Antithrombotic effect of purified caffeine and ethanol extracts of Coffea Liberica Hiern. Leaves in Swiss Albino mice

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Abstract: Coffee is the most widely consumed beverage throughout the world due to its stimulant effect and beneficial health properties. Thrombosis, a frequently occurred symptom of all kinds of cardiovascular diseases, is a leading cause of morbidity and mortality worldwide and thus, it is imperative to discover new thrombolytic agents. This study investigated the antithrombotic potential of purified caffeine and ethanol extracts of *Coffealiberica*Hiern. (Barako coffee) leaves in vivo and in vitrousing Swiss albino mice as models. The extracts were evaluated for in vivo antithrombotic effect by mice tail thrombosis model induced through injection of kappa carrageenan by intraplantar administration. In vitro thrombolytic potential was evaluated through the measurement of anticoagulant effect through prothrombin time (PT) and activated partial thromboplastin time (APTT). The results of the present study indicated that there was a significant inhibition ($p<0.05$) of induced tail thrombosis compared to the negative control group in the groups treated with 150 mg/kg ethanolic extract and 50 mg/kg and 100 mg/kg caffeine after 24 hours. The said doses have sustained their significant antithrombotic activities ($p<0.05$) except for 100 mg/kg caffeine which lost its significance after 48 and 72 hours. Moreover, there were significant increases ($p<0.05$) in the PT level of mice treated with 150 mg/kg ethanolic extract and 50 mg/kg, 100 mg/kg and 150mg/kg caffeine compared to the negative control group. APTT was also significantly prolonged ($p<0.05$) compared to the negative control group in mice treated with 100 mg/kg and 150 mg/kg ethanolic extract and 50 mg/kg and 100 mg/kg caffeine. Another noteworthy finding is that the ethanol extract showed better antithrombotic properties than caffeine and the latter showed insignificant effects in high doses. In conclusion, *Coffealiberica*

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ethanolic and caffeine leaf extracts revealed thrombolytic capabilities which are of importance in thrombotic diseases.

Keywords: *Coffealiberica*, antithrombotic, thrombosis, caffeine, carrageenan, prothrombin time, activated partial thromboplastin time

INTRODUCTION

In normal state, the hemostatic system preserves blood in its fluid state and react to blood vessel injury by clot formation. At instances of such injury, bleeding is halted through the formation of hemostatic plug by platelets and coagulation factors (Mackman, Tilley & Key, 2007). In addition to that, hemostasis is regulated by a balance in the procoagulant and anticoagulant properties of the vascular endothelium (Lee, Yang, Ku, Song,&Bae, 2012). On the other hand, failure to establish an equilibrium may result to either bleeding tendency or thrombosis (Riddel, Aouizerat, Miaskowski, & Lillicrap, 2007).

Thrombosis, along with other serious and life-threatening cardiovascular diseases like stroke, ischemia and coronary heart diseases, continues to be a principal cause of mortality embodying about 30 percent of global deaths (Lindholm &Mendis, 2007). It is an extremely complex process of the formation of a clot, known as a thrombus, in the circulation which can be initiated by blood vessel injury, disturbed blood flow or increased platelet adhesion and aggregation. Moreover, it is often correlated to the progression of atherosclerosis which poses greater threats and eventually higher likelihood of mortality (Arslan, Bor, Bektas, Mericli,& Ozturk, 2011).

It is initiated when excessive quantities of thrombin are formed during pathologic processes overwhelming the regulatory mechanisms of hemostasis. When the vessel wall is breached or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby starting the formation of a thrombus. Exposed collagen activates the buildup of platelets while exposed tissue factor initiates the production of thrombin, which not only converts fibrinogen to fibrin but also triggers platelets (Furie&Furie, 2008).

Despite the availability of various antithrombotic drugs, the current agents being used have some drawbacks like poor compliance, multiple drug interactions and residual platelet hyperreactivity (Weitz, Eikelboom, &Samama, 2012). For instance, aspirin, a well-known antiplatelet drug which also has efficient secondary preventive functions on ischemia, can cause severe hemorrhage and upper gastrointestinal bleeding (Jagtap, Sancheti &Phadke, 2012). Thus, it is imperative to look for new thrombolytic drug sources.

Recently, there has been an emergent interest in the utilization

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of natural agents to prevent and treat many illnesses including cardiovascular disorders (Tachjian, Maria,& Jahangir, 2010). The increasing knowledge of the platelet function mechanism is beneficial to the development of potent antithrombotic and antiplatelet drugs. Performing experiments on mice regarding thrombosis is generated mostly because the receptors and signaling pathways in mouse platelets show striking similarities to the human system with virtually every protein represented and with every cascade appearing to serve similar functions in both species (Sachs &Nieswandt, 2007).

Upon the application of in vitro and in vivo studies in various animals, many plant extracts are found out to be promising candidates for clinical use in treatment of thrombosis (Vilahur, Padro,&Badimon, 2011). Among these are the oil extract of *Mauritiaflexuosa* peel (Fuentes, Perez, Guzman, Alarcon, Navarrete, et al., 2013), *Campomanesiaxanthocarpa* aqueous extract (Klafke, da Silva, Rossato, Trevisan & Walker, 2012), and *Morus alba* leaves ethanol extract (Dong, Hyun, Man, Yoon & Won, 2014).

Like these plants, a lot of beneficial pharmacological actions can be derived from coffee especially its major active component, caffeine. It has been known to have compelling antioxidant activity due to its substantial levels of hydrophilic and lipophilic antioxidants and can increase glucose uptake in cultured human skeletal muscle cells adipocytes (Chu, Chen, Brown, Lyle, Black, et al., 2012). It was found out that caffeine also has a preventive effect against breast tumor growth and recurrence by inhibiting the procarcinogenic effects of active stromal fibroblasts (Al-Ansari & Aboussekhra, 2014). Green coffee bean extract (GCBE) can inhibit fat accumulation through activation of fat metabolism in the liver (Shimoda, Seki & Aitani, 2006). On top of that, coffee drinking (200 ml/ one cup) has been confirmed to decrease platelet aggregation in man. *Ex vivo*, coffee drinking extensively inhibited platelet aggregation stimulated by arachidonic acid and collagen (Natella, Nardini, Belelli, Pignatelli, Di Santo, et al., 2008).

*Coffealiberica*Hiern. is indigenous to tropical West Africa and today is mainly cultivated in Philippines, Malaysia, Indonesia, West Africa, Surinam and Guyana. It is a specie that grows in lowland to lower mountain rain forests or open scrub vegetation and is adapted to a warm and humid environment. Its roasted coffee beans are more popularly consumed in Southeast Asia where the coffee is brewed and drunk with sugar and milk. It is an evergreen, robust tree growing to 20 meters high when not pruned and with glabrous branches. Leaves are dark glossy green and broadly elliptic measuring up to 38 cm long and up to 15 cm wide (Lim, 2013).

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A B

Figure 1. COFFEA LIBERICA(A) tree and (B) leaves

Phytochemical screening revealed that methanolic extracts of the leaves of coffee contain flavonoids (Nayeem, Denny, & Mehta, 2011). The antioxidant capability offlavonoids presents a therapeutic potential in cardiovascular diseases, gastric ulcers, cancer or hepatic pathologies. Their antiviral, anti-allergic actions, antithrombotic and anti-inflammatory properties are also notable (Gallego, Campos & Tuñon, 2007). Furthermore, immature leaves of *C. liberica*contain caffeine synthesized from the obromine. This caffeine is gradually replaced by methyluric acids specifically theacrine at the next growth stage (Ashihara, Sano & Crozier, 2008).

Many studies have been carried out to examine the different effects of coffee bean extracts to health but attention has not been focused extensively on the benefits that can be obtained from coffee leaves. Therefore, the present study aims to: a) investigate the possible antithrombotic effects of caffeine and ethanol extract derived from *Coffealiberica* leaves *in vivo* using carageenan-induced tail thrombosis model in mice, b) evaluate the anticoagulant and thrombolytic effects of purified caffeine and ethanol extracts using several *in vitro* coagulation tests, c) make a comparison between the thrombolytic capabilities of purified caffeine and ethanol extract and d) determine the concentration of the extracts at which the antithrombotic effect is at its maximum for potential clinical use in the treatment of thrombotic diseases. Once the antithrombotic effect derived from *C. liberica* leaves has been attested, it may serve as a safer, more accessible and more affordable alternative to the commercially available thrombolytic agents.

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MATERIALS AND METHODS

Plant material

Fresh immature *Coffealiberica* leaves were collected from Brgy. Pinagtung-ulan, Lipa City, Batangas during the month of June 2014. The leaves along with the fresh beans were identified and authenticated by Mr. Noe B. Gapas, Museum Researcher II of the Botany Division, National Museum, Manila.

Preparation of Sample

Two kilograms of *Coffealiberica* leaves were washed with water to remove extraneous matter (Rahman, Ali,& Ali, 2008), shade-dried at room temperature (20°C) for three days (Verma& Kumar, 2010), cut into small pieces (Parashar, Parashar, Sharma,& Pandey, 2009) and crushed into a fine powder using sterilized mortar and pestle. To achieve uniformity of texture, the powder was screened through sieve no. 60 (Verma& Kumar, 2010).

Liquid-liquid Extraction of Pure Caffeine

Caffeine extraction was according to a procedure proposed by Mohammed & Al-Bayati (2009). Five hundred grams powder of sieved coffee leaves were dissolved in 25ml distilled water. The solution was mixed for four hours using a magnetic stirrer and gently heated to ease the removal of caffeine. Then, it was filtered using a glass filter. In a volume ratio of 25:25 ml, the initially prepared coffee solution was mixed with dichloromethane. It was stirred for 10 minutes. Using a separatory funnel, the caffeine from the solution was extracted. The process was done four times using 25ml dichloromethane at each series and the extracts were stored in volumetric flasks. The caffeine was recrystallized with 5ml hot acetone followed by gradual addition of hexane until the solution appeared cloudy. It was cooled and the crystalline caffeine was collected after evaporation of the solvent under a fume hood (Atehnkeng, Ojiambo, Donner, Ikotun, Sikora, et al., 2008).

Characterization of Pure Caffeine

High Performance Liquid Chromatography (HPLC) Analysis

Comparison of the peak areas and retention time of the isolated compound and standard caffeine (HPLC-grade, *Sigma*) was performed at Lipa Quality Control Center, Lipa City, Batangas, Philippines. It was analyzed by using 50ug/ml concentration of isolated and standard compound in methanol- glacial acetic (95:5 v/v) solvent.

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The peak area was recorded on Shimadzu HPLC model LC-10 using water-methanol-acetic acid (70:27:3 v/v/v) at flow rate 1.0ml/min. Twenty ul of injection volume was eluted in RP C(18) column at room temperature with monitored wavelength of 273nm using diode array UV detector. Solid phase extraction was done prior to the analyses to avoid the obstruction of the column (Verma& Kumar, 2010).

Ethanol extract preparation from COFFEA LIBERICA leaves

Ethanol extract was obtained through a Soxhlet procedure described by Annegowda, Mordi, Ramanathan, Hamdan, & Mansor (2011). Fifty grams of the ground leaf material was extracted using 500ml ethanol (99.5%, v/v) at 70°C for 48 hours using the soxhlet extractor. The liquid extract was filtered and concentrated using a rotary evaporator under reduced pressure at 50°C. The dried extract was stored at 4°C until future utilization.

Experimental Animals

Inbreed male Swiss albino mice weighing between 30 and 40grams were obtained from Department of Pharmacology and Toxicology, University of the Philippines College of Medicine, Ermita, Manila. They were acclimatized for one week prior to the experiments. They were placed in an air-conditioned room with 12/12h light/dark cycle and a temperature of 22 ± 2°C and suppliedwith food and water *ad libitum* (Arslan et al., 2011).

Experimental Design

A total of 48 male mice were randomly grouped into eight (8) with six animals each (Arslan et al., 2011).

Group I: 20% dimethyl sulfoxide (DMSO) – Negative Control
Group II: 50 mg/kg of *C. liberica* ethanol extract dissolved in 20% DMSO
Group III: 100 mg/kg of *C. liberica* ethanol extract dissolved in 20% DMSO
Group IV: 150 mg/kg of *C. liberica* ethanol extract dissolved in 20% DMSO
Group V: 50 mg/kg of *C. liberica* isolated caffeine dissolved in 20% DMSO
Group VI: 100 mg/kg of *C. liberica* isolated caffeine dissolved in 20% DMSO
Group VII: 150 mg/kg of *C. liberica* isolated caffeine dissolved in 20% DMSO
Group VIII: 100 IU heparin sodium – Positive Control

The entire administration was carried out by intraperitoneal injection (Arslan et al., 2011).

Carrageenan-induced mice tail thrombosis model

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Utilizing carrageenan in assessing antithrombotic agents allows visual and direct observation of thrombosis progression in a time-dependent manner. Carrageenan is a polysaccharide polymer manufactured from red seaweeds and commercially used as a gelling and thickening agent. Type I carrageenan contains high amounts of kappa carrageenan, a potent thrombogen that acts through local blood vessel inflammation and endothelial cell injury thereby initiating the release of interleukin-1 and tumor necrosis factor.

One hour after the administration of the test samples, each mouse was injected with 40ul (1%) Type I carrageenan dissolved in NSS by intraplantar administration in the right hind paw. The formation of wine-colored thrombus at the end of the tail was examined and the thrombus lengths were measured after 24, 48 and 72 hours (Arslan et al., 2011).

Sample Collection and Preparation

Blood samples were collected 24 hours after the last treatment (Arslan et al., 2011), from the mice's facial vein through submandibular bleeding technique (Golde, Gollobin,& Rodriguez, 2005) using evacuated tubes containing 3.2% trisodium citrate in the proportion of 1:9 anticoagulant to blood. Each mouse was punctured with a metal lancet at the back of the jaw, very slightly behind the hinge of the jawbones, toward the ear. The blood samples were centrifuged at 1500 x g for 15 minutes at room temperature to obtain platelet poor plasma (Huaco, Werneck, Vicente, Silva, Diez, et al., 2013).

In vitro coagulation assays

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined using a coagulometer (Thrombotimer, BehnkElektronik, Germany), according to the manufacturer's instructions. Prior to testing, one metal ball was placed into each cuvette and allowed to warm for at least 3 minutes before use. For PT assay, 100 ul plasma was pre-warmed for one minute at 37MC. PT reagent from Diagnostica *Stago*, Inc., France (200 ul; prewarmed at 37MC for 10 minutes) was then added to start the coagulation and the clotting time was noted. For APTT assay, 100 ul plasma was pre-warmed for one minute at 37MC. Then, APTT assay reagent from Diagnostica *Stago*, Inc., France (100 ul) was added and incubated for five minutes at 37MC. Thereafter,100ul pre-warmed calcium chloride ($CaCl_2$) solution was added to instigate coagulation and the clotting time was recorded. Both PT and APTT results were expressed in seconds.

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Statistical Analysis

Results were expressed as the mean ± standard error of mean (S.E.M) to show variation in groups. All the data were presented at a 0.05 level of significance employing One-Way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test for demonstrating differences between the control and treatment groups and Tukey's HSD methodfor comparison between the extracts as post-hoc procedures.

RESULTS AND DISCUSSION

I.Ethanolic Extract of C. LIBERICA leaves

Extraction of 2 kilograms of crushed C. LIBERICAleaves resulted to approximately 35 grams of crude extract (1.75% yield) as shown in Figure 2. In a study by Nayeem, et al. (2011), phytochemical screening of methanolic extracts of coffee leaves revealed that they contain flavonoids, alkaloids, glycosides, phenolic compounds, carbohydrates, sterols, tannins and proteins.

Figure 2. Ethanolic extract from C. LIBERICA leaves

Figure 4A and 4B shows the HPLC chromatograms of the standard and isolated caffeine respectively. The retention times of the isolated caffeine at the first, second and third peaks were 1.843, 2.233 and 3.614 minutes, respectively while standard caffeine had retention times of 1.847, 2.225 and 3.614 minutes, respectively. Furthermore, isolated caffeine had a total peak area of 22659352 while standard caffeine had 23266954. Lastly, the isolated caffeine was found out to be 101.03% pure. This clearly shows that the isolated compound is truly caffeine. In relation to this, dichloromethane is the most extensively used solvent for decaffeinating coffee and tea with extracting efficiency of up to 98-99% (Belay, Ture, Redi,&Asfaw, 2008). The results are also

Figure 3. Isolated caffeine sample from C. LIBERICA leaves

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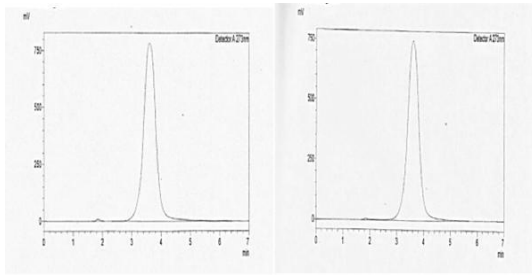
supported by the findings of Verma& Kumar (2010) revealing 99.27% pure caffeine isolated from CAMELLIA SINESIS(tea) leaves using the same extraction method.

II. Comparison of Isolated and Standard Caffeine

In 500 grams of crushed C. LIBERICA leaves, approximately 4 grams of caffeine were isolated (0.08% yield). Figure 3 shows the standard caffeine and the isolated caffeine from the coffee leaves which were subjected to high performance liquid chromatography (HPLC) in order to compare their peak areas and retention times and determine the purity of the extract.



A B

Figure 4. HPLC Chromatogram of (A) Standard Caffeine and (B) Isolated Caffeine from COFFEA LIBERICA leaves using dichloromethane

III. Mice Tail Thrombosis

In vivo antithrombotic capability of the extracts of COFFEALIBERICA leaves were assessed using the extent of inhibition in tail thrombosis length it caused to the mice models. Approximately 8 hours after the intraplantar injection of carrageenan, the thrombosis tail infarction appeared in Group I. In Groups II to VIII, the formation of thrombosis occurred 3-6 hours after. These were seen as wine colored regions at the end of the tail of the mice as shown in Figure 5. A week after the carrageenan injection, the tail thrombosis led to extensive tail necrosis especially at Group I.

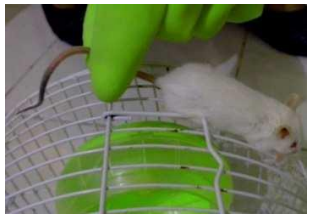


Figure 5. Tail thrombosis formation in a Swiss albino mouse 8 hours after carrageenan injection

Table 1 shows the effect of the treatment with ethanolic and purified caffeine extracts on the length of tail thrombosis after 24, 48

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and 72 hours. The negative control group administered with 20% DMSO resulted in a thrombus length of 3.30 ± 0.24 centimeters after 24 hours. Moreover, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts resulted in tail thrombus lengths of 4.13 ± 0.42, 2.25 ± 0.43 and 1.78 ± 0.23 centimeters, respectively. Meanwhile, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg caffeine extracts yielded thrombus lengths of 1.87 ± 0.24, 1.75 0.18 and 2.97 ± 0.26 centimeters, respectively. Lastly, in the positive control group, treated with heparin sodium, a 1.47 ± 0.18 centimeters thrombus formation was obtained.

Table 1
Effect of treatment with ethanolic and caffeine extracts of COFFEALIBERICA leaves andheparin sodium on lengths of tail thrombosis after 24, 48 and 72 hours

	Length of Tail Thrombosis		
Group	**After 24 hrs**	**After 48 hrs**	**After 72 hrs**
I- Negative Control	3.30 ± 0.24	3.48 ± 0.23	3.70 ± 0.18
II- 50mg/kg Ethanolic Extract	4.13 ± 0.42	4.27 ± 0.40	4.35 ± 0.34
III- 100 mg/kg Ethanolic Extract	2.25 ± 0.43	2.82 ± 0.33	3.33 ± 0.29
IV- 150 mg/kg Ethanolic Extract	1.78 ±0.23	2.20 ± 0.30	2.52 ± 0.27
V- 50 mg/kg Caffeine	1.87 ± 0.24	2.27 ± 0.25	2.53 ± 0.29
VI- 100 mg/kg Caffeine	1.75 ± 0.18	2.50 ± 0.19	2.95 ± 0.25
VII- 150 mg/kg Caffeine	2.97 ± 0.26	3.58 ± 0.30	4.43 ± 0.34
VIII- Heparin Sodium	1.47 ± 0.18	1.63 ± 0.19	1.87 ± 0.17

After 48 hours, the negative control group administered with 20% DMSO resulted in a thrombus length of 3.48 ± 0.23 centimeters. Furthermore, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts resulted in tail thrombus formation of 4.27 ± 0.40, 2.82 ± 0.33 and 2.20 ± 0.30 centimeters, respectively. In addition to that, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg caffeine extracts yielded thrombus lengths of 2.27 ± 0.25, 2.50 ± 0.19 and 3.58 ± 0.30 centimeters, respectively. Finally, in the positive control group, treated with heparin sodium, a 1.63 ± 0.19 centimeters thrombus formation was noted.

The negative control group administered with 20% DMSO resulted in a thrombus length of 3.70 ± 0.18 centimeters after 72 hours. Consequently, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts resulted in tail thrombus formation of 4.35 ± 0.34, 3.33 ± 0.29 and 2.52 ± 0.27 centimeters, respectively. Additionally, the groups treated with 50 mg/kg, 100 mg/kg and 150 mg/kg caffeine extracts yielded thrombus lengths of 2.53 ± 0.28, 2.95 ± 0.25 and 4.43 ± 0.34 centimeters, respectively. Lastly, in the positive control group, treated with heparin sodium, a 1.87 ± 0.17 centimeters thrombus formation was observed.

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Increasing concentrations of ethanolic extract showed progressive decrease in thrombus lengths. On the other hand, caffeine-treated groups showed opposite effects with decreasing inhibition of thrombus formation consequently. Also, it can be noted that there is a constant increase in thrombus lengths over time probably due to the diminishing effects of the experimental treatments as well as with heparin sodium.

Table 2 presents the comparison of the negative control and the concentration of *C. liberica*ethanolic and caffeine extracts on lengths of mice tail thrombosis after 24, 48 and 72 hours of thrombosis induction. After 24 hours, upon comparing the negative control with 50 mg/kg, 100 mg/kg, and 150 mg/kg ethanolic extracts, p-values of 0.2112, 0.0700, and 0.0036 were obtained respectively. Additionally, when compared to 50 mg/kg, 100 mg/kg, and 150 mg/kg caffeine, the resulting p-values were 0.0064, 0.0029, and 0.9370 respectively. Lastly, when compared to positive control, the obtained p-value was 0.0004.

Meanwhile, after 48 hours, upon comparing the negative control with 50 mg/kg, compared to positive control, the obtained p-value was 0.0002. Furthermore, after 72 hours, upon comparing the negative control with 50 mg/kg, 100 mg/kg, and 150 mg/kg ethanolic extracts, p-values of 0.2430, 0.3972, and 0.0141 were obtained respectively. When compared to 50 mg/kg, 100 mg/kg, and 150 mg/kg caffeine, the resulting p-values were 0.0218, 0.0885, and 0.9996 respectively. Lastly, when 100 mg/kg, and 150 mg/kg ethanolic extracts, p-values of 0.3960, 0.8819, and 0.0221 wereobtainedrespectively. Also, when compared to 50 mg/kg, 100 mg/kg, and 150 mg/kg caffeine, the resulting p-values were 0.0246, 0.2579, and 0.2781 respectively. Finally, when compared to positive control, the obtained p-value was 0.0002.

Table 2
Comparison of the negative control and the concentrations of C. LIBERICA ethanolic and caffeine extracts on the lengths of mice tail thrombosis

		After 24 hrs	After 48 hrs	After 72 hrs
	Concentration	p-value	p-value	p-value
I- Negative Control	II- 50mg/kg Ethanolic Extract	0.2112	0.2430	0.3960
	III- 100 mg/kg Ethanolic Extract	0.0700	0.3972	0.8819
	IV- 150 mg/kg Ethanolic Extract	0.0036*	0.0141*	0.0221*
	V- 50 mg/kg Caffeine	0.0064*	0.0218*	0.0246*
	VI- 100 mg/kg Caffeine	0.0029*	0.0885	0.2579
	VII- 150 mg/kg Caffeine	0.9370	0.9996	0.2781
	VIII- Heparin Sodium	0.0004*	0.0002*	0.0002*

**Significant at p-value < 0.05*

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This suggests that the groups treated with 50 mg/kg and 100 mg/kgethanolic extracts and 150 mg/kg caffeine showed no significant inhibition of mice tail thrombosis after 24 hours since they have p-values which are greater than 0.05, whereas the groups administered with 150 mg/kg ethanolic extract, 50 mg/kg and 100 mg/kg caffeine and heparin sodium showed significant inhibition of mice tail thrombosis since p-values were less than 0.05 and thus they exhibited in vivo antithrombotic activity. These effects were also seen after 48 and 72 hours, except for 100 mg/kg caffeine which lost its significance after 48 hours.

It can be implied that 50 mg/kg and 100 mg/kg *C. liberica*ethanolic extracts were not able to significantly prevent thrombus formation since they have lower concentration of the bioactive antithrombotic component. On the other hand, 150 mg/kg ethanolic extract may have the sufficient amount of phenolic compounds such as flavonoids which are noted to have both anti-inflammatory and antithrombotic effects (Kassim, Achoui, Mustafa, Mohd & Yusoff, 2010). These results are also comparable to the findings of Kou, Tian, Tang, Yan and Yu (2006), in which Radix Ophiopogonjaponicus tuber root aqueous extract at doses of 12.5 and 25.0 mg/kg markedly inhibited thrombosis of ICR mice at 48 and 72 hours and slightly inhibited thrombosis at 24 hours after carrageenan injection.

However, contrasting with the ethanolic extract, tail thrombus length decrease in caffeine was not dose dependent. 50 mg/kg caffeine inhibited thrombus formation but 100 mg/kg caffeine lost its significance over time and 150 mg/kg caffeine administration consistently demonstrated increase in thrombus length compared to the negative control rather than inhibition as shown in Figure 7. In relation to this, a study by Tsioufis, Dimitriadis, Vasiliadou, Taxiarchou, Vezali, Tsiamis et al., (2006) claimed that heavy coffee consumption may have the possibility of activating inflammatory pathways, affecting fibrinolysis and producing prothrombotic mechanisms. These are also similar with the findings of Arslan et al. (2011) wherein *Crataegusorientalis* (Hawthorn) ethanolic leaf extract at 200 mg/kg also lost its significance and 300 mg/kg concentration showed decreased significant values ($p<0.05$) after 48 and 72 hours.

IV. In Vitro Coagulation Tests (Prothrombin Time and Activated Partial Thromboplastin Time)

To investigate the potential interactions of *C. liberica* leaves ethanolic extract and isolated caffeine with coagulation factors, the effects of the extracts on coagulation time were evaluated by measuring

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prothrombin time (PT) and activated partial thromboplastin time (APTT). Prothrombin time is a measure of the integrity of the extrinsic and common pathways of the coagulation cascade. It represents the time for a plasma sample to clot after the addition of calcium and an activator of the extrinsic pathway (thromboplastin). Therefore, deficiencies or inhibitors of clotting factors in the said pathways result in the prolongation of the PT (Kamal, Tefferi & Pruthi, 2007). Furthermore, a useful measure of the efficacy of the intrinsic coagulation pathway is the activated partial thromboplastin time (aPTT), measured as the time it takes for a clot to form in the plasma in the absence of tissue factor following introduction of an activator such as kaolin. An abnormally short APTT can indicate a hypercoagulable and is associated with increased risk of venous thrombosis whereas abnormally long APTT may indicate bleeding tendencies (Gaunt, Lowe, Lawlor, Casas & Day, 2013).

Table 3
Coagulation tests results in mice

Groups	PT (sec)	APTT (sec)
I- Negative Control	10.92± 0.15	19.85 ±0.07
II-50mg/kg Ethanolic Extract	10.83±0.28	20.27±0.27
III- 100 mg/kg EE	10.93±0.14	21.05 ±0.28
IV- 150 mg/kg EE	13.78±0.16	31.13±0.29
V- 50 mg/kg Caffeine	12.53±0.21	25.15 ±0.15
VI- 100 mg/kg Caffeine	12.83±0.59	21.33±0.11
VII- 150 mg/kg Caffeine	14.68±0.28	19.48±0.37
VIII- Heparin Sodium	20.33±0.60	38.18±0.19

Table 3 presents the prothrombin time and activated partial thromboplastin time results in mice 24 hours after treatment. In the negative control group, PT was 10.92 ± 0.15 seconds. Administration of ethanolic extract resulted in a PT of 10.83±0.28, 10.93±0.14 and 13.78 ± 0.16 seconds for the doses of 50 mg/kg, 100 mg/kg and 150 mg/kg, respectively. Moreover, PT results for 50 mg/kg, 100 mg/kg and 150 mg/kg purified caffeine were 12.53±0.21, 12.83±0.59 and 14.68±0.28 seconds, respectively. Heparin sodium administration which served as the positive control, resulted in PT of 20.33 ± 0.60 seconds.

In the activated partial thromboplastin time results in mice 24 hours after treatment, the negative control group yielded an APTT of 19.85 ±0.07 seconds. Treatment with ethanolic extract resulted in an APTT of 20.27±0.27, 21.05 ±0.28 and 31.13±0.29 seconds for the doses of 50 mg/kg, 100 mg/kg and 150 mg/kg, respectively. Additionally, APTT results for 50 mg/kg, 100 mg/kg and 150 mg/kg purified caffeine were 25.15 ±0.15, 21.33±0.11 and 19.48±0.37 seconds respectively. Heparin sodium, as the positive control, resulted in APTT of 38.18±0.19 seconds.

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It can be noted that there were dose-dependent increases in both PT and APTT for the ethanolic extracts. On the other hand, this scenario was seen only in PT for the caffeine concentrations since in APTT increasing concentration resulted to lowering of APTT. This may be related to the loss of significance of higher doses of caffeine in thrombus formation inhibition as discussed earlier.

Table 4
Comparison of the negative control and the concentrations of C. LIBERICA ethanolic and caffeine extracts on coagulation tests

		Activated Partial Prothrombin Time	Thromboplastin Time
	Concentration	p-value	p-value
I- Negative Control	II- 50mg/kg Ethanolic Extract	0.9997	0.6950
	III- 100 mg/kg Ethanolic Extract	> 0.9999	0.0054*
	IV- 150 mg/kg Ethanolic Extract	< 0.0001*	< 0.0001*
	V- 50 mg/kg Caffeine	0.0131*	< 0.0001*
	VI- 100 mg/kg Caffeine	0.0024*	0.0005*
	VII- 150 mg/kg Caffeine	< 0.0001*	0.7966
	VIII- Heparin Sodium	< 0.0001*	< 0.0001*

*Significant at p-value < 0.05

Table 4 presents the comparison between the concentrations of purified caffeine and ethanol extracts from *C. liberica* leaves with the negative control group in the coagulation tests. In prothrombin time, upon comparing the negative control with 50 mg/kg, 100 mg/kg, and 150 mg/kg ethanolic extracts, p-values of 0.9997, <0.9999, and <0.0001 were obtained respectively. Additionally, when compared to 50 mg/kg, 100 mg/kg, and 150 mg/kg caffeine, the resulting p-values were 0.0131, 0.0024, and <0.0001 respectively. Lastly, it was compared to positive control and the obtained p-value is <0.0001. This suggests that the groups treated with 50 mg/kg and 100 mg/kg ethanolic extracts have no significant prolongation in the prothrombin time since they have p-values which are greater than 0.05, whereas the groups administered with 150 mg/kg ethanolic extract, 50 mg/kg, 100 mg/kg, and 150 mg/kg caffeine and heparin sodium showed significant prolongation of the prothrombin time since p-values were less than 0.05.

It can be inferred that 50 mg/kg and 100 mg/kg ethanolic extracts have less of the active antithrombotic component found in 150 mg/kg ethanolic extract which caused them to not effectively prolong PT. Meanwhile, caffeine isolates showed increasing significance at increasing concentrations. According to a study by Varani, Portaluppi, Gessi, Merighi, Ongini, et al., (2000), caffeine consumption may lead

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to a reduced platelet aggregability as a result of upregulation of the A_{2A} receptors located on the platelet surface which inhibits the release of granules that would activate additional platelets and the coagulation rabbits. In addition to that, observing the p-values of the treatment groups which showed significant prolongation of PT, it can be cascade.

As expected, heparin significantly prolonged the PT since it exerts its anticoagulant effect by antithrombin-mediated inhibition of thrombin (Factor II) of the common pathway (Kamal, et al., 2007). Significant prolongation of PT at Groups IV, V, VI and VII is similar to the observation of Dub & Dugani (2013) wherein pretreatment with 100 or 200 mg/kg per day of the ethanolic extract of *Oleaeuropaea* leaves for 8 weeks significantly prolonged the prothrombin time in deduced that 150 mg/kg ethanolic extract exhibited a greater degree of prolonging than that of the caffeine treatment groups except 150 mg/kg concentration, possibly due to its phenolic and flavonoid contents which have antithrombotic effects towards the extrinsic coagulation pathway.

In activated partial thromboplastin time, upon comparing the negative control with 50 mg/kg, 100 mg/kg, and 150 mg/kg ethanolic extracts, p-values of 0.6950, 0.0054, and <0.0001 were obtained respectively. Additionally, when compared to 50 mg/kg, 100 mg/ kg, and 150 mg/kg caffeine, the resulting p-values were <0.0001, 0.0005, and 0.7966respectively. Lastly, it was compared to positive control and obtained p-value is <0.0001. This means that the groups treated with 50 mg/kg ethanolic extracts and 150 mg/kg caffeine have no significant prolongation in the activated partial thromboplastin time since they have p-values which are greater than 0.05, whereas the groups administered with 100 mg/kg, 150 mg/kg ethanolic extracts, 50 mg/kg and 100 mg/kg caffeine, and heparin sodium showed significant prolongation of the activated partial thromboplastin time since p-values were less than 0.05.

With these results, it can be deduced that 100 mg/kg and 150 mg/kg ethanolic extracts contain adequate amounts of antithrombotic constituents compared to 50 mg/kg ethanolic extract. 150 mg/kg caffeine showed no significant antithrombotic activity. Treatment with caffeine even showed inversely proportional clotting deceleration effect in relation to dose. Thus, higher doses may even pose prothrombotic risks. With this, it can be assumed that lower doses of caffeine may have more positive effects in preventing thrombosis in accordance to the intrinsic clotting factors. Lastly, it can be noted that 150 mg/kg ethanolic extract and 50 mg/kg and 100 mg/kg caffeine were able to prolong coagulation through extrinsic, intrinsic and common pathways.

As seen in the results, heparin was able to decelerate

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coagulation in APTT as supposed. This occurred due to heparin's binding to antithrombin, inhibition of most serine proteases and most importantly, the activation of factor X and thrombin (Fritsma, Dembitzer, Randhawa, Marques, Van Cott, et al., 2012). Prolongation of APTT in Groups III, IV, V, VI and VIII are comparable with the outcome of the study of Chen, Jin, Wang, Wang, Meng & Wei (2014) in which *Toonamicrocarpa*leaf extract notably prolonged APTT in a dose-dependent manner, thus indicating that it may mainly exhibit anticoagulant activity correlating with the intrinsic coagulation process.

Table 5 presents the comparison between the concentration of purified caffeine and ethanol extract from *C. liberica* leaves between each treatment in prothrombin time. When 50 mg/kg ethanolic extract was compared with 100 mg/kg and 150 mg/kg ethanolic extract, it exhibited p-values of 0.999 and < 0.0001 respectively. When 50 mg/kg ethanolic extract is compared against 50 mg/kg, 100 mg/kg and 150 mg/kg of the caffeine extract the p-values of 0.0275, 0.0053 and < 0.0001 were obtained correspondingly. Furthermore, heparin sodium when compared to 50 mg/kg ethanolic extract resulted to the the p-value of <0.0001. The p-values of 0.999 and <0.0001 were obtained by comparing 100 mg/kg ethanolic extract with 50 mg/kg and 150 mg/kg ethanolic extract, respectively. Additionally, when 100 mg/kg ethanolic extract is compared with 50 mg/kg, 100 mg/kg and 150 mg/kg of the caffeine extracts, the p-values of 0.0457, 0.0093 and <0.0001 were obtained correspondingly. Meanwhile, a p-value of <0.0001 is obtained when 100 mg/kg ethanolic extract is compared with heparin sodium. Comparing 150 mg/kg ethanolic extract with 50 mg/kg and 100 mg/kg ethanolic extract resulted in the same p-values of <0.0001. In addition to that, 150 mg/kg ethanolic extract when compared with 50 mg/kg, 100 mg/kg and 150 mg/kg caffeine extracts resulted in p-values of 0.2143, 0.5462 and 0.6117, respectively. Lastly, comparing 150 mg/ kg ethanolic extract with heparin sodium yielded a p-value of <0.0001.

The comparison of 50 mg/kg caffeine with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extract all resulted in p-values of 0.0275, 0.0457 and 0.2143, correspondingly. Consequently, 50 mg/kg caffeine extract when compared against 100 mg/kg and 150 mg/kg caffeine extracts both resulted in p-values of 0.2143 and 0.0022, respectively. Lastly, a p-value of <0.0001 is also obtained in the comparison of 50 mg/kg caffeine with heparin sodium. When 100 mg/kg caffeine is compared with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts, p-values of 0.0053, 0.0093 and 0.5462 were obtained respectively. Moreover, when 100 mg/kg caffeine extract is compared with 50 mg/kg and 150 mg/kg caffeine extracts, they resulted in p-values of 0.2143 and 0.0123, respectively.

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Table 5
Multiple comparison of the concentrations of C. LIBERICA ethanolic and caffeine extracts between each treatment on Prothrombin Time

Concentration		p-value	Interpretation
II-50mg/kg Ethanolic Extract	III- 100 mg/kg EE	0.9999	Not Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	0.0275	Significant
	VI- 100 mg/kg Caffeine	0.0053	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
III-100 mg/kg Ethanolic Extract	II- 50 mg/kg EE	0.9999	Not Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	0.0457	Significant
	VI- 100 mg/kg Caffeine	0.0093	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
IV-150 mg/kg Ethanolic Extract	II- 50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	0.2143	Not Significant
	VI- 100 mg/kg Caffeine	0.5462	Not Significant
	VII-150 mg/kg Caffeine	0.6117	Not Significant
	VIII- Heparin Sodium	< 0.0001	Significant
V-50 mg/kg Caffeine	II- 50 mg/kg EE	0.0275	Significant
	III- 100 mg/kg EE	0.0457	Significant
	IV- 150 mg/kg EE	0.2143	Not Significant
	VI- 100 mg/kg Caffeine	0.2143	Not Significant
	VII-150 mg/kg Caffeine	0.0022	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
VI-100 mg/kg Caffeine	II- 50 mg/kg EE	0.0053	Significant
	III- 100 mg/kg EE	0.0093	Significant
	IV- 150 mg/kg EE	0.5462	Not Significant
	V- 50 mg/kg Caffeine	0.2143	Not Significant
	VII-150 mg/kg Caffeine	0.0123	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
VII-150 mg/kg Caffeine	II-50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	IV- 150 mg/kg EE	0.6117	Not Significant
	V- 50 mg/kg Caffeine	0.0022	Significant
	VI- 100 mg/kg Caffeine	0.0123	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
VIII-HEPARIN Sodium	II-50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	< 0.0001	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant

**Significant at p-value < 0.05*

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Heparin sodium when compared with 100 mg/kg caffeine extract, yielded a p-value of <0.0001. The comparison of 150 mg/kg caffeine with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extract resulted in p-values of <0.0001, <0.0001 and 0.06117. Consequently, 150 mg/kg caffeine extract when compared against 50 mg/kg and 100 mg/kg caffeine extracts resulted in p-values of 0.0022 and 0.0123. Lastly, a p-value of <0.0001 is also obtained in the comparison of 50 mg/kg caffeine with heparin sodium. Finally, the comparison of all extracts with the positive control, heparin sodium, also led to p-values of <0.0001.

Hence, 100 mg/kg ethanolic extract showed no significant difference (p-value >0.05) indicating that they have the same effect with 50 mg/kg ethanolic extract in PT while the remaining extracts showed significant difference (p<0.05). Also, 50 mg/kg ethanolic extract has the same effect with 100 mg/kg ethanolic extract since it resulted in a p-value >0.05 while the remaining extracts that have resulted in p-values of <0.05 showing significant difference in PT prolongation.

It can be further established that all caffeine concentrations have similar effects in prolonging the PT as with 150 mg/kg ethanolic extract while 50 mg/kg and 100 mg/kg ethanolic extract and heparin sodium produced significantly different effects since these groups showed p-values of <0.05. Moreover, 150 mg/kg ethanolic extract and 100 mg/kg caffeine have the same effect with 50 mg/kg caffeine since they resulted in p-values >0.05 while the remaining extracts that have resulted in p-values of <0.05 showing significant difference in PT prolongation. 150 mg/kg ethanolic extract and 50 mg/kg caffeine showed no significant difference (p-value >0.05) indicating that they have the same effect with 100 mg/kg caffeine extract in PT while the remaining extracts showed significant differences (p<0.05). Also, all the extracts have different effects in PT prolongation with 150 mg/kg caffeine except 150 mg/kg ethanolic extract (p-value >0.05) since they all resulted in p-values of <0.05.

It was also indicated that all the extracts have significantly dissimilar effects with heparin sodium on PT prolongation. Thus, none of them provided equivalent thrombolytic effect as to that of the positive control. In addition to that, it can be inferred that varying doses of *C. liberica*ethanolic and caffeine extracts have dissimilar capabilities of prolonging the prothrombin time. This is in contrast with the findings of Zhang, Du, Wang, Yu and Chen (2009), in which Z-Ligustilide from *Radix Angelica sinensis* oil showed no significant effect on prothrombin time prolongation indicating that its antithrombotic effect may not be associated with the coagulation

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system but rather through the inhibition of platelet aggregation.

Table 6
Multiple comparison of the concentrations of C. LIBERICAethanolic and caffeineextracts between each treatmenton Activated Partial Thromboplastin Time

Concentration		p-value	Interpretation
II-50mg/kg Ethanolic Extract	III- 100 mg/kg EE	0.2954	Not Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	0.0503	Not Significant
	VII-150 mg/kg Caffeine	0.2954	Not Significant
	VIII- Heparin Sodium	< 0.0001	Significant
III-100 mg/kg Ethanolic Extract	II- 50 mg/kg EE	0.2954	Not Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	0.9889	Not Significant
	VII-150 mg/kg Caffeine	0.0008	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
IV-150 mg/kg Ethanolic Extract	II- 50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	< 0.0001	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
V-50 mg/kg Caffeine	II- 50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	< 0.0001	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
VI-100 mg/kg Caffeine	II- 50 mg/kg EE	0.0503	Not Significant
	III- 100 mg/kg EE	0.9889	Not Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant
VII-150 mg/kg Caffeine	II-50 mg/kg EE	0.2954	Not Significant
	III- 100 mg/kg EE	0.0008	Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	< 0.0001	Significant
	VIII- Heparin Sodium	< 0.0001	Significant

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Table 6 (cont.)
Multiple comparison of the concentrations of C. LIBERICAethanolic and caffeineextracts between each treatmenton Activated Partial Thromboplastin Time

Concentration		p-value	Interpretation
VIII-HEPARIN Sodium	II-50 mg/kg EE	< 0.0001	Significant
	III- 100 mg/kg EE	< 0.0001	Significant
	IV- 150 mg/kg EE	< 0.0001	Significant
	V- 50 mg/kg Caffeine	< 0.0001	Significant
	VI- 100 mg/kg Caffeine	< 0.0001	Significant
	VII-150 mg/kg Caffeine	< 0.0001	Significant

**Significant at p-value < 0.05*

Table 6 presents the comparison between the concentration of purified caffeine and ethanol extract from *C. liberica* leaves between each treatment in activated partial thromboplastin time. When 50 mg/kg ethanolic extract was compared with 100 mg/kg and 150 mg/kg ethanolic extract, it exhibited p-values of 0.2954 and < 0.0001 respectively. When 50 mg/kg ethanolic extract is compared against 50 mg/kg, 100 mg/kg and 150 mg/kg of the caffeine extract the p-values of < 0.0001, 0.0503 and 0.2954 were obtained correspondingly. Furthermore, heparin sodium when compared to 50 mg/kg ethanolic extract resulted to the the p-value of <0.0001. The p-values of 0.2954 and <0.0001 were obtained by comparing 100 mg/kg ethanolic extract with 50 mg/kg and 150 mg/kg ethanolic extract, respectively. Additionally, when 100 mg/kg ethanolic extract is compared with 50 mg/kg, 100 mg/kg and 150 mg/kg of the caffeine extracts, the p-values of < 0.0001, 0.9889 and 0.0008 were obtained correspondingly.

Meanwhile, a p-value of <0.0001 is obtained when 100 mg/kg ethanolic extract is compared with heparin sodium. Comparing 150 mg/kg ethanolic extract with 50 mg/kg and 100 mg/kg ethanolic extract resulted in the same p-values of <0.0001. In addition to that, 150 mg/kg ethanolic extract when compared with 50 mg/kg, 100 mg/kg and 150 mg/kg caffeine extracts all resulted in p-values of <0.0001. Lastly, comparing 150 mg/ kg ethanolic extract with heparin sodium yielded a p-value of <0.0001.

The comparison of 50 mg/kg caffeine with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extract all resulted in p-values of <0.0001. Consequently, 50 mg/kg caffeine extract when compared against 100 mg/kg and 150 mg/kg caffeine extracts both resulted in p-values of <0.0001. Lastly, a p-value of <0.0001 is also obtained in the comparison of 50 mg/kg caffeine with heparin sodium. When 100 mg/kg caffeine is compared with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts, p-values of 0.0503, 0.9889 and <0.0001 were obtained respectively. Moreover, when 100 mg/kg caffeine extract is compared with 50 mg/kg and 150 mg/kg caffeine extracts, both resulted

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in p-values of <0.0001. Heparin sodium when compared with 100 mg/kg caffeine extract, yielded a p-value of <0.0001.Comparing 150 mg/kg caffeine extract with 50 mg/kg, 100 mg/kg and 150 mg/kg ethanolic extracts resulted in p-values of 0.2954, 0.0008 and <0.0001 correspondingly. Furthermore, 150 mg/kg caffeine extract when compared with 50 mg/kg and 100 mg/kg caffeine extracts both resulted in p-values of <0.0001. Lastly, the comparison of 150 mg/kg caffeine extract with heparin sodium also resulted in a p-value of <0.0001. Lastly, the comparison of all extracts with the positive control, heparin sodium, also led to p-values of <0.0001.

Hence, 100 mg/kg ethanolic extract, 100mg/kg and 150mg/kg caffeine extracts showed no significant difference (p-value >0.05) indicating that they have the same effect with 50 mg/kg ethanolic extract in APTT while the remaining extracts showed significant difference ($p<0.05$). Correspondingly, 50 mg/kg ethanolic extract and 100 mg/kg caffeine extract have the same effect with 100 mg/kg ethanolic extract since they resulted in p-values of >0.05 while the remaining extracts that have resulted in p-values of <0.05 showed significant difference in APTT prolongation. It can also be noted that all extracts when compared with 150 mg/kg ethanolic extract have different significant effects in prolonging the APTT since all of these extracts showed p-values of <0.05. All the extracts have different effects in APTT prolongation with 50 mg/kg caffeine since they all resulted in p-values of <0.05. Meanwhile, 50 mg/kg and 100 mg/kg ethanolic extracts showed no significant difference (p-value >0.05) indicating that they have the same effect with 100 mg/kg caffeine extract in APTT while the remaining extracts showed significant prolongation ($p<0.05$). 50 mg/kg ethanolic extract has the same effect with 150 mg/kg caffeine since it yielded a p value of >0.05 while the remaining extracts showed significant APTT prolongation with p-values <0.05.

Like in prothrombin time, no extract have shown similar antithrombotic effects as the positive control. It can be noted that heparin sodium is still superior among all treatment groups in prolonging the coagulation test results. Similarly, it can be deduced that the low and high concentrations of *C. liberica*ethanolic and caffeine extracts produce different abilities in prolonging the activated partial thromboplastin time. These findings are correlated to the reports of Davison, Levendal, & Frost (2012) in their study on the effects of *Tulbaghia violacea* on blood coagulation in male Wistar rats. APTT had a 1.2-fold increase relative to the positive control which is attributed to the plant's saponin content which has a positive effect on the prevention of platelet aggregation, blood coagulation and fibrinolysis.

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CONCLUSION

Based on the study, it may then be concluded that *Coffealiberica*ethanolic and caffeine leaf extracts possess antithrombotic property as demonstrated by significant inhibition of tail thrombosis length induced by kappa carrageenan at 150 mg/kg ethanolic extract and 50 mg/kg caffeine concentrations. Furthermore, prolongation in prothrombin time was shown at 150 mg/kg ethanolic extract, 50 mg/kg, 100 mg/kg and 150mg/kg caffeine concentrations. Similarly, activated partial thromboplastin time was prolonged at 100 mg/kg and 150 mg/kg ethanolic extract and 50 mg/kg ad 100 mg/kg caffeine concentrations. These may be of great value in thrombotic states and other related cardiovascular diseases. Additionally, lower doses of caffeine showed antithrombotic properties of greater extent in the in vivo models and intrinsic coagulation pathway in which it showed inversely proportional effects in terms of dose increase. Finally, it can also be established that the ethanol extract possesses better antithrombotic capabilities than the isolated caffeine.

RECOMMENDATIONS

Supplementary researches must be conducted to investigate other plants with high flavonoid and caffeine content. Since the crude extract showed greater thrombolytic effects, phytochemical analysis is recommended to determine the constituents responsible for such. Additional studies should also be undertaken to distinguish the exact antithrombotic mechanism of the extracts along with their anticoagulant properties. Other bioactive components of the plant's leaves like caffeic acid and mangiferin could also be studied for the same effects. Further testing can also be done to confirm if caffeine has prothrombotic dangers at high doses. Moreover, the extracts' hepatotoxicity and nephrotoxicity should be evaluated. Finally, liver function should be assessed to confirm the possible cause of PT and APTT prolongation in the study.

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