4.Platelet increasing potential of *Carica papaya* leaf extract in Murine model.

PLATELET INCREASING POTENIAL OF *CARICA PAPAYA* LEAF EXTRACT IN MURINE MODEL

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4.1 Introduction:

Platelets are the predominating elements in the process of hemostasis and thrombosis, which along with red cells and plasma, form a major proportion of both human and animal blood. Each day in a healthy human body approximately $1x10^{11}$ platelets are produced by the cytoplasmic fragmentation of megakaryocytes (MK), their marrow precursor cells. About $1/3^{rd}$ of platelets are always transiently sequestered in the spleen. Normal count of platelets in healthy individual is approximately 150000-400000/µl of blood. The role of platelets in the process of clotting of blood at the site of interrupted endothelium is exceptional. (Source: https://www.nhlbi.nih.gov/health-topics/thrombocytopenia#Causes.)

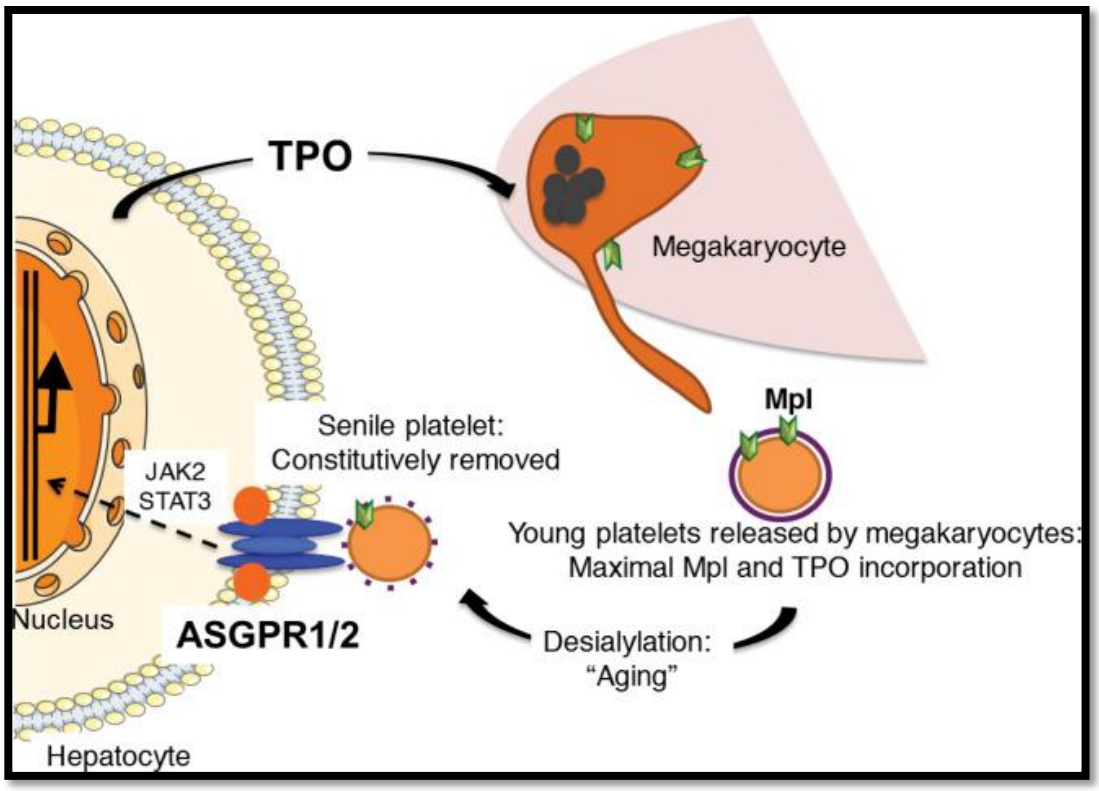

Figure 4.1: Role of Thrombopoetin in Megakaryopsis

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Platelets are packed with secretory granules, which are critical for normal platelet functioning. Among the three types of platelet secretory granules – α–granules, dense granules, and lysosomes, the α–granules are the most abundant and comprise of about 10% of total volume of platelets. There are approximately 50–80 α–granules per platelet, ranging in size from 200–500 μm (Frojmovic & Milton, 1982).

Recent studies have shown that platelets also play an unsuspected role in several other processes such as inflammation (Mannaioni *et al.* 1997; Wagner & Burger, 2003), innate immunity (Semple & Freedman, 2010), neoangiogenesis (Kisucka *et al.* 2006) and tumor metastasis (Karpatkin *et al.* 1981). Platelets circulate in the blood vessels and become activated if there is any bleeding or injury in the body. Collagen from the injured blood vessels signals platelets to become activated and join other components of the system to stop the bleeding (Pignatelli *et al.* 1998). Low blood platelet count can have adverse effects and may become fatal if untreated. The condition of low platelet count clinically known as Thrombocytopenia, is defined as a platelet count less than 150,000/μl. It is considered to be mild when the platelet count is between 70,000 and 150,000/μl, and severe if less than 20,000/μl. Most individuals are asymptomatic if the platelet count is 50,000/μl or greater.

Many factors that cause or induce thrombocyotpenia include, hereditary, certain drugs, chemotherapy, immune reaction towards own platelets, some syndromes like Gilbert's Syndrome, Hemolytic Uremic Syndrome and diseases like dengue. The symptoms may be mild to severe depending upon the underlying cause. These causes are:

- Inadequate thrombopoiesis by Bone Marrow.
- Excessive destruction of normal platelets.

- Abnormally high sequestration of platelets by spleen.

Diseases such as dengue, Idiopathic Thrombocytopenic Purpura (ITP), Thrombotic Thrombocytopenia (TTP) and Systemic Lupus Erythematosus (SLE) result in a low thrombocyte count in the blood. Currently corticosteroids (Jacobs *et al.* 1986), medications like TPIAO, recombinant human TPO (Kuter & Begley, 2002) and platelet transfusions are being utilized for increasing the platelet in thrombocytopenic conditions (Kruskall, 1997). However, some patients have shown resistance to corticosteroids and TPO. Some of these conditions require urgent need of platelet transfusion but there remains perennial shortage of blood and blood components in most of the developing world countries. Also, the platelet products are expensive and among the most misused blood products in developed world (Schofield *et al.* 2003). As very few centers in developing world have platelet separation facility, there is limited availability of platelet products, and hence, there arises a need for alternate, safe and inexpensive mode for increasing platelet. In a recent study, Sathasivam and group has shown that crude papaya leaf formulation was able to increase blood platelet count in mice (Sathasivam *et al.* 2009). However authors have recommended the dose dependent and time dependent studies for the same.

Herbal products are known to have a positive effect against many diseases and are the most popular form of traditional medicines. They formed highly profitable market in the International marketplace and accounted for about US$ 5 billion in 2003-2004 in W. Europe, US$ 14 billion in China and for US$ 160 million in Brazil in 2007. (Fact sheet N°134 December 2008). Traditional healers in Malaysia use *Carica papaya* leaf formulations in palm oil (Vehicle) to increase platelet count (Malaysian folk medicine

reports). Similar results were obtained by Ahmad and group (Ahmad *et al.*, 2011). *Carica papaya* Linn (Caricaceae), known as pawpaw and papaya, is common in tropical and sub-tropical countries. It is a native of the tropics of the Americans and originated from Mexico. Previously known reports demonstrated the presence of Sterols, Tannins, Saponin, Flavonoids, Alkoloids, Phlobatannin, Anthraquionones, Triterpenes in CPLE (aqueous) (Hardisson *et al.* 2001 ;Asaolu *et al.* 2010) Several species of Caricaceae have been used as remedy against a variety of diseases (Munoz *et al.*, 2000; Mello *et al.*, 2008). *Carica papaya* leaf extract have shown to help in wound healing (Nayak *et al.* 2007) and as an antisickling agent (Imaga & Adepoju, 2010). Reports also suggest the anti – tumor and immunomodulatory effects (Otsuki *et al.*, 2010). The leaves of papaya have been shown to contain many active components such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates which are known to increase the total antioxidant power in blood and reduce lipid peroxidation level (Seigler *et al.* 2002).

Use of modern day methods like flow cytometry to evaluate platelet life span has emerged in recent years. And as most flow cytometric studies can be carried out on whole blood, it eliminates the need of isolation of platelets (Lazarus *et al.* 1995). In addition, flow cytometric techniques has been developed which allows the measurement of nearly all the functional capabilities of platelets, such as activation and aggregation and to identify new functions by permitting observation of platelets interacting with leukocytes and measurement of platelet microparticles (Ault, 2001). Young platelets are also referred to as reticulated platelets, due to their RNA content (Harrison, 1997). Flow cytometry can also be useful to determine the age profile of circulating platelets by assessing the

percentage of reticulated platelets in different models in normal and disease states, as it can provide information about production and/or clearance defects.

The aim of current study was to

(i) study the overall platelet increasing property of CPLE in Rat

(ii) *In-vivo* labeling technique and reticulated platelet labeling to distinguish the old and newly formed platelets, after induction with CPLE, in Mice.

4.2 Material and methodology:

Plant material:

Fresh green mature leaves of *C. papaya* were collected from local fields from Vadodara District, Gujarat, India during the months of September-October and were identified by Prof. M. Daniel (Taxonomist), Rtd. Prof. and Ex.Head, Deptartment of Botany, The M.S. University of Baroda, Vadodara. The voucher specimen (BARO/51/2010) is deposited at the herbarium in (Dept. of Botany, The M.S.University of Baroda, Vadodara, India) for future reference.

Preparation of aqueous extract

The leaves of *C. papaya* L. (CP) were shade dried and ground in a food grinder. Around 100 g of powder was boiled with water for 30 min and the filtrate was concentrated to Semisolid form. Final concentration achieved was 35 % w/w. The extracts were stored at 4°C in dried form (Freeze dried) and used for the subsequent experiments.

Experimental Design:

Experiment 1:

Twenty four, 4 month old albino rats of *Wistar* strain weighing 200 – 225 g, were procured from Sun Pharma Advanced Research Centre, Vadodara, India. Animals were housed in polyproplene cages on a 12-h light/dark cycle, food and water were provided ad *libitium*, under the protocols approved by CPCSEA committee of The M.S. University of Baroda (827/ac/04/CPCSEA). Animals were acclimatized for a period of 5 days and were divided in 6 groups; (1) control (2) CPLE at a dose of 10 mg/Kg BW (3) CPLE at a dose of 100 mg/Kg BW (4) CPLE at a dose of 150 mg/Kg BW (5) CPLE at a dose of

1000 mg/Kg BW (6) CPLE at a dose of 3000mg/Kg BW. All doses were suspended in 0.9 % Saline and fed orally with gastric intubation tube. Control group was fed with vehicle only.

Hematological Evaluations:

Blood was collected, after every seventh day, in 2 ml EDTA vacutainers (BD Bioscience) by retro-orbital puncture under mild ether anesthesia before dosing the animals. Blood was utilized for CBC (Complete Blood Count) using Mindray 2800E Automated Haematoanalyser (Mindray instruments, China).

Experiment 2:

Evaluation of life span of platelets using *in vivo* labeling.

Materials:

1. (+) Biotin N-hydroxysuccinimide ester (NHS-biotin) (Sigma Aldrich).
2. Dimethyl sulfoxide (DMSO) (SRL Chemicals, Gujarat).
3. Saline for injection of NHS-biotin: 0.9% NaCl, unbuffered (Clarius Pharma, Gujarat, INDIA).
4. Antibodies: a) LEAF™ Purified anti-mouse CD41 Antibody clone MWReg30 (BioLegend Cat. No. 133910, CA, USA), b) PE anti-mouse CD41 Antibody conjugated to phycoerythrin (PE) Antibody clone MWReg30 (BioLegend Cat. No. 133905, CA, USA).
5. Streptavidin conjugated to allophycocyanin (APC) (BioLegend Cat. No.405207, CA, USA).
6. Aster Jandl citrate-based anticoagulant (AJ): 85 mM sodium citrate dihydrate, 69 mM citric acid, 20 mg/ml glucose, pH 4.6.
7. Platelet wash buffer: 140 mM NaCl, 5 mM KCl, 12 mM sodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0.
8. Platelet buffer: 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$ hexahydrate, 0.5 mM $NaHCO_3$, 10mM glucose, pH 7.4, titrated with NaOH.
9. Needles 25 gauge 5/8 in., syringes 1 ml. (BD Biosciences).
10. Warm water (60^{o} C).
11. Alcohol Swabs.
12. Flow cytometer (BD FACS Aria III, BD Biosciences).

Experimental Setup:

Twenty Four Balb C mice regardless of sex were selected for the study. After acclimatization, the animals were randomly divided into 4 groups each consisting of 6 animals per group. The animals were given free access to food and water for entire period of experiment.

Group 1: Control: Mice were injected 0.9 % saline (*intra-peritoneal)* + 600 µgNHS Biotin *(intra venous)* followed by 0.9% saline through oral intubation.

Group 2: Mice were injected with 4µg of LEAF™ Purified anti-mouse CD41 Antibody intra peritoneal in saline according to Marjon *et al.,(*2009) + 600 µg NHS Biotin *(intravenous)* followed by 0.9% saline through oral intubation after 12 hrs of Antibody induction.

Group 3: Mice were injected 0.9 % saline (*intra-peritoneal)* + 600 µg NHS Biotin *(intra venous)* followed by CPLE at dose of 100mg/kg BW in 0.9% Saline through oral intubation.

Group 4: Mice were injected with 4µg of LEAF™ Purified anti-mouse CD41 Antibody intra peritoneal, according to Marjon *et al.,(*2009) + 600 µg NHS Biotin *(intravenous)* followed by CPLE @ dose of 100 mg/kg BW in 0.9% saline through oral intubation after 6 hrs of Antibody induction.

Procedure:

1. NHS-biotin powder stock was prepared by dissolving NHS-biotin powder (new batch) in DMSO to give a stock of 30 mg/ml.
2. Working NHS–Biotin solution was prepared by diluting the fresh NHS-biotin stock 1/10 in 145mM NaCl at room temperature (RT) (Ensuring that there were no precipitates).
3. 200 ml of NHS-biotin in 145mM NaCl was injected into the tail vein of each mouse (~ 600 µg NHS Biotin).
4. Mice were bled from the tail vein 18h after CPLE dosing by dilating tail veins under heat lamp or with warm water. 5µl blood was taken into 125µl anticoagulant mix (25 µl AJ, 100 µl platelet buffer) and mixture was mixed gently.
5. Samples were centrifuged at 125 x *g* at RT; 100µl of supernatant which contained platelet fraction was collected, while red cell pellet was discarded.
6. CD41-PE antibody and streptavidin-APC (both 1/100 dilution) were mixed in platelet buffer and 100µl of this mixture was added to 100µl of platelets and incubated for 40 min at RT in the dark.
7. Post incubation 500µl of platelet wash buffer was added to each sample and was spun for 6min at 860 x *g*.
8. Supernatant was discarded and platelet pellet (invisible) was re-suspended in 300µl platelet buffer. Samples were then run on Flow Cytometer.
9. 20,000 events were acquired in the platelet gate on a FACS. The platelet population was identified by forward and side scatter.
10. All platelets will be positive for CD41-PE (FL2).
11. Older platelets are Positive for PE and Streptavidin -APC (FL4)
12. Newly formed platelets would be difference of total CD41 positive – Streptavidin (APC) positive

Experiment 3:

Labeling of Reticulated Platelet:

Materials.

1. Heparinized capillaries.
2. EDTA-coated tubes for blood collection (BD Bioscience).
3. Thiazole orange (TO) powder (Sigma Aldrich): Dissolved in methanol at 1mg/ml and stored in aliquots at −20°C in the dark until needed.
4. Anti-mouse CD41 Antibody conjugated to phycoerythrin (PE). Clone: MWReg30 (BioLegend Cat. No. 133905, CA, USA).
5. 1% paraformaldehyde (PFA) in phosphate-buffered saline (PBS).

Experimental Setup:

Group 1: Control animals were injected 0.9 % saline (*intra-peritoneal)* followed by 0.9% saline through oral intubation.

Group 2: Mice were injected 0.9 % saline (*intra-peritoneal)* followed by CPLE at dose of 100 mg/kg BW in 0.9% Saline through oral intubation.

Group 3: Mice were injected with 4µg of LEAF™ Purified anti-mouse CD41 Antibody intra-peritoneal in saline according to Marjon *et al.*(2009), followed by CPLE @ dose of 100 mg/kg BW in 0.9% Saline through oral intubation after 12 hrs of Antibody induction.

Group 4: Mice were injected with 4µg of LEAF™ Purified anti-mouse CD41 Antibody intra peritoneal according to Marjon *et al.*(2009), followed by CPLE @ dose of 100 mg/kg BW in 0.9% Saline through oral intubation after 6 hrs of antibody induction.

The following protocol was followed for fluorescently labelling reticulated platelets using Thiazole Orange (TO).

1. Blood was obtained from mouse by retro-orbital bleeding using heparinized capillaries into EDTA coated tubes.
2. TO in PBS was prepared by taking TO aliquot and diluting it to 0.1mg/ml in PBS.
3. Antibody solution was prepared by diluting CD41-PE 1/40 in PBS.
4. 50ml TO in PBS was mixed with 9ml antibody solution and 1ml blood.
5. Pipetted gently and incubated for 15min at room temperature avoiding light exposure.
6. 1ml 1% PFA in PBS was added to each sample, covered with foil and placed on ice.
7. Samples were run immediately on a Flow Cytometer and gated for platelets (FSC vs. CD41-PE FL2).
8. TO staining can be seen in FL1.
9. Reticulated platelets are TO+ while mature platelets are TO−.
10. Percentage of platelets that are TO + were determined.

4.3 Results:

Total blood profile reveled significant increase (P< 0.05, 0.001) in blood platelet count in all groups with respect to control groups wherein; 100mg and 1000mg CPLE showed maximum increase in platelet count on 28th day of observation with 990333 and 994600 platelets/µl of blood respectively (p<0.001), whereas control group showed 467000l platelets per µl of blood.

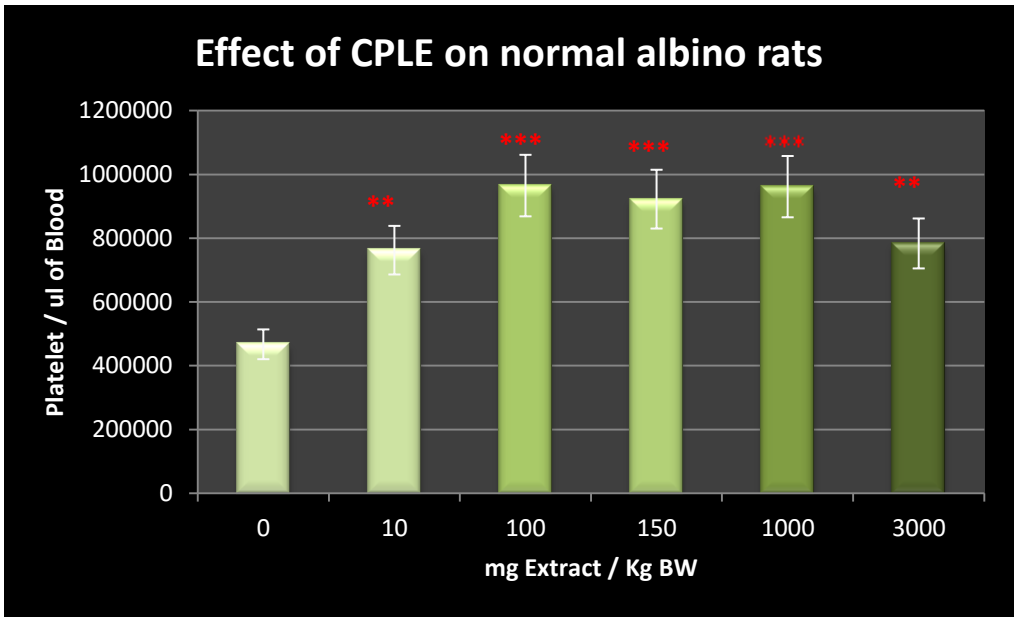
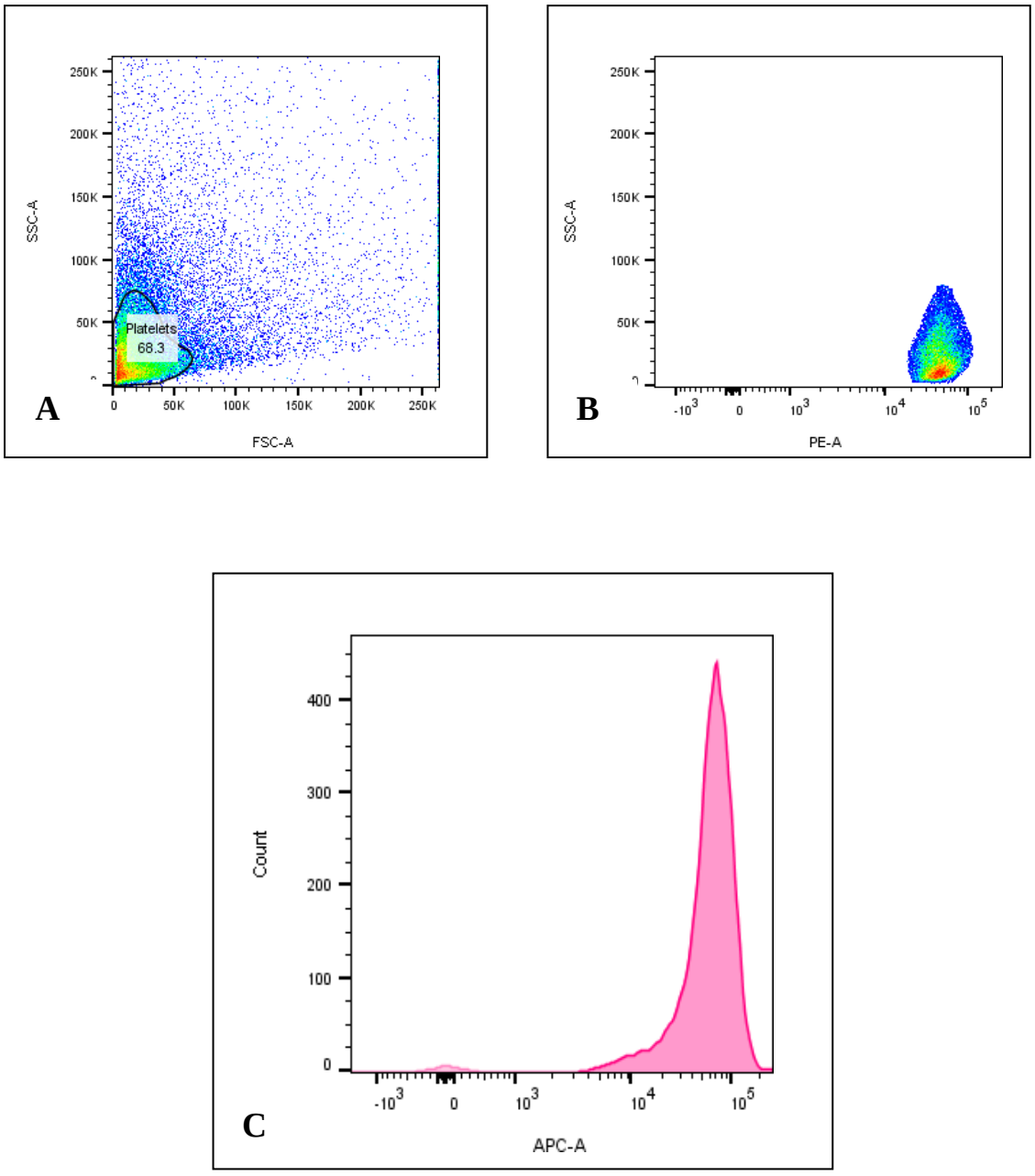


FIG 4.2: Effect of various concentration of CPLE on platelet count in *Rattus rattus norvegicus*

Results are expressed as mean±SE (n=6) where * p<0.05, **p<0.01, p<0.001

Control is compared with Treatment groups

Fig 4.3 Flowcytometric representation of Platelet population in Control group.

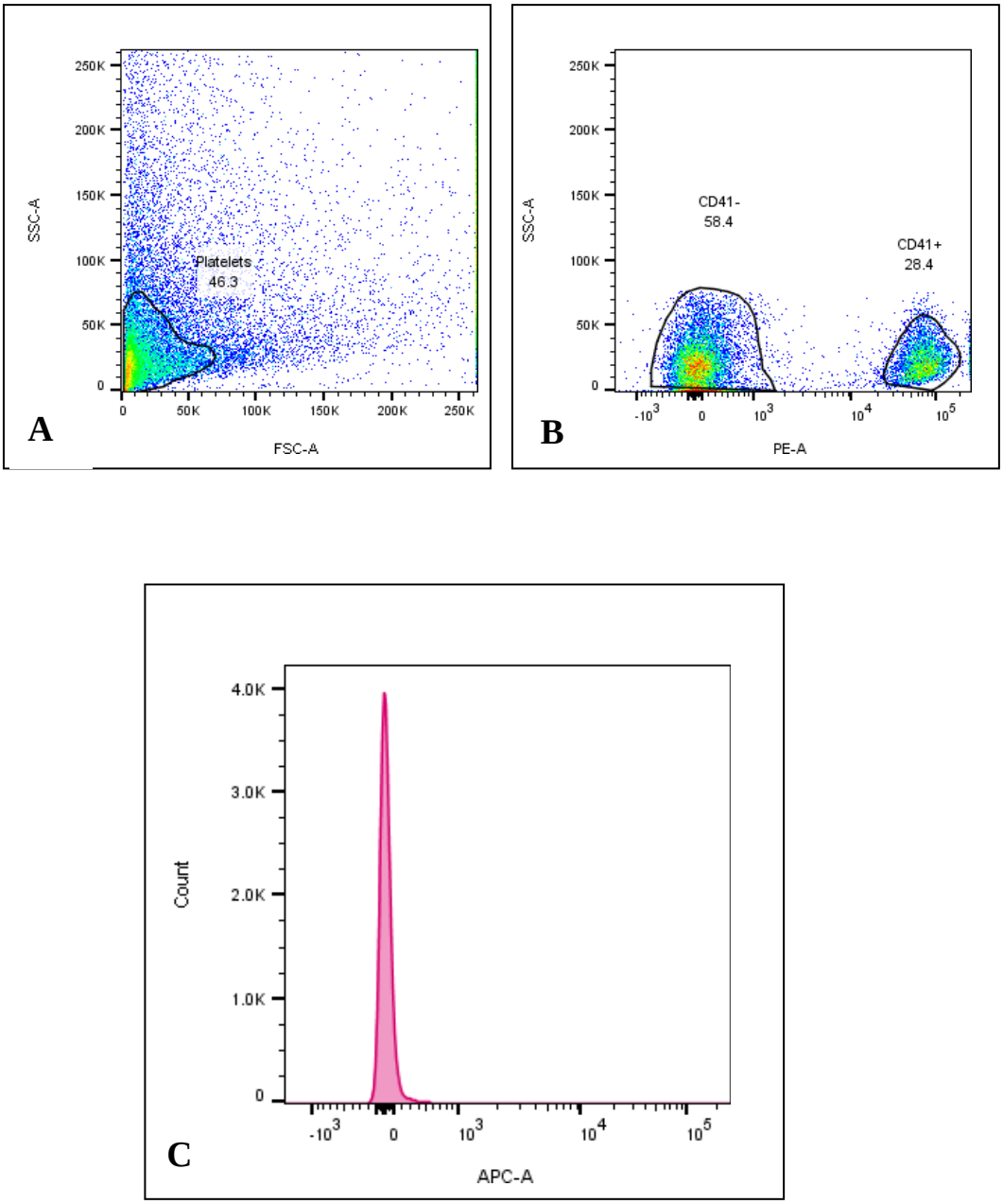


(A) Platelet population was selected via FSC and SCC gating the gated population.

(B) CD41+ (PE) cells (Platelets) are acquired from the population of interest, gated initially**.**

(C) Finally, the number of platelets positive for streptavidin (APC), were reported from the CD41+ population. Results are expressed in cell count**.**

Fig. 4.4: Flowcytometric representation of Platelet population in Antibody (alone) group.

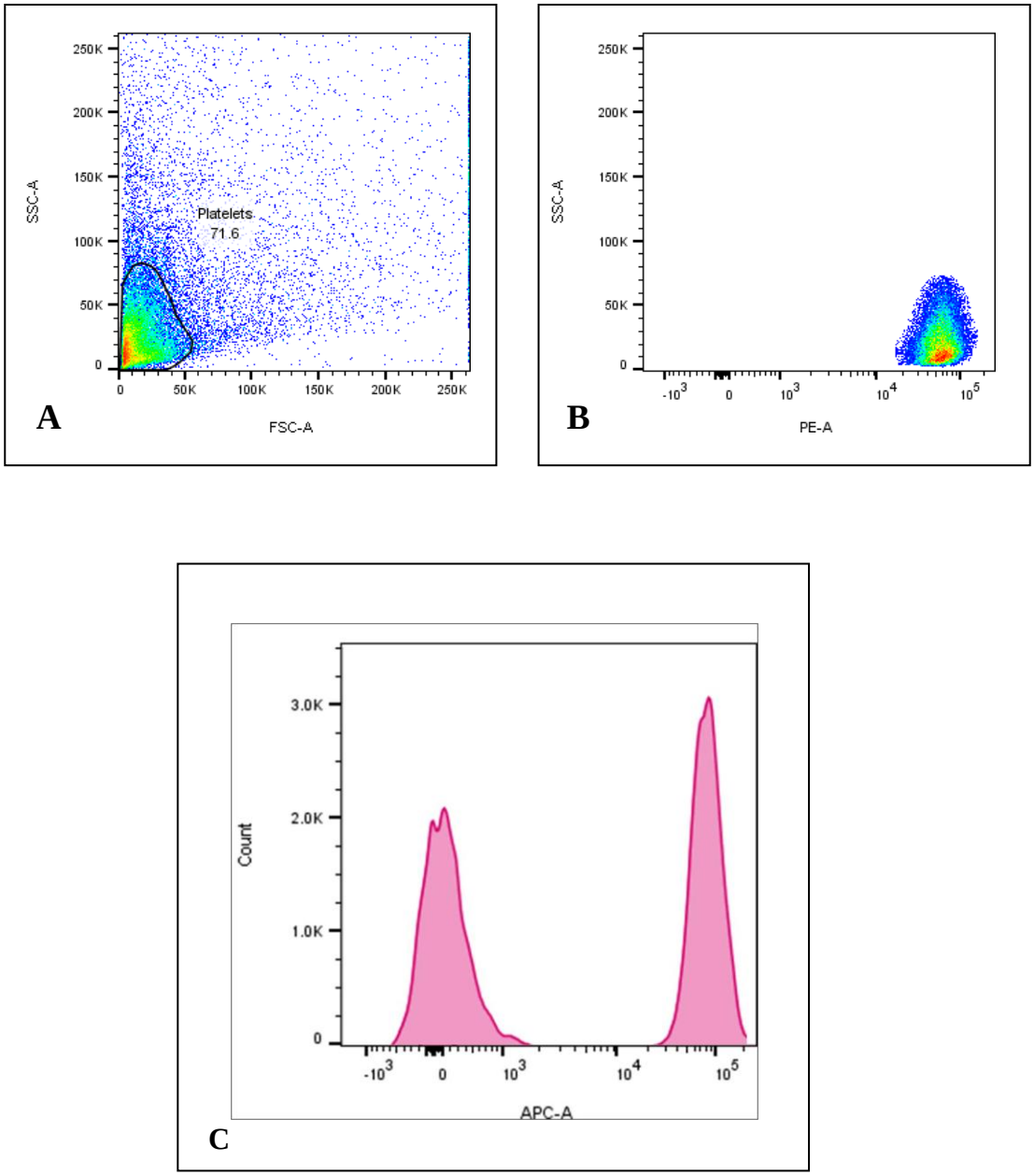


(A) Platelet population was selected via FSC and SCC gating the gated population.

(B) CD41+ (PE) cells (Platelets) were acquired from the population of interest, gated initially.

(C) Finally, the number of platelets positive for streptavidin (APC), were reported from the CD41+ population. Results are expressed in cell count

Fig. 4.5: Flowcytometric representation of Platelet population in Group treated with CPLE.

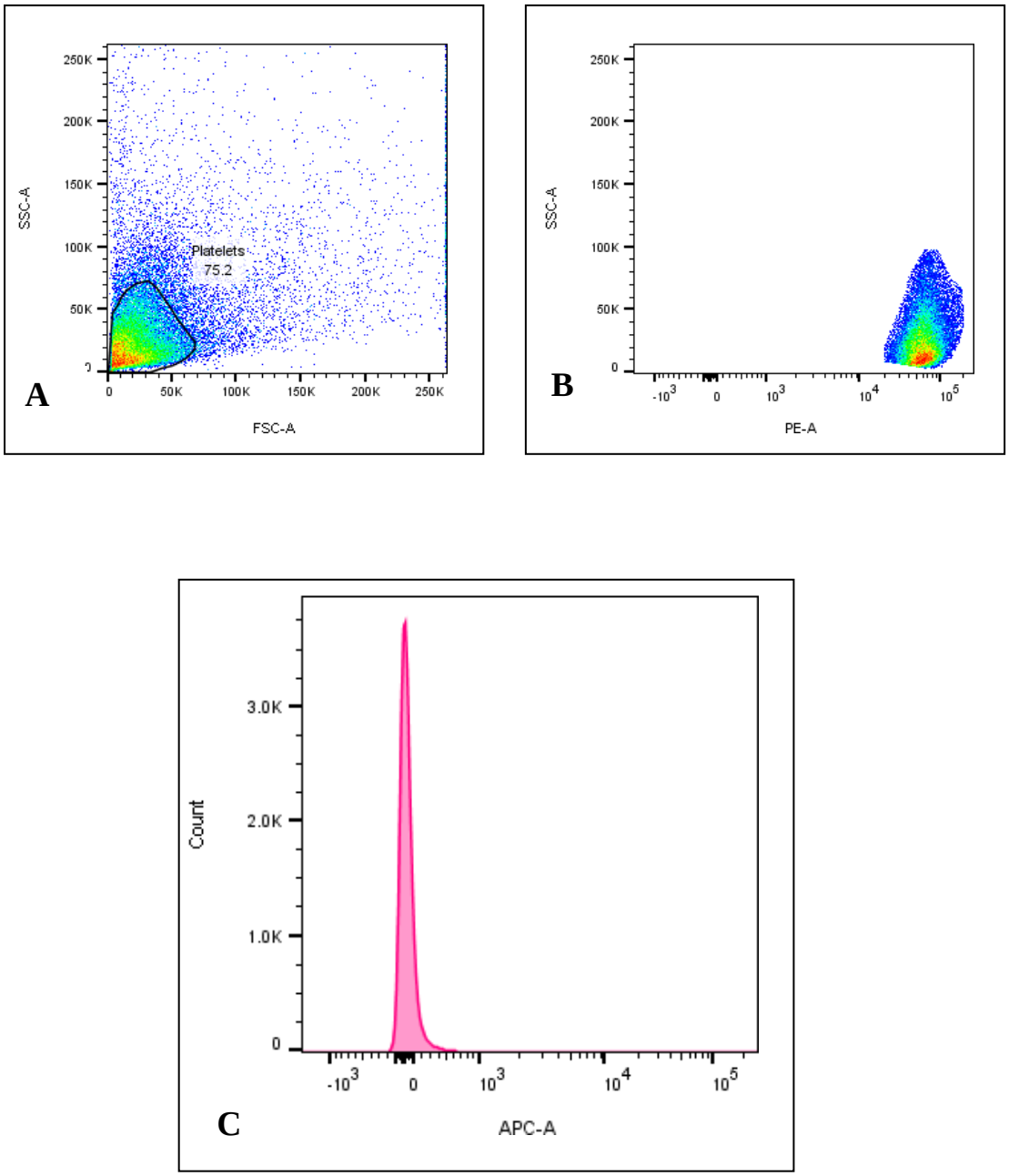


(A) Platelet population was selected via FSC and SCC gating the gated population.

(B) CD41+ (PE) cells (Platelets) were acquired from the population of interest, gated initially.

(C) Finally the number of platelets positive for streptavidin (APC), were reported from the CD41+ population. Results are expressed in cell count

Fig. 4.6 : Flowcytometric representation of Platelet population in Antibody + CPLE group.

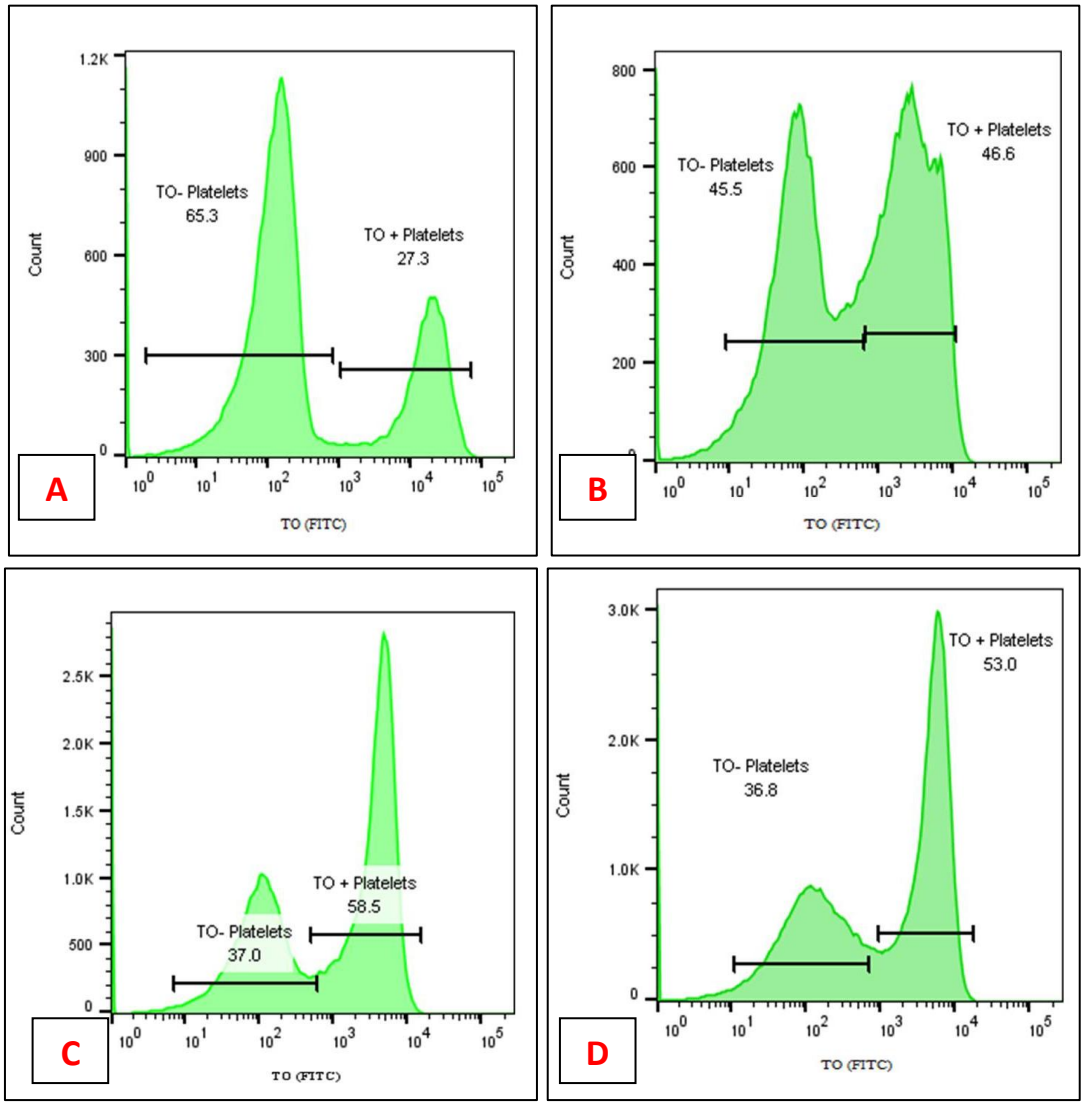


(A) Platelet population was selected via FSC and SCC gating the gated population.

(B) CD41+ (PE) cells (Platelets) were acquired from the population of interest, gated initially.

(C) Finally the number of platelets positive for streptavidin (APC), were reported from the CD41+ population. Results are expressed in cell count

FIG 4.7: Flow cytometric representation of Thiazole Orange (TO) treated platelet, to assess the Reticulocyte count



A shows the control animals, where the normal number of reticulocytes were seen. Around 27.3 % platelets were positive for TO which suggests that newer platelets were released in the blood pool.

B Animals treated with antiplatelet antibody 6 hrs post induction showed decrease in overall population of platelets thus counts were low. To counteract this more population of reticulated platelet are put in circulation suggesting ongoing megakaryopoiesis.

C Animals treated with only CPLE showed increase in megakaryopoiesis as compared to control animals, suggesting potent role of CPLE in increasing blood platelet count.

D Animals treated with both antiplatelet antibody and CPLE revels reversal of the effect of anti platelet antibody which suggests its potency even in antibody induced thrombocytopenia.

Results of flow cytometry revealed, over 95% platelet population to be positive for streptavidin, in control group (2 hrs after NHS biotin injection). They were positive for both CD41-PE and streptavidin –APC flurochrome.

Group 2: Animals subjected to antiplatelet antibody showed immediate decrease in blood platelet. This is evident from Fig.4.4B, which shows significant decline in CD41+ population (about 60%). However there was no steptavidin positive platelet population. This suggests that the CD41+ population were the new platelets formed as a result of megakaryopoiesis

Group 3: Animals, subjected to CPLE, did not show any difference in CD41 Population (Fig.4.5B). However there was new peak of streptavidin negative population that was significantly high, recorded in CD41+ population, suggesting new platelet synthesis and higher megakaryopoiesis with respect to control Fig (4.3C).

Group 4: Animals were subjected to both antiplatelet antibody and CPLE. In this plot there were similar peak of stereptavidin negative cells suggesting antibody completely removed the older population of platelets. However the normal CD41+ population was observed as in control (Fig.4.3B). This suggests that CPLE was able to increase platelet count in our established model of thrombocytopenia.

Further studies using Thiazole Orange (TO), which labels reticulated platelets (young platelets having high RNA content), were visualized on FL1 (Green channel). The results confirmed that CPLE was responsible for the formation of enhanced new platelet population. The population, positive for TO, indicates new platelet in circulation.

4.4 Discussion:

From the various reports published in scientific literature, it appears that *C. papaya* leaf extract does have beneficial properties in dengue (Ahmad *et al.*, 2011; Sarala & Paknikar, 2014). It has been shown to bring about a rapid increase in platelet count. This could be possibly attributed to its membrane-stabilizing property. The flavonoids and other phenols present in the extract have been suggested to provide the beneficial effects (Sharma *et al.*, 2013).

Sathasivam *et al.*, (2009) have reported an increase in platelet count in rats after CPLE treatment. However the study lacked in elaborate dose dependent and time dependent effects of CPLE. Our aim in this study, therefore, was to confirm the general belief that CPLE increased the platelet count if consumed during Dengue fever. A systematic dose dependent and time dependent study reveled that there was significant increase in the platelets at dose as low as 10 mg extract/Kg BW. The higher doses increased the count further with maximum increase between 100 to 1000mg/Kg BW. Our earlier experiment (study 1) has reported no toxic effect of CPLE even at high doses, indicating it can safely be consumed. However, the study lacks answering the main question whether there are new platelets formed after consuming CPLE or the older platelets are not destroyed by the reticulo-endothelial system.

Platelet production is a continuous process and stress conditions stimulate Megakaryopoiesis and platelet production. Therefore it is required to confirm that CPLE induces platelet production over and above the innate mechanism. Hence, present experiment was designed to differentiate new platelets from the old undestroyed ones and prove the role of CPLE as platelet increasing agent. In this study we initially labeled the

platelets with NHS biotin and checked streptavidin (binds to biotin) positive cells from CD41 positive cell population (Platelets are positive for CD41). Antibody induced thromobocytopenic animals and thrombocytopenic animals treated with CPLE were subjected to similar protocol. Platelet population identified with CD41 was checked for reticulocyte population by Thiazole orange. These studies reveled that CPLE extract was inducing the production of platelet significantly.

Our study, therefore, confirms that *Carica Papaya* leaf extract is safe to consume by humans. It would be of great advantage to the patients suffering from Thrombocytopenia caused by various diseases, specially benefitting those patients who require platelet transfusion frequently like that in ITP.

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