

## FIBRINOLYTIC ACTIVITY OF CAFFEIC ACID PHENETHYL ESTER (CAPE): *IN-VITRO* STUDY ON WHOLE BLOOD CLOT

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### ABSTRACT

**Objectives:** CAPE is a phenolic compound found in honey bee product. The present study aimed to determine the fibrinolytic effect of caffeic acid phenethyl ester (CAPE) on whole blood (WB) clot through *in vitro* study.

**Methods:** Standardized WB clots were prepared and incubated in platelet poor plasma (PPP) as control and in 15mM CAPE (diluted in PPP) for varying incubation time: 3, 6 and 9 hours. The plasma from these tubes was measured individually for D-dimer (DD) by immuno-turbidometric method and residual of WB clots were weighed. Repeated measures ANOVA were performed for data analysis.

**Results:** There was significant mean difference of DD between PPP and CAPE groups based on time ( $P < 0.001$ ). The mean DD was higher in the CAPE group compared to PPP groups for all the incubation times (3, 6 & 9 hrs). The mean DD (SD) from PPP (control) recorded at 3, 6 and 9hrs were 0.30 (0.06); 0.41(0.07) and 0.54(0.15)  $\mu\text{g/ml}$  respectively. The mean DD (SD) from plasma containing CAPE at 3, 6 and 9hrs were 0.52 (0.6); 0.56 (0.10) and 1.35 (0.24)  $\mu\text{g/ml}$  respectively.

There was a significant difference of median interquartile range (IQR) of WB clot weight between pre and post incubation in PPP (control) and in CAPE at 3hrs, 6hrs and 9hrs ( $p$  values  $< 0.01$ ). However no significant different of clot weight between control and CAPE treated whole blood.

**Conclusion:** CAPE at 15mM concentration demonstrated fibrinolytic activity based on DD quantitation and not by clot weight by *in vitro* WB clot lysis in this study. CAPE showed time dependent activity and a potential antithrombotic agent requiring further investigation.

**Keywords:** Caffeic acid phenethyl ester, Fibrinolysis, D-Dimer and Whole blood clot.

### INTRODUCTION

The fibrinolytic system is implicated in many physiological and pathophysiological processes [1]. Impairment of fibrinolytic pathway may result in either bleeding tendency or obstruction of the blood flow resulting from inadequate removal of blood clot. This complicates the thrombotic event in atherosclerotic plaque along with other pathological consequences such as in myocardial infarction (MI) and stroke [2, 3].

Previous studies reported various fibrinolytic enzymes in different Asian foods such as Tofuyo, Korean Chung kook-Jang soy sauce, Japanese Natto and edible honey mushroom. These enzymes convert proenzyme plasminogen into serine protease plasmin and play a vital role in fibrinolysis [4, 5]. These results clearly indicate the source of fibrinolytic agents from the natural products. Among them, one of the potential natural compounds is honey bee product which is widely used for its antioxidant property in traditional practice. Till date there is no study ever conducted on fibrinolytic property of honey bee product.

Propolis (bee glue) is a natural resinous material that honey bees collected from plants. Its medical effects include antibiotic, antifungal, antiviral, and antitumor properties. The wide range of propolis activities was mainly attributed due to the presence of bioactive components such as flavonoids, phenolic acids and other derivatives [6-8].

CAPE is a phenolic active component of honeybee propolis extracts which has been reported to have numerous pharmacological activities. It has potent anti-platelet activities [9, 10], immunomodulatory effects and anti-inflammatory activities [11-13]. Researchers reported the antioxidant properties of CAPE which inhibits lipoxygenase activities and NF $\kappa$ B activations [14, 15]. It also reported to have anti-carcinogenic [16, 17], and neuroprotective [18, 19] properties. Polyphenols has reported to have their active role in the treatment of cardiovascular complications in animal models [20].

Traditionally people advice patients on anticoagulant therapy to avoid taking propolis as there will be higher risk of bleeding among them. However there is no scientific evidence for this rationality. This observation suggests for further investigation on

one of the compound in propolis as a preliminary study. Therefore, this study was conducted to investigate the possible effect of selected active compound, (i.e. CAPE) for its fibrinolytic activity by using an *in-vitro* procedure on preformed human *in vitro* whole blood (WB) clot.

### MATERIALS AND METHODS

#### *In vitro* WB clot procedure for fibrinolytic activity studies

The original works of a few researchers were reviewed and studied [21-24]. *In vitro* WB clot procedure to study the fibrinolytic activity was adapted and modified from previously published reports.

The plasma was used as a medium to immerse the WB clot for the purpose of monitoring the coagulation parameters or products. The retracted WB clot when suspended in a plasma milieu became more sensitive to lysis with fibrinolytic agents than in buffer reagent. The plasma proteins that were entrapped in the clots such as plasminogen and fibrin might have contributed to their sensitivity to lysis [25].

#### Preparation of normal pool plasma

This study was approved by the Ethical Committee for human study at Universiti Sains Malaysia. Informed consent was obtained from all voluntary donors involved in this study. Human PPP was prepared and processed strictly using O blood group as source of plasma by collecting about 9 ml of WB from each donor into two tri-sodium citrate tubes (4.5 ml each) The O blood group donors were used as sources of PPP to ensure compatibility. In this study WB clot was created from O blood group individual (however, plasma from A, B and AB blood groups could also be used as sources of plasma because they don't usually cause haemolysis). Blood free from HIV and Hepatitis B (antigen) Ag was drawn from 20 volunteers using evacuated system with multi-sample needle green sterile 21GX1½. Immediately after collection, samples were spun using a centrifuge (Eppendorf, 5810 R-Germany) at 1500g for 15 minutes at room temperature and then the supernatant was spun down at 1200g for 15 min. The procedure was carried out according to the Clinical Laboratory Standardization Institute (CLSI) guideline for coagulation tests.

Next the PPP of all donors were pooled together and the coagulation profile including fibrinolytic parameters were measured to obtain standardized pool PPP especially for prothrombin time, activated partial prothrombin time, fibrinogen, DD, factor VIII activity and von Willebrand factor antigen using coagulation analyzers STA compact [Diagnostic Stago, France] and ACL Elite pro, IL Italy], using specific reagents of the system and according to the instruction provided in the kits.

The pool PPP was kept in small volume 10 ml falcon tube and stored at -80°C.

#### WB clot preparation

Venous blood (4.5ml) was drawn from a healthy volunteer with blood group O. The blood was then transferred into 3 pre-weighed sterile siliconized glass tubes 12x75mm without anticoagulant. It was first allowed to clot at room temperature for approximately 10 min. The tube was covered by parafilm to avoid contamination and haemolysis. It was then incubated at 37°C in water bath controlled temperature (Grant SUB6 England) for 3 hours to ensure complete clot retraction. After the WB clot underwent complete retraction from the edges of the glass tube, the serum was removed using Pasteur pipette. The tubes were dried by using filter paper and each tube with clot was again weighed to determine the clot weight (clot weight=weight of clot containing tube-weight of tube alone) using electronic analytical balance (AND FR-200 MK II, Japan) and the tube containing WB clot was appropriately labelled.

#### Quantitation of DD

The quantitation of DD levels were done by STA Compact coagulation analyzer (STAGO), and were determined photometrically by the immuno-turbidimetric method using Liatest kit. This test was done according to the STAGO, France manufacturer catalogue.

#### Procedure for WB clot lysis incubated in pool PPP

This procedure was used as a control test when time of incubation was monitored for fibrinolytic activity. To each of the tubes containing retracted WB clot, 1 ml of pool plasma was added after thawing at 37°C. The tubes were covered by parafilm and incubated at 37°C for 3, 6 and 9hrs. Following incubation, the plasma was obtained after gentle shaking of the clot and then the plasma was removed into microcentrifuge tube (bullet tube) by Pasteur pipette. Each glass tube containing clot was again weighed post incubation and the difference in weight before and after clot lysis was then subsequently measured. The previously removed plasma containing red blood cells (RBCs) and other particles due to clot lysis was spun at 1200 g for 5min (ependorf 5424- USA) and the supernatant was tested for the DD levels. The procedure was repeated for 10times to assess DD and clot weight changes.

#### Procedures for WB clot lysis incubated in CAPE

Commercial pure CAPE powder was purchased from Sigma Company (Aldrich USA). The powder was dissolved in dimethyl sulfoxide (DMSO) according to the concentration required. In this study 2M concentration was used and prepared as per the manufacturer's instruction. The solution was used as a stock from

which suitable dilution was made to study the fibrinolytic activity using *in vitro* WB clot method. The aliquots were kept in cryogenic vials and stored at -80°C until use. The solution was thawed at room temperature (~22°C) whenever needed and the unused portion was discarded.

15mM CAPE was prepared using PPP as diluents (1 ml) and WB clots were immersed into this concentration. Following the incubation at different times (3, 6 and 9 hours) the DD level and WB clot weight were measured similar as described above. The same procedure was repeated 10 times to assess the changes of DD and the difference in WB clot weight pre and post CAPE treatment.

#### Statistical analysis

Statistical analyses were performed using PASW® Statistics 20 (SPSS, Chicago IL). Data were expressed as mean difference of DD within group analysis followed by pairwise comparison for WB clot lysis, when incubated with plasma and treated with CAPE (time effect). The relationship between them was investigated by using repeated measures ANOVA between groups for treatment effect (regardless of time) and between groups based on the time (time dependent). The WB clot weight between pre and post plasma incubation and CAPE treatment were compared using Wilcoxon Signed Rank test. A \*p value ≤0.05 and \*\*p<0.01 was considered to be statistically significant.

#### RESULTS

There was a significant mean difference of DD within PPP and CAPE groups based on time (F= 421.13, P <0.001). Pairwise comparison with Bonferroni correction showed that there was a significant difference for all comparisons in PPP group, however in CAPE group, the comparison between 3 and 6 hours showed insignificant result as shown in Table1.

The mean DD (SD) for PPP incubation based on time was recorded at 3, 6 and 9hrs were 0.30 (0.06); 0.41(0.07) and 0.54(0.15) µg/ml respectively. The mean DD (SD) for CAPE treatment at 3, 6 and 9hrs were 0.52 (0.6); 0.56 (0.10) and 1.35 (0.24) µg/ml respectively.

From the test of between -subject effects, there was an overall significant of DD among the two groups (F= 284.81, P<0.001). The mean of DD was higher in CAPE group compared to PPP (Table 2).

There was a significant difference of median(IQR) of clot weight between pre and post PPP incubation at 3, 6 and 9 hrs showing pre vs post results at 3 hrs: 0.72 (0.13) vs 0.68 (0.12); at 6hrs: 0.74 (0.11) vs 0.68 (0.10) and at 9hrs: 0.75 (0.14) vs 0.71(0.11) gram respectively (p values <0.01). The clot weight reduction reflects WB clot lysis activity post incubation with PPP irrespective of the time.

There was a significant difference of median (IQR) of clot weight between pre and post CAPE incubation at 3, 6 and 9 hrs showing pre vs post results at 3hrs: 0.79 (0.05) vs 0.74 (0.07); at 6hrs: 0.74 (0.04) vs 0.68 (0.05) and at 9hrs: 0.74 (0.06) vs 0.71 (0.07) gram respectively (p values <0.01). The clot weight reduction post incubation in CAPE indicates WB clot lysis activity but statistically there was no different from the clot weight in the control group. This finding indicates that no different in fibrinolytic activity for both groups based on clot weight (p> 0.05).

**Table1: Comparison of DD levels within group for each incubation in PPP and CAPE according to time.**

Comparison D-Dimer	PPP (control)		CAPE	
	MD(95% CI)/µg/ml	p-value	MD(95% CI)µg/ml	p-value
0hr & 3hrs	-0.12(- 0.16, -0.08)	<0.001*	-0.34(- 0.41, -.27)	<0.001*
0hrs & 6hrs	-0.23(-0.27,-0.19)	<0.001*	-0.38(-0.50,-0.26)	<0.001*
0hrs & 9hrs	-0.36(-0.45, -0.27)	<0.001*	-1.17(-1.45, -0.89)	<0.001*
3hrs & 6hrs	-0.11(-0.16,-0.06)	<0.001*	-0.04(-0.18,-0.09)	>0.95
3hrs & 9hrs	-0.24(-0.35, -0.14)	<0.001*	-0.83(-1.10, -0.56)	<0.001*
6hrs & 9hrs	-0.13(-0.24, -0.02)	0.015*	-0.79(-0.48, -1.09)	<0.001*

\*p value <0.05 is significant

Repeated measures ANOVA within group analysis applied followed by Pairwise comparison with 95% confidence interval adjustment by Bonferroni correction. MD=mean difference

Table 2: Overall mean difference of DD among the control and CAPE treated groups.

Comparison	Mean (95%)	MD(95% CI) $\mu\text{g}/\text{ml}$	p. value
Pool PPP	0.36(0.34, 0.38)	-0.30(-0.33, -0.26)	<0.001*
CAPE	0.65(0.62, 0.69)		

\*p value <0.05 is significant

Repeated measures ANOVA between group analysis was applied F-stat (df) =248.81(2)

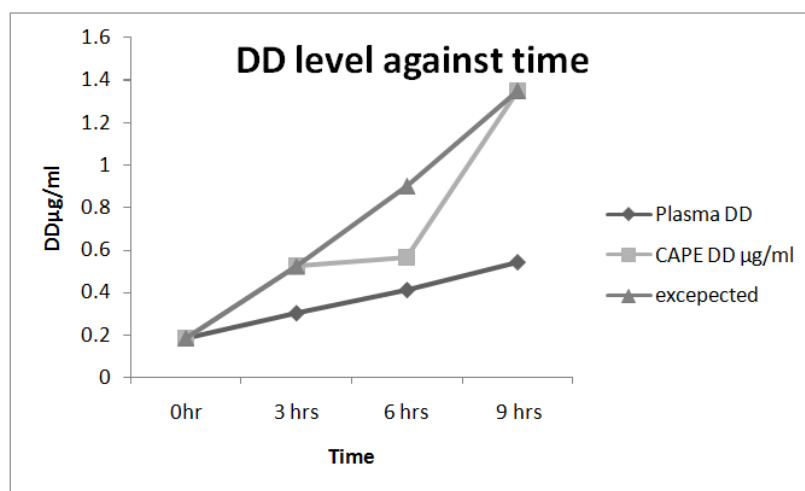


Fig. 1: DD levels against time for control, CAPE and expected results.

## DISCUSSION

The present study used DD quantitation and WB clot weight for the *in vitro* WB clot lysis method to assess the fibrinolytic activity of CAPE. In this study, retracted WB clot was used as fresh WB clot is not recommended for fibrinolytic studies[26]. The major difference between non-retracted and retracted blood clots is the quantity of unbound plasminogen inside the clot (plasminogen/fibrin ratio). Unbound plasminogen is expected to be higher in non-retracted than in the retracted clots. This high ratio (which is as a result of high number of plasminogen molecules in close proximity to fibrin that can bind to partially degraded fibrin) makes the non retracted clots more prone to lysis with any type of activator irrespective of the plasminogen concentration outside the clot. The ratio is even lesser in retracted clots as the pool of plasminogen is mainly located at the external surface of the clot[26]. The antioxidant, immunomodulatory, anti-inflammatory and anti-platelet activities of CAPE have been investigated but the information on haemostatic properties of CAPE is very scarce or none [9, 10, 12, 19].

The finding of this preliminary study revealed a statistically significant mean difference of DD levels in CAPE group when compared to PPP as control at different points of time. The control was used to compare and eliminate the fibrinolytic activity which could occur spontaneously.

The overall mean of DD levels when compared between CAPE and PPP groups regardless to time (treatment effect) is approximately twice of that of PPP as in table 2 which reflected that CAPE at 15mM concentration showed some fibrinolytic properties. The present study correlated with former study using the thrombolytic agents such as rt-PA which cleaves the fibrinogen and fibrin of thrombus into fragments D and E, and these products are represented by the concentration of DD as a specific fibrin degradation product[27].

The increasing trend of DD agrees with the previous report that used haemoglobin and radioactive method to evaluate the clot lysis activity [28, 29]. In figure 1 a graph was drawn to explain the expected DD level where the linearity was not achieved in this study at 6 hour point. There was a technical reason for not getting the expected level of DD. Upon correction the linearity of DD with time was able to be achieved and showed a significant different with the control (PPP). In his study, retracted WB clot incubated in PPP was

used as control test in which the lysis was allowed to occur spontaneously. The concentrations of DD were lower in untreated WB clot (in PPP) than in the CAPE treated clot when the time factor was put into account. This study shows that DD levels increased with time of incubation and is different when compared with CAPE treatment at the given time which were statistically significant. A comparable finding with the plasma clot has been reported previously [27].

The median weights of the WB clot post incubation for both plasma and CAPE treatment were significantly lower than pre plasma incubation. This result correlates with recently published studies [21, 22, 24]. However there was no significant different of clot weight between CAPE and control groups. Hence the activity of fibrinolysis was best seen with DD compared to clot weight suggesting this parameter is more sensitive in detecting fibrinolytic activity at molecular level than the clot weight.

The findings of this study suggest that at 15mM concentration, CAPE induces fibrinolytic process on WB clot as evidence by increasing DD levels compared to the control specimens. However the exact mechanism whether its effect is through direct or indirect fibrinolytic pathway remains for further work in future. The CAPE concentration in honey and propolis was reported in the range of (0.22±2.7 to 2.0±0.8) %w/w±RSD (relative standard deviation) from a number of 43 samples of propolis obtained from different countries using high performance liquid chromatography (HPLC). In some sample CAPE was not detected [30]. Another study performed in Italy reported CAPE concentration in the range of 9.9±0.4 to 12.9±0.7  $\mu\text{g}/\text{mg}$ ±SD weight of crude propolis[6].

The reason of using 15mM of CAPE in this study is due to consistent results obtained from this concentration based on incubation time and also it was the lowest concentration which gives reaction. Antiplatelets effect of CAPE works by inhibiting collagen binding detected by platelet aggregation at CAPE concentration between 15-100  $\mu\text{M}$  in an *in vitro* study. In this respect higher concentration of CAPE was shown to induce fibrinolytic activity in this study. The reason probably relates to the clot weight used in this experiment and the milieu in static plasma may also influence the fibrinolytic activity.

It is possible that fibrinolytic activity induced by CAPE is time dependent based on the ongoing increasing trend of DD when

compared with the control samples at the given time. It is not known whether crude propolis or honey taken orally has a low grade fibrinolytic effect to the body. CAPE showed fibrinolytic activity by DD quantitation using *in vitro* WB clot lysis study based on the procedure described here, but at higher concentration compared to the method described for its antiplatelets effect reported before.

#### CONCLUSION

This preliminary work demonstrated that CAPE can activate fibrinolytic system at high concentration based on the method used in this study. CAPE at 15mM represents time dependent fibrinolytic activity which requires further investigation to confirm its effect as potential antithrombotic agent.

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