

# IN VITRO HAEMOSTATIC ACTIVITY OF RHODOMYRTUS TOMENTOSA (AITON) HASSK. AQUEOUS LEAF EXTRACT

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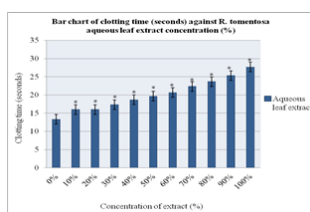
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## Graphical abstract



## Abstract

Potential of *Rhodomyrtus tomentosa* as a native anticoagulant been tested by evaluating the possible haemostatic effect of *Rhodomyrtus tomentosa* aqueous leaf extract in vitro. In vitro coagulation assays such as Thrombin time (TT), Prothrombin time (PT) and Activated partial thromboplastin time (APTT) tests were performed on normal coagulation control plasma spiked with different concentrations of the leaf extract (10 – 100%). Total tannin and flavonoid for all different concentrations of leaf extract was measured using spectrophotometer. The results were analyzed using one way ANOVA followed by post-hoc Dunnet's and Pearson's correlation. The results showed that TT, PT and APTT of normal coagulation control plasma spiked with extract were markedly prolonged. A significant anticoagulant ( $p < 0.05$ ) showed by PT was at lowest concentration (10%), TT was at concentration 50 – 100% and APTT was at concentration 80% - 100%. The clotting times for TT, PT and APTT were significantly correlated ( $p < 0.05$ ) with total tannin and flavonoid. In summary, a potent anticoagulant effect of *Rhodomyrtus tomentosa* significantly higher in extrinsic pathway (PT) and the bioactive compound of tannin and flavonoid attributes to the anticoagulant activity of *Rhodomyrtus tomentosa*.

Keywords: *Rhodomyrtus tomentosa*, anticoagulant, haemostasis, intrinsic pathway, extrinsic pathway

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## 1.0 INTRODUCTION

Thromboembolic diseases are the third most common form of acute cardiovascular diseases and second to cardiac ischemic syndromes and stroke [1]. Researchers had come up with anticoagulant and antithrombotic drugs such as heparin, aspirin and warfarin that had been widely used nowadays as therapy for Thromboembolic diseases [2]. Before the production of synthetic anticoagulant drugs, our ancestors had been used natural sources in preventing these diseases. Withdrawal of bovine product from usage, plus, the usage of porcine-based products that are strictly prohibited in Muslim religion has encouraged the researchers to seek

alternative resources for discovery of novel anticoagulant drugs. Before the production of synthetic anticoagulant drugs, our ancestors have used natural sources in prevention of diseases arising from uncontrolled clotting disorders [3]. Research found that the plants can be the alternative sources for the new anticoagulants agents due to their biological activities [4].

In respond to this, we found that the medicinal plant like *Rhodomyrtus tomentosa* (Aiton) Hassk. or known as "Kemunting" has the potential for further investigation as an anticoagulants agent. *Rhodomyrtus tomentosa* (Aiton) Hassk. is a family of Myrtaceae. It is also known as "Ceylon hill gooseberry". *Rhodomyrtus tomentosa* is a flowering plant, which native to southern and south eastern

Asia, India, east to southern China, Taiwan, Philippines, and south to Malaysia [5]. *Rhodomyrtus tomentosa* leaves are reported to be useful in medicinal in several countries all over the world and the leaves extract of *Rhodomyrtus tomentosa* are found to be useful as antifungal activity, antibacterial activity, antiulcer and antioxidant [6,7]. The leaves also have been found beneficial in treating colic, diarrhoea, dysentery, abscesses, furunculosis, and hemorrhage and the leaves of concentrated decoction are used on wounds, impetigo and abscesses as an antiseptic wash [8]. The Chinese used the leaves of *Rhodomyrtus tomentosa* as a pain killer, while in Indonesia, these leaves are used to heal wounds. In Malaysia, the decoction of *Rhodomyrtus tomentosa* plant with roots is used to treat diarrhoea and heartburn [9].

To date, no study on the haemostatic activity of *Rhodomyrtus tomentosa* been published. The present study aims to evaluate the potential of anticoagulant properties of *Rhodomyrtus tomentosa in vitro* by determining the effect of *Rhodomyrtus tomentosa* aqueous leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) and the phytochemical screenings for the different concentrations of the extract was performed to determine the relationship between phytochemical content with the haemostatic activities of the *Rhodomyrtus tomentosa*.

## 2.0 METHODOLOGY

### 2.1 Plant Material

The *Rhodomyrtus tomentosa* leaves were collected from its natural habitat from Bachok, Kelantan, Malaysia on March 2015.

### 2.2 Plant Extractions

Freshly collected *Rhodomyrtus tomentosa* leaves were washed with tap water and normal saline. Dust free leaves were dried (50°C) for 2 days and ground. 200 grams of powdered betel leaves were soaked in 800 ml aqueous solvent, kept on orbital shaker for 72 hours (3 days) at 100 rpm for cold maceration. The crude extracts were then being filtered (Whitman No.1). About 400 mL of the obtained filtrate was reduced to 40 mL with rotary evaporator at 100°C. The aqueous extracts were kept in centrifuge tube covered with aluminium foil. The final volume of the extract was 40 mL

### 2.3 Total Tannin Determination

Using gallic acid as reference, the total tannin content in *Rhodomyrtus tomentosa* leaf was determined using the Folin-Ciocalteu assay by Tamilselvi et al. [10] with slight modification. Briefly, gallic acid standard (15-75µg/ml) and extracts were

added into separate tubes each containing Folin-Ciocalteu phenol reagent and 6% sodium carbonate, mixed gently and incubated for 90 minutes at 25°C in the dark. Absorbance was measured at 725 nm (Shimadzu). For each test sample, three replicates were tested and the results are expressed as average µg gallic acid standard equivalents per 100g dry weight (µg GAE/100g DW).

### 2.4 Total Flavonoids Determination

Using quercetin as reference, according to Sahu, R. & Saxena, J. [11]. the total flavonoid content was determined using colorimetric assay. Quercetin standard or extracts was added into separate test tubes each containing 10% aluminium nitrate, 1M potassium acetate and methanol. The mixture was thoroughly mixed and incubated for 40 minutes at room temperature in the dark. Absorbance was measured using Shimadzu spectrophotometer at 510 nm (Shimadzu). For each test sample, three replicates were tested and the results are expressed as averaged µg quercetin standard equivalents per 100g dry weight (µg QUE/100g DW).

### 2.5 Determination of Blood Coagulation Parameters

Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were tested against comparable concentrations of Sahu, R. & Saxena, J. leaf extracts (20 mg/ml to 100 mg/ml) different concentrations of *Rhodomyrtus tomentosa* extract with commercial human plasma to measure the anticoagulant activity. All coagulation parameters were performed manually to determine the potential pathways that are being inhibited by the *Rhodomyrtus tomentosa* leaves extracts. Normal saline been used for negative control.

Assays for PT, APTT and TT test were performed according to manufacturer's instructions respectively (R2 Diagnostics Inc, South Bend, USA). Each assay was performed in triplicate and averaged results calculated. Anticoagulant activity was measured and results are reported as clotting time (seconds).

### 2.6 Statistical Analysis

Data collected in this study were subjected to one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's multiple comparison analysis using Statistical Package for the Social Sciences (SPSS) software. The data were considered as statistically significant when p-value was less than 0.05 ( $p < 0.05$ ). One-Way ANOVA test and post-hoc Dunnett were recruited to find the significant values between phytochemical contents (tannin and flavonoid) and coagulation times.

### 3.0 DISCUSSION AND RESULTS

The purpose of this study was to evaluate the possible haemostatic effect of aqueous leaf extract of *Rhodomyrtus tomentosa* *in vitro* and in order to discover the potential of *Rhodomyrtus tomentosa* leaf as an anticoagulant agent. The haemostatic effect of *Rhodomyrtus tomentosa* was determined by Thrombin time (TT), Prothrombin time (PT) and Activated partial thromboplastin time (APTT) tests using normal coagulation control plasma. Based on the results obtained, the aqueous extract of *Rhodomyrtus tomentosa* leaf gave positive effects on haemostasis activity where it prolonged the coagulation time for all coagulation assays TT, PT and APTT. The plasma coagulation time for all different concentration of *Rhodomyrtus tomentosa* aqueous leaf extract were compared to the plasma coagulation time of normal control (normal saline), and the results showed that the aqueous extract of *Rhodomyrtus tomentosa* leaf significantly prolonged PT at lowest concentration (Figure 2). TT test showed a significance anticoagulant ( $p < 0.05$ ) at 50% to 100% concentrations of leaf extract. While, APTT showed a significance anticoagulant ( $p < 0.05$ ) at 80% to 100% concentrations of leaf extract. It can be seen in the Figure 1 and Figure 3. This aqueous extraction showed better extraction for PT as an anticoagulant.

Turning to the comparison between coagulation times and phytochemical contents (tannin and flavonoid) showed that the coagulation times were significantly correlated with the phytochemical contents (tannin and flavonoid) as in Table 1.

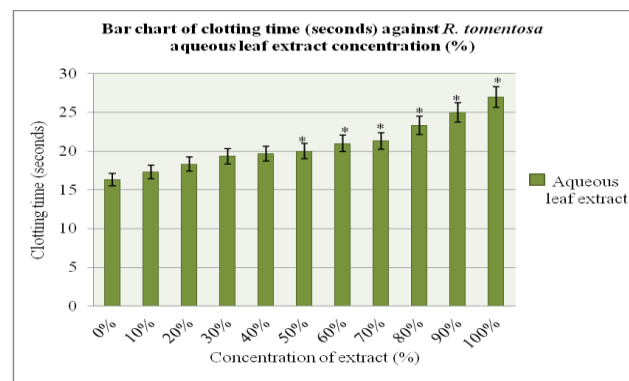
Thrombin time (TT) measures the time consumed for the conversion of thrombin-mediated fibrinogen to fibrin clot (Manicam *et al.*, 2010). The TT was significantly prolonged ( $p < 0.05$ ) at 50%, 60%, 70%, 80%, 90% and 100% of leaf extract concentrations. From the results obtained it suggests that the aqueous leaf extract of *Rhodomyrtus tomentosa* had property which can inhibit the time of fibrinogen conversion to fibrin, thus reduce the level of thrombin in plasma. Move on to Activated partial thromboplastin time (APTT) test revealed significant prolonged at the 80% concentration of aqueous leaf extract whereby as the APTT test measures the 'intrinsic' pathway and 'common' pathways of coagulation, where it assessed the action of factors XII, XI, IX, VIII, X, II and fibrinogen [12]. The higher concentration of *Rhodomyrtus tomentosa* aqueous leaf extract may had anticoagulant properties which can inhibit the activities of coagulation factors in the intrinsic coagulation pathway.

Prothrombin time (PT) test was performed to know the effects of *Rhodomyrtus tomentosa* aqueous leaf extract on coagulation factors involved in the extrinsic pathway. An abnormally prolonged the PT which triggered by anticoagulant agents is associated with the interference of the integrity of coagulation proteins in the extrinsic coagulation

pathway, especially factor VII [13]. Besides, to checks the other function of blood clotting factors involved in the extrinsic and common pathway, such as factors II, VII, X and fibrinogen [14]. In this study, PT results study suggests that *Rhodomyrtus tomentosa* aqueous leaf extract significantly interfere the extrinsic coagulation pathway and the aqueous extraction showed better extraction for PT as an anticoagulant.

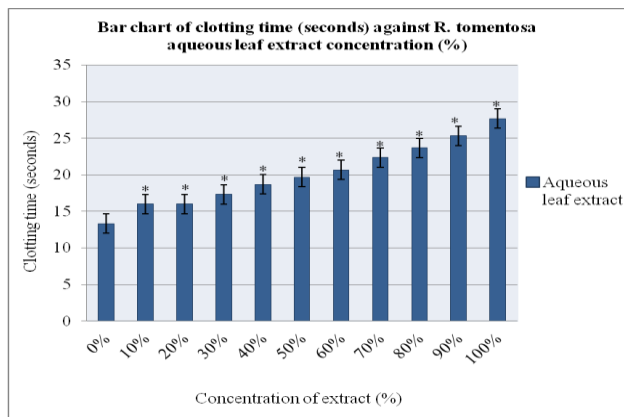
Zooming on phytochemical analysis, total tannin and flavonoid were detected in all different concentrations of *Rhodomyrtus tomentosa* aqueous leaf extract. The comparison between the total tannin and flavonoid with APTT, PT and TT revealed that there were positive relationships between them. As for the APTT, PT and TT tests, the anticoagulant activity of all different concentration of *Rhodomyrtus tomentosa* aqueous leaf extract increased with the increased of total tannin and flavonoid. When analyzed with Pearson's correlation using SPSS 18.0 version, the results showed that there were correlations between mean of APTT, PT and TT with the total tannin and flavonoid. Where, the increasing mean of APTT, PT and TT were significantly ( $p < 0.05$ ) associated with increasing the total tannin and flavonoid (not shown in this paper). The study suggested that the tannin and flavonoid contents contributed to the effect of clotting time of APTT, PT and TT tests. The phytochemical screening *Rhodomyrtus tomentosa* carried out by Lai *et al.* [15, 16] showed the presence of the flavonoid and tannin, in which they are biologically active phytochemicals. The phytochemical screenings of results obtained in this study were correlated with the report by Bamidele *et al.* in 2010 [17] in which the tannins associated with haemostatic activity of *A. conyzoides*. In 2012, Tanko *et al.* [18] revealed that high phytochemical contents of the flavonoid and of mushroom aqueous extract (*Ganoderma lucidum*) do give impact on the haemostatic mechanism in rats.

#### Thrombin Time



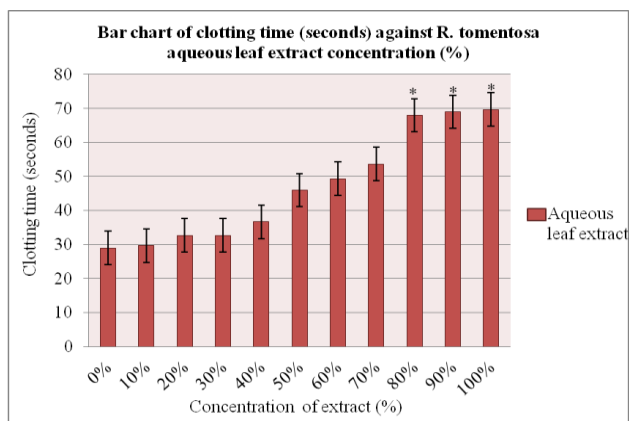
**Figure 1** Outcome findings of thrombin time test in different concentration of *Rhodomyrtus tomentosa* aqueous leaf extract tested with normal coagulation control plasma. \*p value  $< 0.05$  significance. Aqueous leaf extract of *Rhodomyrtus tomentosa* showed significance prolonged thrombin time start from 50% concentration onwards

### Prothrombin Time



**Figure 2** Outcome findings of prothrombin time test in different concentration of *Rhodomyrtus tomentosa* aqueous leaf extract tested with normal coagulation control plasma. 0% is a control value (normal saline). \*p value < 0.05 significance. Aqueous leaf extract of *Rhodomyrtus tomentosa* showed significance prolonged prothrombin time in all concentration

### Activated Partial Thromboplastin Time



**Figure 3** Outcome findings of activated partial thromboplastin time test in different concentration of *Rhodomyrtus tomentosa* aqueous leaf extract tested with normal coagulation control plasma. 0% is a control value (normal control). \*p value < 0.05 significance. Aqueous leaf extract of *Rhodomyrtus tomentosa* showed significance prolonged activated partial thromboplastin time start from 80% concentration onwards

**Table 1** Correlate the mean of thrombin time, prothrombin time and activated partial thromboplastin time with total tannin and flavonoid

| Correlation        |                            | N  | Pearson correlation (r) | p-value              |
|--------------------|----------------------------|----|-------------------------|----------------------|
| Mean time (second) | Mean concentration (µg/ml) |    |                         |                      |
| TT                 | Tannin                     | 10 | 0.835 <sup>a</sup>      | < 0.05 <sup>b</sup>  |
| PT                 | Tannin                     | 10 | 0.855 <sup>a</sup>      | < 0.05 <sup>b</sup>  |
| APTT               | Tannin                     | 10 | 0.825 <sup>a</sup>      | < 0.05 <sup>b</sup>  |
| TT                 | Flavonoid                  | 10 | 0.964 <sup>a</sup>      | < 0.001 <sup>b</sup> |
| PT                 | Flavonoid                  | 10 | 0.990 <sup>a</sup>      | < 0.001 <sup>b</sup> |
| APTT               | Flavonoid                  | 10 | 0.968 <sup>a</sup>      | < 0.001 <sup>b</sup> |

a. Correlation is significant at the 0.01 level (1-tailed)

b. The p < 0.05 indicated significant correlation.

## 4.0 CONCLUSIONS

In conclusion, *Rhodomyrtus tomentosa* leaf extracted with aqueous has potent anticoagulant properties for all coagulation test of TT, PT and APTT. The significance and prominent results were best showed in PT as potential anticoagulant. The bioactive compound like tannin and flavonoid no doubt to be responsible for the anticoagulant activity of this *Rhodomyrtus tomentosa* aqueous leaf extracts. It is recommended to further investigate the possible haemostatic effect of *Rhodomyrtus tomentosa* aqueous leaf extract *in vivo*.

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