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# Evaluation of Clinacanthus nutans leaves extract on Prothrombin Time and Activated Partial Prothrombin Time test

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ABSTRACT

Limitations in existing anticoagulants have prompted a search for the natural origin of anticoagulants. Since there is an increasing need to source for pharmacological and medicinal materials from a plant source, thus an exploratory effort towards identifying and characterizing new anticoagulants from plants is worthwhile. Clinacanthus nutans (C. nutans) commonly known as Sabah snake grass belongs to Acanthaceae family was chosen since it has therapeutic and medicinal benefits. This plant possessed anti-oxidant, anti-microbial, anti-inflammatory, and anti-diabetic activities. This study was carried out to identify the anticoagulant activities of C. nutans leaves in aqueous and methanol extracts at different concentrations based on prothrombin time (PT) and activated partial thromboplastin time (aPTT). The dried leaves of C. nutans were ground into a fine powder and extracted using aqueous and methanol. Anticoagulant assays of PT and aPTT were done on three different concentrations of C. nutans aqueous and methanol extracts (10 mg/mL, 20 mg/mL, and 30 mg/mL) and control group respectively. PT and aPTT of plasma samples were prolonged in both types of extracts ($p<0.05$). This study highlights that the anticoagulant activity of aqueous and methanol extracts of C.nutans affects the intrinsic and extrinsic pathway, possibly due to the inhibitory action of the clotting factors. These results suggested that aqueous and methanol extracts of C. nutans exhibited anticoagulant activity at certain concentrations.

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## INTRODUCTION

Thrombosis or formation of blood clotting is commonly related to disruption and blockage of blood flow. The impact of this blood clotting contributes to the development of many diseases such as stroke, atherosclerosis, and heart attack. Blood coagulation is one of the components of haemostasis (Omodamiro and Ikekamma, 2016). The indicators of blood coagulation are PT and aPTT (Cordier *et al.*, 2012). PT is used to evaluate the coagulation factors V, VII, and X in the extrinsic pathway, while aPTT is used to evaluate the coagulation factors such as VIII, IX, XI, XII, and prekalikrein in the intrinsic coagulation pathway of the coagulation cascade. Current available treatment, such as heparin and warfarin, acts as an anticoagulant in order to treat blood clotting disease (Harter *et al.*, 2015). However, these drugs can cause adverse effects such as bleeding, especially in the elderly, patient with antiplatelet therapy, and patient with other diseases such as cancer, renal, and liver problem (Ageno *et al.*, 2012). Meanwhile, to overcome

these problems, plants can become natural remedies and alternative sources to treat blood coagulation (Harter *et al.*, 2015). Thus, many studies have been explored on the anticoagulant activity of various medicinal plants with the hope to find new and more effective anticoagulant agents (Félix-Silva *et al.*, 2014; Cordier and Steenkamp, 2012). In this study, Clinacanthus nutans (hereafter referred to as C. nutans) belongs to Acanthaceae family was chosen since it has potential therapeutic and medicinal benefits. According to Alam *et al.* (2016), C. nutans is known as 'Sabah snake grass'or 'belalai gajah' in Malay and 'Phaya Yo' in Thai. In Thailand, C. nutans is useful in treating skin rashes, insect and snake biting, herpes simplex virus (HSV) and varicella-zoster virus (VZV) lesions (Sakdarat *et al.*, 2009). The phytochemical studies demonstrated that C. nutans plant has rich sources of active compounds such as flavonoids, glycosides, glycoglycerolipids, cerebrosides, and monoacylmonogalatosyl glycerol that serve as anti-inflammatory, antiviral, antioxidant and anti-diabetic activities (Alam *et al.*, 2016). As of today, there is no study reported on C.nutans anticoagulant activities. Therefore, this study was conducted to evaluate the effect of aqueous and methanol extraction of C. nutans leaves extract on prothrombin time (PT) and activated partial thromboplastin time (aPTT) test on platelet-poor plasma samples.

## MATERIALS AND METHODS

### Preparation of plant material

C. nutans leaves were collected and identified from Alor Setar, Kedah, and used freshly for extraction. The leaves were washed with fresh water and dried under shade at room temperature. The leaves were ground into a powder and stored in a dry, dark place prior to anticoagulant testing.

### Preparation of plant extracts

### C. nutans aqueous extraction

The aqueous extract of C. nutans was prepared according to the method by Intan *et al.* (2015) with some modification. 800 g of C. nutans leaves were cut into small pieces and let to be dried under the sunshade. The complete dried of the leaves pieces were ground into a fine powder. 100 g of the fine powder was weighed on a balance scale (AY220, Shimadzu, Japan) and soaked in 1 L of distilled water (1:10 w/v) at room temperature. Then the mixture was stirred by using magnetic stirrer (IKA C-Mag HS7) in a conical flask and left overnight. After that, the mixture was filtered by using Whatman No.1 filter paper (Whatman, England), and the supernatant was collected. In order to produce a thick syrupy mass crude extract, rotary evaporator (R-215, Butchi rotavaporator, Switzerland) was used to evaporate the filtrates under vacuum reduced pressure at 60°C. The crude extract was kept at -20°C in Scott's bottle prior to further analysis

### C. nutans methanol extraction

The methanol extract of Clinacanthus nutans was prepared according to the method by (Rahim *et al.*, 2016) with some modification. 500 g of C. nutans leaves were cut into small pieces and let to be dried at room temperature. The complete dried of the leaves pieces were ground into a fine powder. 100 g of the fine powder was weighed on a balance scale (AY220, Shimadzu, Japan). The fine powder was soaked in 1 L of 90% methanol at room temperature. Then the mixture was stirred by using magnetic stirrer (IKA C-Mag HS7) in a conical flask and left overnight. After that, the mixture was filtered by using Whatman No.1 filter paper (Whatman, England), and the supernatant was collected. In order to produce a thick syrupy mass crude extract, rotary evaporator (R-215, Butchi rotavaporator, Switzerland) was used to evaporate the filtrates under vacuum reduced pressure at 60°C. The crude extract was kept at -20°C in Scott's bottle prior to further analysis.

### C. nutans solution preparation of Aqueous and Methanol Extraction

The stock of aqueous and methanol extraction of C.nutans were prepared and diluted with sterile distilled water and normal saline, respectively, to a final concentration of 10, 20, and 30mg/ml.

### Platelet Poor Plasma Preparation

The platelet-poor plasma (PPP) was obtained by purchasing the normal control plasma (Diagnostica Stago, France). The samples of normal PPP were stored at -20°C prior to further analysis. Different concentrations of 10, 20 and 30 mg/mL of C. nutans extracts were used to mix with the plasma and incubated in water bath (Memmert, USA) at 37°C for 7 minutes in order to measure the prothrombin time (PT) and activated partial thromboplastin time (aPTT) within 3 hours of sample preparation.

### In vitro Anticoagulant Activity

The sample of normal platelet-poor plasma (PPP) (Diagnostica Stago, France) was divided control group and experimental group. For the negative control group of normal prothrombin time (PT) and normal activated partial thromboplastin time (aPTT), there is no anticoagulant such as heparin or extracts was added, whereas, for positive control,

the tube contained heparin. For the experimental group, the PPP were spiked with different concentrations of C.nutans (10, 20, and 30 mg/mL). The sample was tested in a water bath at 37°C.

The PT test was carried out using STA-Neoplastine® CI Plus (Diagnostica Stago, France) that consists of calcium thromboplastin while the aPTT test using thromboplastin reagent C.K. Prest® 2 (Diagnostica Stago, France) containing a standardized amount of cephalin and silica. Both reagents were reconstituted by adding activator and was mixed gently. Coagulation assay was then performed manually according to the manufacturer's instruction.

### Statistical Analysis

The data of PT and aPTT are shown as mean ± S.E.M.($n$=3), and analysis was performed using a one-way analysis of variance (ANOVA), followed by post hoc Dunnett's multiple range tests to determine the means of significance from the control (SPSS version 20.0). The student t-test was used for independent samples to determine the significant difference of clotting time among different extraction methods. The results with a p-value less than or equal to 0.05 were considered significant (*), $p \leq 0.01$ is highly significant (**), and $p \leq 0.001$ is very highly significant (***).

## RESULTS AND DISCUSSION

### PT and aPTT Clotting time of C. nutans extraction

Table 1 showed the PT (in seconds) for aqueous and methanolic extract at three different concentrations (10, 20 and 30 mg/mL) were significantly higher in comparison to positive control (29.0 ± 0.0) and negative control (12.0 ± 0.0) with p<0.001. While the aPTT (in seconds) for aqueous and methanol extraction at three different concentrations were also significantly higher in comparison to a positive control (34.0 ± 0.0) and negative control (30.0 ± 0.0) with p<0.001.

Table 1 Depicts the aqueous and methanol extract of PT and aPTT at three different concentrations of 10, 20, and 30 mg/mL, respectively. By using the ANOVA and post hoc analysis with Dunnet multiple comparisons test, both aqueos and methanol extract were significantly prolonged (p<0.001) the PT and APTT in a dose-dependent manner in comparison to the normal control plasma.

### Prothrombin Time Between Aqueous And Methanolic Extract

Figure 1 shows the comparison between aqueous and methanol extract of C. nutans on PT (in seconds) at three different concentrations (10, 20, and 30 mg/mL). There was no significant difference of C. nutans extract at 10 mg/mL with p-value 0.3. C. nutans methanolic extract at 20 mg/mL and 30 mg/mL showed a significant prolongation of PT compared to aqueous extract at similar concentrations with a p-value of 0.03 and p-value 0.02, respectively.

### Activated partial thromboplastin time of aqueous and methanolic extract

Figure 2 shows the comparison between aqueous and methanol extract of C. nutans on aPTT (in seconds) at three different concentrations. There was a significant prolonged aPTT of C. nutans methanolic extract in comparison with aqueous extract; where at 10 mg/mL p-value was 0.001. For concentration 20 mg/mL the p-value was 0.008. At concentration 30 mg/mL the p-value was 0.03.

In the present study, the aqueous and methanol extract of C. nutans leaves exhibited a prolonged PT and aPTT at concentrations of 10, 20, and 30 mg/ml. This finding could be associated with interference in the one or more clotting factors of intrinsic and extrinsic coagulation pathways, respectively (Hood and Eby, 2008; Adams and Bird, 2009; Cordier *et al.*, 2012). The efficiency of the extraction and the anticoagulant activity obtained from the extracts were affected by the polarity of the solvents (Chew *et al.*, 2011). The study also mentioned that solvents have characteristics such as low viscosity, low density, and high diffusivity allows them to diffuse easily into the pores of plant materials, thus causing the bioactive components to leach out. The extraction of plants depends on the type of solvent used in the extraction procedure. In addition, methanol has a lower boiling point, which is 64.7 °C compare to water that has a high boiling point, which is 100 °C (Chew *et al.*, 2011). Methanol is a polar solvent that easily dissolved bioactive compounds in plants. Based on the results obtained, both aqueous and methanol solvents exhibited anticoagulant activities, but methanol exhibited a longer anticoagulation time than aqueous solvent. Thus, methanol is a better solvent to identify the anticoagulant activities of C. nutans compare to the aqueous solution.

According to Khoo *et al.* (2014), bioactive compounds such as hexuronic acids, polysaccharides, and polyphenolics contribute to anticoagulant activities. Meanwhile, Kee *et al.* (2008) reported that tannins also displayed anticoagulant and antithrombotic effects. Besides, saponins, starch, alkaloids, and flavonoid also contribute to anticoagulant activities (Ughachukwu *et al.*, 2012). Previous study by Tu *et al.* (2014) revealed that steroids, triterpenoids, cerebrosides, glycoglycero-

**Table 1: PT and aPTT of aquoeus and methanol extraction of C. nutans at three different concentrations**

| | Aqueous | | Methanol | |
|---|---|---|---|---|
| | PT | aPTT | PT | aPTT |
| 10 mg/mL | 34.7 ± 2.1 | 40.3 ± 1.5 | 37.0 ± 3.0 | 50.3 ± 1.5 |
| 20 mg/mL | 40.7 ± 1.2 | 45.7 ± 1.2 | 48.0 ± 3.6 | 60.0 ± 5.0 |
| 30 mg/mL | 45.3 ± 1.5 | 50.0 ± 2.0 | 52.3 ± 2.5 | 62.0 ± 6.2 |
| Positive control (heparin) | 29.0 ± 0.0 | 34.0 ± 0.0 | 29.0 ± 0.0 | 34.0 ± 0.0 |
| Negative control | 12.0 ± 0.0 | 30.0 ± 0.0 | 12.0 ± 0.0 | 30.0 ± 0.0 |
| p-value | <0.001*** | <0.001*** | <0.001*** | <0.001*** |

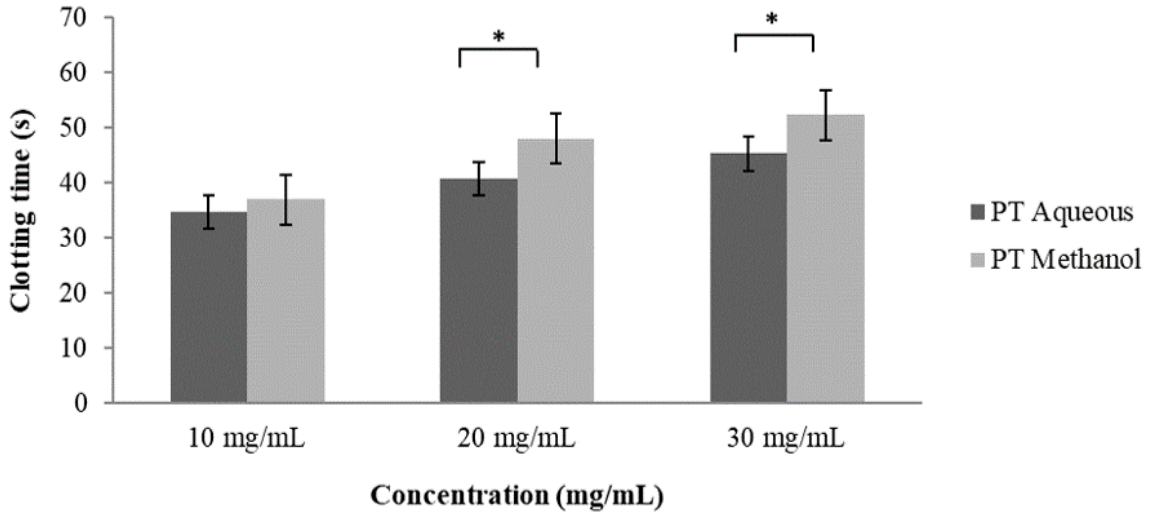


**Figure 1: PT clotting time of plasma on methanol and aqueous extraction of C. nutans. Significance differences of PT test for both aqueous and methanol extraction at three different concentrations (10, 20, and 30 mg/mL) were determined by the Student t-test, whereby p<0.05 was considered significant.**

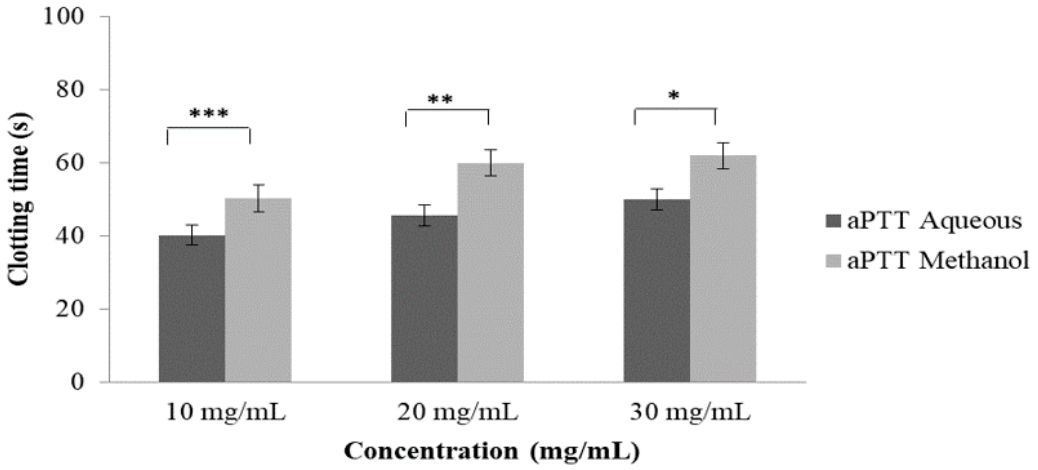


**Figure 2: aPTT clotting time of plasma on methanol and aqueous extraction of C. nutans. Significance differences of aPTT test for both aqueous and methanol extraction at three different concentrations (10, 20 and 30 mg/mL) were determined by Student t-test where by p<0.05 was considered significant, *p<0.05, **p<0.01 and ***p<0.001.**

lipids, glycerides, sulfur-containing glycosides were isolated from C. nutans, whereas Intan *et al.* (2015) mentioned that C. nutans contains phenolic, saponins, flavonoid and tannin which exhibited anti-coagulant effects.

## CONCLUSIONS

The anticoagulant activities of C. nutans were shown in both aqueous and methanol extraction. In this study, the methanol extraction showed higher clotting time compared to aqueous extraction. The PT and aPTT for both extracts exceeded the reference range, thus indicates this plant has an effective anti-coagulant effect. In conclusion, C. nutans may possess as alternative anticoagulants properties in the future. However, further works such as isolation of bioactive compounds that responsible for the anti-coagulation activity need to be done to validate this observation.

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