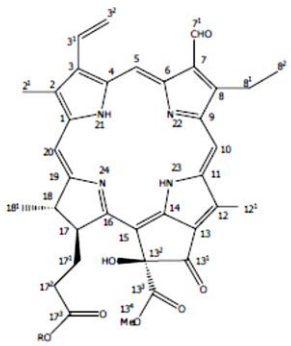
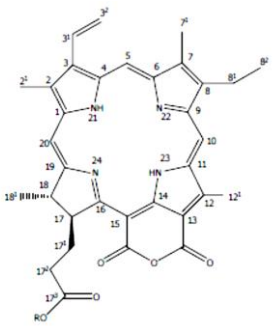
There is no single standardized method for optimal extraction since different extraction method would result in different amount of bioactive compound from different plant material (Galanakis, 2018). A previous study by Khoo *et al*. (2015) indicated that the combination factors of different drying (Freeze dry, oven dry and air dry) and extraction methods (Sonification and soaking) produce a significant effect on phytocompound yield of *C. nutans* leaf and stem extracts. Khoo *et al*. (2015) reported that air dried *C. nutans* leaf extracted through UAE produced extract with the highest phenolic content yield at 7.29 mg GAE/g.

Table 1: Structure of bioactive compounds isolated from *C. nutans*

Name of the compounds	Structure	Reference
Stigmasterol		(Alam et al., 2016)
Beta-Sitosterol		(Alam et al., 2016)

Lupeol		(Alam *et al.*, 2016)
Vitexin		Huang *et al.* (2015)
Isovitexin		Huang *et al.* (2015)
Shaftoside		Huang *et al.* (2015)

Clinamide A		(Tu et al., 2014)
Clinamide B		(Tu et al., 2014)
Clinamide C		(Tu et al., 2014)
2-*cis*-entadamide A		(Tu et al., 2014)
13^2-hydroxy-(13^2-S)-phaeophytin A		(Sakdarat et al., 2009)
13^2-hydroxy-(13^2-R)-phaeophytin A		(Sakdarat et al., 2009)

13^2-hydroxy-(13^2-S)-phaeophytin B		(Sakdarat et al., 2009)
Purpurin-18-phytyl ester		(Sakdarat et al., 2009)

METHODOLOGY/MATERIALS

Reagents and Chemicals

Methanol which was used for extraction was of analytical grade, and it was purchased from Thermo Fisher Scientific. The chemicalrutin was supplied by Sigma-Aldrich, Germany, while sodium nitrate ($NaNO_3$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), 2-Diphenyl-1-picryl hydrazyl (DPPH) and trolox were purchased from Merck, Germany.

Sampling

C. nutans samples were collected from the herbal nursery at the project Plot-Demo UNDP/GEF-IDS which was located at Mile 30 Kimanis, Papar, Sabah, Malaysia. The plants were rinsed with tap water to remove dirt, and then dried with tissue paper. The fresh leaves and stem were then separated for further study.

Preparation of Dried *C. nutans* Samples

The fresh leaves of *C. nutans* were dried to constant weight using three different drying methods.

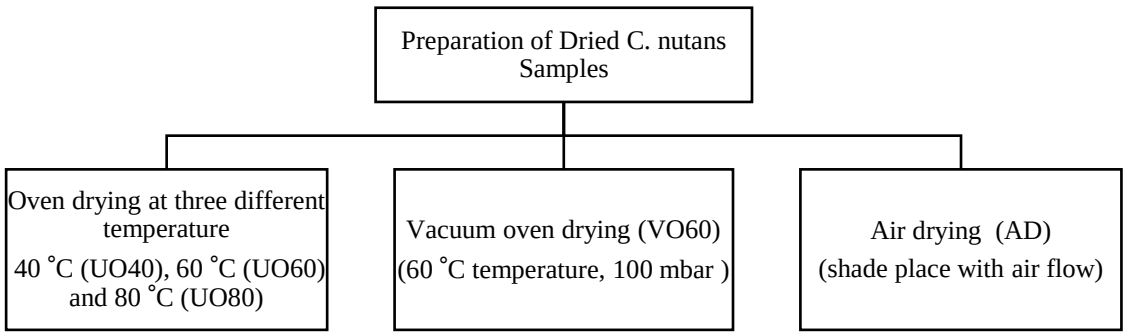


Figure 1: Diagram of the drying methodology

The dried *C. nutans* leaves samples are grinded into fine powder with an electric blender (Model 7010S Waring Laboratory Blender), and then stored in a cold room until required.

Extraction of Bioactive Compounds

The dried samples were extracted using three different extraction techniques which include Soxhlet, maceration and ultrasound assisted solvent extraction.

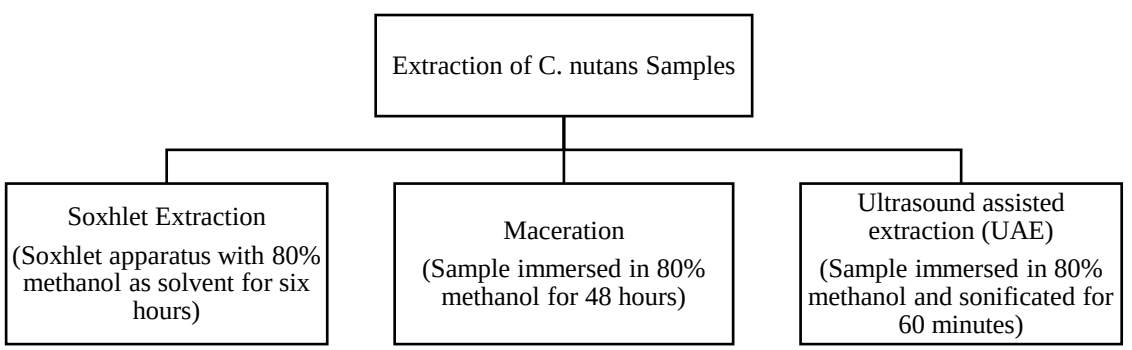


Figure 2: Diagram of the extraction methodology

Soxhlet Extraction: Powdered plant material (10.0 g) was extracted with 100 ml aqueous methanol (methanol: water, 80:20 v/v) in a Soxhlet apparatus. The temperature of the process corresponded to boiling point of solvent used and the extraction time was carried out for six hours. The extract was filtered with Whatman filter paper No. 1, concentrated and evaporated using rotary evaporator (Model RV 10 control V IKA rotary evaporator) and stored in a refrigerator until further analysis.

*Maceration:*The extraction was carried out using powdered plant material (10.0 g) in a conical flask immersed with 50 ml aqueous methanol (methanol: water, 80:20 v/v) and placed on a mechanical shaker at room temperature with constant stirring rate at 120 rpm. The flask was covered with aluminum foil to prevent any evaporation and left for 48 hours. The extract obtained was filtered with Whatman filter paper No. 1, concentrated, evaporated using rotary evaporator (Model RV 10 control V IKA rotary evaporator) and stored in a refrigerator until further analysis.

Ultrasound assisted extraction (UAE): Extractions were performed in an Elmasonic S900H ultrasonic bath with ultrasonic frequency of 37 kHz. 10.0 g of fine *C. nutans* powder was homogenized in a blender with aqueous methanol (methanol: water, 80:20 v/v) at 1:5, sample to solvent ratio as extracting solvents. The homogenized extract was kept in a beaker which was then covered with aluminum foil and sonicated for 60 minutes at room temperature (25 ± 1 °C) as described by Sarega *et al*. (2016). After that, the crude extracts were filtered, concentrated and evaporated using rotary evaporator (Model RV 10 control V IKA rotary evaporator) and stored in a refrigerator until further analysis.

Determination of Total Flavonoid Content (TFC) in Plant Extract

TFC was determined according to the procedure described by Raya *et al*. (2015). Initially, 1 ml of extract was mixed with 0.3 ml $NaNO_3$ in a test tube covered with an aluminium foil, and then left for 5 minutes. Next, 0.3 ml of 10% $AlCl_3$ was added to the mix, followed by addition of 2 ml of 1 M NaOH. The absorbance was measured at 510 nm using a UV-VIS spectrophotometer (Genesys 10S UV-Vis spectrophotometer). The standard curve was prepared with rutin as standard and the results were expressed as rutin equivalent (mg RU/g extract). Three replicates of each sample extract were analyzed.

Evaluation of Antioxidant Activity

The DPPH assay was performed with slight modification according to the procedure described by Marques *et al*. (2017). Briefly, 0.1 ml of extract was added to 3 ml of diluted DPPH solution and incubated in the dark for 30 minutes so that the antioxidants present in the exract would react with the radical. The sample's absorbance was measured at 517nm using a UV-VIS spectrophotometer (Genesys 10S UV-Vis spectrophotometer). The standard curve was prepared with TE and the results were expressed as mg TE/g extract. Three replicates of each sample extract were analyzed.

RESULTS AND DISCUSSION

Total Flavonoid Content of *C. nutans* Leaves

The influence of different drying methods and extraction techniques on TFC of *C. nutans* were shown in Figure 3. Results obtained from this study indicated that combination of different drying methods and extraction techniques had significant effect ($p < 0.05$) on the TFC of *C. nutans* leaves, ranging from 18.29 to 24.53 mg RU/g. The TFC range obtained from this study is in accordance to that of Fong (2015) where TFC from different locations in Malaysia ranged from 10.35 to 34.13 mg QE/g. High temperature had been known to degrade bioactive compounds, and a decrease in flavonoid content at 80 °C showed that the application of high temperature for drying significantly damage heat sensitive bioactive compounds in *C. nutans*. It was observed that oven dried leaves at 80˚C (UO80) obtained the lowest yield for all extraction techniques, ranging from 18.29 to 19.83 mg RU/g. The combination of drying *C. nutans* leaves using universal oven at 60°C and Soxhlet extraction obtained the highest TFC yield at 24.53 mg RU/g. This was followed by vacuum oven-dried samples at 60°C and extracted through UAEat 23.41mg RU/ g. Whereas, the highest flavonoid content from air dried samples was obtained by UAE extraction at 22.92 mg RU/g.

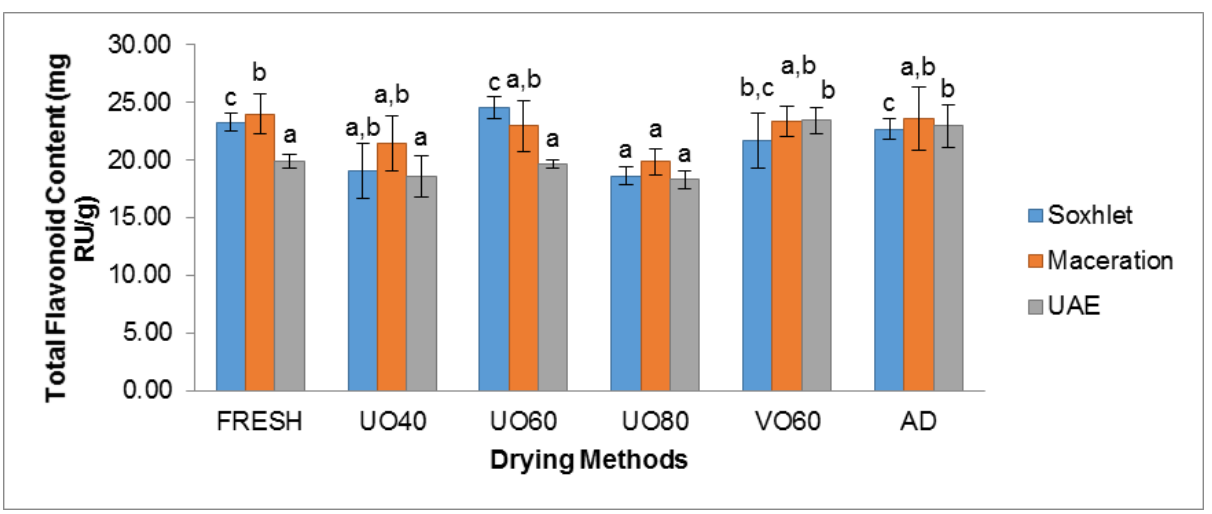


Figure 3. Effect of different drying methods and extraction techniques on TFC of *C. nutans* leaves. Data were reported as means ± standard deviations of triplicate experiments. Bars with different superscript letters ($^{a-c}$) were significantly different ($p < 0.05$) according to Duncan's multiple range tests.
UO40/ UO60/ UO80: Universal oven drying at 40˚C/ 60˚C/80˚C; VO60: Vacuum oven-drying at 60˚C; AD: Air drying in a shaded place with appropriate air flow.

Antioxidant Activity of *C. nutans* Leaves

The antioxidant activity of *C. nutans* leaves was determined using spectrometric method by DPPH assay, and the results were expressed in mg TE per g of dry weight (mg TE/ g). When antioxidant molecules reacted with DPPH free radicals, DPPH accepted a hydrogen atom from the antioxidant which resulted in the reduced form of DPHH, followed by the loss of violet colour (Pisoschi *et al*., 2009). The DPPH radical scavenging activities of *C. nutans* leaves from different drying methods and extraction techniques were shown in Figure 4. The DPPH radical scavenging assay revealed that the combined factor of different drying methods and extraction techniques produced a significant impact on the antioxidant properties of *C. nutans* leaves ($p < 0.05$). The result also indicated that drying method using high temperature resulted in lower antioxidant capacity as seen in samples dried with 80 °C temperature. This similar outcome was seen in a study by Pham *et al.* (2015) that reported a decreased in antioxidant capacity when the oven temperature was increased from 80 °C to 90 °C. This could be due to the degradation of heat sensitive antioxidant compounds when samples were exposed to high temperatures. For this study, the highest DPPH value at 89.73 mg TE/ g was obtained from the extract with the highest TFC yield, which was the combination of oven dried*C. nutans* leaves at 60°C and soxhlet extraction.This was followed by air-dried samplesand vacuum oven-dried samplesat 60°C extracted through maceration at 85.46 mg TE/ g and 83.96 mg TE/ g respectively.The results indicated that flavonoids had contributed to the antioxidant activity of extract since extract with high flavonoid content had higher antioxidant capacity.

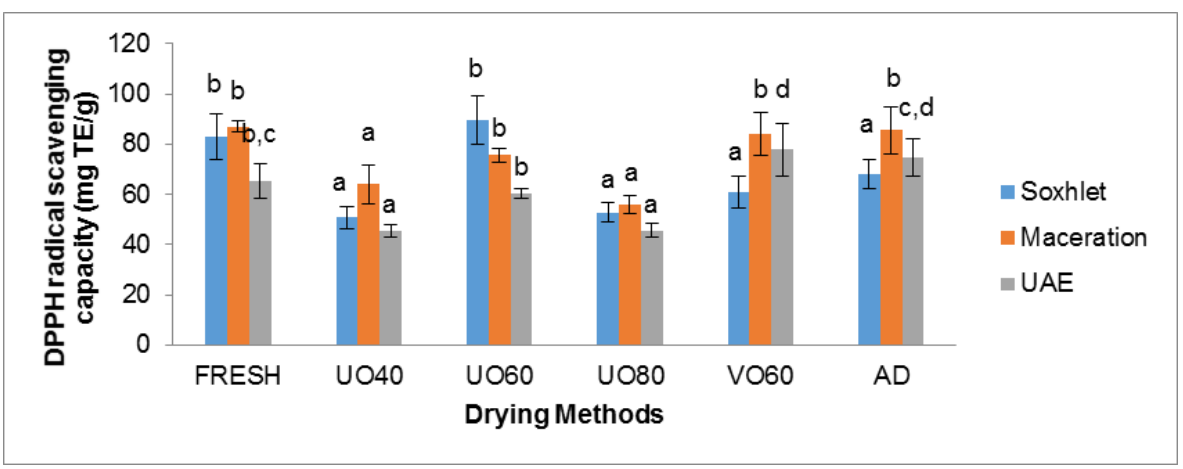


Figure 4. Effect of different drying methods and extraction techniques on antioxidant capacity of *C. nutans* leaves.
The bars were means ± standard deviations of triplicate experiments and those with different superscript letters ($^{a-d}$) were significantly different ($p < 0.05$) according to Duncan's multiple range tests.
UO40/ UO60/ UO80: Universal oven drying at 40˚C/ 60˚C/80˚C; VO60: Vacuum drying at 60˚C; AD: Air drying in a shaded place with appropriate air flow.

CONCLUSION

The study showed that TFC and antioxidant properties of *C. nutans* leaves were significantly affected by different drying methods and extraction techniques ($p < 0.05$). The results demonstrated that bioactive compound can still be retained in samples when suitable drying method was applied to deactivate the enzymes responsible for microbial degradation.For this study, this combination of drying *C. nutans* leaves using oven at 60˚C and soxhlet extraction obtained the highest total flavonoid and antioxidant activity at 24.53 ± 0.95 mg RU/g and 89.73 ± 4.39 mg TE/ g respectively. The results for highest antioxidant activity was followed by air-dried samples and vacuum oven-dried samples at 60°C, extracted through maceration at 85.46 mg TE/ g and 83.96 mg TE/ g respectively.

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