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Sabah snake grass extract pre-processing: Preliminary studies in drying and fermentation

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Abstract. Clinacanthus nutans (Burm. F.) Lindau which also known as 'Sabah Snake Grass' among Malaysians have been studied in terms of its medicinal and chemical properties in Asian countries which is used to treat various diseases from cancer to viral-related diseases such as varicella-zoster virus lesions. Traditionally, this plant has been used by the locals to treat insect and snake bites, skin rashes, diabetes and dysentery. In Malaysia, the fresh leaves of this plant are usually boiled with water and consumed as herbal tea. The objectives of this study are to determine the key process parameters for Sabah Snake Grass fermentation which affect the chemical and biological constituent concentrations within the tea, extraction kinetics of fermented and unfermented tea and the optimal process parameters for the fermentation of this tea. Experimental methods such as drying, fermenting and extraction of *C.nutans* leaves were conducted before subjecting them to analysis of antioxidant capacity. Conventional ovendried (40, 45 and 50°C) and fermented (6, 12 and 18 hours) whole C.nutans leaves were subjected to tea infusion extraction (water temperature was 80°C, duration was 90 minutes) and the sample liquid was extracted for every 5th, 10th, 15th, 25th, 40th, 60th and 90th minute. Analysis for antioxidant capacity and total phenolic content (TPC) were conducted by using 2, 2-diphenyl-1-pycryl-hydrazyl (DPPH) and Folin-Ciocaltheu reagent, respectively. The 40°C dried leaves sample produced the highest phenolic content at 0.1344 absorbance value in 15 minutes of extraction while 50°C dried leaves sample produced 0.1298 absorbance value in 10 minutes of extraction. The highest antioxidant content was produced by 50°C dried leaves sample with absorbance value of 1.6299 in 5 minutes of extraction. For 40°C dried leaves sample, the highest antioxidant content could be observed in 25 minutes of extraction with the absorbance value of 1.1456. The largest diameter of disc that could be observed at 18 hours of fermentation sample had a pile size of 3 cm that had expanded to 5.9 cm of diameter which indicated the microbe's growth.

1. Introduction

Tea development has been evolving since about four thousand years ago which started the discovery of its leaves, Camellia sinensis until advancements in tea processing which enable tea become the most popular drink beverage after water [1,2]. Tea processing involved several steps which starts from the selection of leaves, withering, fermentation for fermented tea-making and sun drying. Green tea,

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Oolong and black teas are among the major teas in the market. Green tea is unfermented tea while Oolong and black teas, respectively are semi-fermented and fermented tea.

Currently, the demand of plant-based products in the market as an alternative to modern medicines to treat and prevent oxidative stress-related health diseases such as cancer, diabetes and other chronic diseases have increased significantly due to some claims of efficacy. These claims are possibly due to the phytochemical content which acts as antioxidants such as phenolic, flavonoids, saponins, tannins and lignin presence in plants. These antioxidants have capabilities to neutralize the effect of free radicals and inhibit the cell damaging oxidation reaction to treat and prevent chronic diseases [3,4]. Oxidative stress which is among the contributor to chronic diseases, where reactive oxygen species (ROS) and reactive nitrogen species (RNS) including free radicals were rapidly produced in human cells that led to the occurrence of cell damage [5,3,6].

Clinacanthus nutans (Burm. F.) Lindau which belongs to Acanthaceae family is a small shrub natively grown in tropical Asian countries [7]. In Malaysia, it is known as 'Sabah Snake Grass' or 'Belalai Gajah'(Elephant trunk) among the locals while in Thailand it is known as 'Phaya Yo' or 'Phaya Plong Thong'[3,7]. *C.nutans* has been used as traditional medicines by Asian natives to treat several of health-related diseases. In Malaysia, it is used as a potent alternative medicine to treat cancer [8]. In Thailand, it is used to treat insect and snake bites, skin rashes, virus-related diseases such herpes simplex (HSV) and varicella-zoster lesions (VZV) [3,7]. Indonesians use it to cure dietary-related health disease such as diabetes and unhygienic-related health disease such as dysentery [3,9].

Previous studies reported that the anti-inflammatory [10], analgesic and antiviral properties against varicella-zoster [11] and herpes simplex type -2 [12] virus were found in *C.nutans* leaves extract. According to one study, antioxidant activities towards free radical-influenced hemolysis are present in ethanolic extract of *C.nutans* leaves [13]. Past studies on natural products have developed steps in preparation, preservation and extraction in order to quantify the yield of phenolics compounds [3,14,15,16,17].

However, fresh plants states easily degrade, thus it, preservation methods are needed. The fresh Lamiaceae herbs water content is approximately 75-80% and to preserve its freshness, moisture content must be less than 15% [18]. Drying methods are among the most popular to preserve sample from further deterioration by microorganisms, preserve its biochemical constituents [19], to conserve the desirable qualities and minimize the storage volume [20].Presently, *C. nutans* plants are sold in plants fair and nursery. Some companies are also producing health drinks from *C. nutans* leaves and shoots. There is study about the effect of drying methods on antioxidant activities of *C.nutans* [3] but no literature is available to study the effect of oven-drying at various temperatures on the phytochemical constituents such as phenolic compounds and flavonoids.

Numerous previous studies have reported the ability of fermentation to mobilize the conjugated phenolics that can enhance the content of phenolic and antioxidant properties in fermented samples [21,22,23]. Previous studies also reported that solid state fermentation tends to enhance the antioxidant properties in legumes or cereals. Products such as carbohydrate-degrading enzymes such as xylanases, cellulases, amylase and estrases were produced by microbes during fermentation which are useful to release the bound phenolic compounds in samples during extraction [24,25,26,23,27,28].

Although many studies have been conducted on the effect of drying and fermentation methods on antioxidant capacity of plant products, there are little to no information available about the effect of oven-dried at various temperatures and the effect of pile fermentation in various fermentation duration on microorganism's growth in *C.nutans*. Therefore, this study was conducted to determine the effect of oven-drying at various temperatures and duration on the antioxidant capacity of the sample and the effect of pile-fermentation method at various durations of fermentation on microorganism's growth in *C.nutans*.

2. Materials and Methods

2.1 Plant Materials

Fresh leaves of *C. nutans* with no physical damage were collected from Block B Faculty of Engineering of UMS, Kota Kinabalu, Sabah. Five grams of fresh leaves were plucked from its main tree, washed and rinsed with tap water and distilled water, respectively to remove dirt. Leaves were then pat dry with kitchen towel to blot the excess water and arranged onto aluminium foil.

2.2 Preparation of unfermented C. nutans tea

The preparation of this tea was adapted from the preparation procedure of *C. nutans* tea according to Barek *et al.* [3] and *Ocimum tenuiflorum* tea by Rabeta and Lai [19] with some modifications. The sample (3g) was dried using an oven (Binder, USA) at 50°C for six hours. This step will be repeated for the next sample with different drying durations (12 and 18 hours) and different oven temperatures (40°C, 45°C and 50°C).

2.3 Preparation of fermented C. nutans tea

The preparation of this tea was adapted from the preparation procedure of *C. nutans* tea according to Barek *et al.* [3] and *Ocimum tenuiflorum* tea by Rabeta and Lai [19] with some modifications. The cleaned fresh leaves (3g) went through a withering process inside the incubator (Memmert, USA) at 25° C for 11 hours to reach 60% of moisture content. The withered leaves then underwent a piling fermentation process inside the incubator (Memmert, USA) at 25° C. The withered leaves were piled with thread inside a ceramic cup and the cup opening was closed with aluminium foil before proceeding with the fermentation process. The duration of the fermentation process inside the oven (Binder, USA) at 30° C to achieve 3° of moisture content in order to stop the fermentation process [29].

2.4 Preparation of leaves infusion

The extraction process was done according to Barek *et al.* [3] which imitated the common tea brewing situations using hot water. The dried unfermented leaves (3 g) were added into 80°C hot distilled water (300 mL) and continuously stirred using a magnetic stirrer. The sample extract (10 ml) was collected at specific durations of 5, 10, 15, 25, 40, 60 and 90 minutes. This process was also used to prepare leaves infusion for dried fermented leaves.

2.5 Determination of total phenolic content

The total phenolic content (TPC) was determined by the Folin Ciocalteu (FC) assay as described by Barek *et al.*, [3] with some modifications. 1 ml of extracted sample was mixed with 0.75 ml of 10-fold diluted Folin-Ciocalteu's reagent. The mixture was left to stand for five minutes at room temperature. 0.75 ml of NaCO₃ (60 g/L) solution was added to the solution. The mixture was left for ninety minutes further at room temperature. The absorbance was read at 725 nm using UV-Vis spectrophotometer (V-650 Spectrophotometer, Jasco, Tokyo, Japan). No calibration curve had been done as this was a preliminary study.

2.6 2, 2-diphenyl-1-pycryl-hydrazyl (DPPH) free radical scavenging activity

The DPPH scavenging activity was determined by the method of Barek *et al.*, [3] with some modifications. The DPPH solution was prepared by mixing the DPPH powder with methanol at a concentration of 250 μ M. The extracted sample solution (2.5 mL) was mixed with 2.5 mL of DPPH solution. The mixtures were incubated in a dark room at 28°C for 30 minutes. DPPH solution in methanol was used as a blank. The absorbance was read at 517 nm with UV-Vis spectrophotometer (V-650 Spectrophotometer, Jasco, Tokyo, Japan). No calibration curve had been done as this was a preliminary study.

2.7 Microbes Growth Study (Paper Disc Diffusion with modification)

Dried leaves were mixed with 50 ml of distilled water and sterilized paper discs. The paper disc was plated on agar and incubated in room temperature at dark place for seven days. Observation and measurement of the disc diameter was carried out the next day and continued doing so for seven days.

3. Results and Discussion

3.1 Total Moisture Content

The moisture content of *C.nutans* leaves were determined by using A.O.A.C method [30]. Two samples of leaves were weighed at 5 g each and dried inside the oven (Binder, USA) at 105°C for 24 hours. The dried leaves were re-weighed to get the dry weight of the leaves. The moisture content of *C.nutans* leaves was found to be 79.64%.

Sample	Moisture Content (%)
А	79.60
В	79.68

Table 1: The moisture content of leaves samples

3.2 Drying Kinetics for Oven-Drying

The fresh leaves were placed inside the oven and dried at 40°C, 45°C and 50°C. The mass of the leaves were recorded at every hour until the mass reading became constant.



Figure 1: Experimental drying curves of C.nutans leaves at different temperatures

Figure 1 illustrates the effect of various oven-temperature and duration of drying on *C.nutans* leaves. At 40°C, the drying rate was minimal and approached equilibrium at 17 hours and the maximum drying rate was at 50°C which approached equilibrium at 4 hours. For all the three samples, the final moisture content was 20%. It also shows that as the temperature increased, the duration for the leaves to reach constant moisture content decreased. This is in line with the drying theory. As the temperature increased, there was an increase in the rate of mass transfer (water) to achieve the similar equilibrium moisture content (approximately 0.98 g water/100g d.m) [31].

3.3 Effect of drying temperatures on phenolic content in unfermented leaves

Figure 2 illustrates the absorbance unit over the duration of extraction on *C.nutans* leaves. The spectrophotometer reading gives the phenolic content value in the quantification of phenolic compounds from plant materials. The 50°C oven-dried leaves had higher absorbance than those dried at 40°C. Processing the samples will affect the content, bioactivity and bioavailability of bioactive compounds [20]. Initial conclusion that can be drawn from this finding was that sample that had been dried at higher temperatures had greater capacity to extract higher phenolic content at shorter duration of extraction.



Figure 2. Effect of different dried sample temperatures on the phenolic content

3.4 Effect of drying temperatures on antioxidant content in unfermented leaves

Figure 3 illustrates the absorbance values over the duration of extraction on *C.nutans* leaves. The 50°C oven-dried leaves had the higher absorbance value than those dried at 40°C. Higher drying temperature reduced the times of extraction and costs of processing. Drying at higher temperatures helped increase the rates of deactivation of enzymatic degradation which was responsible for the loss of antioxidant in samples [21]. The same conclusion can be reached as in phenolic content above.



Figure 3. Effect of different dried sample temperatures on the antioxidant activity

3.5 Effect of pile-fermentation on the growth of microorganism's

Figure 4 illustrates the diameter of discs over the pile sizes of fermented *C.nutans* leaves. The 18 hours fermented leaves and pile size (3cm) produces the largest diameter of disc. At present, the species of microbes are unknown, however in pile fermentation of Guangdong Pu-erh tea showed complexity of microbes communities in which fungi were the dominant microbe [32,33]. Initial conclusion can be drawn from this finding, sample with longer duration of fermentation and thicker piles sizes produce higher microorganism's growth.



Figure 4. Effect of different fermented sample duration on the microorganism's growth

4. Conclusion

It can be preliminarily concluded that increasing the drying temperatures can reduce the duration of drying and can increase the extraction of phenolic and antioxidant contents from *C.nutans* leaves in shorter durations of extraction. Increasing the duration of fermentation and size of piles can induce the development of microorganism's growth. However, more investigation is needed to find the effect of fermentation on phenolic content, antioxidant activity and microorganism's growth in fermented *C.nutans* leaves. Further work will need to be done to get the standard calibration curve for phenolic content and antioxidant activity for *C.nutans* fermented leaves. Works on rate and yield data for the extraction of *C.nutans* fermented leaves are also conducted. The result of this study will be utilised to determine the critical parameters as well as the range for optimal extraction to ensure the standardization of the leaves extract for overall antioxidant content.

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