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Mechanism of Fruit Ethanol Extract of Phoenix dactylifera on Heparin Induced Thrombocytopenia in Rats

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ABSTRACT

Thrombocytopenia occurs frequently in patients with hematologic disorders and other diseases in hospital. Thrombocytopenia may cause bleeding, from mild to severe, which is treated for the patients. The cauese of death in thrombocytopenia cases in hospital is mainly (80%) due to uncontrolled bleeding and lack of platelet concentrates availability. The chemical on drugs that effectively to resolve thrombocytopenia through increase the thrombopoiesis mechanism still questioned. The purpose of this study was to explain the mechanism of the effect of fruit ethanol extract of Phoenix dactylifera L on thrombopoiesis increase in thrombocytopenia. The sample used 30 male rats were divided into 3 groups: negative control (K1): rats were given daily with CMC Na 0,5%; positive control (K2): rats were given heparin and CMC Na 0,5%; and the treatment group (K3): rats were given heparin and fruit ethanol extract of Phoenix dactylifera L dose of 10 mg /200 g BW. Furthermore, the cardiac blood sampling to calculate the amount of blood platelet and examination to bone marrow for expression of Hsp10, IL-6, cMpl, JAK2 with immunohistochemical methods. Result of this study showed that K3 (treatment group fruit ethanol extract of Phoenix dactylifera L) had significantly higher platelet counts (1125.50 ±233.98) than control groups K1 (795.90 ± 224.75) and K2 (409.70 ± 79.67). There were increased expression of Hsp10 in stromal cells, IL6 in macrophages, cMpl and JAK2 in the cells of megakaryocytes. From the results of this study concluded that fruit ethanol extract of Phoenix dactylifera L is a potential treatment for increase thrombopoiesis on heparin induced thrombocytopenia in rats

Key words: Thrombocytopenia, *Phoenix dactylifera* L, Hsp10, IL6, cMpl, JAK2

INTRODUCTION

Thrombocytopenia occurs frequently in patients with hematologic disorders and other diseases in hospital. Thrombocytopenia is marked by the decrease in blood platelet count to less than 150,000/uL, caused by decreased platelet production, increased platelet destruction, increased platelets sequestration, and other conditions that cause the loss of the number of blood platelets. Thrombocytopenia may cause bleeding, from mild to severe, which is threatening for the patient [1].

The cause of death in the majority (80%) cases of thrombocytopenia in hospitals are due to uncontrolled bleeding and lack of availability of platelet concentrates. The chemical on drugs that effective to resolve thrombocytopenia through increase the thrombopoiesis mechanism still questioned [2].

The expression of Hsp 10 reported decreases in ITP patients who experienced thrombocytopenia and found elevated in normal bone marrow examination [3]. The results of other studies, Hsp 10 expression is increased in normal bone marrow examination. For that we need further identification on Hsp 10 as chaperones who are in megakariocytic lineage precursors and a role in the stimulation of progenitor cell differentiation of megakaryocytes [4]. Besides Hsp 10, IL6 expression decreased in mouse bone marrow thrombocytopenia, it is necessary to further identification of the roles and IL6 in stimulating thrombopoiesis [5].

Thrombopoiesis regulated influenced by cMpl as thrombopoietin receptor. Expression of c-Mpl has an important role in the stimulation of thrombopoiesis, which increase the activity of endomitosis and maturation of megakaryocytes [6]. Increased endomitosis process can occur via intracellular signal transduction Janus Kinase 2 (JAK2), which has an important role in the activation and deployment of intracellular signal transduction from cell surface receptors to the nucleus / JAK-STAT pathway [7]. Therefore, the identification of Hsp 10, IL6, c-MPL and JAK2 trombopoiesis be important in stimulating further studied as a treatment for thrombocytopenia through the ethanol extract date fruit [8,9].

The purpose of this study was to explain the mechanism of the effect of date fruit ethanol extract (*Phoenix dactylifera* L) on thrombopoiesis increase in thrombocytopenia by blood sampling taken from heart to platelet counting and sampling taken from bone marrow to examining the expression of Hsp 10, IL6, cMpl, Jak2 using immunohistochemistry.

MATERIAL AND METHODS

Preparation of fruit ethanol extract of *Phoenix Dactylifera* L: Preparation of fruit ethanol extract of *Phoenix dactylifera* L in lab Pharmacy, University of Widya Mandala Surabaya Indonesia. Preparation was done by taking a date (without seeds) and then a date cut into small pieces (soft), was added to the jar and then soaked with ethanol 70% as much as three liters and then stirred until smooth and then a jar closed for 5 days (maceration). Intake of fruit extracts dates with funnel bucher drawn by using a suction pump. Results extract obtained is then dried to be included in laburotary vacuum evaporator and carried up into extracts / condensed (temperature 40 ° C) for 1 hour.

Unit of Experimental: This study was a laboratory experimental; Posttest Only Control Group Design. Male rats, aged 3 months, BW 190-200 g, a total of 30 were divided into 3 groups : K1 (negative control group), all test animals in this group were not induced by heparin and only standard feed and CMC Na 0,5% 5 ml /200 g BW rat/day during the studies; K2 (positive control group), after is injected with heparin 270 U/200 g BW rat / day (subcutaneous), given for 2 days, so that the two groups of rats thrombocytopenia and the next day all the rats were standard feed and CMC Na 0,5% 5 ml /200 g BW rat/day, for 2 days; and K3 (treatment group) is injected with heparin 270 U/200 g BW rat/day (subcutaneous), given for 2 days futhermore were given CMC Na 0,5% 5 ml /200 g BW rat/day and fruit ethanol extract of *Phoenix dactylifera* L 10 mg /200 g BB rat/day, with a long time of 2 days. On the fifth day, rats in all groups (K1, K2 and K3) were sacrificed by anesthesia using ketamine HCl doses of 5 mg /200 g BW intramuscular. Furthermore, the cardiac blood sampling to calculate the amount of blood platelet with hematologic analizer (Sysmex KX-21) and examination to bone marrow for expression of Hsp10, IL-6, cMpl, JAK2 with immunohistochemical methods.

Taking blood and bone marrow preparations: The last day of the study (fifth days), all mice were sacrificed by means of injection anesthetic ketamine at a dose of 5 mg/200 g BW rat/day (intramuscular). Surgery performed on the thorax region, so the region is open thorax and the heart and blood vessels visible ascending aorta. Next take heart blood / ascending aorta with a 3 ml syringe and accommodated in vacutainer containing EDTA, and then performed surgery on a rat's femur bone marrow retrieval. Rat femur right and left, cut with scissors mouse bone longitudinal and transverse bone marrow tissue then it taken using a needle insulin micro-fine nano 4 mm (No. 32) with a thickness of 2x2x2 mm. Furthermore, the bone marrow is placed on the object glas then made smears and paraffin blocks after that was soaked in formalin [10].

Eexamination of Platelets: Examination of blood platelets do in Labkesda Surabaya. Examination of blood platelets with the technique as follows; tubes containing EDTA and placed blood appropriate place on the appliance hematology analyzer Sysmex KX-21. Samples are aspirated through the aperture into the transducer chamber. Platelets are calculated based on the method of Discrimination Circuit (DC) changes in the voltage direct current resulting in the appearance pulse or pulses of electricity. Charge electric field will be detected based on the magnitude of the pulsation and displayed in paper prints out. The result of the calculation in units of the number of platelets / mm^3.

Immunohistochemistry examination: Immunohistochemical examination conducted in the laboratory of pathology Faculty of Veterinary Medicine Surabaya. Immunohistochemical examination techniques as follows; preparations of rat bone marrow smear staining with peroxidase performed using a mouse monoclonal antibody and specific. Mixture do deparaffinisasi use 2 times xylol, xylol eliminated with absolute ethanol ranging up to 70%, the last with running water for 10-15 minutes, put in a 3% H2O2 solution for 30 minutes, washed with PBS pH 7.4 for 3 times. To remove debris by 0.025% trypsin, then washed 3x PBS. Reacted with *HSP10, IL-6, c-MPL and JAK2 monoclonal antibody,* were incubated for 24 hours at a temperature of 40 C in a humidity chamber. Washed 3 times with PBS @ 5 minutes, then added peroxidase label streptavidin and incubated for 1 hour. Washed 3 times with PBS, then treated with the substrate diamino benzidine (DAB), which created a new and incubated for approximately 30 minutes at room temperature with shaken. Washed with distilled water, added Mayer's hematoxylin for 6 minutes. Washed with running water, then with distilled water. Dried preparations (aerated), spilled entelan and covered with a coverglass. Observe the light microscope for the counting process (determined microscopically with a magnification of 1000 times in 5 visual fields.

RESULTS

Effect of fruit ethanol extract of *Phoenix Dactylifera* L on Platelet Count

Based on the results, the p value was $p < 0,001$, there was difference between groups in platelet count. The mean of platelet count in K3 was higher than K1 and K2 (Table 1).

Table 1: Effect of fruit ethanol extract of *Phoenix dactylifera* L on Platelet Count

Groups	Platelet count (mm^3)				p
	Mean	Median	Min	Max	
K1 (Control)	795, 90[a]	731,00	526	1161	
K2 (Heparin)	409,70[b]	408,00	298	608	< 0,05
K3 (Heparin + *Phoenix dactylifera* L)	1125,50[c]	1144,50	799	1437	

Superscript within each column indicate significant difference between the means (p < 0.05)

Effect of fruit ethanol extract of *Phoenix dactylifera* L on Hsp 10 expression

Based on the results, the p value was 0.040 ($p < 0.05$): there was difference between groups in cells expressing Hsp10. The mean of K3 was higher than K1 and K2 (Table 2).

Table 2: Effect of fruit ethanol extract of *Phoenix dactylifera* L on Hsp 10 expression

Groups	Hsp10				(p)
	Mean	SD	Min	Max	
K1 (Control)	4,60[a]	1,77	2,00	8,00	
K2 (Heparin)	2,40[b]	1,71	0,00	5,00	0,05
K3 (Heparin + *Phoenix dactylifera* L)	7,80[c]	3,11	2,00	12,00	

Superscript within each column indicate significant difference between the means (p < 0.05)

The results of IHC examination of bone marrow during the 2-day study in 3 groups. The expression and Hsp 10 color intensity was higher in fruit ethanol extract of *Phoenix dactylifera* L (K3) than K1 and K2 (Figure 1).

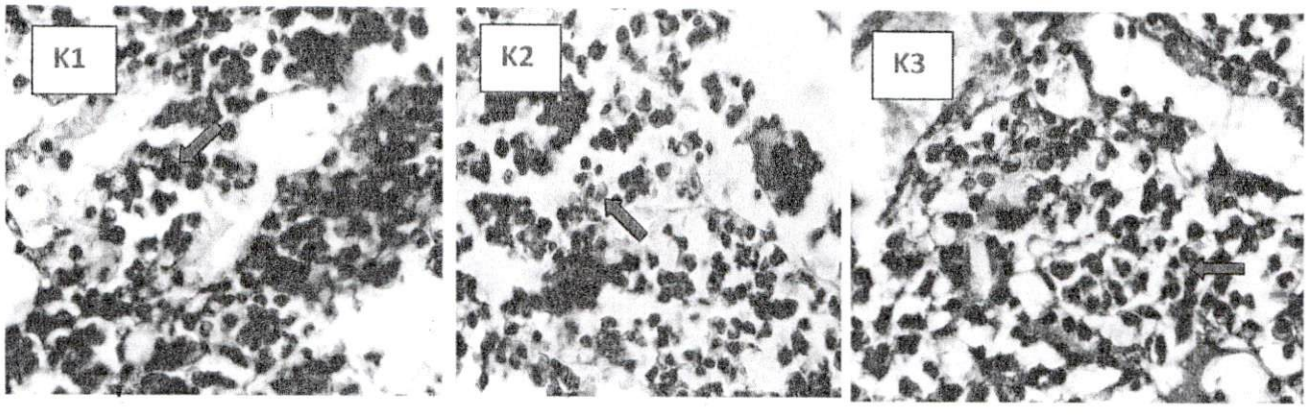

Fig.1: Differences in the expression of Hsp 10 in stromal cells K1, K2, and K3 with immunohistochemical staining 1000x magnification (arrows indicate cells expressing Hsp10).

Effect of Date Fruit Ethanol Extract on IL6 expression

Based on the results, the p value was $p < 0,05$: there was difference between groups in cells expressing IL6. The mean of K3 was higher than K1 and K2 (Table 3).

Table 3. Effect of fruit ethanol extract of *Phoenix dactylifera* L on IL6 expression

Groups	IL6				p
	Mean	SD	Min	Max	
K1 (Control)	$5,40^a$	1,64	4	9	
K2 (Heparin)	$2,70^b$	2,16	0	7	< 0,05
K3 (Heparin + *Phoenix dactylifera* L)	$8,60^c$	2,91	4	12	

Superscript within each column indicate significant difference between the means ($p < 0.05$)

The results of IHC examination of bone marrow during the 2-day study in 3 groups. The expression and IL6 color intensity was higher in ethanol extract of the date fruit (K3) than K1 and K2 (Figure 2).

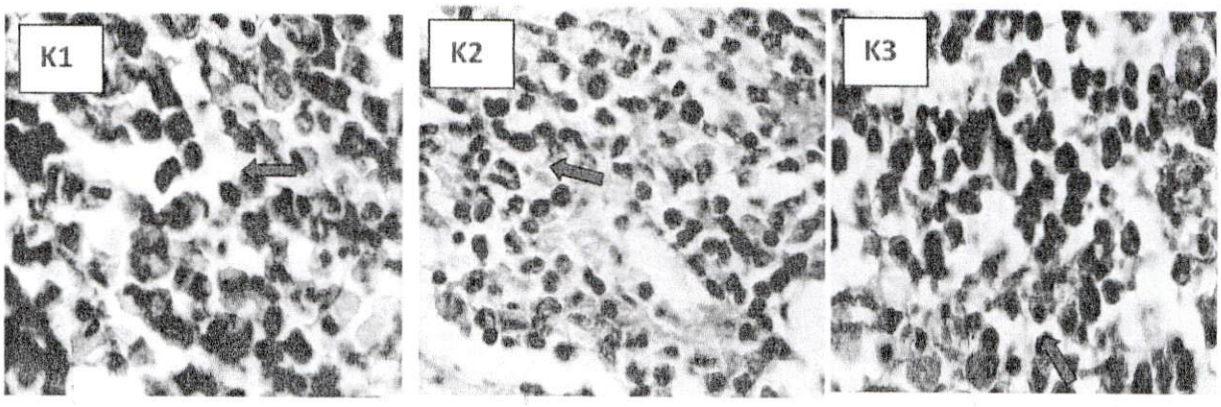

Figure 2: Differences in the expression of IL6 in Macrophag cells K1, K2, and K3 with immunohistochemical staining 1000x magnification (arrows indicate cells expressing IL6).

Effect of Date Fruit Ethanol Extract on cMpl expression

Based on the results, the p value was $p < 0,05$: there was difference between groups in cells expressing cMpl. The mean of K3 was higher than K1 and K2 (Table 4).

Table 4: Effect of fruit ethanol extract of *Phoenix dactylifera* L on cMpl expression

Groups	cMpl				p
	Mean	SD	Min	Max	
K1 (Control)	$3,40^a$	2,27	2	8	
K2 (Heparin)	$1,00^b$	0,94	0	3	< 0,05
K3 (Heparin + *Phoenix dactylifera* L)	$6,40^c$	3,62	3	12	

Superscript within each column indicate significant difference between the means ($p < 0.05$)

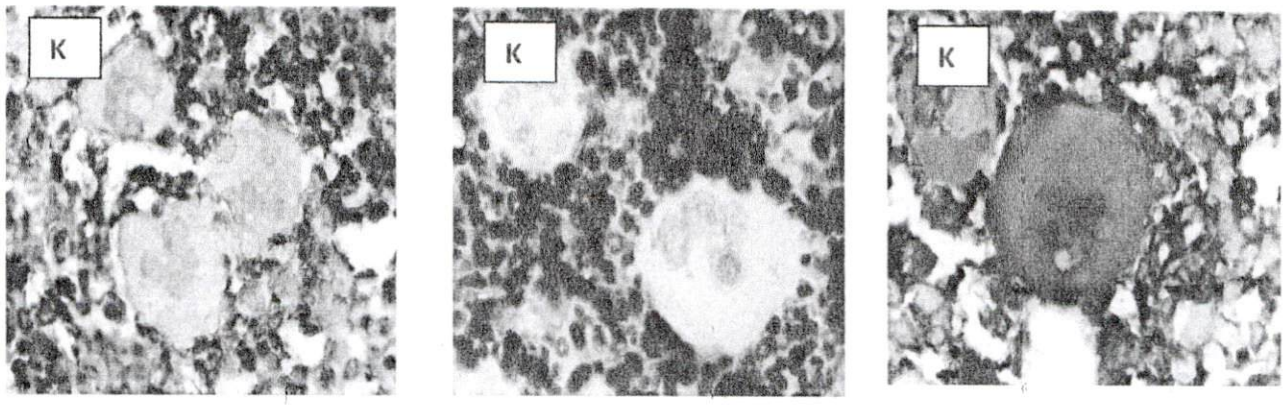

Figure 3: Differences in the expression of cMpl in Megakaryocyte K1, K2, and K3 with immunohistochemical staining 1000x magnification (arrows indicate cells expressing cMpl).

The results of IHC examination of bone marrow during the 2-day study in 3 groups. The expression and cMpl color intensity was higher in fruit ethanol extract of *Phoenix dactylifera* L (K3) than K1 and K2 (Figure 3).

Effect of Date Fruit Ethanol Extract on Jak2 expression

Based on the results, the p value was p p < 0,001 (p < 0,05): there was difference between groups in cells expressing Jak2. The mean of K3 was higher than K1 and K2 (table5).

Table 5: Effect of fruit ethanol extract of *Phoenix dactylifera* L on Jak2 expression

Groups	Jak2				p
	Mean	SD	Min	Max	
K1	3,90[a]	2,60	1,00	8,00	
K2	1,60[b]	1,83	0,00	5,00	< 0,05
K3	6,60[c]	1,89	4,00	10,00	

Superscript within each column indicate significant difference between the means (p < 0.05)

The results of IHC examination of bone marrow during the 2-day study in 3 groups. The expression and JAK2 color intensity was higher in fruit ethanol extract of *Phoenix dactylifera* L (K3) than K1 and K2 (Figure 4).

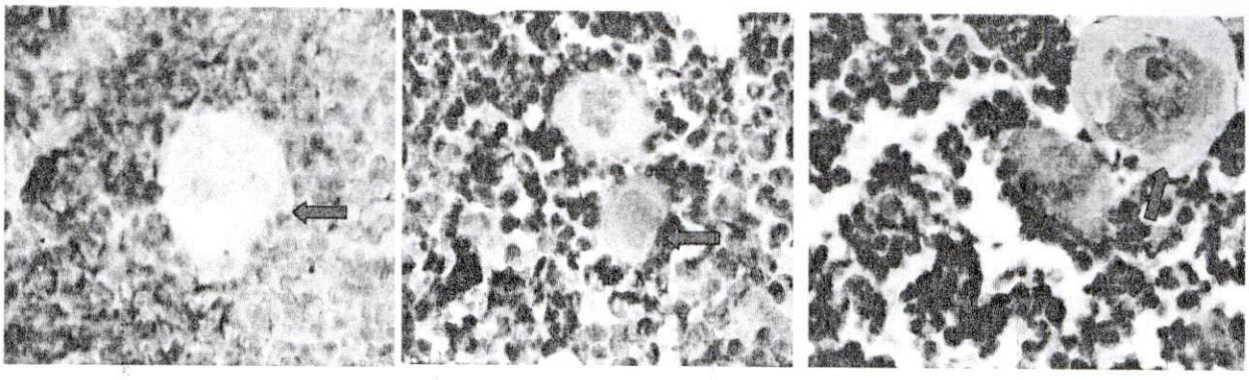

Fig 4. Differences in the expression of Jak2 in megakaryocyte K1, K2, and K3 with immunohistochemical staining 1000x magnification (arrows indicate cells expressing Jak2).

DISCUSSION

This study found that the correlation test results showed that fruit ethanol extract of *Phoenix dactylifera* L has a positive relationship and contribute to increased thrombopoiesis through expression of Hsp10, IL6 and cMpl. That mechanism because of the fruit ethanol extract of *Phoenix dactylifera* L that contains flavonoids which bind to sugar (flavonoid glycoside). The content of flavonoid glycosides include fructose and glucose that is higher than that of date fruit to another, so that the date fruit stimulates transduction signaling pathways cells, activation of signaling pathways cells can stimulate macrophages, including in producing interleukin 6 (IL6) to maintain cell survival as well as for the development of cell / proliferation [11,12]. Flavonoid glycosides in the date fruit also increase the sensitivity of cell receptors. One of them is the receptor c-MPL has an important role in regulating the process thrombopoiesis [13,14]. The fruit ethanol extract of *Phoenix dactylifera* L also has potential as an energy source in a series of metabolic processes of the body cells so as to activate the heat shock factor (HSF) and induces the heat shock protein (HSP) [15]. Especially HSP 10 which are in megakaryocytic lineage precursors [4].

While the results of path analysis IL6 expression have a positive impact and contribute to increased expression of JAK2, meaning that every increase in the expression of IL6 will be followed by increased expression of JAK2. The mechanism of IL-6 in stimulating thrombopoiesis can be directly on the cell or through the activation pathway megakaryocytes Janus Kinase2 (JAK2). JAK2 function to develop and maintain the viability of the cells, by activating the mechanism of intracellular signal transduction molecules, causing the cell nucleus proliferative effects (endomitosis) and maturation of megakaryocytes [16]. Platelet production can be increased with an increase in core replication/endomitotik and raise the volume of the cell cytoplasm of megakaryocytes. Improved process endomitotik megakariosit cells will be followed by an increase in the blood of platelets [17].

CONCLUSION

The fruit ethanol extract of *Phoenix dactylifera* L dose 10 mg/200 g BW rat/day increased thrombopoiesis through the increased expression of Hsp10 in stromal cells, increased expression of IL6 in macrophages, increased

expression of cMpl and expression of JAK2 in the cells of megakaryocytes. The mechanism that the fruit ethanol extract of *Phoenix dactylifera* L dose of 10 mg /200 g bw / day increase thrombopoiesis through the increased expression of IL6 and JAK2. The increased expression of JAK2 had a greatly contribute of the platelet production.

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