The Potential Role of Thrombopoietin and Interleukin-6 in the Thrombocytosis Effect of \textit{Carica papaya} Leaves

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**ABSTRACT**

Dengue fever is endemic in tropical urban developing regions worldwide. Thrombocytopenia is an important clinical feature which may result in bleeding. However, there is no specific treatment for Dengue-induced thrombocytopenia. \textit{Carica papaya} leaves (CPL) is a popular remedy in South East Asia to treat Dengue-induced thrombocytopenia. Development of CPL into pharmaceutical therapeutic agents is not forthcoming due to lack of rigorous scientific evidence and unknown mechanism of action. This study investigated the role of thrombopoietin (TPO) and interleukin (IL-6) in the thrombocytosis effect of CPL in vivo. These experiments were conducted using busulfan-induced thrombocytopenic rats. Treatment of aqueous and methanol extracts of CPL at 600mg/kg were administered orally for 7 consecutive days and serum platelet count was determined intermittently until day 15. At the end of experiments, serum Thrombopoietin (TPO) and IL-6 levels were determined by ELISA. Both aqueous and methanol extracts of CPL significantly increased platelet count compared to the control groups ($x^2 (2) = 25.373$, $P = 0.00$). Investigations into the mechanism of thrombocytosis showed that TPO and IL-6 levels were increased compared to controls but was not statistically significant ($H (3) = 5.339$ $P = 0.149$) ($H (3) = 4.412$ $P = 0.220$) respectively. This study is the first to document the thrombocytosis effect of both aqueous and methanol CPL extracts in a rodent model system. Our findings showed that aqueous extract of CPL demonstrated an increase of TPO and IL-6 levels. We suggested that the possible mechanism could be linked with the upregulation of major thrombopoietic cytokines such as TPO and IL-6.

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**INTRODUCTION**

Dengue fever is endemic in tropical urban developing regions worldwide. The disease burden is huge with significant socio-economic consequences (Bhatt \textit{et al.}, 2013). Thrombocytopenia of moderate degree is a common finding associated with DF and occurs in 26 to 50% of the classical picture in adults (Halstead, 2007). Although thrombocytopenia may result in bleeding tendencies ranging from mild gum bleeding to fatal haemorrhagic shock, there is very little correlation between thrombocytopenia and the occurrence of
severe bleeding in dengue patients (Lye et al., 2009). Nonetheless, there is no specific treatment for Dengue-induced thrombocytopenia. Carica papaya leaf (CPL) is a popular remedy in South East Asia to treat Dengue-induced thrombocytopenia (Subenthiran et al., 2013). Development of CPL into pharmaceutical therapeutic agents is not forthcoming due to lack of rigorous scientific evidence and unknown mechanism of action.

The thrombocytosis effect of CPL is well documented in rodent model and clinical trial. Sathasivam et al. (2009) have shown that suspension of CPL in palm oil revealed significant increase of platelet counts in mice. Fresh CPL extracts also showed significant increase in platelets and RBC counts in mice (Dharmarathna et al., 2013). Interestingly, Gammulle et al. (2012) revealed higher platelet counts in hydroxyurea-induced thrombocytopenia Wistar rats following administration of fresh CPL concentrate. Patil et al. (2013) observed similar findings in cyclophosphamide-induced thrombocytopenic rats with an increase of platelet count and decreases of clotting time following administration of CPL aqueous extract. CPL extracts was found to exert similar platelet augmentation effects with Psidium guajava extracts in cyclophosphamide-induced thrombocytopenic rats (Bordoloi et al., 2016). Clinical trials of CPL was conducted on 80 patients with dengue fever and were randomized into two groups; one group received CPL extract capsules with the standard treatment, whereas the other group received only standard treatment for dengue. According to this study, the platelets increased faster in patients administered with CPL capsule (Yunita et al., 2012).

In a study by Subenthiran et al. (2013) involving 228 patients diagnosed with dengue fever and dengue hemorrhagic fever, half of the cases received CPL juice for three consecutive days while the rest remained as the control. Blood monitoring showed a considerable increase in the platelet count among the intervention group as well as the expression of the ALOX 12 and PTAFR genes. In addition, Gadhwal et al. (2016) suggested that CPL is useful in dengue treatment and may prevent complication of thrombocytopenia as demonstrated by increased platelet counts, reduced hospitalization period and platelet transfusion requirement among dengue patients receiving CPL capsule.

However, mechanism by which CPL was able to induce thrombocytosis is very limited and unclear. Zunjar et al. (2016) demonstrated the role of the alkaloid carpaine in CPL thrombocytosis. Ranasinghe et al. (2012) observed membrane stabilization property of CPL which may help prevent platelet lysis. CPL also inhibited a protease involved in viral assembly affecting virulence which led to improved thrombocytosis (Senthilvel et al., 2013). CPL has been found to contain antioxidants and free radical scavenging property which is thought to affect thrombocytosis (Okoko and Ere, 2012). Previously, we have performed phytochemical analysis of CPL, in which 19 various compounds were detected in aqueous extract whilst methanol extract contains 24 compounds (Abdelrahim et al., 2019). CPL was found to have phenolic and flavonoids antioxidant compounds such as luteolin hexoside, dicafeoylquinic acid, apigenin, chrysoeriol, p-coumaroyl malate, o-feruloylquinic acid, naringenin methyl ether, isorhamantin-30-glucoside, o-cafeoyskimic acid and isoquercetin acetate (Abdelrahim et al., 2019). During thrombopoiesis, cytokine thrombopoietin (TPO) which is the main regulator of platelet production binds to surface receptor c-Mpl and regulates the process of platelet formation through various downstream signaling such as PI-3 kinase-Akt, MAPK, and ERK1/ERK2 (Yu and Cantor, 2012). In addition, IL-6 stimulates thrombocyte production by increasing thrombopoietin (TPO) secretion in the liver (Kaser et al., 2001). Sharma et al. (2019) observed an increase in TPO and IL-6 in CPL aqueous extract treated rats but was not significant. This observation needs validation. Using busulfan-induced thrombocytopenia rat model, we investigated CPL thrombocytosis mechanism through determination of TPO and IL-6 levels by both aqueous and methanol based extracts.

MATERIALS AND METHODS

CPL extracts

CPL powdered leaves of Indian origin were obtained from Nutricargo, (New Jersey, USA). CPL aqueous extract was prepared using decoction extraction method whereby methanol extract was prepared using maceration method. For aqueous extract, 200g of powdered leaves were dissolved in 2L distilled water and heated at 70°C for 1hr following filtration through Whatman filter paper no 1 (Maidstone, UK) using a funnel. The filtrate was collected and further heated at 60-70°C until volume was less than halved (~600ml). The concentrated extract was then dried in the oven at 40-50 ºC for three days before being stored at room temperature for further use (Adenowo et al., 2014). For methanol extraction, 600g of powdered leaves were soaked in 6L of methanol (John Kollin, Midlothian, UK) for 24 hrs and filtered through Whatman (Maidstone, UK) filter paper no 1. The filtrate was subsequently con-
centrations of platelet count were performed in the four groups. Group A served as sham control, was not treated with busulfan and only received distilled water. All rats in Group B, C and D were given busulfan to induce thrombocytopenia. For negative control, rats in Group B received distilled water. In treatment groups, Group C received 600mg/kg of aqueous Carica papaya leaves extract (AQ CPL) and Group D received 600mg/kg of methanol Carica papaya leaves extract (ME CPL).

Experimental design and procedures

All rats were randomly distributed into four experimental groups (n = 8) which received different treatments (Figure 1). In this study, busulfan-induced thrombocytopenic rats were used by giving intraperitoneal injection of busulfan to produce significant reduction in platelet counts of Sprague-Dawley rats. Busulfan is an alkylating anti-cancer agent with myeloablative properties and activity against non-dividing marrow cells and possibly, non-dividing malignant cells (Hassan, 1997). Group A served as sham control, was not treated with busulfan and only received 2ml distilled water as treatment. Groups B, C, and D were given busulfan on day 0 and day 3 for induction of thrombocytopenia. Busulfan solution (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) was prepared at a final concentration of 10 mg/ml in polyethylene glycol (Kuter and Rosenberg, 1995). A total dose of 20mg/kg BW freshly prepared busulfan was administered by intra-peritoneal (IP) injection for each rat. Group B served as negative control receiving 2ml distilled water as treatment. Group C received 2ml of CPL aqueous extract while group D 2ml CPL methanol extracts both at 600mg/kg BW. Treatment of either distilled water or CPL extracts were administered through oral gavage using a 5ml syringe with a stainless steel ball tipped 16G needle (Harvard Apparatus Ltd., Sevenoaks, Kent, UK). All treatments (distilled water and CPL extracts) were administered once daily from day 7 to 15. Platelet count was performed on days 0, 3, 7, 11 and 15 from blood collected through cardiac puncture. Rats were anaesthetized using Avertin which was prepared by combining 2.5g tribromoethanol (Sigma-Aldrich, Missouri, USA), 10ml absolute ethanol (John Kollin Corporation Midlothian, UK), 1.5 ml tertamyl alcohol (Fisher scientific, Massachusetts, USA) and 108.5ml normal saline to make up 120ml anaesthetic solution by IP injection. 0.5ml blood was extracted from each rat by cardiac puncture and kept in sodium citrate tube. Platelet count was determined using Neubauer’s improved Haemocytometer (Hawksley Ltd; England) according to standard protocol. TPO and IL-6 levels were determined by ELISA (Wu et al., 2010) using specific kits (Elabscience Co. Ltd. Maryland, USA) which were read using SunriseTM microplate reader (Tecan AG Switzerland).

Figure 1 shows design of experimental group. All animals were divided into 4 groups. Group A was a positive control group and only received distilled water. All rats in Group B, C and D were given busulfan to induce thrombocytopenia. For negative control, rats in Group B received distilled water. In treatment groups, Group C received 600mg/kg of aqueous Carica papaya leaves extract (AQ CPL) and Group D received 600mg/kg of methanol Carica papaya leaves extract (ME CPL). Statistical Analysis

Where relevant, data are shown as mean values with standard errors of the mean (S.E.M.). Platelet count data were analysed using repeated measures general linear models (rmGLM) in SPSS (version 19.0) with TIME after introduction of experimental treatment being fitted as within-subject factor and TREATMENT (non-busulfan induced control, busulfan-induced control, CPL aqueous extract, CPL methanol extract) as a between subject factor. The Huynh–Feldt adjustment to the degrees of freedom was used to interpret significance on the side of caution when the data did not meet the requirements of Mauchley’s Test of Sphericity. Bonferroni and LSD post hoc multiple comparison tests were performed for each treatment group against each other. In addition, Kruskal-Wallis test was performed for data points on day 7 and day 15 of experiment. ELISA result for thrombopoietin and IL-6 levels was tested using Kruskal –Wallis test. A P value equal to or less than 0.05 was accepted as indicating a significant difference. All statistical models were checked for approximately normal distribution of residuals.

RESULTS

Thrombocytosis effect of CPL extracts

At the beginning of experiment (day 0) all groups had a comparative mean platelet count which ranged between 66.5 ± 1.4 × 10^6/μL and 71.8 ± 1.8 × 10^6/μL. With time the mean platelet count dif-
Analysis by repeated measures ANOVA revealed highly significant main effect of treatment (CPL extracts versus controls) ($F_{1,3}=340.994 \ P = 0.00$). Following induction with busulfan on days 0 and 3 there was a clear reduction of mean platelet count on day 7 in groups B, C and D (25 ± 1.01 × 10⁶/μL, 26.7 ± 0.7 × 10⁶/μL and 27.5 ± 0.5 × 10⁶/μL respectively) while group A which was not treated with busulfan remained constant at 69.7 ± 1.6 × 10⁶/μL (see Figure 2) and this difference was statistically significant ($H(3) = 15.839 \ P = 0.01$). Following treatment of CPL extracts the mean platelet count on day 15 in both CPL treated groups C and D had 127.9% and 83.8% increase respectively while group B (treated with distilled water) had a 16.6% decrease in mean platelet count. This difference was statistically highly significant ($H(3) = 25.373 \ P = 0.00$).

Figure 2 shows effect of oral administration of *Carica papaya* leaves aqueous and methanol extract on thrombocytopenia induced rat platelet counts (mean ± SEM, n=7/group). Platelet counts were measured at Day 0, 3, 7, 11 and 15.

### Effect of CPL extracts on TPO level

Mean TPO level was increased in the group which were treated with busulfan+CPL aqueous (51.9 ± 16.8 pg/ml) while both control groups (distilled water and busulfan+distilled water) had lower mean TPO levels (36.9 ± 8.2 pg/ml and 41 ± 12.1 pg/ml respectively) (see Figure 3A). However this was not statistically significant ($H(3) = 5.339 \ P = 0.149$). In contrast, mean TPO level of the group treated with busulfan+CPL methanol was lowest compared to all other groups (17.8 ± 4.5 pg/ml) (see Figure 3A).

### Effect of CPL extracts on IL-6 level

Mean IL-6 level was increased in the group which were treated with busulfan+CPL aqueous (95.6 ± 8 pg/ml) while both control groups (distilled water and busulfan+distilled water) had lower mean IL-6 levels (76.6 ± 8.6 pg/ml and 76.7 ± 18.1 pg/ml respectively) (see Figure 3B). However this was not statistically significant ($H(3) = 4.412 \ P = 0.220$). In contrast, mean IL-6 level of the group treated with busulfan+CPL methanol was lowest compared to all other groups (63 ± 9 pg/ml) (see Figure 3B).

### DISCUSSION

We first validated the thrombocytosis effect of CPL in vivo using chemotherapy induced rat model. This is consistent with others who demonstrated similar effect either using busulfan (*Zunjar et al., 2016*) or different bone marrow suppressant like cyclophosphamide (*Patil et al., 2013; Akhter et al., 2015*) and carboplatin (*Tahir et al., 2014*). Both aqueous and methanol extracts were significantly increased compared to controls with CPL aqueous with better efficacy. CPL may cause an increase in the platelet count by different mechanisms mediated by mul-
multiple active components such as papain, chymopapain, alkaloids, flavonoids, flavanols, benzylglucosinolate, and tannins. It is suggested that these compounds stimulate the megakaryocytes to produce sufficient numbers of platelets to maintain a suitable platelet count in mammals, particularly during chemotherapy (Tahir et al., 2014). CPL contains cardiac glycosides, anthraquinones, carpaine, pseudocarpaine, and phenolic compounds (Zunjjar et al., 2016). Previous reports suggested that CPL extracts have beneficial properties in increasing platelet count in dengue patients as well as in the murine animal model. 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., 2019). Previous study reported the presence of myricetin, caffeic acid, trans-furic acid and kaempferol in standardized CPL aqueous extract which increases platelet count in cyclophosphamide-induced thrombocytopenic rats (Anjum et al., 2017). To the best of our knowledge, for the first time solvent based CPL extraction was also able to induce significant thrombocytosis. This may indicate the role of polar bioactive compounds in CPL thrombocytosis. The validation of TPO and IL-6 role in thrombocytosis however was inconclusive. Although there was arithmetical increase of IL-6 and TPO in CPL aqueous extract treated rats this was not statistically significant as what was observed by Sharma et al. (2019). Our results reported that CPL methanol showed a trend in reducing both IL-6 and TPO although not significant. These findings suggested that the mechanism of increased thrombocyte production in response to CPL aqueous extracts could be linked with the upregulated thrombopoietic cytokines such as IL-6 and TPO. Since TPO is the major cytokine involved in megakaryopoiesis and thrombopoiesis, it is possible that CPL enhances thrombocyte production by first increasing IL-6 expression in stem cells and leukocytes, which in turn enhances the production of TPO in the liver, leading to an increased rate of thrombocyte production. In addition, an increase in cell proliferation could also be aided by an increase in the production of stem cell factor. The previous report suggested that stem cell factor acts in synergy with other cytokines, such as TPO, to increase the proliferation of immature progenitor cells which may contribute to thrombocyte production (Aziz et al., 2015). This may suggest that either the bioactive compound responsible for thrombocytosis is non-polar in nature or indeed some of the active agents in methanol extract inhibit TPO production. Another possibility will be that polar components present in CPL methanol extract may have suppressed TPO and IL-6. For example polar flavonoid like apigenin decreased IL-6 (Hougee et al., 2005). Furthermore, Abdelrahim et al. (2019) detected luteolin and apigenin in CPL methanol extract but not in the aqueous extract. Although there have been few studies demonstrating the thrombopoietic effects of CPL in vivo, none of those studies elucidated by which this effect is being mediated due to various reasons. For example, there was a great challenge to establish an ideal thrombocytopenia state in animal
model due to toxicity of busulfan. Validation of the role of TPO and IL-6 in CPL thrombocytosis mechanism however, was inconclusive due to the only relative demonstration of TPO and IL-6 increase. Therefore, additional studies are necessary using different method of thrombocytopenia induction on animal model, as well as determination of cytokines level before and after CPL treatment.

CONCLUSION

Our study has shown that the thrombocytopoietic effects of CPL may have been mediated by the stimulation of TPO and IL-6 which are important mediator of thrombocytopenia. To date, this finding is first documentation of thrombocytosis effect of both aqueous and methanol CPL extracts in thrombocytopenia rodent model system. Therefore, it is suggested that CPL has the potential to be used in the treatment of thrombocytopenia, as the treatment was only started after the animal model was confirmed to be thrombocytopenic. However, more study is required to determine the exact mechanism of CPL on thrombocytopoiesis from molecular level and its potentials to treat thrombocytopenia in Dengue fever.

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Conflict Of Interest

The authors declare that they have no conflict of interest for this study.

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