

## Evaluation of Anticoagulant Property of Aqueous and Ethanolic Extracts of *Morinda citrifolia*

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**Abstract:** Anticoagulant is an agent used to treat patients with cardiovascular diseases by preventing new clots formation. It works by inhibiting blood from clotting, therefore preventing progression of thrombosis. Heparin, the animal based polysaccharides is a widely used anticoagulant has many adverse effects. This study aimed to evaluate the effects of *Morinda citrifolia* Aqueous Extract (MCAE) and Ethanolic Extract (MCEE) on plasma coagulation *in vitro*. Platelet Poor Plasma (PPP) from fifty healthy volunteers was incubated with different concentrations of extracts (10, 20, 30, 40 and 50 mg mL<sup>-1</sup>) and subjected for clotting assays of Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). The samples that been incubated by MCAE and MCEE showed prolongation of PT and APTT. These findings indicated that *Morinda citrifolia* extracts has anticoagulant effect *in vitro* and also suggest that it may become a potential plant based anticoagulant which is should be effective and safe for clinical need in dealing with patient with cardiovascular disorders.

**Key words:** *Morinda citrifolia*, plant based polysaccharide, coagulation assays, anticoagulant activity, MCAE

### INTRODUCTION

Blood coagulation is a cascade of an enzymatic event initiated in response to tissue damage which ultimately leads to the formation of blood clots. Blood coagulation factors and platelet are two main components involve in ensuring formation of stable blood clot. Dysfunction of this process especially in relation to platelet is a known risk factor of many Cardiovascular Diseases (CVDs). Animal polysaccharides especially heparin is an example of a widely used anticoagulant for the treatment and prevention of thrombosis (Johnell *et al.*, 2002). Aspirin is the most commonly used anti-platelet drugs. Warfarin is an oral anticoagulant. Studies have reported a life threatening complications associated with the usage of these drugs (Hankey and Eikelboom, 2006; Greinacher and Warkentin, 2006).

Polyphenolic-polysaccharides isolated from higher plants do not contain sulfate groups and their anticoagulant activity is due to the presence of hexuronic acids residues, like GlcA or GalA and its derivatives (Yoon *et al.*, 2002). *Morinda citrifolia* has a long history of traditional used in Hawaiian and Tahitian Islands (Samoylenko *et al.*, 2006). Major compound in *Morinda citrifolia* plants include alkaloid, scopoletin,

polysaccharides, potassium, vitamin C, terpenoids, alkaloids and anthraquinones (nordamnacanthal, morindone, rubiadin and anthraquinone glycoside) (Wang *et al.*, 2002). Some of these compounds have contributed to wide range of the therapeutic effects like antibacterial, antiviral, antifungal, antitumor, antihelminth, analgesic, anti-hypertensive, anti-inflammatory and also with immune enhancing effects of *Morinda citrifolia* (Wang *et al.*, 2002).

*Morinda citrifolia* belongs to Rubiaceae Family, the plants known as being rich in polysaccharides. Scopoletin, a coumarin group is another known major component of *Morinda citrifolia* (Wang *et al.*, 2002). Obasi had postulated Ca<sup>2+</sup> reserves scopoletin induced prolongation of blood clotting time. Heparin, a widely used anticoagulant is a muco-polysaccharide derived from porcine intestinal mucosa (Heparin, 2011). Major life threatening complication, risk of contamination with pathogen and unsuitable usage among muslim due to religion's matter are among the drawback of its usage. There is a clinical need for new plant based potent anticoagulant which is should be effective and safe. Researchers were interested in assessing the nature of anticoagulant property of the *Morinda citrifolia* fruits on human blood.

## MATERIALS AND METHODS

**Extract preparation:** Commercially available *Morinda citrifolia* extract in dry powder form was obtained from Malaysian Agricultural Research and Development Institute (MARDI).

**Ethanol extract preparation:** The ethanolic extract of *Morinda citrifolia* was prepared according to the method described by Pongnaravane *et al.* (2006) with slight modification. *Morinda citrifolia* dried fruit powder of 120 g has been extracted by maceration process. The 4000 mL 99.8% of undenatured ethanol was used as a solvent at room temperature where the ethanol was exchanged daily for 3 consecutive days. The combined filtrates were evaporated under vacuum reduced pressure at 40°C using a rotary evaporator to produce a thick syrupy mass crude extract. The crude extract was diluted in saline to prepare extract stock solutions at different concentration of 10, 20, 30, 40 and 50 mg mL<sup>-1</sup>. These stock solutions had been kept this way at 4°C.

**Aqueous extract preparation:** The aqueous extract of *Morinda citrifolia* was prepared according to the method of Thoo *et al.* (2010) with some modifications. Dried *Morinda citrifolia* fruit powder of 100 g were soaked in 1000 mL of distilled water and was shake vigorously by using a shaker for 15 min at room temperature. The mixture was then sonicated for 1 h and refrigerated for 3 days. Mixture was filtered and kept in -80°C. Then, it was subjected for freeze dry to obtain its powder form of the extract. The saline dilution of the extract at different concentration of 10, 20, 30, 40 and 50 mg mL<sup>-1</sup> were prepared and stored as extract stock solutions. These stock solutions had been kept this way at 4°C.

**Total Phenolic Content (TPC) of the extracts:** TPC of the extracts were measured by using Folin-Ciocalteu Method described by Ismail *et al.* (2004). All samples and readings were prepared and measured in triplicate. Gallic acid was used as standard. The 0.2 mg mL<sup>-1</sup> stock standard solution of gallic acid was prepared by dissolving 100 mg of dry gallic acid in 500 mL of distilled water. Working standards of between 0.025 and 0.15 mg mL<sup>-1</sup> were prepared by diluting the stock solution with distilled water. The extract was prepared at concentration of 25 mg mL<sup>-1</sup>. Total 100 µL of extract was transferred into a test tube and 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10 fold with deionised water) was added and mixed. The mixture was allowed to stand at room temperature for 5 min. Then, 0.75 mL of 6% (w/v) sodium carbonate was added to the mixture and mixed gently. After standing at room temperature for 90 min, the absorbance was read at 725 nm using UV

spectrophotometer. Results are expressed as milligrams of Gallic Acid Equivalent (GAE) per 100 g of *Morinda citrifolia*.

**Sample collection:** This study was conducted in UPM after obtaining UPM ethical approval (UPM/FPSK/PADS/T7-MJKEtikaPer/F01(LECT\_FEB\_08)01). Fifty healthy volunteers who were not on any medication including herbal or supplement were recruited. Respondents who are on medication or had history of taking any medication or supplement were excluded. Thirty volunteers (14 males and 16 females) were recruited for the ethanol based extract (MCEE). Twenty healthy respondents (10 males and 10 females) were involved in analyzing the coagulant assay of aqueous based extract (MCAE). Total 9 mL of venous blood was collected in 1 mL 3.8% trisodium citrate solution (9:1, v/v) from all respondents.

**Sample preparation:** The sample preparation was prepared according to the procedure describe by Laffan and Manning (2010). The blood sample was centrifuged at 1500 g for 10 min to obtain the Platelet Poor Plasma (PPP). The plasma was subjected for baseline coagulation assays, PT and APTT. The remaining plasma was mixed and incubated with different extract concentrations of 10, 20, 30, 40 and 50 mg mL<sup>-1</sup> (1:1, v/v) for 7 min at 37°C before subjected for PT and APTT measurements. All blood samples were tested within 3 h of blood collection.

**Assay for Prothrombin Time (PT):** The 100 µL of samples (plasma and the various plasma-extract mixtures) were added to the reaction channel which contained a reaction vial and it was incubated for 180 sec. Total 200 µL of thromborel S reagent was added to the samples. The experiments were carried out in duplicates.

**Assay for activated Partial Thromboplastin Time (APTT):** The 100 µL of samples (plasma and the various plasma-extract mixtures) were added to the reaction channel containing a reaction vial. After 180 sec incubation period, 100 µL of Actin FS reagent was added. This mixture was re-incubated for another 180 sec. About 100 µL of calcium chloride reagent was then added to activate the intrinsic clotting cascade and the time (in sec) from this addition to clot formation was defined as the APTT. The experiments were carried out in at least, duplicates.

**Statistical analysis:** One way ANOVA test was used to analyze the data. The p>0.05 will be taken as significant (SPSS, 2010).

**RESULTS AND DISCUSSION**

The present study demonstrated that both ethanol and aqueous based forms of *Morinda citrifolia* crude extracts appear to have anticoagulant property *in vitro*. Both form of *Morinda citrifolia* crude extracts showed a concentration-dependent manner anticoagulant property. Determination of total phenolics showed that both MCEE and MCAE contain rich sources of polyphenolic compounds. Their total concentration in MCEE was 244 mg GAE/100 g while total concentration in MCAE was 406 mg GAE/100 g.

**Effect of *Morinda citrifolia* extracts on PT *in vitro*:** At the lower concentrations of the extracts, no influence on clotting process was observed. The mean baseline PT values of the respondents were varied between 9.4 and 14.2 sec with the mean of 11.38±1.15 sec. The mean PT values in the presence of 10 mg mL<sup>-1</sup> of both MCEE and MCAE were 14.24±2.05 and 13.27±2.11, respectively which statistically was not significant difference (p>0.05). The anticoagulant effect was initially observed at the concentration of 20 mg mL<sup>-1</sup> where the mean PT value for MCEE and MCAE were 18.93±2.64 and 19.83±5.54 sec, respectively. Prolongation of the PT values of various concentration of both MCEE and MCAE were significant as compared to the control group with the p value of >0.05 (Table 1). PT assays were markedly prolonged with the increment of the extracts concentration of 30, 40 mg mL<sup>-1</sup> where the mean PT of MCEE was of 31.75±8.07 and 58.27±15.69 sec, respectively. Similar findings were observed with MCAE where at concentration of 30 and 40 mg mL<sup>-1</sup>, the mean PT value were 32.64±12.03 and 55.97±14.54 sec, respectively. The blood coagulometer failed to measure any reading with both form of extracts concentration of 50 mg mL<sup>-1</sup>. There were statistically significant differences of the mean PT value between the

baseline (control) as compared to the *Morinda citrifolia* crude extracts concentration of 20, 30 and 40 mg mL<sup>-1</sup> with all the p<0.05.

**Effect of *Morinda citrifolia* extracts on Activated Partial Thromboplastin Time (APTT) *in vitro*:** The baseline mean APTT values of respondents ranged between 24.1-34.3 sec with a mean of 30.06±1.27 sec. The mean APTT value in the presence of 10 mg mL<sup>-1</sup> of both MCEE and MCAE were of 33.70±3.65 and 32.08±2.16 sec, respectively. Both MCEE and MCAE did not show significant differences of the APTT assays with the concentration extracts of 10 mg mL<sup>-1</sup>. The effect of *Morinda citrifolia* crude extracts on APTT assays were as shown in Fig. 1. At the concentration of 20 mg mL<sup>-1</sup> up to 40 mg mL<sup>-1</sup>, significance differences from the baseline (control group) was observed with p<0.05. The mean APTT value of MCEE at 20, 30 and 40 mg mL<sup>-1</sup> were 46.36±7.58, 81.55±16.97 and 118.03±10.18, respectively. Similar results were observed with MCAE where the mean APTT values were 41.91±4.48, 71.29±18.27 and 114.74±11.53. At the concentration extracts of 50 mg mL<sup>-1</sup>, the blood coagulometer was failed to measure the APTT value of both forms of extracts.

Blood clotting is a complex process involving coagulation factors, platelet and collagen in blood vessels. PT and APTT assays are the common exercise to check for any disturbance in extrinsic or intrinsic pathway

Table 1: Prothrombin Time (PT) with various concentrations of both ethanol and aqueous based *Morinda citrifolia* crude extracts

Extract	Concentration (mg mL <sup>-1</sup> )	Prothrombin time (sec)
Control	-	11.38±1.15 <sup>a</sup>
MCEE	10	14.24±2.05 <sup>a</sup>
	20	18.93±2.64 <sup>b</sup>
	30	31.75±8.07 <sup>c</sup>
	40	58.27±15.69 <sup>d</sup>
	50	>60
MCAE	10	13.27±2.11 <sup>a</sup>
	20	19.83±5.54 <sup>b</sup>
	30	32.64±12.03 <sup>c</sup>
	40	55.97±14.54 <sup>d</sup>
	50	>60

Each value represents the mean±SD. The same alphabet are not significantly different (p<0.05) within groups using ANOVA test. The bold values indicate the clot was not observed in measured sample. No statistical tests were performed. MCEE: Ethanol based Extract; MCAE: Aqueous Extract

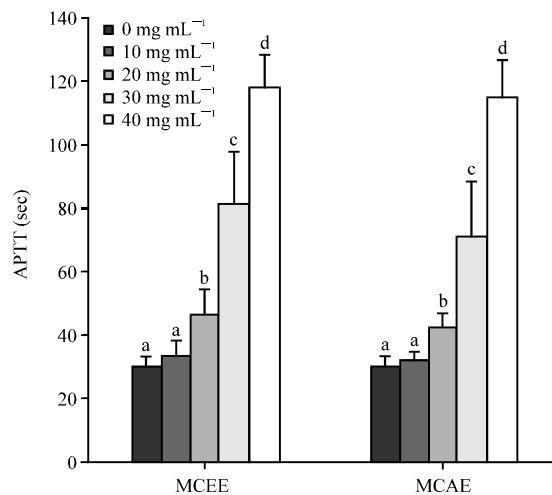


Fig. 1: APTT with various concentrations of both ethanol and aqueous based *Morinda citrifolia* crude extracts. Each value represents the mean±SD. The same alphabet are not significantly different (p<0.05) within groups. The results were analyzed using ANOVA test. MCEE: Ethanol based Extract; MCAE: Aqueous based Extract

of this complex enzymatic event, respectively. Prolongations of the assays suggest a possibility of the disturbance in this complex coagulation cascade. The PT and APTT tests are used to distinguish between the effect of the test agents on the extrinsic and intrinsic pathways (Brown, 1988). Prolongation of the PT values suggests a disturbance of the coagulation factors in the extrinsic pathway: factor V, VII, X, prothrombin and fibrinogen (Laffan and Manning, 2010). All coagulation factors except factor VII and XIII are thought to act on intrinsic pathway. Therefore, prolongation of the APTT values suggestive of wider range of coagulation factors is involved. These two clotting assays will be also affected in the presence of circulating inhibitor towards the coagulation factors and inhibition of the  $Ca^{2+}$  or phospholipids action (Laffan and Manning, 2010). Marked prolongation of the PT and APTT can also be seen with low levels of fibrinogen.

Anticoagulant is an agent that inhibits blood from clotting (Mourao and Pereira, 1999). It is used to treat patients with thrombosis by preventing new clots from forming. Anticoagulants have been widely used both in therapeutic processes and *in vitro* medical treatments (Mourao and Pereira, 1999). In medical, certain conditions require different anticoagulants with distinct characteristic. Heparin, sulfated polysaccharide, a major anticoagulant effect by inactivating thrombin and activated factor X (factor Xa) through an Antithrombin (AT) dependent mechanism. Heparin acts as a catalyst to markedly accelerate the rate at which antithrombin III (heparin cofactor) neutralizes thrombin and activated coagulation factor X (Xa). Antithrombin III generally neutralizes activated the coagulation factors by slowly and irreversibly complexing stoichiometrically with them. However, in the presence of heparin it neutralizes these factors almost instantaneously (Laffan and Manning, 2010).

The polysaccharide phenolic protein complex had been found to exhibit both anticoagulant and procoagulant effects on blood coagulation system (Pawlaczyk *et al.*, 2010). Indeed, Pawlaczyk *et al.* (2011) showed that phenolic-polysaccharide isolated from *Erigeron canadensis* L. potentially useful in anticoagulant and anti-platelet therapy. Prolongation of both PT and APTT with dose dependent concentrations of *Morinda citrifolia* crude extracts in this study probably also contributed by phenolic-polysaccharides content of it.

Polyphenolic compounds are a wide group of organic secondary plant metabolites. They have been classified into several classes including hydroxybenzoic acids, hydroxycinnamic acids, coumarins, xanthenes, stilbenes, anthraquinones, lignans and flavonoids (Manach *et al.*, 2004).

Coumarin is the organic compound of a class of naturally occurring phytochemicals found in many plant species. It is derived from variety of plants to be manipulated as chemical in particular anticoagulants. Dicumarol, a coumarin glycoside better known as warfarin is the most commonly used oral anticoagulant medication (Morton, 1992). Phenolic compounds have been found to be the major group of functional micronutrients in *Morinda citrifolia* juice. Damnacanthal, scopoletin, morindone, alizarin, aucubin, nordamnacanthal, rubiadin, rubiadin-1-methyl ether and other anthraquinone glycosides have been identified in *Morinda citrifolia* (Morton, 1992; Dittmar, 1993; Dixon *et al.*, 1999; Wang and Su, 2001). Ikeda *et al.* (2009) proposed content of scopoletin which is coumarin derivative as the quality evaluation and control of *Morinda citrifolia* products. In the blood coagulation cascade, coumarin works by inhibiting the  $Ca^{2+}$  activity. Prolongation of the coagulation assays of PT and APTT demonstrated in this study most probably caused by the interference of procoagulant action of  $Ca^{2+}$  by phenolic compounds found in these crude extracts of *Morinda citrifolia*.

## CONCLUSION

The usage of *M. citrifolia* is very wide from treating external to internal ailments. In the present research, researchers proposed that the *Morinda citrifolia* extracts has a potential plant based anticoagulant property. However, further study on the bioactive compound specific content and the exact mechanism of these *Morinda citrifolia* crude extracts and *in vivo* study need to be evaluated for further confirmation.

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