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Effect of *Ocimum sanctum* (Tulsi) aqueous leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) of human plasma

Abstract — Conventional anticoagulant therapy is the mainstay of medical treatment for deep vein thrombosis disorders. However, there are many complications associated with these agents such as bleeding. Hence, the search for novel anticoagulant derived from natural substances such as plants origin is in high demand nowadays. *Ocimum sanctum* (*O. sanctum)* also known as *Ocimum tenuiform* (OT), tulsi or holy basil from the family of Lamiaceae has been widely used for thousands of years in Ayurveda and Unani systems to cure or prevent a number of illnesses such as headache, malaria, ulcers, bronchitis, cough, flu, sore throat and asthma. The objective is to investigate the effect of *O. sanctum* (Tulsi) aqueous leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) in human plasma. Coagulation activity of *O. sanctum* was measured via PT, APTT and TT assay in citrated plasma collected from thirty-six healthy regular blood donors. The plasma was tested against different concentrations of *O. sanctum* aqueous extract as follows: 0.1mg/ml, 0.5 mg/ml and 1.0 mg/ml. Result shows the aqueous extract of *O. sanctum* prolonged the PT and APTT assays (p<0.05) but had no effect on TT assay (p>0.05). The gas chromatography-mass spectrometry (GC-MS) analysis had identified the linolenic acid at 1-10% of ethanol and aqueous concentration at different retention time which was responsible for the coagulation activities of *O. sanctum* in human plasma. This study suggests that *O. sanctum* does affect coagulation activity in human plasma and can be potentially used as naturally derived anticoagulant products in the future.

Received 10 March 2017.
Revised 22 June 2017.
Accepted 03 July 2017.
Published Online 24 July 2017

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***Keywords*: *Ocimum sanctum*, linolenic acid, anticoagulant, GCMS, coagulation assays**

1 INTRODUCTION

Based on World Health Organization (WHO), about 80% of total world's populations especially in Asia are dependent on traditional medicine for primary healthcare [1]. Herbal plants are considered as important sources of medicine from which large number of therapeutic drugs are obtained due to their chemical diversity. Drugs derived from plants are considered effective, safe and economical as compared to conventional therapy which has wider therapeutic window and side effects. The phytochemicals from herbal plants are found to have biological activities such as antioxidant, antimicrobial and anticoagulant properties [1]. Therefore, the use of herbal medicine provides an alternative to overcome the limitations of available anticoagulants such as warfarin and heparin which have bleeding complication, as well as uncertainty of the newer anticoagulant drugs dosing in some patient populations such as patient with underlying chronic diseases [2].

One of the plants which has medicinal value belongs to the genus *Ocimum,* from the family Lamiaceae, which is widely distributed in the Indian sub-continent [3]. *O. sanctum* has been widely used in Ayurveda and Unani systems to cure or prevent a number of illnesses such as headache, malaria, ulcers, bronchitis, cough, flu, sore throat and asthma [4]. In addition, it also has been proven to have multifarious medicinal properties such as wound healing [5], anticancer [6], antioxidant [7], anti-inflammatory [8] and anticoagulation in animal model [9].

O. sanctum has many curatives uses that the early Indians considered this plant as highly sacred and worth worshipping and hence gave given the name Sacred Tulasi or Holy basil [3]. Being so rich of benefits, having tremendous medicinal potentials and as there is no studies carried out to determine blood coagulation activity

of *O.sanctum* in human plasma and no toxicity effects has been observed *in vitro* has prompted us to study this plant. This study was performed to determine the coagulation properties of aqueous extract of *O.sanctum* in human plasma by measuring the PT, APTT and TT as basic coagulation tests.

2 MATERIALS AND METHODS

2.1 Plant Materials

O.sanctum leaves were collected from a residential area (no: 2004 Jalan Belida, Taman Seluang, Kulim, Kedah). The plant was examined by a taxonomist at the Herbarium Unit of School of Biological Science, University Sains Malaysia, Pulau Pinang. Voucher specimen number (11628) was given by the USM Herbarium unit. The leaves were washed with tap water and dried in industrial oven (KIMAH, Penang, Malaysia) for 6 days. Then, the dried leaves were pulverized with herb grinder (ZM200 RETSCH, Haan, Germany) and fine powder was obtained. The powder was stored in Schott bottle at room temperature until further use. Linolenic acid standard was purchased from Sigma Aldrich (L2376).

2.2 Leaf Extraction

Firstly, 46g of powdered *O.sanctum* was added into a beaker containing 600ml of ethanol (Analytical grade, QReC,) with the ratio of 1:2(w/v). The solution was homogenized in an ultrasonic sonicator (Wise Clean, Wertheim, Germany) for 30 minutes at 1000 kHz to 2500 kHz. Then, the *O.sanctum* leaf extract was centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) for 25 minutes at 4000rpm. After centrifugation, the pellets were retained and extracted again with the same ratio of 1:2 (w/v). Then, the combined supernatant was filtered using filter paper on filter funnel and evaporated in rotary evaporator (EYELA, Tokyo, Japan) at 60°C, 180hPa until a green paste form is obtained. The pellets were re-extracted with distilled water (dH_2O) using the same method as above and dried in freeze dryer (EYELA, Tokyo, Japan) until a powdery form is obtained. The ethanol and aqueous extracts were weighted and stored in a fridge at -20°C until further use. The percentage of yield for ethanol and aqueous extractions were calculated using this formula:

$$\text{Percentage of yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of extracted leaves}} \times 100\%$$

2.3 Donor Recruitment and Blood Collection

Thirty-six regular blood donors (*n*=36) were recruited for this study and 3ml of peripheral blood sample was collected in citrated tubes from the diversion pouch of the blood donation bag by the medical officer or nurses at Clinical Trial Complex (CTC), Advanced Medical and Dental Institute (AMDI). Inclusion criteria for the blood donors include the age range between 18-60 years old and not on any medication such as anticoagulants, antiplatelet, fibrinolytic agents, antibiotics as well as not on any herbal, vitamin or mineral supplements within 7 days before blood donation. Consent to participate in this study was obtained prior to blood sample collection. This protocol was authorized by Research Ethics Committee (Human) (HREC), Universiti Sains Malaysia (USM) (USM/JEPeM/15120573).

2.4 Sample Preparation

The blood samples were centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 4,500 rpm for 15 minutes via refrigerated centrifuge at 4°C to obtain platelet poor plasma (PPP). The obtained plasma was treated with different concentration of *O. sanctum* aqueous extracts (0.1, 0.5 and 1.0 mg/ml) with the ratio of 1:1 (plasma: extract) and incubated for 5 minutes at 37°C.

2.5 Measurements of PT, APTT and TT

The baseline coagulation screening tests PT, APTT and TT, were measured by STA coagulation analyzer (Diagnostica Stago, France). Normal saline was used as negative control. PT was measured using STA-Neoplastine CI plus reagent that contained calcium thromboplastin and the result was compared with normal standard. Meanwhile, the STA-PTT Automate reagent and 0.025M calcium chloride were used to measure APTT. In addition, STA-Thrombin reagent was used to measure TT assay to determine fibrin clot formation. Duplicates were prepared for each assay. All tests were performed within 4 hours after blood collection. The remaining samples were discarded with the consent of the HREC of USM.

2.6 GCMS Analysis

The methods for the preparation of GCMS samples were adopted from previous work with some modifications [10]. A 1g sample of aqueous extraction of *O. sanctum* was diluted in 10ml of Ethanol (HPLC grade) to obtain a 10% concentration of extract which became the stock solution. A 1ml aliquot of the stock solution was then transferred to a test tube and labeled as 10%. Meanwhile, the remaining stock solution was diluted in ethanol to obtain 1%-9% concentrations. A total of 10 different concentrations were prepared (1%-10%). The stock solution for ethanol extract was prepared by using the same method as above. Linolenic acid standard was used as positive control while aqueous and ethanol extract was used as negative control. Apart from that, 100 ppm of linolenic acid standard solution was prepared in 10 mL of ethanol. Then, the solutions were vortexed for 60 seconds. Meanwhile, for the aqueous extract, solvent was removed under Nitrogen (N_2) stream in a dry block heater at 35°C. Then, the Nitrogen stream was removed once the aqueous extract forms dried residues and re-dissolved in 10ml of ethanol. Next, the solutions were filtered using nylon syringe filter (13nm x 0.45µm pore size) and stored in GC screw cap vials (Agilent, United State). Subsequently, the samples were heated at 60°C for 1 hour and occasionally swirled. Following that, the samples were allowed to cool down at room temperature prior to GCMS analysis.

GCMS analysis setting was adopted from Botinestean *et al*. (2012) with some modifications [11]. The linolenic acid standard, ethanol and aqueous extracts were analyzed using Agilent GCMS 7890A (Agilent, CA, United State) spectrometer. HP-5 Ms capillary column (30.0 m x 0.25m x 0.25 µm) were used to analyze the samples. The temperature was set within 50°C – 250°C with 4°C /min. The temperature for the detector and injector was set at 280°C. Helium gas was used as carrier gas with a flow rate of 1.5 psi. About 2µl of linolenic acid standard, ethanol and aqueous extract were injected into GCMS. The compounds analyzed via GCMS were then identified and compared using the National Institute of Standards and Technology (NIST) database. Fig. 1 shows the GC-MS chromatogram of linolenic acid standard at retention time of 43.40 minutes and Fig. 2 shows the comparison of GCMS chromatogram between the linolenic acid standard (Figure 2a) and NIST Mass spectrometry Data center (Figure 2b).

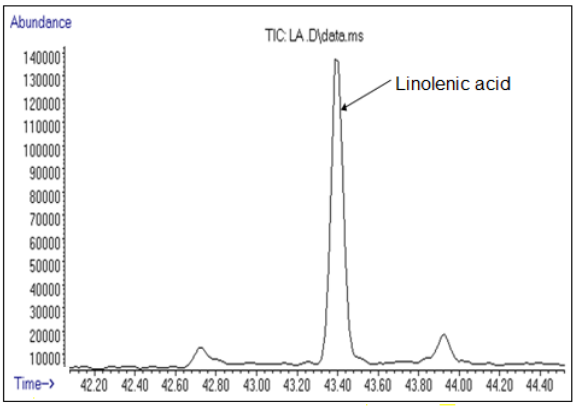

Figure 1: GCMS chromatogram of Linolenic acid standard at retention time of 43.40 minutes.

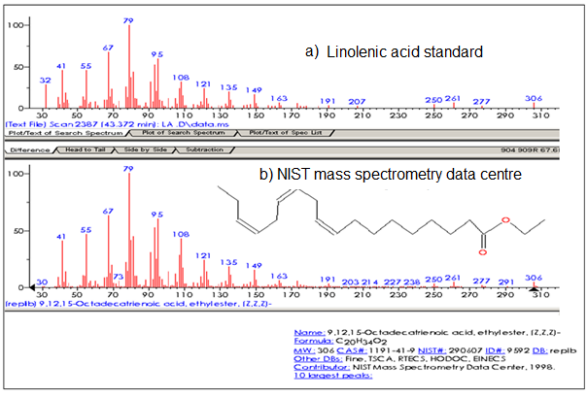

Figure 2: Comparison of GCMS chromatogram between the (a) linolenic acid standard versus (b) NIST mass spectrometry data center.

2.7 Statistical Analysis

Statistical Analysis was performed by using SPSS version 22.0. The values for coagulation screening test (PT, APTT and TT) were compared with baseline value. The mean of all the data were summarized according to different concentrations of aqueous extract (0.1, 0.5 and 1.0 g/ml). Data were analyzed and reported as mean and standard deviation (SD) by comparison to the baseline value using One Way Anova Test. A values of $p<0.05$ was considered as statistically significant.

3 RESULTS

3.1 Extraction

Ethanol and water give different yield of extraction of *O. sanctum* due to difference in polarity index. The ethanol extract of *O. sanctum* was obtained in dark green paste form with a percentage yield of 3.80%. Meanwhile, the aqueous extract of *O.sanctum* was obtained in brown powder form with a percentage yield of 13.3%.

3.2 Blood Coagulation Activities

Basic coagulation screening tests were performed such as PT, APTT and TT to investigate the *in vitro* coagulation activity of *O.sanctum* on human plasma. The mean and standard deviation (SD) of baseline (0.0 mg/ml) PT value was 12.3 seconds and 0.51. The mean PT at extract concentration of 0.1mg/ml, 0.5mg/ml and 1.0mg/ml were statistically significant (p <0.05) when compared to mean PT of baseline value. In all the aqueous concentration of extract, PT increases at 0.1mg/ml and 1.0 mg/ml but slightly decreased in 0.5 mg/ml as shown in Table I. The normal range used in this study for PT assay was 12.2-14.2 seconds based upon reference from Diagnostica Stago. All PT results in different aqueous concentration were more than 14.2 seconds.

Meanwhile, the mean and standard deviation (SD) of baseline (0.0 mg/ml) APTT values were 37.2 seconds and 3.39. The mean APTT at extract concentration of 0.1mg/ml, 0.5mg/ml and 1.0mg/ml were statistically significant (p<0.05) when compared to mean APTT of baseline value. In all the aqueous concentration of extract, APTT increases at 0.1mg/ml to 1.0 mg/ml but slight decreases at 0.5 mg/ml as shown in Table II. The normal range used in this study for APTT assay was 31.7-44.0 seconds based upon Diagnostica Stago. All APTT results in different aqueous concentration were more than 44.0 seconds.

For TT, the mean and standard deviation (SD) of baseline (0.0mg/ml) was 15.0 seconds and 0.54. The mean TT at extract concentration of 0.1mg/ml, 0.5mg/ml and 1.0mg/ml were not statistically significant (p>0.05) as compared to mean TT of baseline value.

Table I: Mean clotting time of Prothrombin Time (PT) assay at different concentration of extract

Prothrombin Time (PT) Assay				
Concentration of extract (mg/mL)	**n**	**Mean clotting time (SD)**	**F-statistic (df)**	***P*-value**
0.0	36	12.3 (0.51)	139.025 (3)	<0.05
0.1		17.1 (1.28)		
0.5		16.5 (1.21)		
1.0		16.7 (1.35)		

One-way ANOVA test
Only mean clotting time between '0.0mg/ml and 0.1mg/ml', '0.0mg/ml and 0.5mg/ml' and also '0.0mg/ml and 1.0mg/ml were significantly different by post hoc test Bonferroni's procedure.

Table II: Mean clotting time of Activated Partial Thromboplastin Time (APTT) assay at different concentration of extract

Activated Partial Thromboplastin Time (APTT)				
Concentration of extract (mg/mL)	**n**	**Mean clotting time (SD)**	**F-statistic (df)**	***P*-value**
0.0	36	37.2 (3.39)	53.355 (3)	<0.05
0.1		49.0 (5.02)		
0.5		48.1 (4.94)		
1.0		49.2 (5.52)		

One way ANOVA test
Only mean clotting time between '0.0mg/ml and 0.1mg/ml', '0.0mg/ml and 0.5mg/ml' and also '0.0mg/ml and 1.0mg/ml were significantly different by post hoc test Bonferroni's procedure.

Table III: Mean clotting time Thrombin Time (TT) assay at different concentration of extract

Thrombin Time (TT)				
Concentration of extract (mg/mL)	**n**	**Mean clotting time (SD)**	**F-statistic(df)**	***P*-value**
0.0	36	15.0 (0.54)	0.491 (3)	>0.05
0.1		14.8 (0.61)		
0.5		14.9 (0.62)		
1.0		15.0 (0.66)		

One-way ANOVA test
The mean clotting time were not significant (p> 0.05) by post hoc test Bonferroni's procedure

4 DISCUSSION

Anticoagulants such as warfarin is the drug of choice to treat thrombosis. However, the main adverse effect associated with warfarin is bleeding which occurred in about 7.2 and 1.3 per 100 patient-years according to meta-analysis [12]. Apart from that, the newer anticoagulant drugs such as apixaban and edoxaban which have wider therapeutic windows, do make the laboratory monitoring more difficult. In addition, these newer drugs also have uncertainty of drugs dosing in some patient's populations such as patient with underlying chronic diseases [2]. Hence, herbal medicines are widely used throughout the world as an alternative therapy to conventional anticoagulant. Besides, herbal medicines are cost effective and has fewer complications as compared to conventional anticoagulants [1, 3].

In this study, a choice of two solvent systems (ethanol and water) were used to obtain crude extracts of *O. sanctum.* Ethanol and water are polar protic solvents which extract flavoids, alkaloids, saponins and polyphenols [13]. This study showed the percentage yield of extraction in aqueous extract was higher (13.33%) compared to ethanol extract (3.80%). In this study, *O. sanctum* was extracted using ultrasound extraction at various frequencies from 20 kHz to 2000 kHz to increase the permeability of cell wall as well as to produce the cavities. However, the problem with ultrasound extraction is that this method may cause formation of free radicals from ultrasound energy hence disturbing the drug molecules in the plant [14].

In this study, the coagulation activity of *O. sanctum* was measured based upon the basic coagulation screening test such as PT, APTT and TT. Even though two types of extracts were prepared, only aqueous extract was used to study the coagulation activity. This is because water is a universal solvent and is proven to have stronger anticoagulant/antimicrobial activities than non-polar solvent such as ethanol [15]. In 2012, Akremi and co-workers had found that PT and APTT for the aqueous extract of *Citrullus colocynthis* leaves have stronger anticoagulation activity of plasma when compared to organic extract [15]. Another study by Félix-Silva and co-workers showed that leaf extract of *Jatropha gossypiifolia* L. (Euphorbiaceae) has significant anticoagulant activity by prolonging the APTT [16].

Coagulation tests that were performed in this study were PT, APTT and TT. PT reflects the extrinsic and common coagulation pathway whereas APTT reflects the intrinsic and common coagulation pathway in haemostasis process. Another basic screening test for coagulation factor activity is international normalized ratio (INR) which was introduced to standardize the PT results across different laboratories [16]. In this study, INR was not performed because PT alone is able to reflect the clotting factor activity mainly factor VII [17]. Reduction of clotting factor activities by < 30-40% would affect both PT and APTT [18]. This study showed that both PT and APTT were significantly prolonged in aqueous concentration of *O. sanctum* from 0.1-1.0mg/mL.

The prolongation of PT and APTT might be due to presence of linolenic acid compound which exhibits anticoagulation activity [9]. According to Kadian and Parle, the linolenic acid is found in fixed oil of seeds of *O. sanctum* [19]. In a study conducted by Rehan *et al*. (2001) linolenic acid was extracted from *O. sanctum* prolonged bleeding time which is due to inhibition of platelet aggregation [9]. Meanwhile, a study carried out by Barja *et al*. (1994) showed that long term consumption of dietary linolenic acid decreases the serum thromboxane and increases the bleeding time by 40% in both humans and animals [20, 21]**.**

Another basic coagulation screening test that was performed in this study was TT which measures the fibrin polymerization process [18]. This study showed that TT changes at baseline and at different aqueous concentration of 0.1-1.0mg/ml were not statistically significant and all the measurements were within normal range. It can be concluded that there was no effect of *O. sanctum* leaf extract on fibrin polymerization process.

Thus, by considering the impacts of anticoagulant agents which may lead to morbidity and mortality, it is important that these drugs are replaced with natural solution derived from plants such as *O. sanctum* which has also showed anticoagulant properties by prolonging both the intrinsic and extrinsic clotting time pathway [18].

Different classes of phytochemical bioactive compounds were previously identified and isolated from *O. sanctum* which was associated with the pharmacological activities of the plants [22]. The absence or presence of these

bioactive compounds in ethanol and aqueous extract were based upon the chemical group type and polarity index of solvents [15]. In this study, the bioactive compounds of *O.sanctum* were analyzed via GCMS based on their retention times.

Since, the bioactive compound responsible for anticoagulation properties of *O. sanctum* was linolenic acid which was proven by previous study, the aim of GCMS analysis in this study was to identify the presence of the linolenic acid at various concentrations (1-10%) of ethanol and aqueous extract. GCMS analysis was used because it has broad application in identifying biological components in drug and vegetables metabolites [23]. However, the use High Pressure Lipid Chromatography is encouraged for future study to isolate and quantify other bioactive compounds which can be responsible for coagulation activity.

Linolenic acid was identified for 1-10% of ethanol and aqueous concentration of *O. sanctum* but at different retention time. This indicates that the linolenic acid is present in ethanol and aqueous leaves extracts despite the various concentrations without any change in the chemical properties. Fig. 3a represent the overall GCMS chromatogram of 1-10% of ethanol and aqueous extracts of the leaves *O. sanctum.* Fig. 3b showed the GCMS of linolenic acid standard.

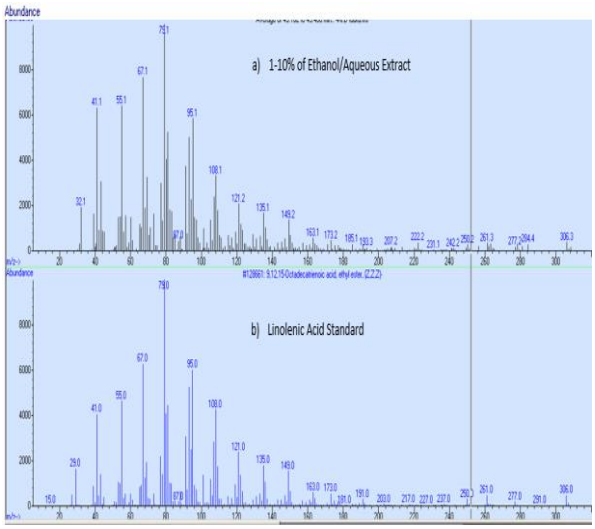

Figure 3: Overall GCMS chromatogram of (a) 1-10% of ethanol and aqueous extract of the leaves *O. sanctum* in comparison to (b) linolenic acid standard.

5 CONCLUSION

In this study, we have found that the aqueous extract of *O.sanctum* showed anticoagulation effects in human plasma by prolongation of both PT and APTT assays. However, for TT assay there was no changes in mean clotting time which indicates that *O. sanctum* does not affect the rate of conversion of fibrinogen to fibrin formation. This finding led to the conclusion that *O. sanctum* leaves may act as an anticoagulant and thus, potentially may replace the current conventional anticoagulant drugs. Despite that, further study is needed to look at which clotting factors are affected the most by *O. sanctum* leaf extract; either the intrinsic clotting factors, the extrinsic clotting factors or the common pathway coagulation factors.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENT

This study was funded by an AMDI Student Incentive and USM Research University Grant (1001/CIPPT/811322). We would like to thank Mr.Firdaus, Mr.Mohd Najwan and Dr. Rohayu Hami for their priceless effort and support.

AUTHORSHIP

All authors have made substantial contributions to all of the following: [1] the conception and design of the study, or acquisition of data, or analysis and interpretation of data **[MG, SSN and MR]**, [2] drafting the article of revising it critically for important intellectual content **[MG, SSN, MR and NAS]**, [3] final approval of the version to be summated **[MG, SSN, MR and NAS]**.

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