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Guava leaf juice effect towards number of megakaryocytes in bone marrow of thrombocytopenic mice

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ABSTRACT

BACKGROUND
Dengue virus infection that most frequently occur in tropical and subtropical regions can cause many symptoms, one of which is a decrease in thrombocyte count. Recent studies showed that guava leaf extract can increase the thrombocyte count in rats. The present study aimed to determine the effect of guava leaf juice in increasing the number of megakaryocytes in the bone marrow of thrombocytopenic mice.

METHODS
This study was of experimental design. The study subjects were 24 mice (*Mus musculus*). The mice were randomly divided into four groups that were subjected to intervention for 14 days. Group 1 was given guava leaf juice (56 mg/kg) and quinine (14 mg/kg), group 2 guava leaf juice (56 mg/kg) only, group 3 was given quinine (14 mg/kg) and group 4 distilled water. After 14 days, from the bone marrow of the femoral bones of each of the mice, hematoxylin eosin stained histologic preparations were made. Anova test was used to analyze the data.

RESULTS
The mean megakaryocyte count per field of view in group 1 (2.83), group 2 (3.30), group 3 (2.24) and group 4 (2.93). Anova test results for all groups showed significant differences between groups ($p<0.05$). The mean megakaryocyte count was increased in group 1 compared to group 3, but the difference was statistically not significant ($p=0.206$).

CONCLUSION
Guava leaf juice can increase the megakaryocyte count in the bone marrow of thrombocytopenic mice. This suggests a potential role of guava leaf juice in improving the platelet count in thrombocytopenic disorders.

Keyword: Guava leaf juice, megakaryocyte, mice, thrombocytopenia.

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INTRODUCTION

Dengue virus (DENV) is transmitted by *Aedes aegypti* mosquitoes, especially in tropical and subtropical regions. This virus can cause dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome. In 2010 there were 2.2 million case reports worldwide and in 2015 this number increased to 3.2 million cases. [1-3] There were approximately 50 to 100 million symptomatic cases every year in Asia, followed by Latin America and Africa, and these countries became the regions with the greatest number of cases, which is 75% of total cases every year. [4,5] In Indonesia there were 126,675 DHF cases in 2015, of which 1,229 died, whereas in 2014 there were 100,347 cases, of which 907 died.[6] Thrombocytes come from megakaryocytes as precursors, which are giant discoid shaped cells with diameters of 1-3 micrometers and are usually found in bone marrow.[7,8] DENV can cause thrombocytopenia by infecting endothelial cells in bone marrow. The infected cells become necrotic, release toxic cells and activate the coagulation and fibrinolytic systems that can reduce hemopoiesis and endothelial cell thrombogenicity.[3] In addition, DENV infection induces platelet consumption due to disseminated intravascular coagulation, increases platelet destruction through apoptosis, lysis by the complement system and by the involvement of antiplatelet antibodies.[9-11]

The early phase of DHF displays hypocellularity and attenuation of megakaryocyte maturation. This can be caused by direct lesions of progenitor cells by DENV, infected stromal cells and changes in bone marrow regulation. In thrombocytopenia there are increased amounts of thrombopoietin (TPO), a cytokine that binds to the TPO receptor c-MPL (myeloproliferative leukemia virus oncogene) to regulate megakaryopoiesis.[9]

Psidium guajava is a tropical plant of the *Myrtaceae* family that can grow to 35 feet, and is recognizable by its brown and peeled off bark. Guava leaves contain carotenoids, anthocyanins, essential fatty acids, lecithins, alkaloids, phenols, saponins, tannins, triterpenes, flavonoids (particularly quercetin) and vitamin C.[12,13] Quercetin acts as an antioxidant that affects cell differentiation. Quercetin can also increase the cytokines granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) that stimulate megakaryopoiesis and can increase the number of megakaryocytes.[12,14,15] A previous study showed that guava leaf juice extract can increase the number of thrombocytes in rats.[14]

Another report showed that guava leaf extract increases the number of platelets in cyclophosphamide-induced thrombocytopenia in rats. However, the role of megakaryocytes in increasing the platelets is still poorly understood. [16,17]

To evaluate the effect of guava leaf juice on the role of megakaryocytes in thrombocyte production, we performed this study to determine the megakaryocyte count in the bone marrow of the femoral bones of thrombocytopenic mice after guava leaf juice administration.

METHODS

Study design

The study was of experimental design and was carried out at the Division of Cell Biology, Department of Anatomy, Physiology and Cell Biology, Faculty of Medicine and at the Biosystems Laboratory, Faculty of Mathematics and Science, Padjadjaran University, from January-August 2017.

Study subjects

The subjects of this study were 24 mice (*Mus musculus*) divided into four groups. The number of subjects was obtained using Federer's formula $(N-1)(T-1) \geq 15$ where N is the number of subjects and T is the number of intervention groups. The inclusion criteria of this study were eight to twelve-weeks old male mice weighing 20-30 grams. The exclusion criteria were mice that died or became ill during the adaptation period. The dropout criteria were mice that died or became ill during the intervention.

Guava leaf juice administration

Six mice of each intervention group were placed in one cage in the Biosystems Laboratory, Faculty of Mathematics and Science, Padjadjaran University. The cages were equipped with chaff as bedding, ventilation, feed and water storage. The standard feed was given ad libitum. The mice were adapted for seven days, then they were randomly assigned to four groups. Group 1 was given 56 mg/kg guava leaf juice and 14 mg/kg quinine, group 2 was given 56 mg/kg guava leaf juice, group 3 was given 14 mg/kg quinine, and group 4 was given 1 ml of distilled water.[14] The administration was done using orogastric tubes. Quinine tablets were ground using pestle and mortar then dissolved in distilled water. The intervention was given for fourteen days, and then the mice were sacrificed by cervical dislocation

Preparation of guava leaf juice

The guava leaf juice was made as previously described.[14] Guava leaves were obtained from the arboretum, Faculty of Mathematics and Sciences, Padjadjaran University. The leaves were cleaned with water, rinsed in distilled water and blended in a juicer after addition of distilled water until smooth and the dregs were removed with a strainer. The ratio of distilled water and guava leaves was 1:1 (w/v), and the juice was kept at 4^{o}C in the refrigerator.

Histologic analysis

After the mice were terminated, their femoral bones were taken for histologic preparations. The histologic preparations were made in the Cellular Biology Preparation Room, Faculty of Medicine, Padjadjaran University. The histologic preparations were then stained with hematoxylin-eosin.[12,18] The histologic preparations were read under the microscope at 100 times magnification. Every slide was read in three different random parts. From each slice, megakaryocytes were read in three different fields of views of the total visible area.

Statistical analysis

Data distributions were tested using Kolmogorov-Smirnov test and homogeneity of variance was tested using Levene test. Because the data were not normally distributed and had homogenous variance, we used the Kruskal-Wallis test with the value of $p<0.05$ being considered statistically significant. Since the Kruskal-Wallis test result was significant, we used the Mann-Whitney test to determine the differences between groups.

Ethical clearance

Ethical clearance for this research study was given by the Research Ethics Committee, Faculty of Medicine, Padjadjaran University under number 972/UN6/C1/3/2/KEPK/PN/2016.

RESULTS

Data obtained from histologic preparations of the femoral bones can be seen in Table 1 and Figure 1, showing differences in mean megakaryocyte count. To determine whether the between-group differences were statistically significant or not, first we analyzed the data distribution and homogeneity of variance. The data distribution was analyzed using the Kolmogorov Smirnov test and the result was that the data was not normally distributed ($p<0.05$).

The variance was analyzed using the Levene test and the result was that data had homogenous variance ($p>0.05$). Because the data were not normally distributed and had homogenous variance, we used a non-parametric

Table 1. Megakaryocyte count by treatment groups

	Treatment group				p value
	G1	G2	G3	G4	
Megakaryocytes	2.83 ± 0.89	3.30 ± 0.29	2.00 ± 0.42	3.32 ± 0.98	0.206

G1=Guava leaf juice and quinine; G2=Guava leaf juice; G3=Quinine; G4=Distilled water

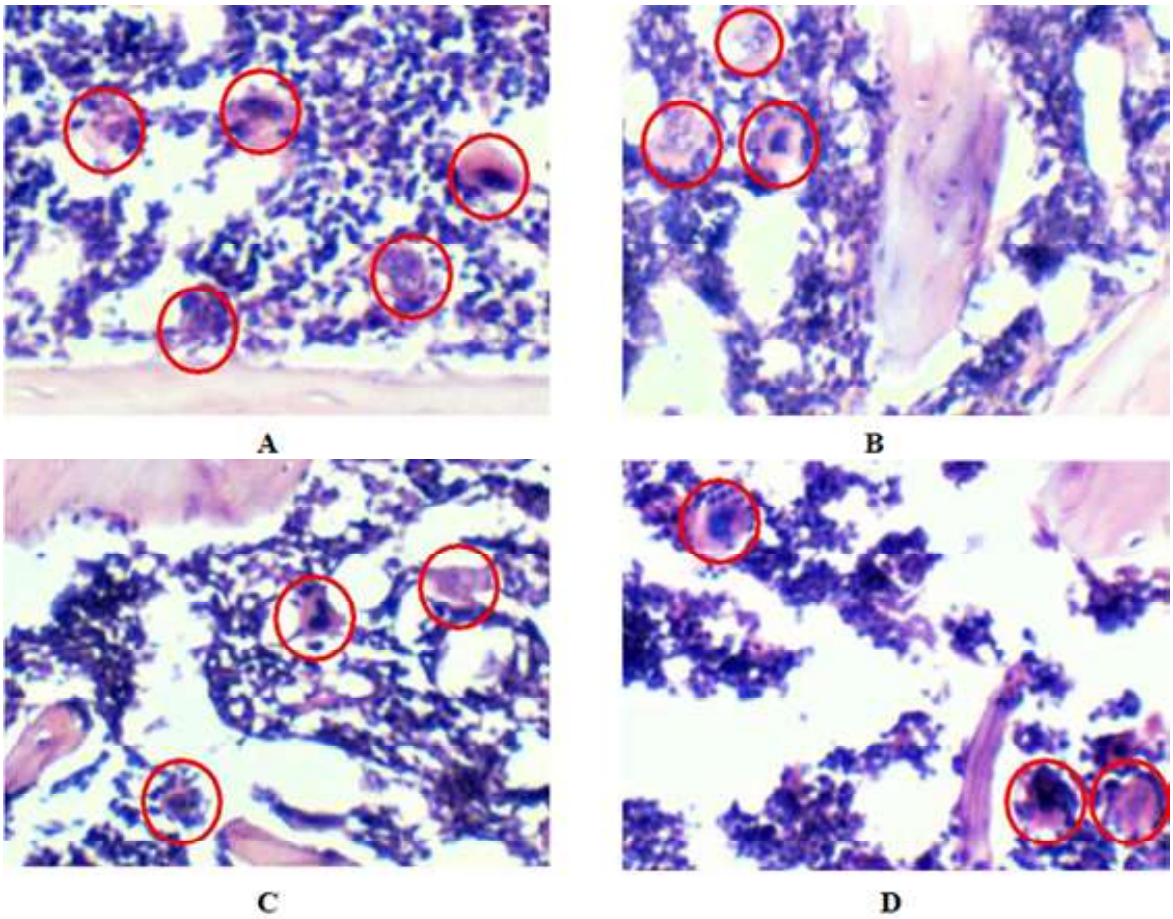

Figure 1. Histologic preparation of femoral bone marrow (A) Group 1; (B) Group 2; (C) Group 3; (D) Group 4
After guava leaf juice treatment, mice were sacrificed and the femoral bones were collected for HE staining. Quantitative observation of megakaryocytes in the bone marrow showed that the megakaryocyte count in the group receiving guava leaf juice and quinine (A) was increased compared to the quinine group (C). Furthermore, there is no statistically difference between the guava leaf juice group (B) and the distilled water group (D). Microscopic images at 10x magnification where red circles indicate megakaryocytes

test, i.e. the Kruskal Wallis test. The result of the Kruskal Wallis test was shown to be significant ($p = 0.021$).

In Table 1, the mean megakaryocyte count of group 1 (2.83) is greater than that of group 3 (2.24) and that the mean megakaryocyte count of group 2 (3.3) is greater than that of group 4 (2.93). To determine whether the differences between the means of group 1 and group 3 and between the means of group 2 and group 4 were significant or not we used the Mann Whitney test. The result of the Mann Whitney test shown in Table 2 demonstrates that there was no significant difference between group 1 and group 3 ($p>0.05$) and also between group 2 and group 4 ($p>0.05$).

DISCUSSION

In this study quinine was used to induce thrombocytopenia and decrease the megakaryocyte count in mice. Quinine can cause thrombocytopenia by recognizing Von Willebrand

Table 2. Mann Whitney Test

Groups	p value
G1 G2	0.671
G3	0.206
G4	0.644
G2 G3	0.022
G4	1.00
G3 G4	0.020

G1=Guava leaf juice and quinine; G2=Guava leaf juice; G3=Quinine; G4=Distilled water

factor or the glycoprotein receptors Ib/IX or IIb/IIIa. These receptors are found on thrombocytes and megakaryocytes. Quinine- dependent antibody can reduce megakaryocyte viability by apoptosis induction, and inhibit the expression of markers that decrease proplatelet production.[19,20] Reduction in megakaryocyte viability and apoptosis causes the decrease in megakaryocyte numbers in bone marrow. Our present study showed that mean megakaryocyte count wreas decreased after quinine treatment (G1 and G3) (Table 1).

Furthermore, there was an increase in mean megakaryocyte count in the thrombocytopenia mice model after guava leaf administration (G2) (Table. 1). This phenomenon could be explained by the fact that guava leaf contains the flavonoid quercetin, saponins and also vitamin-C.[17,21,22] Quercetin could act as antioxidant affecting cell differentiation[14] and increasing granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). Finally, all these pathways stimulate megakaryopoiesis and are followed by increases in megakaryocyte count.[23,24]

In addition, the mean megakaryocyte counts from the normal control group (G4) and the groups were given guava leaf juice (G1 and G2) did not show any significant differences. This indicates that guava leaf could not affect the number of megakaryocytes in normal mice. It is concluded that the components in guava leaf do not affect the homeostasis of megakaryocytes formation. On the other hand, megakaryocyte formation is influenced by systemic thrombopoietin, which then binds to c-MPL on megakaryocytes to stimulate the formation of megakaryocytes. In the thrombocytopenic condition, the levels of circulatory thrombopoietin are increased, so it binds to c-MPL to help improve megakaryocyte production. [9] On the contrary, in the normal condition, the platelet count is within normal limits and the platelets bind to circulatory thrombopoietin followed by no increment of megakaryocyte production.[22]

The limitation of this study was that no previous study had shown the optimum dose, while our study only used one dose of guava leaf juice. Another limitation was that the observations at the histological examination were restricted to counting the number of megakaryocytes in the femoral bone.

Although the results were not statistically significant, administration of guava leaf juice may still increase the number of megakaryocytes. This condition could increase the number of thrombocytes, and hopefully could reduce the risk of fatal cases in thrombocytopenia cases like dengue hemorrhagic fever.

Further studies are needed to find out the optimal dose and the toxicity of guava leaf. It is also necessary to conduct a new study to investigate the molecular mechanism of guava leaf in increasing the number of megakaryocytes.

CONCLUSION

Guava leaf juice can increase megakaryocyte number in bone marrow of thrombocytopenic mice model. This suggests a potential role of guava leaf juice in improving the platelet count in thrombocytopenic disorders.

CONFLICT OF INTEREST

Competing interest: no relevant disclosure.

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CONTRIBUTORS

NA contributed to design of the study, performed the experiment and wrote the manuscript. AHA performed the experiment and wrote the manuscript. ARR contributed to the design of the study. All authors read and approved the manuscript.

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