The Potential Role of Thrombopoietin and Interleukin-6 in the Thrombocytosis Effect of 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. 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 (x² (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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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, Gadhiwal 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. Ranaasinghe 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, isorhamntin-30-glucoside, o-cafeoyshikimic acid and isoquercetrin 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 °C 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-
centrated using a rotary evaporator maintained at 55°C. The concentrated extract was dried at 65°C for 10 days before being stored at 4°C for further use.

**Experimental Animals**

A total of 32 male Sprague-Dawley rats aged 7-8 weeks weighing between 180-250g were purchased from Forensic Alchemist Resources and Trading (Shah Alam, Malaysia) and acclimatized for ten days before initiation of any procedure. The animals were housed in Accela (Prague, Czechia) Optitrat individual ventilated cages system with suitable conditions for an experiment such as temperature-controlled room (22 ± 2°C) and 12-hour light-dark cycle. Rats were provided with standard rodent food and water ad libitum. Rats were weighed regularly before and during treatment for the monitoring of their health status. Animal ethical clearance was obtained from Universiti Kebangsaan Malaysia (UKM) animal ethics committee [UKMAEC/20-0402010-CAT-1].

**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. 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 × 10⁶/μL and 71.8 ± 1.8 × 10⁶/μL. With time the mean platelet count dif-

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**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).
Figure 1: Design of experimental group

fered between the groups.

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 \((25 \pm 1.01 \times 10^6/\mu L, 26.7 \pm 0.7 \times 10^6/\mu L and 27.5 \pm 0.5 \times 10^6/\mu L respectively)\) while group A which was not treated with busulfan remained constant at \(69.7 \pm 1.6 \times 10^6/\mu L\) (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 \pm 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 \((51.9 \pm 16.8 \text{ pg/ml})\) while both control groups (distilled water and busulfan+distilled water) had lower mean TPO levels \((36.9 \pm 8.2 \text{ pg/ml} \text{ and } 41 \pm 12.1 \text{ pg/ml} \text{ respectively})\) (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 \((17.8 \pm 4.5 \text{ pg/ml})\) (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 \((95.6 \pm 8 \text{ pg/ml})\) while both control groups (distilled water and busulfan+distilled water) had lower mean IL-6 levels \((76.6 \pm 8.6 \text{ pg/ml} \text{ and } 76.7 \pm 18.1 \text{ pg/ml} \text{ respectively})\) (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 \((63 \pm 9 \text{ pg/ml})\) (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-
Figure 3: Effect of oral administration of *Carica papaya* leaves (CPL) aqueous and methanol extract on (A) thrombopoietin (TPO) concentrations (pg/ml) and (B) IL-6 levels (pg/ml) in busulfan-induced thrombocytopenia rat

multiple active components such as papain, chymopapain, alkaloids, flavonoids, flavanols, benzylglucosinolates, 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 (Zunjar 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-ferulic 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 thrombocytopoiesis. 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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