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Inhibitory Effect of Rhodomyrtus tomentosa Methanolic Leaves Extract on P-selectin Platelet Aggregation of ABO Blood Group by ELISA Method

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Abstract
In light of discovering the new antiplatelet from the natural source, this study was undertaken to determine the inhibitory effect of *Rhodomyrtus tomentosa* methanolic leaves extracts on P-selectin platelet aggregation of the ABO blood group. Whole blood samples from each blood group were collected tested with different concentration of leaves extract by using ELISA method. The inhibition was considered to occur if the values of concentration P selectin detected below 1.56 ng/ml. Instead of inhibiting the P-selectin expression as antiplatelet, *Rhodomyrtus tomentosa* accelerates the P-selectin expression as an agonist in a dose-dependent manner. In conclusion, no antiplatelet effects were seen.

Keywords: P-selectin, *Rhodomyrtus tomentosa*, platelet aggregation, ABO Blood Group

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1.0 Introduction

Thromboembolic disease is one of the leading cases that related to cardiovascular, and platelet mechanism, thus medical practitioner had produced synthetic alternative products such as heparin, warfarin and aspirin that function as antiplatelet and antithrombotic. These thromboembolic disease continue to have a severe socio-economic impact on the cost of health care to patients and their families which directly will have an impact on the quality of life (Sambamoorthi, Tan, & Deb, 2015).However, over centuries ancient ancestors commonly used herbal plants to cure disease back in time while medical practitioner found that by producing natural resource products safer and has an outstanding role in medicine. They have been either used directly applied to the injuries site or after undergoes certain chemical modification processes (Ojiako, 2014). The natural remedies have been discovered treating different kinds of diseases.

One of the natural remedies is *Rhodomyrtus tomentosa* because of the multi-benefits to cure disease. *R.tomentosa,* a flowering plant known as Kemunting in Malaysia belongs to Myrtaceae family. *R.tomentosa,*also was known as *Rose myrtle* is originated from South East Asia and the plant already discovered and utilized in traditional medicines(P. Wu et al., 2015). Various parts from this plant, from fruits, flowers, and leaves are used as a medical herb. The previous study stated that this plant contained a high volume of phenolic and flavonoids compound that plays a significant role in preventing cardiovascular diseases and can act as anti-inflammation and antioxidant. It can also use as a painkiller, treat wounds, heartburn and to treat urinary tract in China (Abd Hamid, Mutazah, Yusoff, Abd Karim, & Abdull Razis, 2017).

Thus, to determine the potential of *R.tomentosa,*as an antiplatelet agent, this study was carried out. The inhibitory effects in different concentration of antagonists were evaluated using whole blood group and focus on P-selectin expression in platelet aggregation. Therefore, ABO blood group were used as the sample to determine the inhibitory effect of the antagonist on P-selectin in platelet aggregation.

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2.0 Literature Review

2.1 Phytochemical components of *Rhodomyrtus tomentosa*

Phenolic compounds play a role in the prevention of cardiovascular diseases, diabetes mellitus and cancers. Based on previous epidemiological studies, phenolic compound plays an important role in disease associated with chronic inflammation and oxidative stress (Cicerale, Lucas, & Keast, 2012). Meanwhile, Abd Hamid et al., (2017) reported that there are present of various components in this plant including flavonoids, triterpenes and tannins that give a response in an antioxidant property. By consuming fruits that rich with antioxidant property can act as an effective agent for prevention of mortality and cancer incidence (Rahman, 2007). Besides, the previous study shows that this plant contains steroids, flavonoids and alkaloids compounds. Flavonoids and alkaloids are found in secondary metabolites and are widely used in the form of the drug to treat diabetes, skin disorders and anti-inflammation (Hasibuan, Ilyas, & Hanum, 2015). A study by Wu et al., (2015) in China revealed that *R.tomentosa* berries contain phenols, flavonoid, organic acids, quinones and other constituents are widely used as functional ingredients. It also an economical source of plants that helps in promoting health benefits. Meanwhile, flavonoids are the major group of secondary metabolites that widely found in leaves, flowers and seeds of the plant. It is already well known that flavonoids give the response in antiplatelet, anti-allergic, anti-bacterial and anti-inflammatory (Faggio et al., 2017).

2.2 P-selectin as platelet aggregation marker

P-selectin also called CD62P is a class of adhesion molecules of selectins that aid in platelet adhesion during inflammation. P-selectin is a family from CAM molecules family that associated with interaction among platelets, leukocytes and endothelial cells at the sites of vascular injury (Blann, 2014; Ho, Jou, Wu, & Hsu, 2012). There are lots of adhesion molecules which discovered platelet activation marker that is on set during inflammation such as platelet activation-dependent granule to external membrane protein (PADGEM) and granule membrane protein 140 (GMP-140). Most of previous study from (Ho et al., 2012; Lalko et al., 2003; Sloan, Sloan, & Cannon, 2006; Voss, Morgenstern, Waas, & Matzdorff, 2006; J. Wu et al., 2016), reported that P-selectin always used as detection marker in platelet aggregation and platelet activation study. This is because the soluble form of P-selectin has a physiologic function that mediates the initial leukocyte adhesion during acute inflammation and only expressed upon stimulation that makes it preferable in a study of platelet aggregation.

3.0 Methodology

3.1 Plant materials & reagents

Human sSELP (Soluble P-Selectin) ELISA kit from Elabscience company was used in this study. Two kilogram (kg) harvested *R.tomentosa le*aves were taken from DKaduk Herb & Floral Nursery, Kajang and Tanah Merah, Kelantan. The leaves were collected in box and plastic bags. The leaves were sent for identification and authentication at Forest Research Institute Malaysia (FRIM) with sample number PID 190417-11.

3.2 Preparation of *Rhodomyrtus tomentosa* leaves extract

A modification from Mordmuang & Voravuthikunchai, (2015) study, the leaves were washed and cleaned using tap water and normal saline. The leaves were dried under shade at room temperature for 2 days and grounded in an electric blender into powders. 200 grams of leaves powder were soaked in 800 ml of 99.9% methanol in 1L Schott's bottle with agitation on an orbital shaker for 72 hours (3 days) at 110 rpm. After 3 days' extraction, the extracts were filtered using Whattman filter paper No. 1 to get the pure mixture of extraction. 800 ml of filtered extracts were reduced under pressure 375rpm at 40℃ using rotary evaporator. The methanolic extracts were kept in centrifuge tube covered with aluminium foil and stored at 2-4℃ prior testing. The crude extracts approximately 5 grams were dissolved in 10% dimethyl sulphoxide (DMSO). 100% DMSO was diluted with distilled water as 1ml of 100% DMSO diluted with 9ml distilled water. 1 gram of crude extract was dissolved in 1 ml of 10% DMSO to form a stock solution. The diluted crude extracts were stored in microcentrifuge tube at 2-6℃ until used. The diluted crude extracts with 10% DMSO were considered as 100% concentration of *R. tomentosa*. Then, further dilution was obtained from the stock solution to form five different concentration of plant extract (20%, 40%, 60%, 80% and 100%).

3.3 Sample collection

Ethical approval was given by UiTM Research Committee (600-IRMI 5/1/6). Blood samples were drawn on 13 April 2018 from four healthy Medical Laboratory Technology (MLT) students, studying in UiTM Puncak Alam. All volunteers were ensured to be in good condition and healthy. Volunteers were strictly free from any medications contained aspirin or antiplatelet drugs within 2 weeks before blood collection. Volunteers were given written consent form before participation. 3ml of blood was collected in 3.2% sodium citrate tube as guided by Faculty of Health Sciences, UiTM Ethics Committee. All samples were stored at 2-8℃ until used within 5 days.

3.4 ELISA method

To study the inhibition of P-selectin expression, samples were prepared in a microcentrifuge tube with 75µl whole blood and 25µl of different concentration of *R.tomentosa* extracts. Samples were done in duplicate by each concentration leaves extract. Firstly, 75µl whole blood was pipetted in a microcentrifuge tube and 25µl of different concentration of *R.tomentosa* (20%, 40%, 60%, 80%, 100%)

was added as an antagonist to inhibit the platelet aggregation. 100µl of samples prepared were then pipetted in a microplate coated well. To detect captured P-selectin, 100µl Biotinylated Detection Ab Working Solution was pipetted in the wells after 90 minutes incubation of samples. Followed by washing 3 times with 350µl of wash buffer by soaking each well 1 to 2 minutes. 100µl HRP Conjugate working solution was added with 30 minutes incubation. The color reactions were initiated by adding 90µl Substrate reagent and stopped with 50µl Stop solution. P-selectin binding was evaluated by measuring the absorbance at 450nm on Multimode Microplate Reader (Infinite M200).

4.0 Findings

This data were presented descriptively by measuring the mean and standard deviation for each concentration of samples. Graph and table below showed the standard concentration and absorbance provided by this assay. The standard curve was plotted and concentration of samples was compared to the standard curve.

Table 1. Standard concentration and OD values

Concentration Standard (ng/ml)	**100**	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**0**
OD	2.732	1.795	0.962	0.524	0.228	0.115	0.082	0.063
Corrected OD	2.669	1.732	0.899	0.461	0.165	0.052	0.019	0

A standard curve was a plot by setting y-axis as absorbance (optical density) and x-axis as Human sSELP Concentration expressed in the reference standard and samples. The absorbance was subtracted to the average zero standard optical density to get corrected optical density. The average reading of standard and samples should be subtracted.

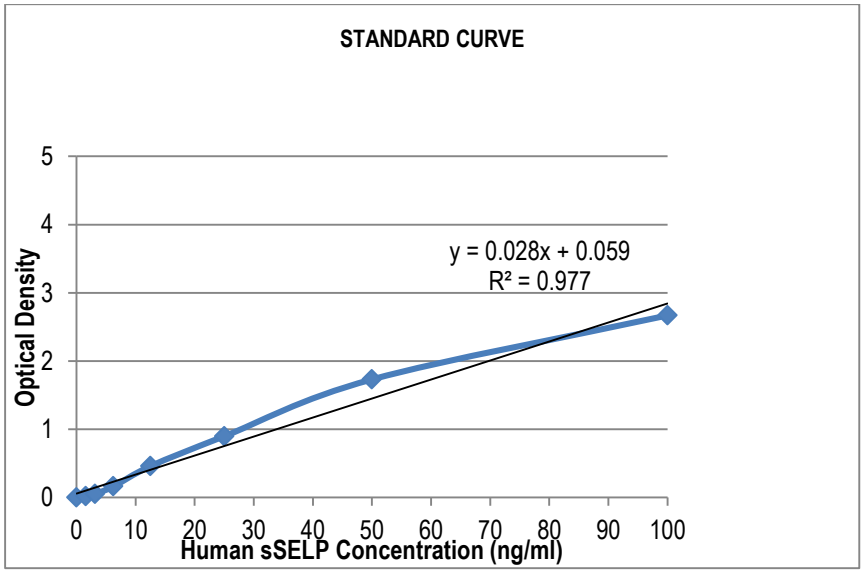

Figure 2: Standard curve of OD standard against concentration standard

Table 2. Mean concentration sample of blood group A

Concentration of leaves extract (%)	**Means of sample concentration ± Standard Deviation (SD) A1**	**Means of sample concentration ± Standard Deviation (SD) A2**	**Mean concentration samples of blood group A ± Standard Deviation (SD)**
20	55.313 ± 8.465	79.896 ± 8.597	67.605 ± 17.383
40	90.565 ± 2.218	86.658 ± 4.934	88.612 ± 2.763
60	87.680 ± 20.074	89.464 ± 8.495	88.572 ± 1.261
80	4.360 ± 0.488	36.083 ± 44.309	20.222 ± 22.432
100	5.352 ± 1.409	51.641 ± 9.233	28.497 ± 32.731

The data was tabulated descriptively by using Microsoft Excel by showing five different concentrations of *Rhodomyrtus tomentosa* leaves extract in ABO blood group towards P selectin inhibition. All the data are presented as mean and standard deviation.

Table 3. Mean concentration sample of blood group B

Concentration of leaves extract (%)	Means of sample concentration ± Standard Deviation (SD) B1	Means of sample concentration ± Standard Deviation (SD) B2	Mean concentration samples of blood group B ± Standard Deviation (SD)
20	3.241 ± 1.388	38.704 ± 9.743	20.972 ± 25.076
40	9.278 ± 4.740	48.055 ± 4.269	28.667 ± 27.420
60	54.074 ± 1.205	54.185 ± 6.495	54.130 ± 0.079
80	57.889 ± 0.629	52.185 ± 6.757	55.037 ± 4.033
100	47.889 ± 0.052	52.167 ± 12.492	50.023 ± 3.025

The data was tabulated descriptively by using Microsoft Excel by showing five different concentrations of *Rhodomyrtus tomentosa* leaves extract in ABO blood group towards P selectin inhibition. All the data are presented as mean and standard deviation.

Table 4. Mean concentration sample of blood group AB

Concentration of leaves extract (%)	Means of sample concentration ± Standard Deviation (SD) AB1	Means of sample concentration ± Standard Deviation (SD) AB2	Mean concentration samples of blood group AB ± Standard Deviation (SD)
20	25.241 ± 2.435	35.889 ± 11.104	30.565 ± 7.529
40	37.630 ± 28.494	49.185 ± 5.133	43.408 ± 8.171
60	43.685 ± 9.088	52.963 ± 0.262	48.324 ± 6.561
80	51.0925 ± 1.022	44.796 ± 4.688	47.944 ± 4.452
100	62.37 ± 21.370	42.315 ± 13.383	52.343 ± 14.181

The data was tabulated descriptively by using Microsoft Excel by showing five different concentrations of *Rhodomyrtus tomentosa* leaves extract in ABO blood group towards P selectin inhibition. All the data are presented as mean and standard deviation.

Table 5. Mean concentration sample of blood group O

Concentration of leaves extract (%)	Means of sample concentration ± Standard Deviation (SD) O1	Means of sample concentration ± Standard Deviation (SD) O2	Mean concentration samples of blood group O ± Standard Deviation (SD)
20	29.177 ± 22.078	49.788 ± 26.096	39.483 ± 14.574
40	47.702 ± 6.410	28.565 ± 19.382	38.134 ± 13.532
60	59.338 ± 10.250	21.515 ± 1.170	40.427 ± 26.745
80	41.874 ± 0.051	17.756 ± 0.839	29.815 ± 17.054
100	57.378 ± 40.087	18.439 ± 15.897	37.909 ± 27.534

The data was tabulated descriptively by using Microsoft Excel by showing five different concentrations of *Rhodomyrtus tomentosa* leaves extract in ABO blood group towards P selectin inhibition. All the data are presented as mean and standard deviation.

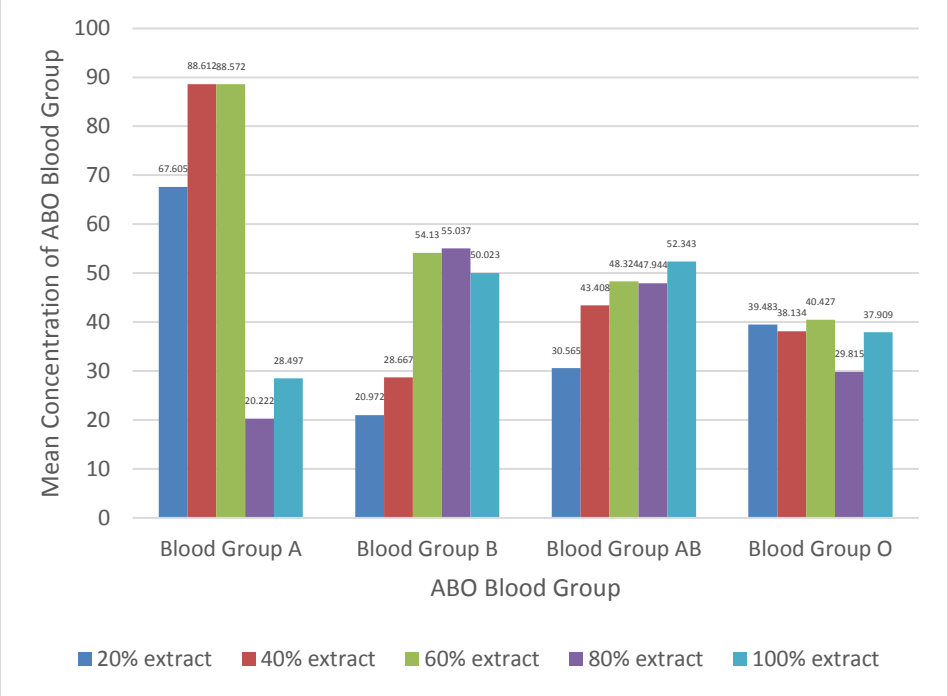

Figure 2. Mean concentration of ABO blood group
The histogram of five different concentrations of *Rhodomyrtus tomentosa* leaves extract in ABO blood group towards P selectin inhibition. All the data are presented as mean and standard deviation.

5.0 Discussion

Cardiovascular diseases are the closely related to disorder of platelet malfunction and it has been reported in many countries including Malaysia. Study by (Aziz et al., 2015) on the incidence and prevalence of acute stroke in Malaysia. Stroke can be treated by using antiplatelet agents such as aspirin. As mentioned in (Rosafio et al., 2017) noncardiogenic stroke can be treat with some of antiplatelet agent which it will reduce the risk of stroke or even death. However, it will be more risky in those patients whose resistance towards some of commercialized antiplatelet.

Guidelines for the Primary Prevention of Stroke by (Goldstein et al., 2011) mentioned that aspirin is not safely used in preventing a first stroke person who has low risk of stroke which may due to dosage suggested can make them more suffered to be in serious condition. Therefore, this study was carried out by using natural source, which is *R.tomentosa,*. By exploring the natural sources can enhance the quality of product, safely used by patients and less harmful side effects. From previous study, this plant has multiple biological properties and has potential to become natural antiplatelet drug. Study by (Olas, Wachowicz, Stochmal, & Oleszek, 2002) showed that phenolic compound in *R.tomentosa,*can give effect on platelet aggregation as the phenols will enhance the inhibitory activity. Flavonoids component present in plants can inhibit platelet aggregation mechanism (Faggio et al., 2017). Study aim to determine the inhibitory effects of different concentrations of *R.tomentosa,*leaves extract on platelet aggregation by using ELISA method. Samples P-selectin expression of ABO blood group been explored to observe inhibitory effect of extract. P-selectin expression must be suppressed upon inhibition activity respond as it is a marker of platelet aggregation.

The result of this study was evaluated by referring to the detection range provided by the kit instead referred to control that was established by a slight modification from a previous study (Salles et al., 2010). The control itself does not show the inhibitory effect, as the final color of the assay was yellow after adding stop solution, which indicates there was still present of P-selectin in control. Thus, the sample concentration cannot be determined by referring to the control. The positive control was made by adding 25µl of 2µM aspirin in 75µl whole blood respectively. The commercial aspirin was used to evaluate the inhibitory effect; however, the concentration of aspirin may not be strong enough to inhibit the P-selectin in samples. In future, the production of control can be established by increasing the concentration of aspirin to ensure the inhibition occurs.

The previous study in healthy Chinese population stated that volunteers from blood group A were detected significantly associated with lower circulating of P-selectin expression compared to another blood group (Zhang, Xu, Zhuang, & Chen, 2016). Following the study, the lower expression of P-selectin gave significant inhibition in platelet aggregation. Contradict to this study, the detection of P-selectin relatively in high expression within blood group A than other blood groups. There was no consistency reading for all samples. However, there was increasing pattern up to 60% concentration leaves extract for blood group A, B and AB. The reading drop at 80% leaves extract concentration may cause by new sample preparation of 80% and 100% concentration. The new sample preparation was prepared due to sudden clotting of the sample while loading samples into well plate. Samples blood group A, B and AB showed increasing reading pattern despite sudden drop at the particular concentration. On the other hand, the reading for samples blood group O showed contradict result from the others. The reading was decreasing from lower concentration extract of 20% to 80% concentration.

The decreasing reading may cause caffeine intake by volunteer throughout the week of blood collection. The following findings also accord with the previous study done by Bachmair, Ostertag, Zhang, & De Roos, (2014) in Netherland that stated there was caffeine effect in platelet aggregation.

Though platelet is crucial in hemostasis and wound healing, nevertheless, the previous study showed evidence of excessive platelet activation could directly relate to pathological disorders such as hypertension and risk of vascular disease. Excessive platelet can also contribute to the progression of thrombotic disease (El Haouari, López, Mekhfi, Rosado, & Salido, 2007; El Haouari & Rosado, 2008). Following a recent study by Zhang et al., (2016), the elevated expression levels of P-selectin in healthy individuals remained unexplained. This study also showed an increasing reading of P-selectin absorbance with increasing concentration of extracts. However, the expression values of P-selectin can affect by variation in the various population, varied distribution of ABO blood group within races that can contribute to the differential expression level.

Moreover, several factors might be interfered the assay reading such as temperature, incubation time, blood collection and preparation of leaves methanolic extract (Qi, Yatomi, & Ozaki, 2001). A constant temperature during incubation or while preparing sample is important to ensure the bioactive compounds in samples is in good condition. It is possible, after blood collection, the metabolic condition of platelet were altered due to shut off of oxygen, glucose and other essential factors supplied for metabolic maintenance of platelet. On the other hand, while performing ELISA, the timing between sample loading and incubation may give slight interference to the reading. Besides, the preparing of the leaves also extracts crucial to maintaining the particular phytochemical components in the leaves extracted by methanol during soaking. The soaking time also plays a major role to ensure the complete soaking occur, too long, too short timing or too high concentration of methanol may affect the extraction.

6.0 Conclusion & Recommendation

In conclusion, the findings showed no inhibitory effect on P-selectin expression in samples by this plant. Samples showed increasing pattern in P-selectin expression by this ELISA kit for each concentration of leaves extract. From the result obtained, *R.tomentosa,*leaves extract did not show any antiplatelet properties in contrast with the evidence provide by previous study stated this plant can be antiplatelet agent. In other words, this finding can be suggested to be natural accelerator for platelet aggregation study as it reveals that instead of inhibition effect, *R.tomentosa,*leaves extract can act as agonist due to increasing P selectin expression. As recommendation, further study on this plant can be done by using either in vivo or in vitro method.

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