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# Effect of Various Fermentation Stages on Antioxidative Activity of Belalai Gajah (*Clinacanthus nutans*) Teas

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**Abstract:** Belalai gajah or Sabah snake grass, with the scientific name, *Clinacanthus nutans* (*C. nutans*) Lindau (Family: Acanthaceae) is a small shrub that belong native to tropical Asia countries. *C. nutans* has been medically recognized to be effective in the treatment of skin diseases and infection. This study was conducted to evaluate and compare the antioxidant activity of methanolic extract from dried (non-fermented), green (least fermented), oolong (semi-fermented), and black (fermented) tea made from *C. nutans*. The antioxidant activities of sample extract were determined by using free radical scavenging activity (DPPH), ferric thiocyanate (FTC), and thiobarbituric acid (TBA) tests. Dried *C. nutans* showed the highest antioxidant activity in the three antioxidant tests. There was no significant different ($P$>0.05) between the dried *C. nutans* and the BHT (synthetic antioxidant) in FTC method. Green *C. nutans* teas showed relatively low DPPH radical scavenging activity, but, it comparatively high antioxidant potential according to FTC and TBA methods. On the other hand, oolong and black *C. nutans* teas have relatively high DPPH radical scavenging activity than green *C. nutans* teas, but low antioxidant potential according to FTC and TBA methods.

**Keywords:** *Clinacanthus nutans*, antioxidant activity, free radical scavenging activity (DPPH), ferric thiocyanate (FTC) test, thiobarbituric acid (TBA) tests

## 1. Introduction

Belalai gajah or Sabah snake grass, with the scientific name, *Clinacanthus nutans* (*C. nutans*) Lindau (Family: Acanthaceae) is a medical herb that found in tropical forest in Asia countries. In a recent study, the pharmacological properties of *C. nutans* used in South East Asia, especially Thailand, an alcoholic extract of the fresh leaves of *C. nutans* is used externally for treatment of skin rashes, snake and insect bites, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions [1], and also as a folk medicine for cancer treatment. Moreover, the extracts from the leaves of *C. nutans* were reported to possess analgesic and anti-inflammatory activities [2], herpes simplex viros type-2 [3], and antiviral activities against varicella-zoster virus [4]. Due to the original and natural properties, nowadays, many people are increasingly and widely used the traditional medicines, particularly herbal medicines during the last two decades [5]. However, the number of people that been reported that experiencing negative health consequences had also been increasing which cause by the use of herbal medicine [6]. Therefore, determining the actual antioxidant activity in the traditional medicines is important to show the real actual usage and the value of the herbal medicines. In the market today, there are a few commercial *C. nutans* tea, but the products do not indicate to which fermentation state that the tea is. Moreover, the antioxidant activity and flavonoid contents of green, oolong, and black made from *C. nutans* has never been done and compared before. Hence, this study was carried out to generate knowledge on the antioxidant activity of the dried, green, oolong, and black teas that

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made from *C. nutans*. In the meantime, provide information on the effect of fermentation process to the antioxidant activity of the *C. nutans*.

# 2. Materials and Methods

## 2.2. Plant material

The plant *C. nutans* was collected from Kuala Terengganu. Authentication of the plant was done by Mr. Muhamad Razali bin Salam from the Department of Biology Science, Faculty of Science and Technology, University Malaysia Terengganu.

## 2.2. Preparation of *C. nutans* teas

The method to produce dried *C. nutans* was modified from the method of Saleem et al. [7]. The fresh plucked *C. nutans* leaves was washed and cleaned. The drying process was carried out by oven dried at 45 ℃ for 2 days. The green, oolong, and black *C. nutans* tea was produced according to the method of Karori [8].

## 2.3. Extraction

Extraction was carried out according to the method used by Niranjan et al. [9].

## 2.4. Antioxidant activity

- **DPPH free radical scavenging activity**

The free radical scavenging activity by plant extract was done according to the method of Mon et al. [10]. The antioxidant activity of the plant extracts and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical activity. The diluted working solutions of the test extracts were prepared in methanol. Alpha-tocophenol and BHT was used as standard in 1-100 µg/ml solution. 0.1mM of DPPH was prepared in methanol and 2 ml of this solution was mixed with 1 ml of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 518 nm using spectrophotometer. Methanol (1 ml) with DPPH solution (0.1mM, 2 ml) was used as blank. The optical density was recorded and percentage of inhibition (% of inhibition) was calculated.

- **Ferric thiocyanate (FTC) test**

The standard method as described by Osawa and Namiki [11] was used to further determine the antioxidant activities of sample methanolic extracts. To 0.1 ml of plant extract, 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate was added. Precisely 3 minutes after addition of 0.1 ml of $2x10^{-2}$ M (0.002M) ferrous chloride in 3.5% hydrochloride acid to the reaction mixture, the absorbance of red colour was measured at 500 nm each 24 hours until one day after absorbance of the control reach maximum. The optical density was recorded and percentage of inhibition (% of inhibition) was calculated.

- **Thiobarbituric acid (TBA) test**

The TBA test was conducted according to the method of Ottolenghi [12]. The same samples as prepared for the FTC method were used. Samples (4 mg or 4 ml) in 99.5% was mix with 2.51% linoleic acid in 99.5% ethanol (4.1 ml), 0.05 M phosphate buffer, pH 7.0 (8ml), and distilled water (3.9 ml) and keep in screw cap containers under dark conditions at 40 ℃ in water bath. To 1 ml of this solution was added aqueous trichloroacetic acid (2 ml) and aqueous thiobarbituric acid solution (2 ml). This mixture was then being placed in a water bath 10 minutes. After cooling, it was centrifuged at 3000 rpm for 20 minutes. Absorbance of supernatant was measured at 532 nm. Antioxidant activity was based on the absorbance on the final day of FTC method. The control and standard were subjected to the same procedure as the sample except for the control, where there was no addition of sample (blank). As for the standard, 4 mg of sample were replaced with 4 mg of alpha-tocophenol or BHT. The optical density was recorded and percentage of inhibition (% of inhibition) was calculated.

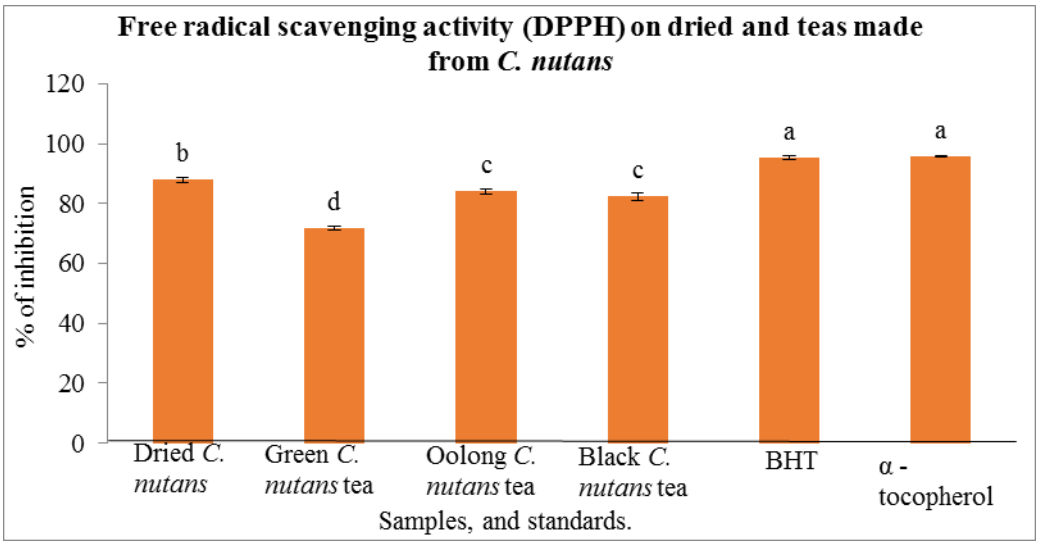
## 2.5. Statistical analysis

All treatments and analysis was carried out in duplicates. All results were reported as mean ± standard deviation. One-way ANOVA was carried out to determine the significant different of antioxidant activity of dried and teas made from *C. nutans* by using Minitab 14.0 statistical software. The significant differences between means were determined by using Fisher's Multiple Comparison statistical analysis ($P < 0.05$).

## 3. Results and Discussion

### 3.1. DPPH free radical scavenging activity

Antioxidants are not effective if added to the product after the onset of oxidation. The radicals intermediated generated are also responsible for oxidation propagation. An effective scavenger will definitely favorably influence the inhibition of the whole oxidation process [13]. Figure 1 shows the free radical scavenging activity (DPPH) of dried, green, oolong, and black teas made from *C. nutans*. Dried *C. nutans* showed the highest percentage of inhibition compared to the other *C. nutans* teas but significantly ($P<0.05$) lower than that of standards butylated hydroxyl toluene (BHT) and alpha-tocopherol. There was no significant ($P>0.05$) different in percentage of inhibition between oolong *C. nutans* teas and black *C. nutans* teas. It is interesting to note that the green *C. nutans* tea exhibited the lowest inhibition percentage than the oolong and black *C. nutans* teas. All the sample extracts have significantly different ($P<0.05$) with the standards.




Values are mean ± standard deviation of two replications. Value with the same letter (a, b, c) are not significantly ($P>0.05$) different, between samples.

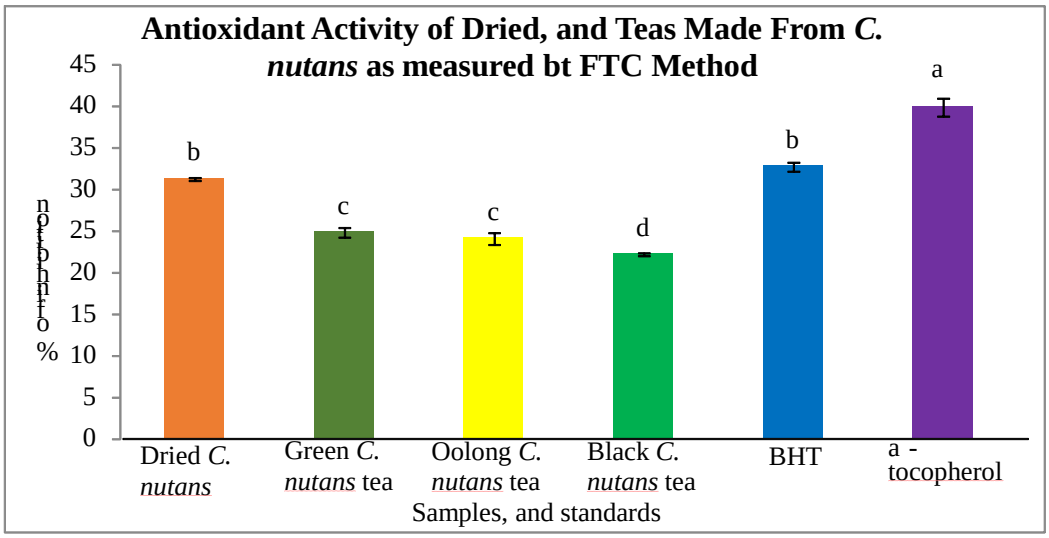
Fig. 1: Free radical scavenging activity (DPPH) of dried, green, oolong, and black teas made from *C. nutans*.

Non-fermented herbal tea *Strobilanthes crispus* (*S. crispus*) exhibits the higher scavenging effect than the fermented herbal tea (*S. crispus*) [1]. Generally, the content of catechins in tea (*C. sinensis*) is related to the degree of fermentation of tea during processing [14]. Thus, the content of catechins in teas is in the order green tea > oolong tea > black tea. But, the variable in antioxidant activity of the teas (*C. sinensis*) may not be completely attributed to the content of catechins [14]. This means that the variable in antioxidant activity of the tea might not be entirely come from the catechin content alone. Black tea leaves (*C. sinensis*) are subjected to crushing and a full fermenting process where catechin derivatives are oxidized, resulting in the formation of the polymeric compounds, thearubigins (TRs) and theaflavins (TFs) [15], which also plays an important role in antioxidant activity.

### 3.2. Ferric thiocyanate (FTC) test

Figure 2 shows the antioxidant activity (percentage of inhibition) of dried, green, oolong, and black teas made from *C. nutans* as measured by FTC method.

Results shows that dried *C. nutans* was significantly ($P$<0.05) highest in inhibition percentage than the other *C. nutans* teas. The percentage of inhibition of linoleic peroxidation of dried *C. nutans*, green, oolong, and black *C. nutans* teas were 31.21 ± 0.18, 24.82 ± 0.58, 24.06 ± 0.72, and 22.18 ± 0.18%, respectively. None of the plant extracts showed absorbance values greater than the negative controls (without plant extracts) at the end point of both methods, indicating the presence of antioxidant activity. However, all the sample extracts exhibited strong antioxidant activity as determined by the FTC methods, surpassing the activity of the standard commercial antioxidants, alpha-tocopherol and BHT. There was no significant ($P$>0.05) different in percentage of inhibition between the dried *C. nutans* and the BHT.




Values are mean ± standard deviation of two replications. Value with the same letter (a, b, c) are not significantly ($P$>0.05) different, between samples.

Fig. 2: Antioxidant activity of dried, green, oolong, and black teas made from *C. nutans* as measured by FTC method.

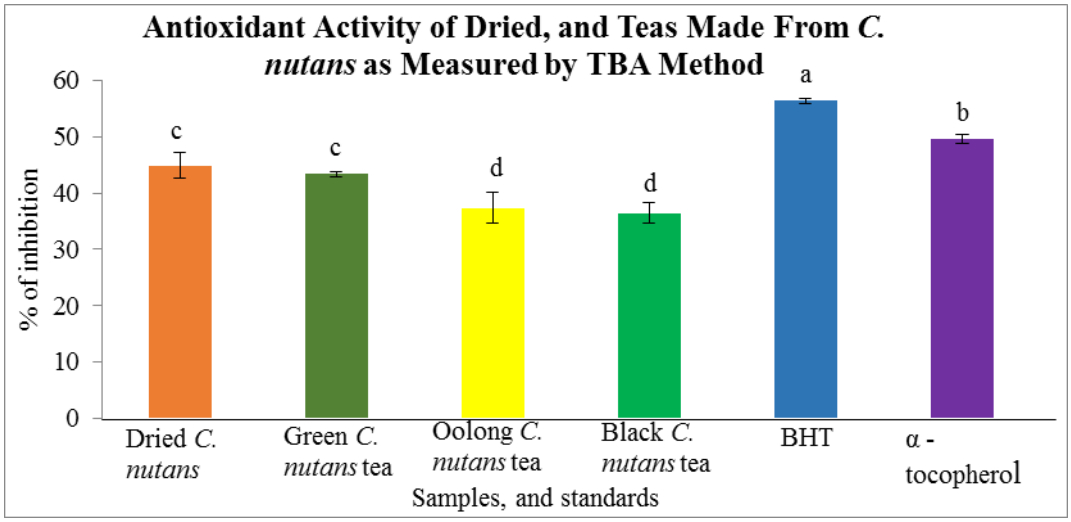
Consequently, dried *C. nutans* (non-fermented) shows the highest percentage of inhibition as compared to the other *C. nutans* teas. This condition may be due to the deterioration of phenolic substances during fermentation in fermented tea [16]. Green tea (*C. sinensis*) flavanols have been classified according to their stereochemical configurations into "catechins" (2, 3-trans, such as catechin, CG, GC, and GCG) and "epicatechins" (2, 3-cis, including EC, ECG, EGC, and EGCG). The total catechin (polyphenol) content found in green tea was higher than that in black tea (*C. sinensis*). The higher total catechin content (EGCG, EGC, EC, +C and ECG) in green tea showed significant influence on the antioxidant activity (8). In addition, the antioxidant activity was higher in tea extracts containing high levels of EGCG, EGC, EC, +C and ECG [8]. The presence of large amount of catechins make the green tea (*C. sinensis*) becomes the most powerful antioxidant tea as compared to oolong and black tea [17].

## 3.3. Thiobarbituric acid (TBA) test

The FTC test determines the amount of peroxide at the initial stage of oxidation while TBA test is used to measure the secondary product of oxidation [18]. In general, the antioxidant by TBA method is higher than that of FTC method. This might be signifying that the amount of peroxide in the initial stage of lipid per oxidation is less than the amount of peroxides in the secondary stage [19]. Moreover, the secondary product is much more stable for a period of time [20]. Figure 3 shows the antioxidant activity of dried, green, oolong, and black teas made from *C. nutans* as measured by TBA method. There was no significant ($P$>0.05) different between inhibition percentage of the dried *C. nutans*, green *C. nutans* teas and between oolong, black *C. nutans* teas in TBA test.

In overall results, green *C. nutans* teas (least fermented) showed relatively low DPPH radical scavenging activity as compared to dried, oolong, and black *C. nutans* teas, but, it comparatively high antioxidant potential according to FTC and TBA methods. On the contrary, oolong (semi-fermented) and black

(fermented) *C. nutans* teas have relatively high DPPH radical scavenging activity than Green *C. nutans* teas, but low antioxidant potential according to FTC and TBA methods. These differences might be due to their different antioxidant mechanisms or variations in their ability to scavenge free radicals [21]. Different mechanism involved in the determination methods, structures of the different phenolic compounds and possible, due to the synergistic effects of different compounds may also cause the difference in antioxidant activities [22]. Moreover, the differences between the various processes of manufacture result in differences in the polyphenol profile between green, oolong, and black tea [8].




Values are mean ± standard deviation of two replications. Value with the same letter (a, b, c) are not significantly different ($P$>0.05), between samples.

Fig. 3: Antioxidant activity of dried, green, oolong, and black teas made from *C. nutans* as measured by TBA method.

It is interesting to note that dried *C. nutans* (non-fermented) showed the highest antioxidant activity in the three antioxidant test (DPPH, FTC and TBA). Non-fermented herbal tea (*S. crispus*) exhibits the higher antioxidant activity than the fermented herbal tea (*S. crispus*) [16]. In addition, other polyphenol present in the extract, plus vitamins and mineral might contributed to the antioxidant activity of the various fermentation stages of herbal teas [16]. Pannangpetch et al. [23] found that the ethanolic extract of *C. nutans* has *in vitro* high antioxidant activity. These results indicate the possibility of employing the *C. nutans* extract as an antioxidant substance to ameliorate the oxidative damage.

## 4. Conclusion

In this study, different types of *C. nutans* teas have been made and compared with dried *C. nutans*. The dried, and *C. nutans* teas was extracted with methanol. The antioxidant activities of sample extract were determined by using Free radical scavenging activity (DPPH), Ferric thiocyanate (FTC), and Thiobarbituric acid (TBA) tests. Dried *C. nutans* showed the highest antioxidant activity in the three antioxidant test (DPPH, FTC and TBA). In addition, there was no significant different ($P$>0.05) between the dried *C. nutans* and the BHT (synthetic antioxidant) in FTC method. Green *C. nutans* teas showed relatively low DPPH radical scavenging activity, but, it comparatively high antioxidant potential according to FTC and TBA methods. On the other hand, oolong and black *C. nutans* teas have relatively high DPPH radical scavenging activity than Green *C. nutans* teas, but low antioxidant potential according to FTC and TBA methods. However, it is not possible to conclude whether the same compound is responsible for the antioxidant activity in all extract tested. The result of this study would be able to serve as reference that dried *C. nutans* and different fermentation stages of *C. nutans* teas potentially have an active antioxidant and could be used as healthy beverages in pharmaceutical industry.

## 5. Acknowledgements

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