IN-VITRO ANTICOAGULANT EFFECT OF ORTHOSIPHON STAMINEUS BENTH EXTRACTION ON COAGULATION ASSAYS

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ABSTRACT

O.stamineus, commonly known as Misai kucing, is widely grown in Southeast Asian and usually used for herbal tea. O.stamineus contains flavonoids. Flavonoids have been reported to exhibit a wide range of biological effect, including anti-platelet actions. This can be potentially had medicinal value for an anticoagulant effect against human blood. This study aims to find out whether O.stamineus extract can affect platelet function results and to compare the effect of different concentrations of O.stamineus on platelet function using normal commercialised human plasma in vitro method. Healthy, fresh of O.stamineus leave cut into smaller part and initially dries on microwave oven at 60oC, after that blender into dried powder. Extract the O.stamineus leave into two solvent, which is methanol and aqueous. Prepare the concentration for both methanol and aqueous extraction 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Measured coagulation assays (PT,aPTT and TT) manually using the in-vitro method. The results for the coagulation test, which are PT, aPTT and TT shows all the concentration showed prolong mean for the entire test compared to control for methanol and aqueous extraction. A significant and excellent correlation between O.stamineus methanol extract and aqueous extraction concentrations PT, aPTT and TT and all p-values are significant. O.stamineus leave extract has been proven to prolong the coagulation time of PT, aPTT and TT. Further study should be proceeded to confirm which component or bioactive compound is exactly the one interfere or inhibit coagulation process.

Keywords: Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), flavonoids.

INTRODUCTION

Antithrombotic drugs or anticoagulants play an important role in preventing thromboembolic disorder such as disseminated intravascular coagulation, pulmonary embolism, strokes and heart attack. However, the use of most commonly antithrombotic drugs, heparin nowadays has several limitations and gives side effects such as bleeding complication [1].

O.stamineus, commonly known as Misai Kucing, is widely grown in Southeast Asian and the leaves of this plant are used usually for herbal tea [2]. Previous studies had shown that more than twenty phenolic compounds were isolated from *O.stamineus* plant including lipophilic flavones, flavonol glycosides and caffeic acid derivatives such as rosmarinic acid and 2, 3-dicaffeoyltartaric acid, were identified and quantified by HPLC [3-5]. Furthermore, *O.stamineus* contains Flavonoids. Flavonoids have been reported to exhibit a wide range of biological effects, including anti-inflammatory, anti-allergic, vasodilators and antiplatelet actions [6]. However, there has been no study done on the effect of *O.stamineus* on plasma coagulation. From the review of related literature, the effect of *O.stamineus* has been shown on a diuretic, and to treat rheumatism, abdominal pain, kidney and bladder inflammation, edema, gout and hypertension.

One of the possible solution to solve this problem is to find out an alternative source of anticoagulant from its natural origin. This can be implemented by investigating natural medicinal plant that potentially had medicinal value or specific plants that have an anticoagulant effect against human blood. Therefore, this study aims to investigate whether *O.stamineus* extract can affect platelet function results and to compare the effect of different concentrations of *O.stamineus* on platelet function using normal commercialised human plasma *in vitro* method.

MATERIALS AND METHODS

Plant extraction

Healthy, fresh leaves of *O.stamineus* was bought at the herbal stall in Batu Pahat, Johor and was used in this study. The *O.stamineus* leaves were cut into a smaller part and initially dry on microwave oven at 60°C for at least three days. After that, the dry *O.stamineus* leaves were ground using an electric blender into dried powder. Then, it stored in airtight closed bottles until required.

Aqueous extraction

50 grams of the powdered sample of *O.stamineus* leaves were soaked in 500 ml of distilled water (dH_2O) in a 2000 ml beaker at room temperature. The sample solution was shaken by a gentle agitation every 3 hours for 24 hours. Then, the solution was filtered into a conical flask using then Whatman no. 1 filter paper which performed at room temperature. 250ml of the obtained filtrate was boiled in a beaker until its volume reduces to 25ml. The final filtrate solution was concentrated at 10 times to get an end product.

Methanol extraction

50 grams of ground *O.stamineus* leave were weighed using analytical balance and was suspended in 500 ml of methanol (CH_3OH) in a 2000 ml beaker at room temperature. The solution was shaken and mixed by a gentle agitation in every 4 hours for 24 hours. The suspension was filtered using gauze and then Whatman no. 1 filter paper into a conical flask to remove methanol soluble material. Then, the filtrate solution was evaporated in a rotary evaporator at 60°C to remove residual solvents. Next, the product of evaporation was stored in screw bottle. The dried extract was diluted in Dimethyl Sul Foxide (DMSO) (1:50 w/v) and considered as the stock solution with 100% concentration/strength.

Measurement of coagulation assays Prothrombin time (PT), Activated partial thromboplastin time (aPTT), Thrombin time (TT)

Three clotting assays used to estimate the anticoagulant activities which are activated partial prothrombin time (PT), thromboplastin time (aPTT), and thrombin time (TT). Coagulation assays, which are PT, aPTT and TT are used as a screening test procedure to investigate the anticoagulant activity in extraction product [7]. The plasma used in this coagulation study was commercialised human plasma (Coag-Norm ®). It is in powder form and diluted with 1ml of distilled water. The clotting time in each assay was recorded manually using a stopwatch. The reason why three separate assays are used is to investigate at which stage does the blood clotting pathway is inhibited.

Normal commercialized plasma samples were mixed with different concentration of *O.stamineus* leave extract (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) by ratio of 4: 1[8].

Data Analysis

In this study, the correlations of methanol and aqueous *O.stamineus* leave extract with *in vitro* coagulation activities were examined in the mean of the extract concentration relationship with the coagulation time. The correlation results were obtained using the Pearson correlation test available in SPSS 17.0 software. A correlation test was used to measure the strength and direction of a linear relationship between the concentration of *O.stamineus* leave and plasma coagulation time (PT, aPTT, and TT). This correlation was determined by the Pearson correlation coefficient(r).

RESULTS

Coagulation test

Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) –methanol extraction

Coagulation assays PT, aPTT and TT were used as a screening test procedure to investigate the anticoagulant activity in extraction product [7]. Table 1 and Figure 1 shows the results of prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and mean for control and 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% methanol extraction of *O.stamineus* leave. The entire test is repeated two times for each concentration. The normal prothrombin time (PT) for control is 16 sec while the pathology prothrombin time (PT) for control is 25 sec. The normal activated partial thromboplastin time (aPTT) for control is 33 sec while the pathology activated partial thromboplastin time (aPTT) for control is 75 sec. The normal thrombin time (TT) for control is 14 sec while the pathology thrombin time (TT) for control is 22 sec. All the concentration showed a prolong mean for the entire test compared to control

Table 1 Levels of PT, APTT and TT at various concentrations of *O.stamineus* methanol extract compared with control

Sample		PT (sec)	aPTT (sec)	Thrombin Time (sec)
Control	Normal	16	33	14
	Pathology	25	75	22
Concentration (%)				
0	Test 1	18	37	17
	Test 2	18	33	15
	Mean	**18**	**35**	**16**
10	Test 1	19	38	44
	Test 2	19	40	42
	Mean	**19**	**39**	**43**
20	Test 1	21	45	59
	Test 2	23	45	57
	Mean	**22**	**45**	**58**
30	Test 1	27	54	90
	Test 2	25	56	90
	Mean	**26**	**55**	**90**
40	Test 1	41	78	162
	Test 2	43	80	160
	Mean	**42**	**79**	**161**
50	Test 1	44	117	195
	Test 2	44	115	193
	Mean	**44**	**116**	**194**
60	Test 1	56	200	220
	Test 2	56	200	222
	Mean	**56**	**200**	**221**

Table 1 (cont.)

Sample		PT (sec)	aPTT (sec)	Thrombin Time (sec)
70	Test 1	71	202	250
	Test 2	73	204	250
	Mean	**72**	**203**	**250**
80	Test 1	104	246	250
	Test 2	106	244	254
	Mean	**105**	**245**	**252**
90	Test 1	195	>300	>300
	Test 2	195	>300	>300
	Mean	**195**	**>300**	**>300**
100	Test 1	>300	>300	>300
	Test 2	>300	>300	>300
	Mean	**>300**	**>300**	**>300**

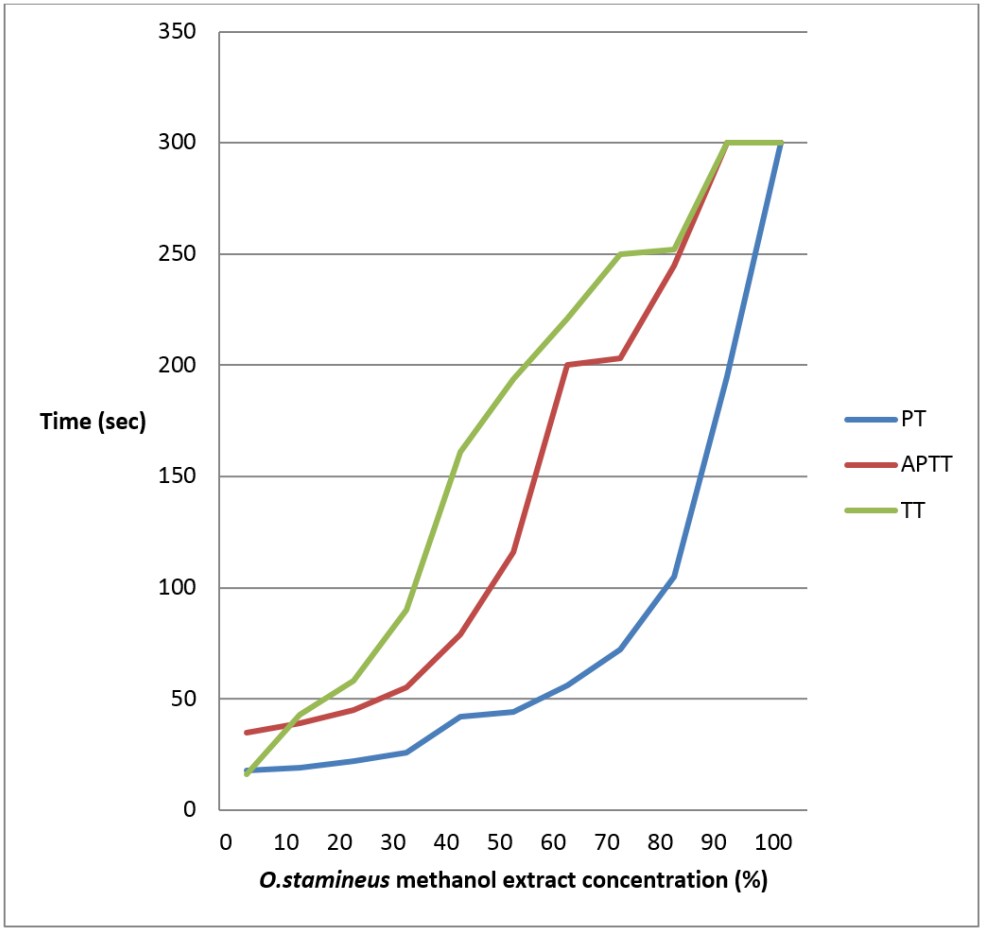

Figure 1 Line graph approximate average Prothrombin time (PT), Activated partial thromboplastin tine (aPTT) and Thrombin time (TT) of against the concentrations of *O.stamineus* methanol extract

Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) –aqueous extraction

Table 2 and Figure 2 shows the results of prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and mean for control and 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% methanol extraction of *O.stamineus* leave. The entire test is repeated two times for each concentration. The normal prothrombin time (PT) for control is 16 sec while the pathology prothrombin time (PT) for control is 25 sec. The normal activated partial thromboplastin time (aPTT) for control is 33 sec while the pathology activated partial thromboplastin time (aPTT) for control is 75 sec. The normal thrombin time (TT) for control is 14 sec while the pathology thrombin time (TT) for control is 22 sec. All the concentration showed a prolong mean for the entire test compared to control except for prothrombin time (PT). It starts to prolong after concentration of 60% to 100%.

Table 2 Levels of PT, aPTT and TT at various concentrations of *O.stamineus* aqueous extract compared with control

Sample		PT (sec)	aPTT (sec)	Thrombin Time (sec)
Control	Normal	16	33	14
	Pathology	25	75	22
Concentration (%)				
0	Test 1	12	42	18
	Test 2	14	44	22
	Mean	**13**	**43**	**20**
10	Test 1	12	45	34
	Test 2	12	45	34
	Mean	**12**	**45**	**34**
20	Test 1	13	51	37
	Test 2	13	51	37
	Mean	**13**	**51**	**37**
30	Test 1	12	53	50
	Test 2	15	53	50
	Mean	**13**	**53**	**50**
40	Test 1	14	60	51
	Test 2	14	62	53
	Mean	**14**	**61**	**52**
50	Test 1	18	69	54
	Test 2	16	69	54
	Mean	**17**	**69**	**54**
60	Test 1	23	102	55
	Test 2	23	104	55
	Mean	**23**	**103**	**55**
70	Test 1	22	125	58
	Test 2	24	125	58
	Mean	**23**	**125**	**58**

Table 2 (cont.)

Sample		PT (sec)	aPTT (sec)	Thrombin Time (sec)
80	Test 1	23	80	64
	Test 2	25	80	66
	Mean	**24**	**80**	**65**
90	Test 1	32	82	75
	Test 2	32	84	77
	Mean	**32**	**83**	**76**
100	Test 1	32	90	106
	Test 2	32	92	106
	Mean	**32**	**91**	**106**

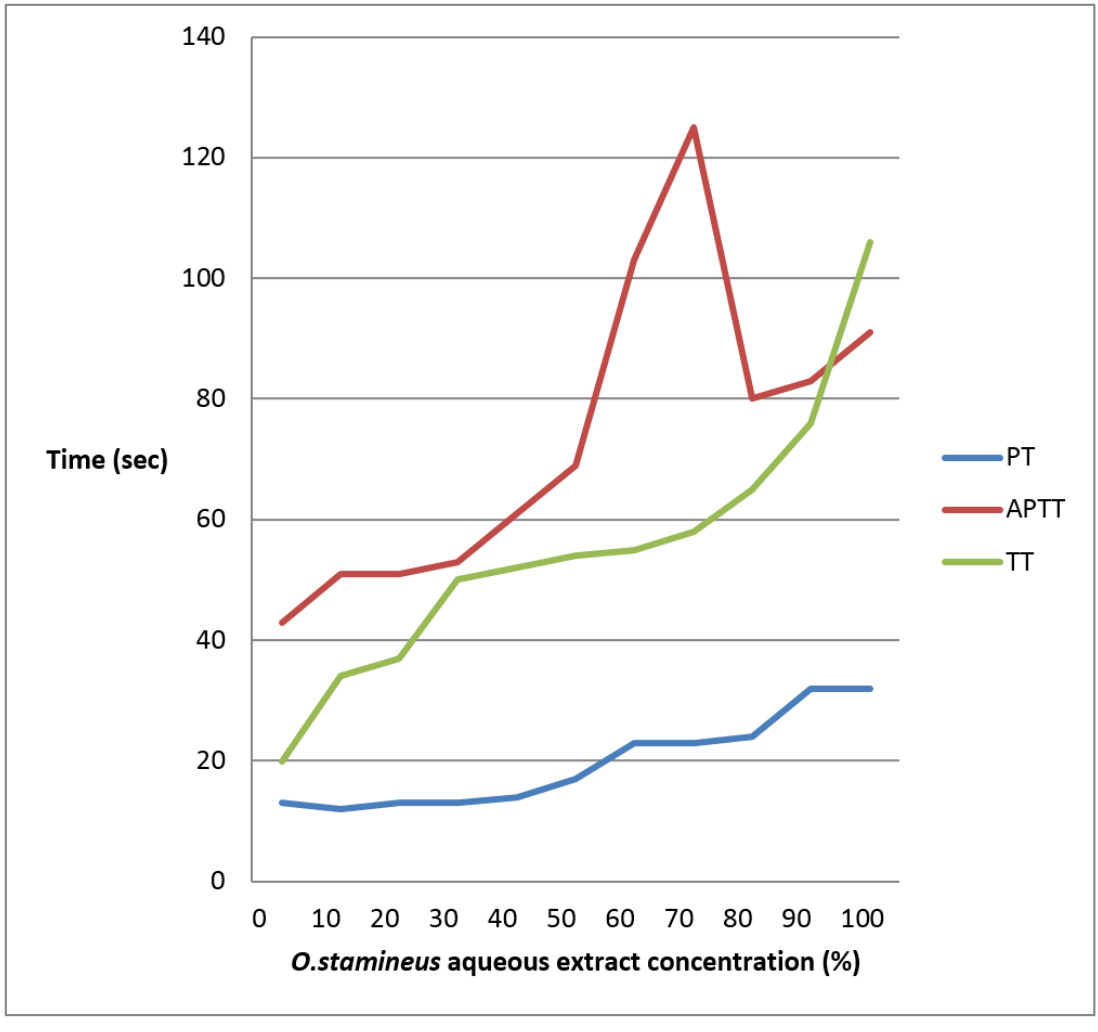

Figure 2 Line graph comparative average Prothrombin time (PT), Activated partial thromboplastin tine (aPTT) and Thrombin time (TT) of against the concentrations of *O.stamineus* aqueous extract

Correlation between concentrations of O. stamineus leaves extracts and in-vitro coagulation activities in plasma

In this study, the correlations of methanol *O.stamineus* leave extract with *in vitro* coagulation activities were examined in the mean of the extract concentration relationship with the coagulation time. The correlation results were obtained using the Pearson correlation test available in SPSS 17.0 software. A correlation test was used to measure the strength and direction of a linear relationship between the concentration of *O.stamineus* leave and plasma coagulation time (PT, aPTT, and TT). This correlation was determined by the Pearson correlation coefficient(r).

excellent correlation (r=0.969) with *O.stamineus* methanol extract concentration. In the other hand, a significant (p<0.01), perfect positive correlation (r=0.985) between *O.stamineus* methanol extract concentrations and thrombin time (TT).

As for the aqueous extraction, all the coagulation tests (PT, aPTT and TT) showed a very significant and excellent correlation with aqueous *O.stamineus* extract concentration as the p values were less than 0.01 and the Pearson correlation coefficient (r) was more significant than 0.76. The r value for PT was 0.876, aPTT was 0.769 and 0.937 for TT.

Table 3 Comparison of p-value and Pearson correlation coefficient between Prothrombin time (PT), Activated partial thromboplastin time (aPTT) and Thrombin time (TT) for methanol and aqueous extraction method

Coagulation test	P value	Pearson correlation coefficient
Methanol extraction method		
Prothrombin time (PT)	0.001	0.835
Activated partial thromboplastin time (APTT)	0.000	0.969
Thrombin time (TT)	0.000	0.985
Aqueous extraction method		
Prothrombin time (PT)	0.000	0.876
Activated partial thromboplastin time (APTT)	0.006	0.769
Thrombin time (TT)	0.000	0.937

p-value significant <0.01

Based on Table 3, there is significant (p<0.01), excellent positive correlation (r=0.835) between *O.stamineus* methanol extract concentrations and prothrombin time (PT). For activated partial thromboplastin time (aPTT), there is also a significant (p<0.01), positive

Overall, the *O.stamineus* methanol extract concentration proposed higher correlation than aqueous extract for both aPTT and TT test but not for the PT test, but still in excellent or perfect correlation.

Prothrombin time test is a screening test for haemostasis, which is performed when there is a presentation of the hemorrhagic condition such as epistaxis, ease of bleeding, haemoptysis or haematemesis. It is also done to monitor anticoagulant therapy and as pre-operative preparation to prevent from severe bleeding during or after operation [9]. The prothrombin time (PT) is a screening test to explore the integrity of extrinsic and common coagulation pathways [9]. In this study, all ten concentrations prepared from *O.stamineus* leave for methanol extraction to prolong the clotting time of plasma in prothrombin time test but for aqueous extraction, it starts to prolong at the concentration of 60% compared to the normal control. Clotting time for normal commercial plasma is used as a control, which is 16 sec. This means that the concentrations of *O.stamineus* leave affect the inhibition of extrinsic pathway in the PT test.

Activated partial thromboplastin test is a screening test for haemostasis, which is also performed when there is a presentation of hemorrhagic state and to assess anticoagulant therapy receives by the patient. However, it is in contrast with PT in which it has done to measure the intrinsic and common coagulation pathway [10]. The principle of the aPTT test involves measuring the clotting time after addition of phospholipids, calcium and intrinsic factor pathway to patient plasma [9]. In this study, methanol and aqueous extractions of *O.stamineus* leave prolong the activated partial thromboplastin time when the coagulation factor is added to the plasma. However, in aqueous extraction, the highest time for clotting at 70% concentration, which is 125 sec and goes decline in time for 80%, 90% and 100% of concentration. Maybe be, this is because the optimum or the highest clotting time at the concentration of 70% in aqueous extraction. For methanol extraction, it shows a relatively extreme prolong mean prothrombin time in every concentration and the highest clotting time are at 90% and 100% which is both are more than 300 compared to control, 33 sec.

Thrombin is an enzyme that helps in regulating the process of coagulation by having stimulatory and inhibitory feedback of its effect. It also helps in converting fibrinogen to fibrin and forming a clot as the end product. Thus, thrombin time assay is used to assess the interface of thrombin to fibrinogen cleavage [11]. The regulation of thrombin is important for controlling the action of thrombin and controlling the overall coagulation system. Thrombin time should be performed when a prolongation in PT and aPTT are idiopathic. Both of the products of methanol and aqueous method show an extreme prolongation of thrombin time compared to normal (14 sec). For methanol extraction, the mean prothrombin time of 90% and 100% concentration show the highest clotting time, which is more than 300 sec. While for aqueous extraction, 100% concentration shows the highest clotting time, which is 106 sec. This suggests that *O.stamineus leave the* methanol and aqueous extraction products can inhibit the activity of thrombin or reduced the level of thrombin in plasma.

According to the correlation test results, the *O.stamineus* leave extract produce by aqueous and methanol extraction methods gave approximately similar anticoagulant effect on the commercial plasma. However, the correlation test value for methanol extraction method is just slightly higher from the aqueous extraction method for aPTT and TT tests in term of direction and strength. A s for PT, the aqueous extraction give higher correlation test value compared to the methanol extraction method. This suggests that methanol extraction method produces an extract with a better anticoagulant effect for TT and aPTT test and aqueous extraction method produces an extract with a better anticoagulant effect for PT test.

Based on the result, all the test for both aqueous and methanol extraction give significant p-value ($p<0.01$). It means that there is a significant difference between the mean time of coagulation assays between the concentration of methanol extraction and aqueous extraction methods. This result suggests that both extraction methods products give different effect against plasma in the three assays used (PT, aPTT, and TT). The methods of chosen depend on what do we want to analyse and isolate from the leave of *O.stamineus.*

CONCLUSION

O.stamineus leave extract has been proven to prolong the coagulation time of prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). A significant and good correlation between *O.stamineus* methanol extract and aqueous extraction concentrations with prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). It shows that some compounds in leaves of *O.stamineus* did possess anticoagulant activity

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