ORIGINAL ARTICLE

In Vitro Antiatherothrombotic Effects of Rhodomyrtus Tomentosa Extract as Potential Anticoagulant From Natural Bioresources

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ABSTRACT

Introduction: Anticoagulants from natural origins can be a good source for the treatment of haemostatic disorders due to compelling scientific evidence that the consumption of dietary anticoagulants or phytochemicals with anticoagulant properties can ultimately reduce or eliminate the risks of thromboembolic diseases. This study aimed to elucidate the novel anticoagulant from natural resources; the plant rose myrtle *Rhodomyrtus tomentosa* (Aiton) Hassk.), locally known as '*kemunting*' in Malaysia. **Methods:** The antioxidant and anticoagulant properties of *Rhodomyrtus tomentosa* leaves ethanol extract (RT-EE) were investigated *in vitro*. Total phenolics (TPC) and flavonoid (TFC) contents of the RT-EE were measured using the Folin-Ciocalteu and colorimetric assays, respectively, with gallic acid and quercetin as standards. Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), and fibrinogen (FIB) were measured *in vitro* for haemostatic activity using normal coagulation plasma. **Results:** In 1 mg/mL of (RT-EE), the phenolic content was 10.82 ± 0.673 mg of gallic acid equivalent (GAE)/g, the flavanoid content was 2.40 ± 0.501 mg of quercetin equivalent (QE)/g. APTT, PT and TT were significantly ($p < 0.001$) prolonged in both RT-EE and piceatannol (PIC), with concentrations ranging from 15.62 up to 1000 mg/mL, and an average of more than 20 sec, 140 sec, 17 sec, and 20 sec, respectively. Pearson's correlation test revealed the APTT and PT tests significantly ($p < 0.001$) correlated with TPC, while TFC significantly ($p < 0.001$) correlated with PT. **Conclusion:** Both PIC and RT-EE showed antithrombotic properties, and RT-EE are attributed to TFC and TPC. Therefore, it is recommended to elucidate the antithrombotic potential through further *in vivo* studies.
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Keywords: *Rhodomyrtus tomentosa*, Haemostasis, Anticoagulant, Phytochemicals

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INTRODUCTION

The ornamental species *Rhodomyrtus tomentosa* (Aiton) Hassk. (*R. tomentosa*) belongs to the Myrtaceae family. *R. tomentosa* is found in India, China, Malaysia, the Philippines, Thailand, Vietnam and Indonesia, among other countries in Southern and Southeast Asia (1). Traditional remedies have been the primary health treatment of choice for almost 80% of the global population (2). Since ancient times, medicinal plants have been utilized due to their wide range of bioactive compounds responsible for numerous therapeutic activities to prevent and cure various types of diseases (3). According to Lai et al. (4), the leaves, buds, roots and fruits of *R. tomentosa* have long been utilised as traditional medicine in Malaysia and China. *R. tomentosa's* unripe fruits are used to cure diarrhoea and dysentery. Meanwhile, ripe fruits help the immune system function more effectively (5). Besides, *R. tomentosa* is also used to treat indigestion, gastralgia, hepatitis, enterogastristis and rheumatoid arthritis (6). In Thailand, Bangladesh and Vietnam, *R. tomentosa* has been used for inflammatory and contagious diseases such as colitis, diarrhoea, boils and haemorrhage (7).

In Singapore, the leaves are used as a pain reliever, the roots, theiate heartburn, and the seeds as a tonic and anti-venom for snake bites (8).

Previous studies have demonstrated that the Myrtaceae family possesses antioxidant and anticoagulant activities (9, 10,11). Active components of the plants derive their anticoagulant effects by acting at different sites of the coagulation cascade. The coagulation cascade is composed of intrinsic, extrinsic, and common routes that involve pro- and anticoagulant proteins, the fibrinolytic system, platelets, and the vascular endothelium, all of which are critical components of the haemostatic response (12). Depending on the three enzyme complexes of prothrombin, factor X and Vitamin K, thrombin formation is generated by a negative feedback loop through initiation and amplification stages (13).The prothrombin time (PT) test determines the levels of factor VI, X, V, prothrombin and fibrinogen via extrinsic and common pathways (14). The activated partial thrombin time (APTT) test measures factors VIII, IX, XI and XII in addition to factors X, V, prothrombin and fibrinogen via the intrinsic and common pathway. The thrombin time (Ts test carried out to determine the polymerized fibrin conversion rate from soluble fibrin by a thrombin inducer (15).

Next, anticoagulant activities have been found in a previous study, as shown by the presence of) tes phytochemicals in *R. tomentosa* (10). A recent study by Marmitt et al.(16) demonstrated an anticoagulant activity of *Myrciaria plinioides* ethanol leaves extract in extrinsic pathway, which was attributed to its high phenolic compound (17) and the plant belongs to the Myrtaceae family. *R. tomentosa* berry extracts are high in phenolics and flavonoids, according to the findings by Maskam et al. (18). On the other hand, phenolics are associated with antioxidant properties as well as health-promoting agents due to the finding of piceatannol as its principal constituent in *R. tomentosa* (19). Flavonoids possess many medicinal benefits such as anti-bacterial, antiplatelet, anti-inflammatory and others (20). Despite the fact that *R. tomentosa* has a plethora of medicinal properties recorded in the literature, its haemostatic action has received very little attention. Therefore, the purpose of this study was to assess the haemostatic activity of *R. tomentosa* ethanol extract in vitro by examining its influence on prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT). The determination of total flavonoid contents and total phenolic contents of *R. tomentosa* ethanol leaves extract were also done. This study's outcomes may be beneficial in discovering novel bioactive ingredients or natural anticoagulants. Furthermore, it may provide a better understanding of the haemostatic mechanism of *Rhodomyrtus tomentosa* (Aiton) Hassk. in humans.

MATERIALS AND METHODS

Plant material and plant extractions

The *R. tomentosa* leaves were purchased from D' Kaduk Herbs and Floral Nursery located in Kajang, Selangor (GPS; 2°58'20.1"N 101°45'40.4"E) and authenticated at the Forest Research Institute Malaysia with the voucher identification number of PID 050319-05. The freshly collected *R. tomentosa* leaves were washed with tap water and normal saline. Leaves were washed with water and oven-dried (40°C) for three days (21). Next, dried leaves were ground in a mechanical blender (Philips blender HR2094/00, Malaysia) into powder. The powdered dried leaves (50g) of *R. tomentosa* were extracted with 500 mL of 95% ethanol and were placed on an orbital shaker with a speed of 100 rpm for seven days. The solvent layers were collected and filtered using No. 1 Whatman filter paper. The filtered extracts were evaporated to dryness in a rotary evaporator at 40℃ by adjusting the pressure to less than 70 mbar to obtain the crude leaves extract. Finally, the crude leaves extract was freeze-dried until crystalline residue was formed. The obtained standardized leaves extract was denoted as *R. tomentosa* ethanolic leaves extract of (RT-EE). RT-EE extract was kept at - 20℃ until further use. Prior to the experiment, the RT-EE was diluted to four concentrations of 15.62, 62.5, 250, and 1000 µg/mL (based on efficacy and nontoxic concentrations) in normal saline to be used in the anticoagulant assays.

Phenolic content determination

Blainski's method with slight modification was used to determine the total phenolic content in RT-EE using the Folin-Ciocalteau test (22). Briefly, gallic acid standard (15-75 µg/mL) and extracts were added into tubes, each containing Folin-Ciocalteau's phenol reagent and 6% sodium carbonate. The mixture was gently shaken before being incubated in the dark for 90 minutes at 25°C. At 725 nm, the absorbance was measured (Shimadzu). The results were represented as an average of gallic acid standard equivalents per 100 g dry weight (g GAE/100 g DW) after three replicates.

Flavonoids content determination

A colorimetric test with quercetin as a reference was used to evaluate the total flavonoid concentration in RT-EE. The samples (quercetin standard or extracts) were added into separate test tubes, each containing 10% aluminium nitrate, 1 M potassium acetate and methanol. The mixtures were mixed adequately before being incubated in the dark for 40 minutes at room temperature. A spectrophotometer was used to measure the absorbance at 415 nm (Shimadzu). For each test sample, three replicates were examined, with the results given as the average of quercetin standard equivalents per 100 g dry weight (g QUE/100 g DW).

Human plasma

Commercial human plasma (REF00539, Stago)

A pool of lyophilized normal human plasma was used as the sample in this study.

Determination of blood coagulation parameters

Prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) were tested against various concentrations of RT-EE (15.625, 62.5, 250 and 1000 µg/mL) with normal human plasma to measure the anticoagulant activity. All coagulation parameters were performed manually to determine the possible inhibition pathways by the plant extracts. Lyophilized control plasma was utilised as a positive control, while normal saline was used as a negative control. The PT, APTT, and TT test assays were carried out as directed by the manufacturer (R2 Diagnostics Inc, South Bend, USA). Each assay was carried out three times, and the average of the data was calculated. The anticoagulant activity was assessed, and the results were expressed as seconds of clotting time.

Statistical analyses

Statistical Package for the Social Sciences (SPSS) software was used to do a one-way analysis of variance (ANOVA) and post-hoc Dunnet's multiple comparison analysis on the data obtained from the study. The data were presented as average ± standard deviation. A p-value of < 0.001 was used to denote statistical significance.

RESULT

Total phenolic and total flavonoids constituents of *R. tomentosa* leaves ethanol extracts (RT-EE)

Overall, the total phenolic content of *R. tomentosa* leaf extracts was higher than flavonoids, with five times flavonoid concentration (Table I). Figures 1 and 2 represent the standard curve obtained to determine the total phenolic and flavonoid in the RT-EE.

Table I – Total phenolic and total flavonoid contents of *R. tomentosa* leaf extracts (RT-EE)

Type of extraction solvent	*Total phenolics (mg GAE/g dry weight)*	*Total flavonoids (mg QUE/g dry weight)*
Ethanol	10.8 ± 0.673	2.41 ± 0.501

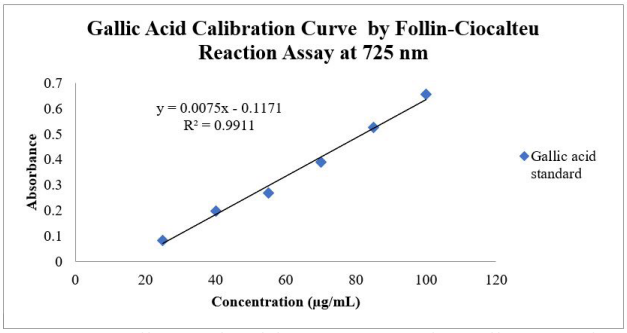

Figure 1 - Gallic acid calibration curve by Follin-Ciocalteu reaction assay at 725 nm.

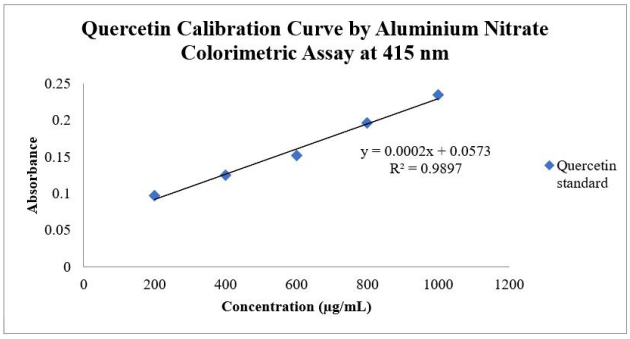

Figure 2 - Quercetin calibration curve by Aluminium nitrate colourimetric assay at 415

Screening of PT activities in piceatannol and *R. tomentosa* leaf extracts (RT-EE)

Anticoagulant activities for both piceatannol and RT-EE tested with commercial human plasma showed prolongation of prothrombin time in all concentrations tested (Figure 3). Similar prolongation times at 20 seconds were observed in 15.625, 62.5, 250 and 1000 µg/mL of piceatannol and RT-EE, respectively.

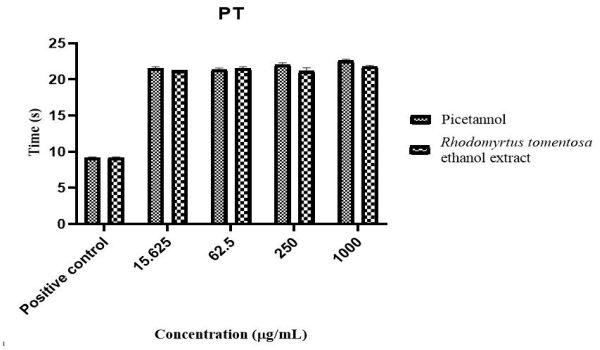

Figure 3 – Effects of *R. tomentosa* ethanol extract on prothrombin time (seconds). Prolonged prothrombin time of piceatannol and R. tomentosa ethanol extract on human plasma at 15.625, 62.5, 250 and 1000 µg/mL with $p < 0.001$ compared to positive control.

Screening of APTT activities in piceatannol and *R. tomentosa* leaf extracts (RT-EE)

Prolongation of activated partial thromboplastin time (APTT) starting from 15.625, 62.5, 250, and 1000 µg/mL of piceatannol and RT-EE extract were ten times higher than the positive control (17 seconds) (Figure 4). A similar pattern of piceatannol effect on APTT could be observed. In contrast, the RT-EE results on the APPT test were different between each concentration but were not statistically significant between each other.

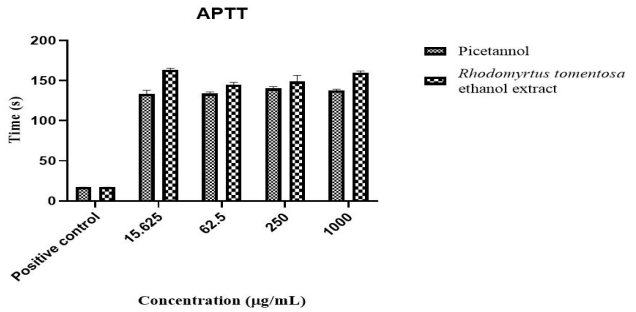

Figure 4 – Effects of *R. tomentosa* ethanol extract on activated partial thrombin time (seconds). Prolonged prothrombin time by piceatannol and *R. tomentosa* ethanol extract on human plasma at 15.625, 62.5, 250 and 1000 µg/mL with $p < 0.001$ compared to positive control.

Screening of TT activities in piceatannol and *R. tomentosa* leaf extracts (RT-EE)

As portrayed in Figure 5, thrombin times (TT) of piceatannol and RT-EE showed a profound increase in all concentrations of 15.625, 62.5, 250 and 1000 µg/mL with $p < 0.001$.

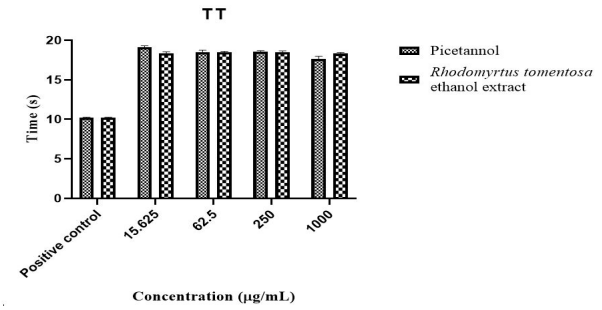

Figure 5 – Effects of *R. tomentosa* ethanol extract on thrombin time (seconds). Prolonged prothrombin time by piceatannol and *R. tomentosa* ethanol extract on human plasma at 15.625, 62.5, 250 and 1000 µg/mL with $p < 0.001$ compared to positive control.

DISCUSSION

Coagulation systems are complex mechanisms involving intrinsic and extrinsic pathways and several elements found in the plasma and tissues. Scientists are increasingly interested in developing new anticoagulant medicines from natural sources, especially because plants are widely utilized throughout the world to cure various ailments. According to Amirou et al. (23), given the existence of antithrombotic drugs with established efficacy, research into novel bioactive natural substances that interfere with platelets and plasmatic coagulation is intensifying. Anticoagulants play a vital role by affecting these pathways and reducing the risks of blood clots. There have been progressive discoveries of novel ethanol leaf extract as a potential anticoagulant alternative, as observed by PT, APTT and TT tests using commercial human plasma.

PT is a test that measures the extrinsic coagulation pathway, which consists of factors V, VII, X, prothrombin and fibrinogen, and is associated with vitamin-K dependent factors. Our study demonstrated that *R. tomentosa* ethanol extract could strongly interfere with PT for more than 20 seconds in all concentrations ranging from 15.625 – 1000 µg/mL, suggesting a powerful anticoagulant effect by *R. tomentosa*. The results are better compared to the findings by Torres-Urrutia et al. (24) for Sultanina (12.8 secs) and Torontel grapes (12.7 secs), as well as by Manicam et al. (25) for *M. malabathricum* (20.0 secs). The anticoagulant effect was achieved by inhibiting factors or components in the clotting cascade of the extrinsic pathway. Torres et al. (24) reported that the alcohol extract showed more prolongation values compared to the aqueous extract. Manicam et al. (25) claimed that the effect of aqueous extract was far superior to that of alcohol extract since it is clear that hot water may extract the phytochemicals responsible for the activity, implying that the active molecules extracted at this temperature are thermally labile.

Conversely, except for factors VII and XIII, APTT engaged in intrinsic pathway components and all clotting factors. Results for the APTT test showed increased prolongation by RT-EE compared to piceatannol. Similar findings reported that *M. malabathricum* had more effects on the intrinsic pathway (25). In a recent study, hexane extraction of *A. sarcocolla* revealed significant anticoagulant activity at higher concentrations (26). In another experiment, *Beta vulgaris* was found to have an APTT > 120 s, indicating that it had a substantial anticoagulant effect (27). Likewise, *P. granatum* altered intrinsic coagulation factors, as prolonged APTT is caused by inherent coagulation factor deficiencies (28). Next, in APTT and PT experiments, both sulfonated series displayed anticoagulant activity that increased with polymer concentrations and degree of substitution, according to a chemical reaction reported by Safa et al. (29). In agreement with Wang et al. (31), anticoagulant activity rose as polymer concentrations and substitution degrees increased; disulfonated derivatives were shown to be superior to monosulfonated derivatives.

Captivatingly, similar prolongation times were seen in the TT test, given that no significant dose-dependent effects were observed. The root bark of *Juglans regia,* on the other hand, considerably lengthened the TT and

lowered fibrinogen levels in vitro and *ex vivo* without affecting with the APTT or PT(23).

R. tomentosa is one of the medicinal plants that have been numerously studied for their phytochemical components. The present study showed that ethanol extracts yield total flavonoids constituents of 2.41 ± 0.501 g dry weight (Table I). The result can be better explained by the fact that flavonoid compounds were primarily found in 70% ethanol extraction due to their polarity (30). At the same time, total phenolic gave higher results than flavonoids at 10.8 ± 0.673 g dry weight. Flavonoids produced from plants have been shown to have antithrombotic properties (31). The antithrombotic activity can be linked to the high concentration of total polyphenols and flavonoids, as shown in other scientific reports (32). *Myrciaria plinioides* belong to the Myrtaceae family showed a significant amount of the total phenolic content (65.07 mg GAE/g extract) (33), and the ethanol leaf extract of *M. plinioides* was able to prevent the extrinsic pathway of the blood coagulation cascade whilst having no impact on the intrinsic pathway *in vivo* study. Although the extract did not directly inhibit FVIIa, it did inhibit FXa and thrombin, two of the extrinsic cascade's primary products (16).In another *in vivo* study, *Syzygium cumini*, which belongs to the Myrtaceae family, has prolonged APTT and represents the extrinsic pathway (11). In 2018, Osunsanmi et al. revealed that betulinic acid derived from Melaleuca bracteata was observed to induce thrombin platelet aggregation attenuated by the isolated compounds and aspirin, affecting the common coagulation pathway (34).

Coagulation assay results showed that piceatannol and *R. tomentosa* extract significantly prolonged all parameters (APTT, PT and TT). *Thymus atlanticus* extracts were found to prolong APTT, PT, and TT, demonstrating that they hindered the activity or synthesis of factors implicated in the intrinsic and extrinsic pathways and fibrinogen synthesis (35). Consistent with the current findings, *Bacillus mojavensis* A21 lipopeptides also prolonged the PT, APTT and TT (36). The APTT, PT, and TT were all dose-dependent by a sulfated polysaccharide from *Globularia alypum* (GASP), showing that GASP blocked both intrinsic and extrinsic blood clot pathways (37).

On three classical coagulation assays of APTT, PT, and TT, purified chondroitin sulphate showed effective *in vitro a*nticoagulant action, prolonging blood coagulation time (38). Uniquely, in this study, different concentrations did not produce varying effects, thus suggesting that the activities may not act in a dose-dependent manner.

CONCLUSION

Based on the remarkable anticoagulant activities demonstrated, it can be said that *R. tomentosa* possesses significant ($p < 0.001$) antithrombotic effects. Specifically, APTT, TT, and PT showed strong positive antithrombotic activities of *R. tomentosa* extract. This study found that *R. tomentosa* contained high levels of phenolic compounds as well as flavonoid compounds. Nevertheless, the study was conducted as a preliminary test. Therefore, further in vivo studies are needed to clarify the anticoagulant properties of *R. tomentosa.* Anticoagulant studies by using other extraction methods can also be done, thus identifying the best extraction method to produce the highest anticoagulant activities.

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