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Full Length Research Paper

In vitro anticoagulant activities of *Melastoma malabathricum* Linn. aqueous leaf extract: A preliminary novel finding

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Limitations of existing anticoagulants have prompted a search for novel agents of natural origin. Fundamental to this research was the observation that the aqueous leaf extract of *Melastoma malabathricum* Linn. possesses potent anticoagulant property. *In vitro* coagulation assays such as activated partial thromboplastin time (aPTT), prothrombin time (PT) thrombin time (TT) and mixing studies were performed on citrated plasmas of healthy volunteer donors spiked with different concentrations of the leaf extract (100 - 1000 µg/ml). The results showed that aPTT of plasma samples spiked with extract was markedly prolonged in a concentration-dependent manner ($p < 0.001$), but was otherwise for PT and TT. Both types of mixing studies corrected the initially prolonged aPTT to normal range. The extract exhibited no inter-gender variability in its anticoagulant activity. This study highlights that the anticoagulant activity of *M. malabathricum* aqueous leaf extract affects the intrinsic pathway of the coagulation cascade by causing clotting factor(s) deficiency.

Key words: *Melastoma malabathricum* Linn., anticoagulant activity, mixing studies.

INTRODUCTION

There has been an accelerated rate of mortality and morbidity stemming from various chronic diseases worldwide over recent years. In view of this, the contribution of vascular coagulopathies to the alarming number of deaths yearly cannot be overlooked because thrombosis was identified as one of the leading causes of myocardial infarction, stroke and pulmonary embolism (Mackman, 2008). Hence, anticoagulants play a pivotal role as agents for the prevention and treatment of thromboembolic disorder (Hirsh et al., 2005; Hirsh et al., 2007). For more than five decades, anticoagulant drugs consisting of heparins, vitamin K-antagonists, and their derivatives have been the major players in the clinical setting. Although their efficacy remains undisputed, the deleterious life-threatening side effects of these drugs have also been well documented (Stone et al., 2007; Bounameaux, 2009).

The cost of developing a novel anticoagulant is also given due consideration, whereby a cheaper yet effective alternative would be of immense welcome (Spyropoulos, 2008; Pawlaczyk, 2009). In addition, there is a pressing need for an orally available anticoagulant agent to replace warfarin (Franchini and Mannucci, 2009). At present, lavish focus is being given to potent anticoagulant sources hailing from natural origin. There is compelling scientific evidences demonstrating that the consumption of dietary anticoagulants or phytochemicals with anti-coagulant properties can ultimately reduce or eliminate the risks of thromboembolic diseases (Matsubara et al.,

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2001; Guglielmone et al., 2002). New discoveries in medical science are reaffirming much of the old herbal lores in order to extend the horizons of botanical medicine. Hence, anticoagulants of natural origin furnish a good source for the aforementioned disorders (Wang, 1999).

We found in this preliminary study that one such potential candidate for anticoagulant agent is *Melastoma malabathricum* Linn. This wild shrub belongs to the family of *Melastomataceae* and can be found growing in various parts of Peninsular Malaysia (Whitmore, 1972). *Melastoma*, well-known as ‘senduduk’ among the local folks, can grow up to 13 feet high in the tropics and remains perennially evergreen (Susanti, 2007). Indigenous people use various parts of this versatile medicinal plant therapeutically to treat various maladies.

In this work, we employed a series of functional clot-based *in vitro* screening and confirmatory assays to validate its anticoagulant activity. Effects of the *Melastoma* aqueous leaf extract on inter-gender variability were also evaluated.

MATERIALS AND METHODS

Plant material

Healthy, matured green leaves of *Melastoma* were collected fresh from its natural habitat along Lebuh Silikon, University Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Young shoots and stems were not included in the sampling. A voucher specimen (SK1512/07) was authenticated by a botanist and deposited at the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, University Putra Malaysia, Serdang, Selangor, Malaysia.

Leaf extraction

Hot water extraction

Freshly procured *Melastoma* leaves were washed under tap water, air-dried and pulverized to powder. Approximately 500 g of the powdered leaves were extracted with 1 L of deionized water for 5 h at 100°C under reflux. The hot water liquid extract was cooled to room temperature (25°C) prior to filtration in a glass-sintered vacuum filter (Sartorius, Göttingen, Germany) using Whatman Grade No.1 filter papers (Whatman, USA) to remove plant debris. The filtrate was then concentrated to dryness *in vacuo* in a rotary evaporator (Büchi Labortechnik AG, Switzerland), freeze-dried and stored at -20°C until further use.

Cold water extraction

Freshly cleaned, ground leaves of *Melastoma* were macerated in deionized water (1:2 w/v) for 5 hours at room temperature (25°C). The filtration of the extract in a glass-sintered vacuum filter preceded removal of water under reduced pressure in a rotary evaporator and freeze-dried. The dried powdered form of the extract was stored at -20°C until further use.

Methanol extraction

Pulverized leaves of *Melastoma* were macerated in methanol (Ajax Finechem, Australia) of analytical grade (1:2, w/v) for 5 hours at room temperature (25°C). The organic extract was then filtered with Whatman Grade No.1 filter papers, concentrated *in vacuo* at 50°C, freeze-dried and kept at -20°C until further use.

Screening of blood donors

Blood samples were drawn from healthy volunteer donors (n = 36) of both genders (ages 18 - 50 years old) after screening *via* questionnaire for familial history of cardiovascular diseases and other major coagulopathies. Blood samples of shortlisted donors were subjected to basic hematology screening tests to exclude those with abnormal results. Written donor consent forms for blood collection were given to all respondents and signed. An ethical clearance was obtained prior to this study from the Medical Research Ethics Committee of the Faculty of Medicine and Health Sciences, University Putra Malaysia, Serdang, Selangor, Malaysia. All procedures were carried out in strict compliance with the Declaration of Helsinki (Williams, 2008).

Blood collection and plasma sample preparation

Blood samples were drawn *via* venipuncture at the *antecubital fossa* of forearm of donors (n = 36). Nine parts of the blood were mixed with one part of 0.109M trisodium citrate. Samples were centrifuged at 2000 g for 10 min at room temperature (25°C) to obtain platelet poor plasma (PPP). Plasma was separated from its cellular components and aliquots were stored in -20°C until use.

Measurements of activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT)

The activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT) assays were carried out in a STA Compact coagulation analyzer (Diagnostica Stago, Asnieres Surseine, France). Plasma samples were spiked with different concentrations of *Melastoma* leaf extracts (100 - 1000 µg/ml), heparin (Sigma, St. Louis, MO, USA) and deionized water. Deionized water was used as vehicle control (Athukorala, 2006).

In brief, a STA-PTT Automate reagent (Diagnostica Stago, France) containing a standardized amount of cephalin and silica was used to measure aPTT in the presence of 0.025 M $CaCl_2$. The STA-Neoplastine® CI Plus (Diagnostica Stago, France), which comprised of calcium thromboplastin, was used to measure PT. Finally, TT, which was used to assess fibrin clot formation in the presence of a pre-determined amount of thrombin, was measured with the STA-Thrombin (Diagnostica Stago, France) reagent.

Mixing studies

A mixing study is instrumental to determine the underlying cause for the unexplainable prolongation of aPTT or PT in plasma samples of patients in clinical settings (Chang et al., 2002). It is generated based on the corresponding assay(s), which is abnormally prolonged. In this experimental design, an aPTT mix was carried out.

Table 1. Yields of different extracts of *Melastoma malabathricum* Linn. leaf and their effects on blood coagulation parameters at 1 mg/ml.

Sample	**Yield (%)	*#aPTT (s)	*#PT (s)	*#TT (s)
Control		38.9 ± 0.5	13.3 ± 0.5	20.1 ± 0.2
Hot water	28.8 ± 0.1^{x}	180 ± 0^{x}	20.0 ± 1.3^{x}	43.2 ± 0.1^{x}
Cold water	14.3 ± 0.6^{y}	120 ± 0.9^{x}	16.4 ± 0.4^{x}	20.1 ± 0.8
Methanol	18.9 ± 0.1^{z}	108 ± 0.7^{x}	14.2 ± 1.7	21.3 ± 0.1

** Values are means of three determinations, on a dry weight basis ± SD. * Values are expressed as means ± SD (n=3). $^{x-z}$ Means of yields (%) with different superscription in same column are significantly different ($p < 0.05$). a-g Means of coagulation parameters with different superscription in each column differ significantly ($p < 0.05$) from the control. aPTT= activated partial thromboplastin time; PT= prothrombin time; TT= thrombin time; s= seconds. # For the preliminary analysis of aPTT, PT and TT, the maximum cut-off time recorded by the coagulation analyzer was 180 s.

Immediate mixing studies

Pooled normal platelet poor plasma (NPP) and PPP spiked with different concentrations of hot water *Melastoma* leaf extract (100 - 1000 µg/ml) were mixed at a ratio of 1:1. Immediately after preparation, the aPTT of the 1:1 mix was tested in the STA Compact coagulation analyzer and clotting time was recorded.

Incubated mixing studies

Normal citrated PPP (NPP) and test plasma (PPP) with different concentrations of *Melastoma* hot water leaf extract (100 - 1000 µg/ml) were incubated separately for 2 h at 37°C. Then the NPP and spiked plasma samples were mixed in a 1:1 ratio after the 2 h incubation period and aPTT of the mix was determined in an automated coagulation analyzer.

Rosner index

Rosner index for mixing studies was calculated as follows:

$$\text{Index} = \frac{\text{CT of 1:1 mix} - \text{CT of NPP}}{\text{CT of test plasma}} \times 100$$

Where,
CT = Clotting time
NPP = Normal pooled plasma

An index less than 15 (<15) indicates coagulation factor(s) deficiency in the corrected plasma samples, whereas, index more than 15 (>15) indicates the presence of an inhibitor that caused the initial prolongation of aPTT (Rosner et al., 1987).

Statistical analysis

Data are expressed as mean ± standard deviation (S. D). Statistical analysis involved a one-way analysis of variance (ANOVA) followed by post-hoc Dunett's multiple comparison for determination of differences in mean. A value of *P* less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

Extraction and screening of *Melastoma* leaf extracts for anticoagulant activities

Water and methanol were employed to extract the fresh leaves of *M. malabathricum* Linn. in this study. The yields of dried plant extracts relative to wet weight of 1 kg fresh plant materials are shown in Table 1. The results for all three extracts vary significantly ($p < 0.05$). Leafy materials extracted with hot water gave the highest yield of 28.8%. Methanol extraction yielded 18.9% of extract, followed by cold water extract with 14.3%.

Table 1 also depicts the screening results of extracts for anticoagulant activities. The blood coagulation assays routinely used in screening procedures consisted of aPTT, PT and TT (Bates and Weitz, 2005). In reference to Table 1, all three coagulation parameters were significantly prolonged ($p < 0.05$) in plasma with hot water extract compared to the normal control plasma. The PT and TT measurements were 20 ± 1.3 s and 43.2 ± 0.1 s, respectively. A striking observation was that the plasma samples with hot water extract tested for aPTT did not clot at all, as evidenced by the maximum cut-off time recorded at 180s. Both cold water and methanol extracts also prolonged aPTT in a significant fashion ($p < 0 .05$) with 120 ± 0.9 s and 108 ± 0.7 s, respectively, in comparison to the 38.9 ± 0.5 s of control plasma. Similar to the hot water extract, the PT of cold water extract was prolonging significantly ($p < 0.05$) in comparison to the control. On the other hand, these extracts did not affect the TT significantly.

Effects of hot aqueous *Melastoma* leaf extract on clot-based assays

Hot water extract was chosen based on its anticoagulant potency that surpassed the other extracts in the

screening procedure. The aPTT, PT and TT results obtained for the investigation of anticoagulant properties of hot aqueous extract of *Melastoma* leaves are presented in Figures 1, 2 and 3, respectively, with heparin as positive control. Figure 1 depicts the prolongation of aPTT in a concentration-dependent fashion from 100 - 1000 µg/ml of extract. Significant anticoagulant activity was recorded for samples with extract beyond 400 µg/ml in comparison with control (plasma sample spiked with deionized water). A most striking observation from Figure 1 is that the extract prolonged aPTT beyond 300 s at 900 and 1000 µg/ml, and this was comparable to that of 5 µg/ml heparin. The cut-off time to measure clotting times was 300 s; beyond which the plasma samples were rendered non-coagulable.

For the purpose of statistical analysis, the results were compared in relative to the control, plasma with deionized water (Jurd, 1995; Berry et al., 2002; Athukorala, 2006). The control plasma was found to record an aPTT of 64.3 and 60.7 s for females and males, respectively. On the other hand, the aPTT of normal pooled plasma (NPP) was 41.0 and 40.5 s for females and males, respectively. It is noteworthy that the aPTT of NPP were significantly different ($p < 0.05$) from the control plasma. Figures 2 and 3 illustrate the PT and TT trends exhibited by the extract and heparin at different concentrations. Contrary to aPTT, no significant effects were observed for PT and TT at the tested extract concentration range. However, TT was significantly ($p < 0.001$) prolonged at the highest concentration of extract (1000 µg/ml). Overall, the PT assay recorded the lowest coagulation inhibitory activity for hot water *Melastoma* extract.

Mixing studies

The effects of two different types of mixing studies on the aPTT profiles of male and female plasma samples spiked with a range of aqueous *Melastoma* leaf extract are as shown in Figures 4 and 5, respectively. Interestingly, the addition of 50% NPP to 50% test plasma corrected the initially prolonged aPTT approximately within 10% of the normal range (31.0 - 46.6 s). Both types of mixes, which are the immediate and timed-incubation mixings, exhibited a similar pattern of correction, without significant difference ($p > 0.05$) in their clotting times. Initially, the aPTT of 100 µg/ml extract in male plasma samples was 63.0 s. However, immediate 1:1 mix corrected the aPTT to 44.3s and the aPTT of timed mix measured 44.7s at this extract concentration. No significant variations ($p > 0.05$) existed between the immediate and timed incubation mix tests. The female plasma samples also demonstrated a similar aPTT mixing profiles (Figure 5). An initially prolonged aPTT of 69.6 s at 100 µg/ml extract was normalized to 45.6 s when subjected to immediate mixing. Likewise, the aPTT tested after incubation recorded 48.6 s, which was not significantly different from its immediate mix counterpart ($p > 0.05$). In brief, initially prolonged aPTT at all extract concentrations were normalized with the addition of equal volume of NPP and there were no significant disparities between the sexes as well as the mix types ($p > 0.05$). The Rosner index of all samples spiked with the extract were less than 15 for both male and female respondents (Table 2).

DISCUSSION

The extraction of medicinal plant materials involves the use of various solvents based on their ability to extract bioactive compounds of different solubility and polarities (Liu, 2003). However, disproportionate emphasis has been placed on the organic solvent extractants, thus overlooking the potential use of water (Raskin and Ripoll, 2004). Cognizant to this aspect, extractions using both water and organic solvent i.e. methanol were carried out in the present study. The net dried weight yields of the leaves extracted with hot water, cold water and methanol in this experiment showed that the highest yield was recovered from hot water extraction method and this too, was the extract that exhibited the most potent anticoagulant properties for all parameters tested in the *in vitro* screening. These results highlight the biochemical nature of the active compounds in *Melastoma* leaves that might contribute to anticoagulation. Since it is evident that hot water seemed to be able to extract the phytochemicals responsible for the activity, presumably, the active compounds extracted at this temperature are thermally labile.

This method of extraction using water as a solvent has been highly lauded by the phytoscience community partly due to the effort to minimize the use of organic solvents (Ong et al., 2006). In addition to the nature of water as a universal solvent, it is an attractive option owing to its environmentally friendly, non-flammable and non-hazardous attributes (Mendonça-Filho, 2006). The efficiency of hot water as the best extractant was also reported earlier for the extraction of medicinal glycosides from *Venomica longifoloa* leaves (Suomi et al., 2000) and isolation of quercetin in onion skin (Wach, 2007).

A growing fascination for natural anticoagulants discoveries stemming from the overwhelming consumer response seeking remedies devoid of unfavorable side effects has prompted the execution of this study (de Medeiros et al., 2000; Trento et al., 2001; Low, 2008). Consequently, *Melastoma* leaf extract proved a potential herbal-based anticoagulant candidate and demonstrated remarkable activities when subjected to a series of *in vitro* coagulation screening procedures. These measures

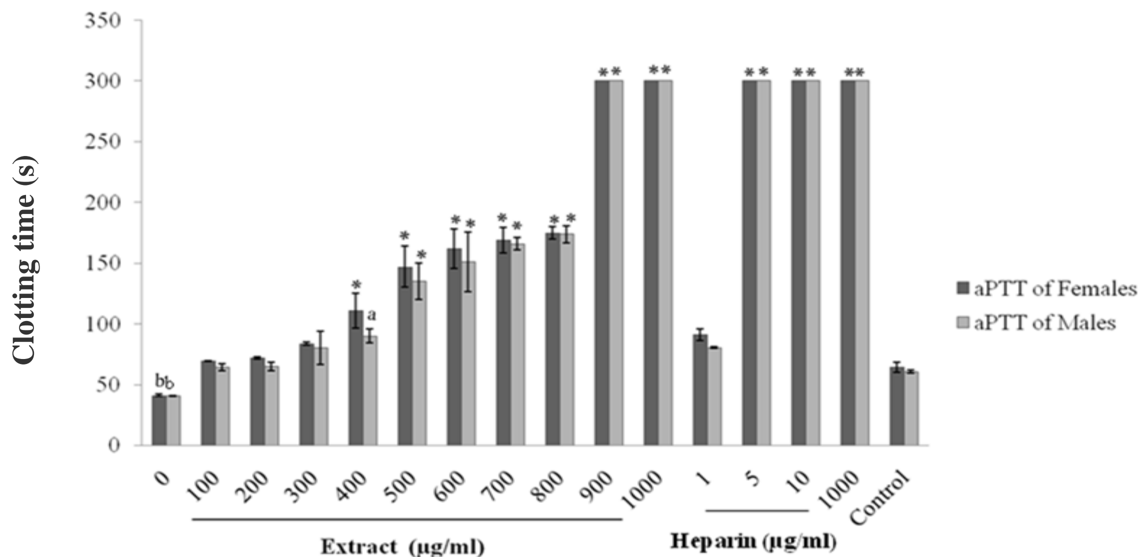

Figure 1. Activated partial thromboplastin time (aPTT) of plasma with different concentrations of *M. malabathricum* Linn. aqueous leaf extract and heparin of both male and female respondents. 0 µg/ml denotes normal control plasma without extract or heparin. Heparin sodium salt from porcine intestinal mucosa (140 USP units mg-1) used as reference. Deionized water used as control (vehicle). The bars represent the mean ± S.D. of 30 independent experiments. *p < 0.001, significance compared with control. a-b p < 0.01, significance compared with control. If no coagulation was recorded, a value of 300s was assigned for statistical analysis purposes.

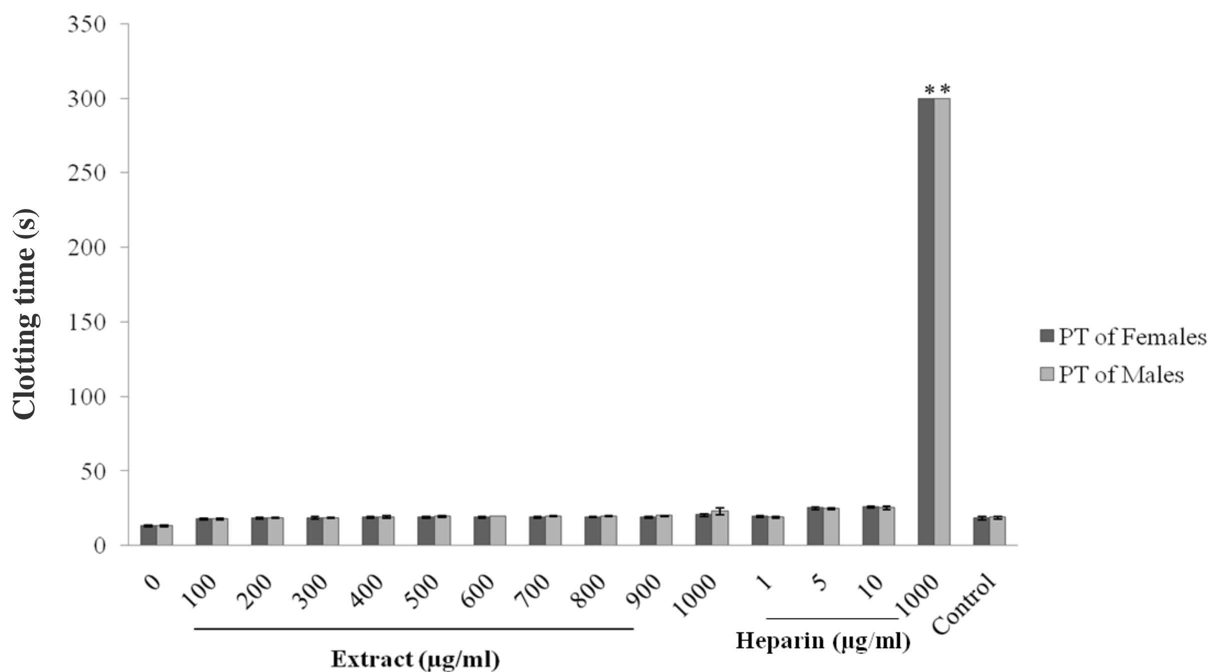

Figure 2. Prothrombin time (PT) of plasma with different concentrations of *M. malabathricum* Linn. aqueous leaf extract and heparin of both male and female respondents. 0 µg/ml denotes normal control plasma without extract or heparin. Heparin sodium salt from porcine intestinal mucosa (140 USP units mg^{-1}) used as reference. Deionized water is used as control (vehicle). The bars represent the mean ± S.D. of 30 independent experiments. *p < 0.001, significance compared with control. If no coagulation was recorded, a value of 300 s was assigned for statistical analysis purposes.

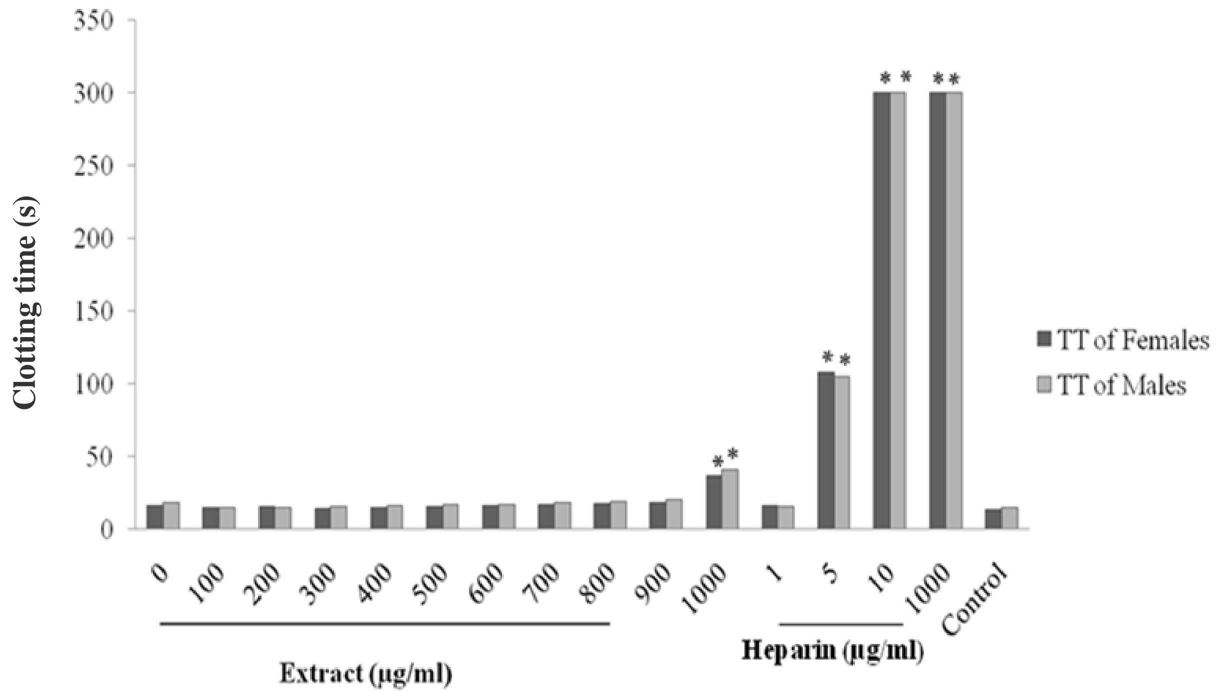

Figure 3. Thrombin time (TT) of plasma with different concentrations of *Melastoma* malabathricum Linn. aqueous leaf extract and heparin. 0 µg/ml denotes normal control plasma without extract or heparin. Heparin sodium salt from porcine intestinal mucosa (140 USP units mg^{-1}) used as reference. Deionized water is used as control (vehicle). The bars represent the mean ± S.D. of 30 independent experiments. *p < 0.001, significance compared with control. If no coagulation was recorded, a value of 300 s was assigned for statistical analysis purposes.

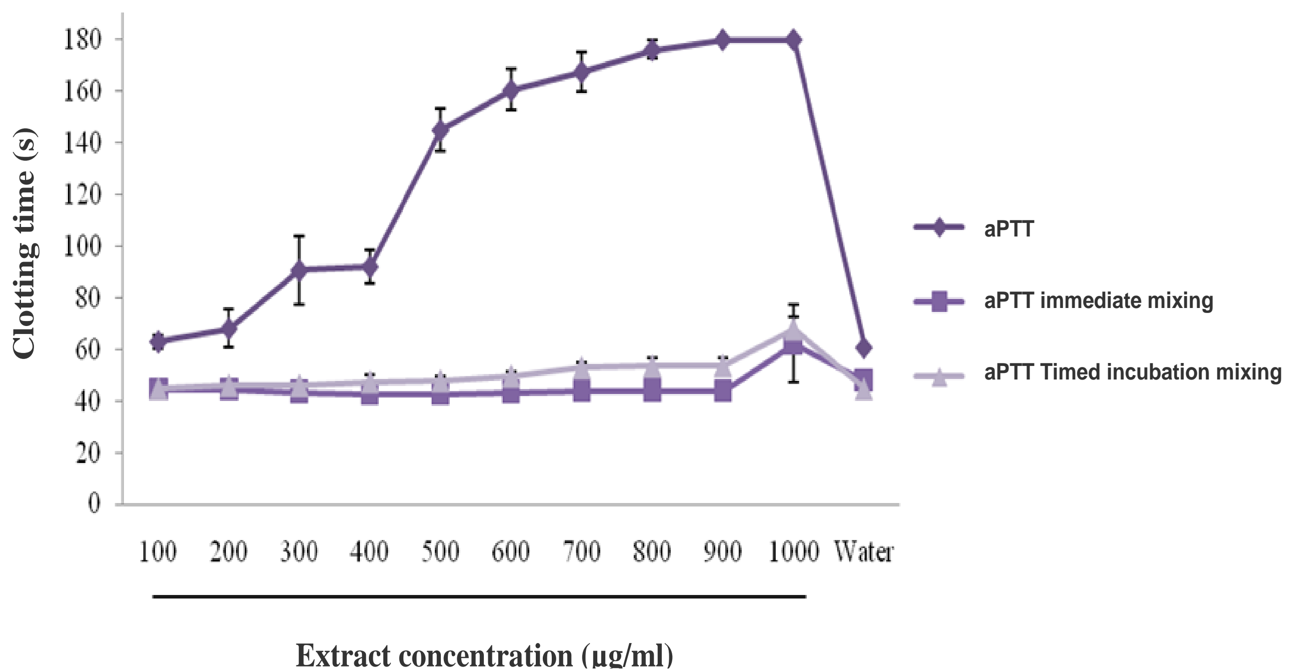

Figure 4. Activated partial thromboplastin time (aPTT) and aPTT mixing studies profiles of male respondents. The values represent the mean ± S.D. of 18 independent experiments. Deionized water is used as control vehicle.

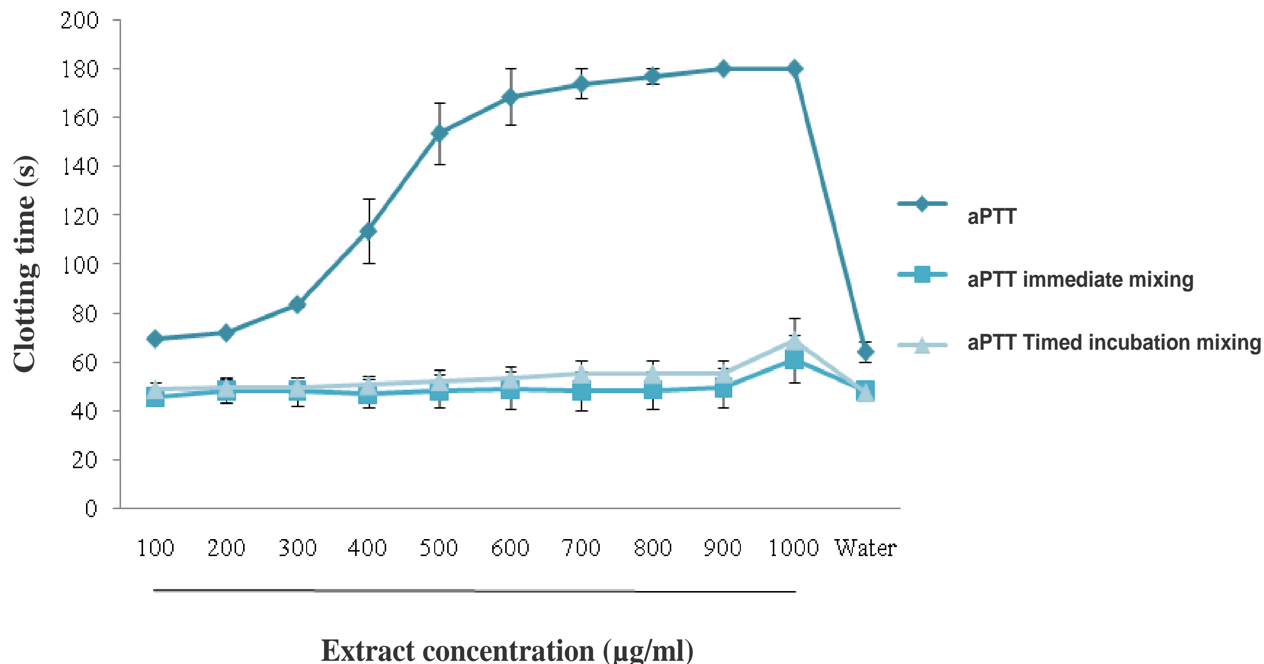

Figure 5. Activated partial thromboplastin time (aPTT) and aPTT mixing studies profiles of female respondents. The values represent the mean ± S.D. of 18 independent experiments. Deionized water is used as control vehicle.

Table 2. Rosner index for aPTT mixing studies of plasma samples spiked with different concentrations of hot aqueous *M. malabathricum* Linn. leaf extract.

Extract concentration (µg/ml)	Immediate mix*		Timed incubation mix*	
	Male	Female	Male	Female
100	4.02 ± 1.53^{aA}	6.50 ± 1.65^{aC}	6.10 ± 1.33^{aBC}	8.99 ± 0.65^{aD}
200	4.46 ± 2.33^{aA}	5.70 ± 0.16^{bC}	5.54 ± 2.10^{bAC}	7.63 ± 1.02^{aD}
300	2.51 ± 0.35^{bA}	4.07 ± 0.34^{bC}	4.63 ± 1.69^{cBC}	6.01 ± 0.66^{bD}
400	1.25 ± 1.3^{bA}	5.71 ± 5.86^{bC}	4.03 ± 0.20^{cB}	5.24 ± 1.18^{bcC}
500	1.03 ± 1.07^{bA}	4.92 ± 5.50^{bCD}	4.54 ± 0.80^{cBC}	5.14 ± 2.04^{bcD}
600	1.43 ± 0.66^{bA}	4.94 ± 5.51^{bCD}	5.23 ± 1.49^{bBC}	4.56 ± 0^{cD}
700	1.41 ± 1.17^{bA}	4.37 ± 5.42^{bC}	6.06 ± 1.6^{aB}	5.04 ± 0.47^{bcD}
800	1.68 ± 1.02^{bA}	4.31 ± 4.77^{bC}	6.24 ± 2.12^{aB}	4.75 ± 0.66^{cC}
900	1.39 ± 0.53^{bA}	4.64 ± 4.72^{bC}	3.92 ± 1.20^{cB}	5.14 ± 0^{bcC}
1000	6.8 ± 2.62^{cA}	8.33 ± 3.02^{cB}	6.94 ± 2.46^{aA}	10.30 ± 0.13^{dC}

* Values are mean ± S.D. of triplicates (n=18 respondents per group). $^{A-D}$ Means with different superscription in the same row are significantly different ($p < 0.05$). $^{a-d}$ Means with different superscription in the same column are significantly different ($p < 0.05$).

are referred to as functional clot-based assays due to their ability to assess the formation of fibrin mesh as the ultimate end-product of the clotting process (Leadley et al., 2000; Bates and Weitz, 2005). Hot aqueous extract of *Melastoma* leaves, whose anticoagulant effectiveness is comparable to that of its commercial counterpart, heparin, showed a positive concentration-dependent inhibition of clot formation with significant prolonged aPTT.

An abnormally prolonged aPTT triggered by anticoagulant agents is associated with interference in the intrinsic coagulation pathway. This test is especially sensitive to the levels of factors VIII, IX, XI, and XII and to some extent can detect deficiencies of factors X, V and II (Smythe, 2004; Tripodi et al., 2004; Hoffman and Monroe, 2005; Koch and Biber, 2007). Conversely, the PT assay monitors the integrity of coagulation proteins,

especially factor VII (FVII), in the extrinsic coagulation pathway (Duxbury and Poller, 2001). Thrombin time, as its name imply, measures the time consumed for thrombin-mediated fibrinogen conversion to fibrin clot (Gou et al., 2003).

This investigation was therefore narrowed down to the intrinsic coagulation pathway. Immediate and timed incubation mixing studies were ensued to verify the underlying nature of aPTT prolongation by the *Melastoma* extract. Theoretically, the addition of NPP with normal levels of coagulation factors to test samples that showed abnormal results, would be able to normalize the clotting time if the abnormality stemmed from factor deficiency (Ledford-Kraemer, 2004). On the contrary, this mixing does not exhibit 'correction' if an inhibitor is present in the test samples (Derksen and de Groot, 2004).
Misclassifications of patient plasmas with mild factor deficiencies as inhibitors based solely on immediate mix results are prevalent in clinical settings and this phenomenon warrants serious attention. Thus, the use of timed incubation mix type was deemed vital in our investigation to address the issue of false positives for factor deficiencies (Fritsma, 2003). Another typical case would be the reverse of the latter, in which samples with FVIII inhibitors were misdiagnosed with severe factor deficiency according to immediate mix reports. However, further investigations with timed mix method helped to correctly classify the case (Kasper, 1991; Brandt et al., 1995). Incubated mix allows time-dependent inhibitors (if any) in the test plasma to exert their effects after a period of incubation. In the current study, inhibitory activity on clotting factor(s) was ruled out as both mix types employed in this investigation were able to correct the aPTT of the test plasmas.

Finally, equal importance was given to the interpretation of data obtained from the mixing studies, apart from comparative graphical representations. The Rosner Index used to analyze the results of mixing studies relative to individual sample pointed to factor deficiency(s) as the core reason pertaining to aPTT prolongation in the plasma spiked with *Melastoma* leaf extract, thus reiterating the former theory of factor(s) deficiency. Samples with an index of <15 are considered to be deficient in coagulation factor(s) (Ledford-Kraemer, 2004) and *vice versa* for samples suspected with an inhibitor (Derksen and de Groot, 2004). This index will point to either one of these factors as the underlying cause for the abnormal prolongation of aPTT. Thus, in this study, the apparent contributing phenomenon for the abnormality in extract-spiked plasmas may duly be factor deficiency.

In conclusion, the findings from this study highlight that the *Melastoma* leaves extracted with hot water possessed the most potent anticoagulant properties comparable to heparin. This extract affects the clotting factor(s) in the intrinsic pathway by markedly reducing their levels, in concert with prolonged aPTT and corrections in mixing studies. No distinct inter-gender variations were reported for all parameters tested. Further investigations are underway to isolate the bioactive compound(s) responsible for the anticoagulant activity as well as the determination of the coagulation factor(s) affected. This natural source may be a cheaper yet potential alternative anticoagulant agent in the future owing to its abundant raw material availability and simple extraction methods.

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