จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย

กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

การศึกษาผลของพิษงูเขียวหางไม้ต่อเกร็ดเลือด

โดย

สุพรรณ สุขอรุณ

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Study the effect of green pit viper (*Trimeresurusalbolabris*) Venom on platelet morphology

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ชื่อโครงการวิจัย Study the effect of green pit viper (*Trimeresurus albolabris*) venom on platelet morphology

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เดือนและปีที่ทำการวิจัยสำเร็จ ธันวาคม 2549

บทคัดย่อ

อุบัติการณ์ การถูกงูเขียวหางไหม้กัดเพิ่มขึ้นทุกปี ผลหลังจากการถูกกัด มักมีเลือดออก เพราะพิษของงูเขียวหางไหม้มีคุณสมบัติเหมือนสาร thrombin ได้มีรายงานกล่าวว่าผู้ที่ถูกงูเขียวหางไหม้กัดมีปริมาตรของเกร็ดเลือดลดลง (Mean Platelet Volume: MPV) พร้อมๆกับจำนวนลดลงด้วย ในการศึกษาครั้งนี้เป็นการศึกษาในหลอดทดลอง เพื่อดูว่าพิษของงูเขียวหางไหม้มีผลทำให้ MPV ลดลง จำนวนเกร็ดเลือดลดลง และดูรูปร่างว่ามีการเปลี่ยนแปลงอย่างไรด้วยกล้องจุลทรรศน์อิเลคตรอน การศึกษาทำโดยการล้างเกร็ดเลือดให้ปราศจากสาร fibrinogen แล้วผสมกับพิษงูเขียวหางไหม้ นำไปวัดค่า MPV ด้วยเครื่อง flow cytometry และดูรูปร่างด้วยกล้องจุลทรรศน์อิเลคตรอน(SME) ผลการศึกษาพบว่า MPV ลดลง (8.9 $\pm$ 1.2 fl และ 4.8 $\pm$ 1.3 fl $P < 0.05$) จำนวนเกร็ดเลือดลดลง (216 $\pm$ 102 x 10^9/L และ 43.4 x 10^9/L $P < 0.05$) ขนาดของเกร็ดเลือดที่ดูจากกล้องจุลทรรศน์อิเลคตรอน มีขนาดเล็กลง มีขนาดเพียง 1.1-1.2 ไมครอน จึงสรุปได้ว่าพิษงูเขียวหางไหม้มีผลทำให้เกร็ดเลือดมีขนาดเล็กลง สอดคล้องสิ่งที่เห็นในกล้องจุลทรรศน์อิเลคตรอน และยังมีส่วนทำให้จำนวนของเกร็ดเลือดลดลงอีกด้วย

คำสำคัญ : Green pit viper, venom, platelet morphology

Project title: THE EFFECT OF GREEN PIT VIPER (*TRIMERESURUS ALBOLABRIS*) VENOM ON PLATELET MORPHOLOGY

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Abstract

The incidence of venomous snake bites increases in every year in Thailand, especially by green pit viper. Consequence after the bite is bleeding because of its thrombin-like property. There is a report mentioned that the mean platelet volume was decreased in those who had been bitten by this snake. In this study is to investigate the effect of green pit viper (*Trimeresurus albolabris*) on platelet volume (MPV), number of platelets and morphology in vitro. The test carried out by washing platelet in phosphate buffer pH 7.2 so as to get rid fibrinogen, and then washed platelets were mixed with green pit viper venom. The mean platelet volume and number of platelets were determined by flowcytometry and its morphology was examined by scanning electron microscope (SEM) respectively. The results showed that the number of platelets were decreased dramatically ($216 \pm 101 \times 10^9$ /l and $78.1 \pm 43.4 \times 10^9$ /l $P < 0.05$) and the MPV was also decreased (8.9 ± 1.2 fl and 4.8 ± 1.3 fl, $P < 0.05$).The morphology of platelets is smaller than normal which ranges from 1.1- 1.2 micrometers. In conclusion, the green pit viper venom can directly effect on platelet morphology especially decreasing platelet volume and numbers.

Key words: Green pit viper, venom, platelet morphology

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Introduction

The green pit viper (*Trimeresurus albolabris* and *Trimeresurus macrops*) is a common venomous snake in Thailand. This venomous snake has increased its important because the biting rates were increased dramatically up 73.58 % (Dumavibhat , 1977).The venom has thrombin-like effect in vitro and causes a defibrination syndrome in vivo (Visudhiphan *et al.*, 1981). Clinical features of this venomous snake bites vary from asymptomatic to fatal bleeding. The venom of *Trimeresurus albolabris* can increase fibrinolytic activity by shortening euglobulin time (Kamnerdnond and Jitprommeta, 2004).Recently, there was a report (Rojnuckarin , *et al.,* 1999) studied a group of patients who had been bitten by green pit viper(*Trimeresurus albolabris* and *Trimeresurus macrops*). The study found that fibrinolytic system activation was very common as indicated by low plasminogen , low antiplasmin and elevated fibrin-fibrinogen degradation product levels (FDP). Concerning platelet determination, significant decrease in total platelet count and mean platelet volume (MPV) were demonstrated in envenomous blood. The changes might be partly due to the effect of green pit viper toxin on platelet morphology (Soogarun, *et al.,* 2003). Alboaggregin-A (AL-A) is a venom toxin of this snake, 50 kDa consists of 2α and 2β subunits. It has been reported that alboaggregin-A has activity at the collagen receptor GPVI in addition to GPIbα, and evidence is provided that this contributes to protein tyrosine phosphorylation, shape change, and GPIIb-IIIa dependent aggregation (Asazuma *et al,*2001). Alboaggregin B (AL-B), is another toxin has been isolated by ion-exchange chromatography. It can agglutinate platelets without the need for $Ca2^{+}$ or any other cofactor. The purified protein showed an apparent molecular mass about 23 kDa under nonreducing conditions. Ristocetin did not alter the binding of AL-B to platelets or affect AL-B-induced platelet agglutination (Peng *et al,* 1991). AL-B has its chain identity to a varity of toxins such as echicetin and botrocetin (Andrews RK,*et al,* 1996). Among them may be representing the chance of cross reacting of antivenom that produced by one of these toxins. Alboaggregin C (AL-C) , there are 14-18 kDa subunits its function is similar to AL- B. Alboluxin is another toxin with potent platelet activator, it induced a tyrosine phosphorylation in platelets that resembles those produced by collagen. It activates platelets via both GPIb and GPVI (Du Xiao-Yan,*et al,* 2002). Albolabrin can also obtain from this snake : homologies with the RGDS domain of fibrinogen and von Willebrand factor.(Williams *et al,* 1990). It is a 7.5-kDa cysteine-rich protein. It belongs to a family of RGD-containing peptides, termed disintegrins, recently isolated from the venom of various vipers and discovered to be potent inhibitors of both platelet aggregation and cell-substratum adhesion (Soszka T,*et al,*1991). The problem has been arisen when

antivenom produced from Trimeresurus albolabris can have cross reaction with the same species even in different country (Pakmanee *et al,* 1998), it must have some homologies. In this study is to investigate the question of decreased mean platelet volume (MPV) and number of platelet that occurred in vivo by performing platelet rich solution without fibrinogen mixed with green pit viper venom in vitro determining the MPV by flow cytometry then observed platelet morphology by electron microscope.

Materials and Methods

1. Lyophilized crude venom (*Trimeresurus albolabris*) was obtained from snake farm of Thai Red Cross. One milligram of crude venom was dissolved in normal saline solution (NSS) as described earlier (Soogarun , *et al.*, 2005)
2. Platelet concentrate without fibrinogen prepared by using 10 ml of EDTA blood mixed with 150 ml of 0.1 M phosphate buffer pH 7.2 (40.5 ml of 0.2M dibasic sodium phosphate and 9.5 ml of monobasic sodium phosphate then added equal volume of distilled water) (Common buffer undated). The solution was then centrifuged in refrigerated centrifuge at 3,000 x g for 15 minutes. The supernatant was discarded and added another 145 ml. gentle agitation was made so as to disperse clumping platelets. The solution was re-centrifuged 50 x g for 10 minutes. The platelets in supernatant were used to measure the MPV and platelet count by flow cytometry (Technicon H 3) . The number of platelets subjected to this experiment must not less than 100 x 10^9 / l. Small amount of red cells can be found in the supernatant.
3. This process was repeated for twenty times. The average platelet parameters were then calculated and reported.
4. Study the morphology of platelet by fixing the mixture of platelets and venom with 2.5% of glutaraldehyde for 4-6 h, then observed the morphology by scanning electron microscope (SEM)

Results

Before treatment the platelets were within normal limits at 216 ± 101 x 10^9 /l while the MPV was 8.9 ± 1.2 fl. After addition of green pit viper venom to the platelet solution at one minute, the number of platelets decreased dramatically from 216±101 x 10^9/ l to 78.1 ±43.4 x 10^9 / l, the fall is significant difference with $P < 0.05$. The MPV was also decreased from 8.9 ± 1.2 fl to 4.8 ± 1.3 fl, this is also significant difference with $P < 0.05$ as shown in table 1. The decreased MPV and number of platelet are concomitantly occurred at the same time as seen in fig.2 and fig. 1. The electron micrograph, the platelets appear regular shape with smooth surface, ranging from 1.4-2.0 micrometers. Because of red cells still existed in the supernatant , thus the study can verify that red blood cells have smooth surface, round shape with disc-like sphere measuring ranged from 5-6 micrometers in diameter (Figure 3). After addition of green pit viper venom to the platelet solution at one minute, the red blood cells are irregularly shaped with multiple cytoplasmic projections. Most red cells show shrinkage and having diameters ranging from 3-4 micrometers. The platelets are also reduced in their diameters which range from 1.1-1.2 micrometers. Their surfaces are irregular and rough. Most platelets adhered closely to one another (Figure 4).

Discussion

This study found that the numbers of platelet were decreased after the exposure of green pit viper venom. The decreased platelets in vivo may in part the result of direct reaction of venom and some of which were consumed by clotting formation. Once the patients received large amount of venom could cause severe consequences as seen in some certain cases came with severe bleeding. This study also supported the previous report mentioned that the decreased MPV in vivo may be due to the snake venom (Soogarun *et al.,* 2003). One might think that larger platelets were consumed by clotting formation. In fact, it was impossible because in this tested system there was no fibrinogen existed thus if clotting formation occurred by platelet itself it would be clogged in the flow cytometry even small clot ones. However, it was postulated that it might be resulting in decreased MCV as suspected in the previous report (Wiwanitkit &Suwansaksri, 2001). However, that report did not mention whether thalassemia trait had been ruled out. This study found that the red cell morphology treated with green pit viper venom had morphologic changed very much like those treated with Russell's viper venom that reported by Nopathorn , but incase of Russel's viper increased hematocrit significantly but did not in the previous report. And such altered morphology was observed immediately at 1 minute and reached maximum at 30 minutes (Nopathorn , *et al.,* 1998, Soogarun, *et al.,* 2005). The decrease of platelet at the first minute may be due to cell lysis and some of them can tolerate and persistence in toxic environment thus beyond that time both graphs were constant. However, the research should carry on further study in this matter. In this study could not give impression which toxin was the cause. Anyway, this study had performed non – denaturing electrophoresis after SDS-PAGE could not achieve. And now we know the the complexity of green pit viper toxins.

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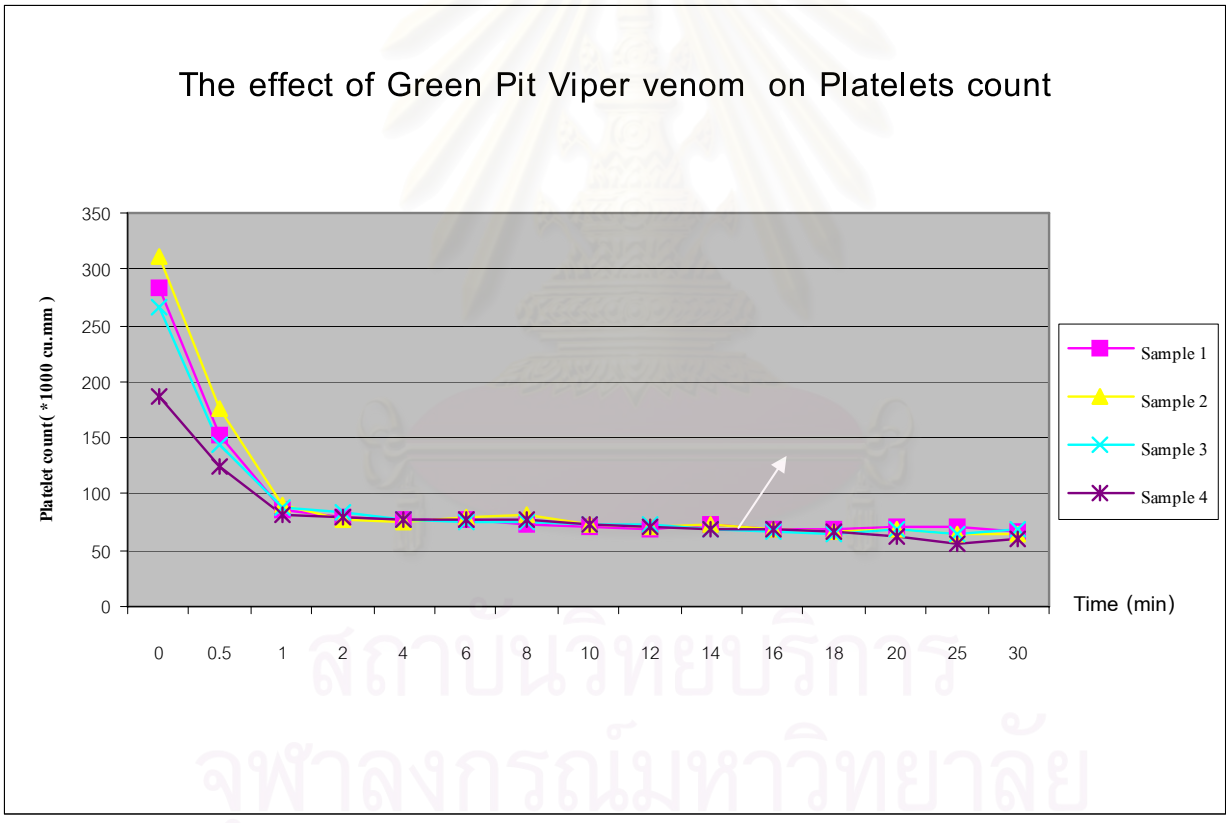
Table 1. Changes occurred after treatment the platelets with green pit viper venom

Parameters	Before treatment	Post treatment	P value
Platelets	216 ± 101 x 10^9/l	78 ± 43.4 x 10^9/l	< 0.05
MPV	8.9 ± 1.2 (fl)	4.8 ± 1.3 (fl)	< 0.05

Fig. 1 Showing platelet numbers after addition of green pit viper venom (1mg/ ml in normal saline solution) to platelet rich solution for 30 minutes



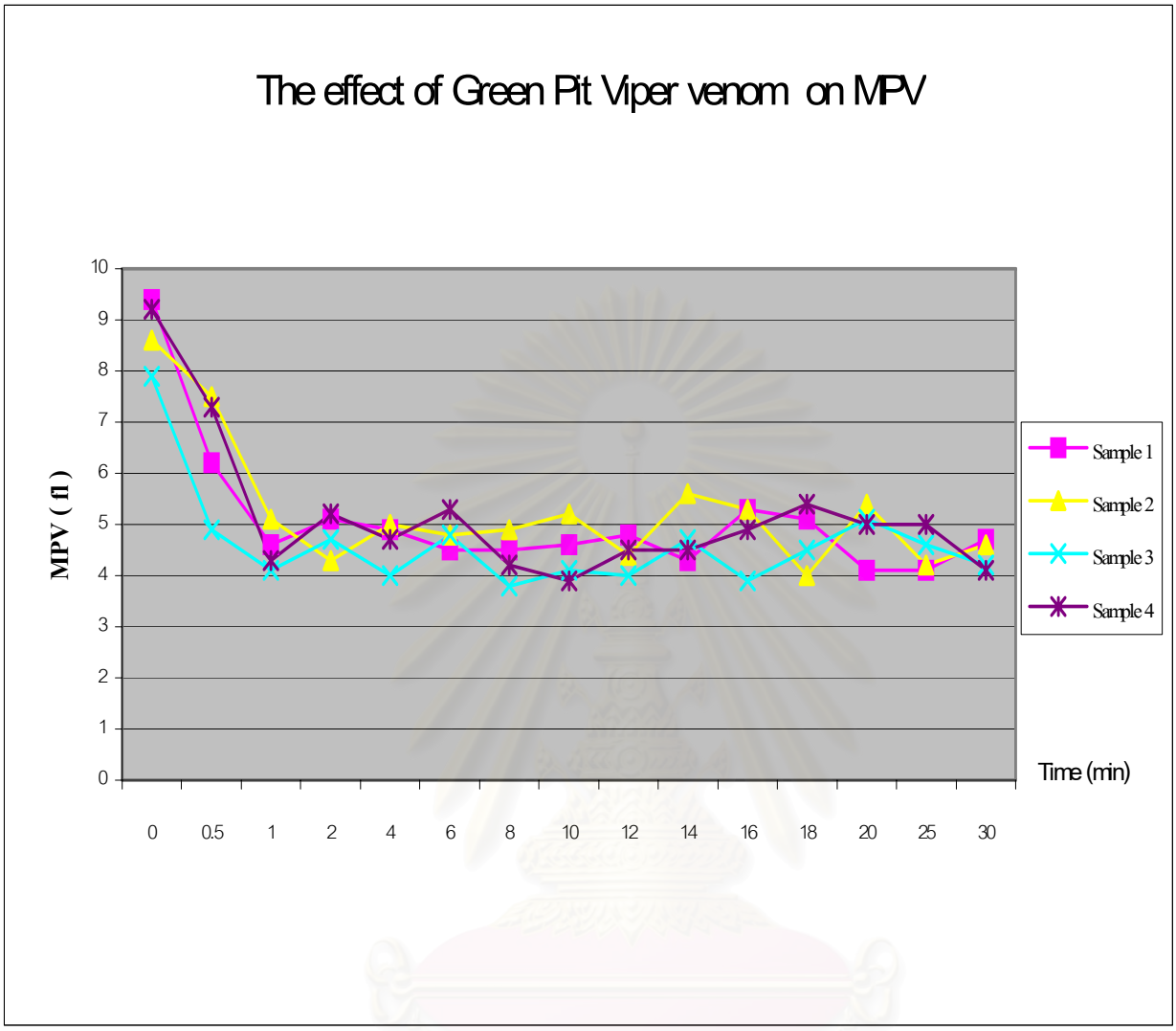


Fig.2. Showing the Mean Platelet Volume (MPV) after addition of green pit viper venom (1mg/ ml in normal saline) for 30 minutes

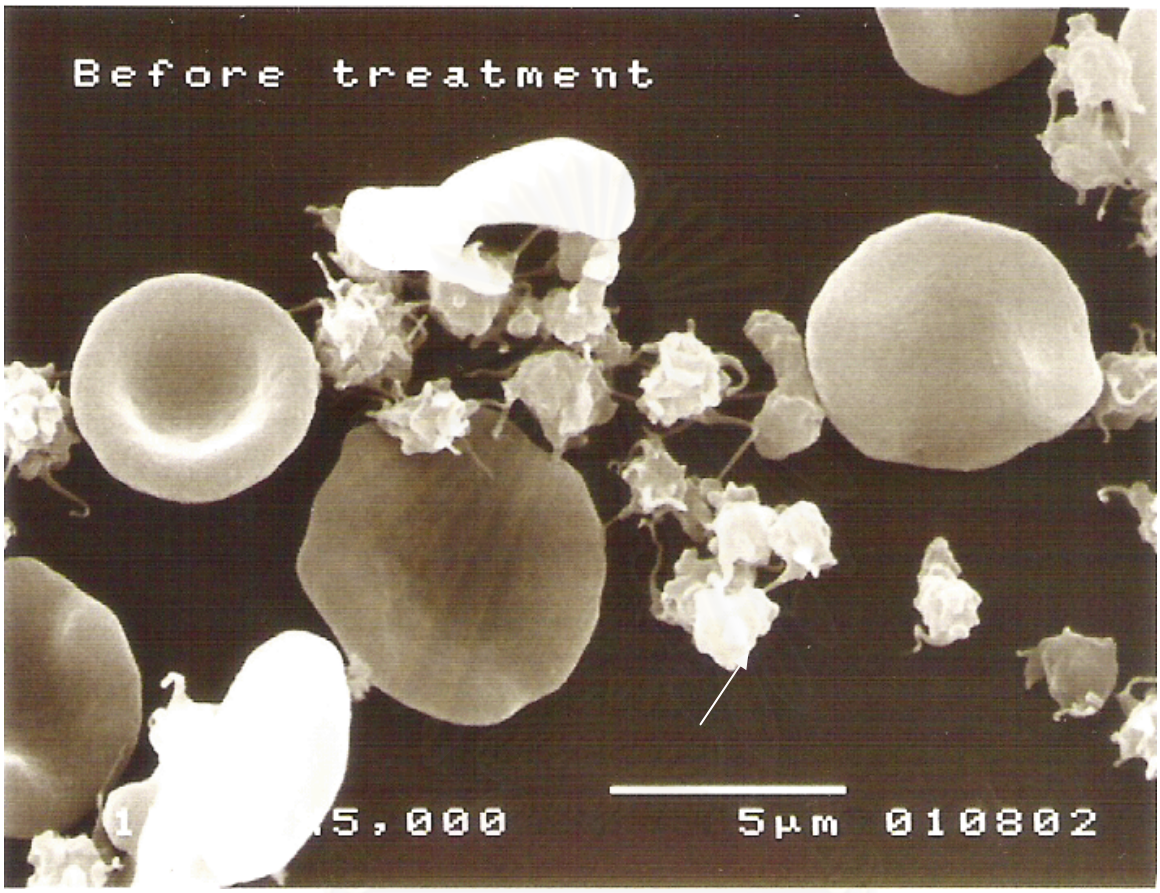


Fig. 3 Electron micrograph of platelets and red blood cells before treatment with green pit viper venom .Platelets appear regular shape with smooth surface ranging from 1.4-2.0 micrometers. Red blood cells have smooth surface, round shape with disc-like sphere measuring from 5-6 micrometers before treatment with green pit viper venom

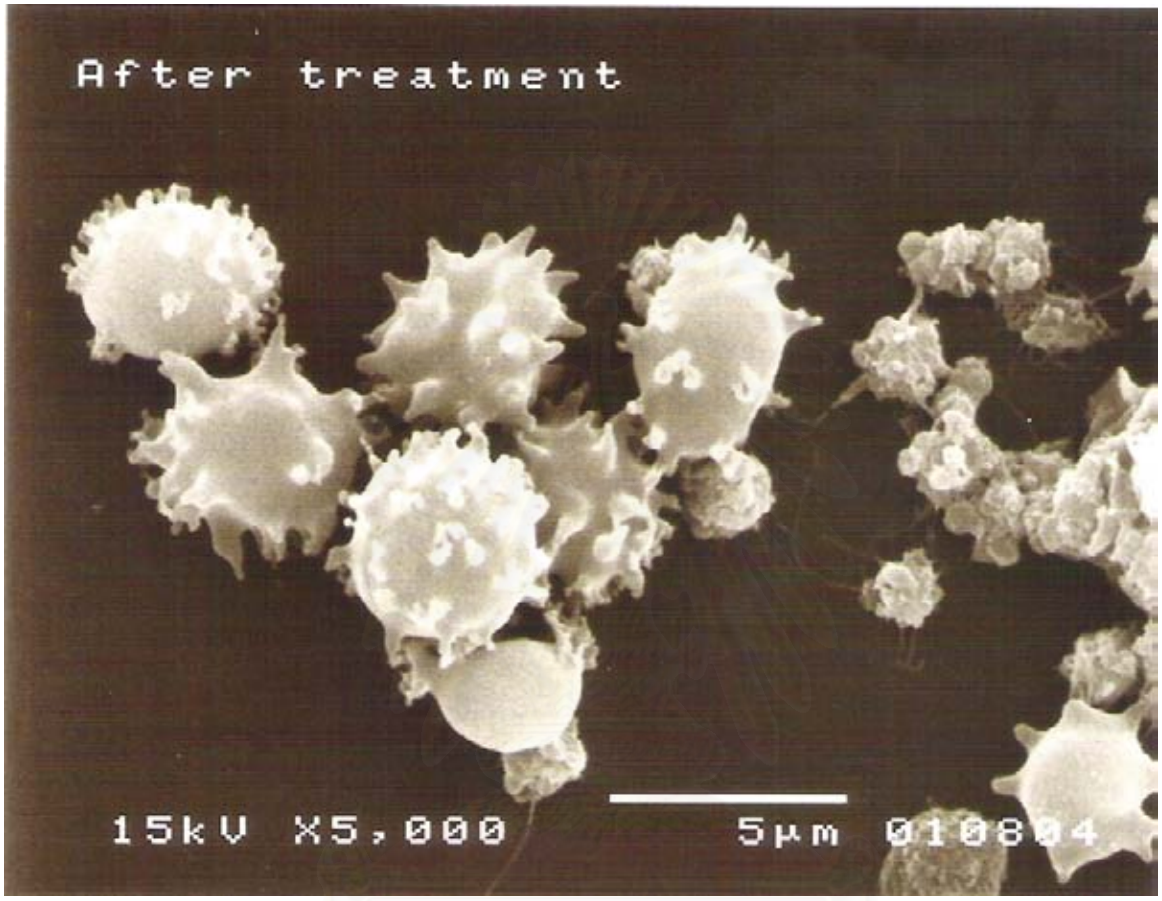


Fig. 4 Electron micrograph of platelets after treatment of green pit viper venom, their surfaces are irregular and rough, diameters ranged from 1.1-1.2 micrometers and most of them adhered closely. Red blood cell showing sphero-echinocytes