Research Article

Anticoagulant and antiplatelet properties of the latex of unripe fruits of *Carica papaya* L. (Caricaceae)

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ABSTRACT

Background: Anticoagulants have found use clinically in the management of coagulation disorders. The aim of this study therefore was to ascertain the anticoagulant and antiplatelet properties of the latex of the unripe fruits of *Carica papaya* (CPUFL) using *in vitro* and *in vivo* models.
Methods: CPUFL was screened for phytochemicals. The time taken, for 100 µL quantities of plasma or whole blood mixed with 100, 300, and 600 µg of CPUFL and standard assay reagents, to form clots in the prothrombin time (PT), activated partial thromboplastin time (aPTT), and clotting time tests were determined (using 1 mg rivaroxaban, 50 IU heparin, or plasma as references). The time taken for cessation of induced marginal ear vein bleeding of New Zealand White rabbits pre-treated orally with either CPUFL (4-12 mg/kg), 2 mg/kg aspirin, or 1 ml/kg distilled water, or 1.5 mg/kg heparin intraperitoneally for 30 mins was also determined.
Results: Saponins, tannins, glycosides, terpenoids, flavonoids, and alkaloids were present in CPUFL. Treatment with CPUFL (100-600 µg), increased both PT and aPTT significantly (p≤0.01-0.0001). There was also a significant increase (p≤0.0001) in clotting time of whole blood at 600 µg/100 µL. CPUFL treatment (4, 8, and 12 mg/kg) showed a dose-dependent increase (p≤0.0001) in bleeding time. Effect between CPUFL, heparin, and aspirin treatment were not significantly different.
Conclusion: The latex of unripe fruits of *C. papaya* L. exhibited anticoagulant and antiplatelet properties suggesting its potential usefulness in the management of blood coagulation disorders.

Keywords: Activated partial thromboplastin time, Bleeding time, Clotting time, Marginal ear vein, Prothrombin time

INTRODUCTION

Anticoagulants are chemical agents that inhibit blood clotting.[1] They are categorized broadly as; agents that inhibit clotting factors in the intrinsic pathway through enhancing the effect of antithrombin III (AT III) (e.g., unfractionated heparin and low molecular weight heparin), agents that exert their anticoagulation effect by inhibiting Vitamin K reductase which invariably prevents the gamma-carboxylation of glutamic acid residue of factors II, VII, IX, and X (e.g., warfarin), agents that inhibit specific clotting factors (e.g., rivaroxaban - factor Xa inhibitor, or dabigatran - thrombin inhibitor), and agents that prevent

platelet activation and aggregation thereby preventing primary hemostasis (e.g., aspirin, ticlopidine, tirofiban, and clopidogrel).[2] Anticoagulants have found use clinically in the management of cardiovascular disorders.

In 2010, there were 52.8 million deaths globally with non-communicable diseases accounting for 34.5 million of these deaths.[3] Cardiovascular diseases were the leading contributor to the deaths caused by non-communicable diseases with thrombosis being the most common underlying pathology of the three major cardiovascular disorders, i.e., ischemic heart disease (acute coronary syndrome), stroke, and venous thromboembolism. Ischemic heart disease (7.0 million

deaths) and stroke (5.9 million deaths) collectively caused one in four deaths worldwide.[3]

Drugs currently used as anticoagulants have various limitations, thus being far from ideal.[4] Warfarin has numerous interactions with diet and other medications, requires complex individualized dosing, and has a delayed onset of action.[5] Unfractionated heparins and low-molecular-weight heparins, which require parenteral administration, pose a risk of heparin-induced thrombocytopenia, osteoporosis, and hemorrhage.[6] Bleeding is the major toxicity of the antiplatelet agents, but thrombocytopenia and neutropenia can also occur.[2] Such significant drawbacks make these agents problematic and inconvenient for clinicians and patients alike, thus prompting research into novel drugs that could offer such advantages as oral mode of administration, more predictable anticoagulant response, greater specificity with no requirement for AT action, and no need for routine patient monitoring.[7-9] Traditionally, some medicinal plants have shown good anticoagulant effects comparable to orthodox drugs.[10] There is, therefore, a need to scientifically validate these effects for laboratory and medical practice. One such plant with this property is *Carica papaya*.

C. papaya L. (Caricaceae) has been extensively used in Ghana and other parts of the world as medication for various diseases including cardiovascular disorders. Major chemical compounds responsible for *C. papaya* remedies include vitamins, minerals, polysaccharides, proteolytic enzymes, citric acid, alkaloids, flavonoids, and proteins such as chemopapain, papain, and carpain. Different concentrations of those bioactive compounds render different parts of papaya a wide spectrum of medicinal uses.[11] The present study, therefore, examined the anticoagulant and antiplatelet properties of the latex of unripe fruit of *C. papaya* by *in vitro* and *in vivo* experimental protocols.

METHODS

Collection of latex of unripe fruit of C. papaya

Fresh latex of the unripe fruit of *C. papaya* L. was collected from locally grown plants at Tepa, in the Ahafo Ano North District, of the Ashanti Region of Ghana, after pictures of the flowers, fruits, and whole plant had been sent to the herbarium of the Herbal Medicine Department of Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST) for verification. Between 0600 and 0800 hrs each day, 4-6 longitudinal incisions, 3 mm deep, were made on the unripe mature fruit surface (from fruit stalk end to the tip of the fruit) using a stainless steel knife. The exuded latex was allowed to run down the fruit and drip into collecting devices (plastic trays) raised on the trunk of the tree (Figure 1). The incisions were repeated 4 times at 3 days interval.[12,13] The collected latex was spread on trays and left for drying at

Figure 1: Collection of the latex of the unripe fruit of *Carica papaya*.

40°C for 14 hrs. The latex was grinded using a laboratory grinding machine producing a greenish or gray powder and labeled *C. papaya* unripe fruit latex (CPUFL).

Drugs and chemicals used

The drugs and chemicals used in this study included: heparin (Mayne Pharma, Espana), soluble aspirin tablets 75 mg (Bristol laboratories Ltd., UK), rivaroxaban tablet 10 mg (Bayer Pharma AG, Berlin, Germany).

Animals

New Zealand White rabbits (1.3-2.4 kg) of either sex were used. These were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana. The rabbits were kept in standard metallic cages in the animal house under ambient humidity, temperature, and light conditions. They were allowed access to normal rabbit feed from Agricare Ltd., Tanoso, Kumasi and water *ad-libitum*.

Qualitative analysis of phytochemicals

Standard methods used for preliminary phytochemical screening were as described by Sofowora.[14]

In vitro anticoagulation assay

Blood (5 ml) each was drawn from the marginal ear vein of healthy rabbits by vein puncture. Nine parts of blood collected were mixed with one part of 3.2% sodium citrate. The blood was centrifuged at 5000 rpm for 10 mins, and the supernatant was collected immediately using a plastic pipette into a plastic tube (FL medical, Italy) and maintained at 2-8°C. It was then used for prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests.

Effect of CPUFL on PT

PT test was performed by the standard PT assay Kit (Cypress Diagnostics, Langdorp - Belgium). 100 µL quantities of plasma were mixed with 100, 300, or 600 µg of CPUFL and incubated for 2 mins at 37°C. Clotting was then induced by the addition of 200 µL of the thromboplastin reagent (calcium rabbit brain thromboplastin: a freeze dried extract of rabbit brain thromboplastin, combined with calcium chloride, buffer, and stabilizers). The time of the formation of a clot was determined using a stopwatch. The determinations were also carried out using plasma and 1 mg rivaroxaban as controls. Seven determinations of the processes were done.

Effect of CPUFL on aPPT

This assay was performed using the standard aPTT assay Kit (Cypress diagnostics, Belgium). 100 µL quantities of plasma were mixed with 100, 300, or 600 µg of CPUFL after which 100 µL of pre-warmed aPTT assay reagent (lyophilized rabbit brain cephalin and micronized silica in buffered medium with stabilizer) was added and incubated for 3 mins at 37°C. Then 100 µL of 0.025M $CaCl_2$ was added and the time taken for the formation of the clot was recorded. The above procedure was repeated using only the plasma to serve as control and using heparin 50 IU as standard. The test was repeated seven times.

Effect of CPUFL on clotting time

Whole blood (5 ml) drawn from the marginal ear vein of the rabbit was divided into five plastic test tubes (FL medical, Italy) labeled I-V each with a quantity of 100 µL. In a water bath with gentle shaking: test tube I (positive control) was tested to determine the normal clotting time; test tube II (1 mg/100 µL rivaroxaban) was treated with 1 mg rivaroxaban to serve as reference; Test tubes III, IV, and V (CPUFL tests) were treated with 100, 300, and 600 µg of CPUFL. All the samples were incubated at 37°C for 1 min after which clotting was induced by adding 100 µL of 0.025 M $CaCl_2$ to counteract the sodium citrate and allow clotting to proceed. The time of the clot formation was recorded.

Effect of CPUFL on the bleeding time

An *in vivo* anticoagulant effect of CPUFL was investigated as previously described by Elg et al.,[15] with modifications. In this determination, rabbits were put into six groups (n=5) and pre-treated orally with either CPUFL (4, 8, or 12 mg/kg; p.o.), aspirin (2 mg/kg; p.o.), heparin (1.5 mg/kg; i.p), or distilled water (1 ml/kg; p.o.) for 30 mins after which the marginal ear vein was picked with a twist lancet (Yancheng Huida Medical Instruments Co., Ltd., China) to cause bleeding. The bleeding vein was gently blotted with Whatman No. 1 filter paper, every 5 sec until cessation of bleeding (no bleeding for 1 min) was noted. The observation time was limited to 10 mins. Care was taken that no pressure

was exerted on the bleeding vein that could cause hemostasis. A baseline bleeding time was determined before any drug treatment.

Statistical analysis

GraphPad Prism for Windows Version 5 (GraphPad® Software, San Diego, CA, USA) was used for all statistical analysis. All results are presented as mean±standard error of mean. Differences between groups were analyzed using one-way analysis of variance followed by Sidak's multiple comparisons *post-hoc* test when comparing control with treatment groups.

RESULTS

### Qualitative phytochemical analysis

The analysis revealed the presence of flavonoids, alkaloids, condensed tannins, saponins, cardiac glycosides, and terpenoids in CPUFL.

Effect of CPUFL on PT

The baseline PT obtained for the blood sample was 10.79±1.14 sec. After treatment with CPUFL (100-600 µg), PT values increased significantly (p≤0.01-0.001) and concentration-dependently; effects were similar to that of 1 mg rivaroxaban (Figure 2).

Effect of CPUFL on aPTT

The baseline aPTT was 41.43±4.68 sec. CPUFL treatments (100, 300, and 600 µg/100 µL caused significant (p≤0.0001)
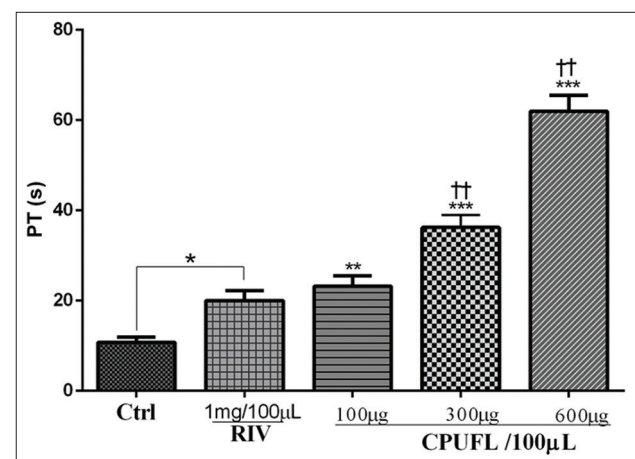
Figure 2: The effect of *Carica papaya* unripe fruit latex, and rivaroxaban on prothrombin time. Values plotted are means±standard error of mean; (n=7). ***p≤0.001; **p≤0.01; *p≤0.05 compared to control (one-way ANOVA followed by Sidak's multiple comparisons *post-hoc* test), ††: p ≤ 0.01; comparison between Rivaroxaban and the concentrations of CPUFL.

and concentration-dependent increments in aPTT. A similar effect was seen with heparin (Figure 3).

Effect of CPUFL on clotting time

Treatment of blood samples with CPUFL resulted in an increase in the clotting time of whole blood, but this was significant (p≤0.0001) at a concentration of 600 μg/100 μL as compared to the control. This effect was similar to the 1 mg/100 μL rivaroxaban treatment (Figure 4).

Effect of CPUFL on bleeding time

CPUFL treatment (4, 8, and 12 mg/kg) showed a dose-dependent increase (p≤0.0001) in bleeding time compared to the control. There were also dose-dependent significant differences (p≤0.01) in effects between CPUFL, heparin, and aspirin treatments (Figure 5).

DISCUSSION

In recent years, naturally occurring chemical substances of plant origin have attracted interest as possible treatments for coagulation disorders such as stroke and myocardial infarction.[16] This study was, therefore, conducted to assess the anticoagulant, and antiplatelet properties of the latex of the unripe fruit of *C. papaya* L. This was to be achieved by investigating the effect of CPUFL on; PT, aPTT, clotting time of whole blood and bleeding time in rabbits.

Results indicated that CPUFL prolonged PT in a manner similar to rivaroxaban; an oral direct Factor Xa inhibitor.[17]

Factor Xa inhibition reduces the thrombin burst in the propagation phase of the coagulation cascade since the conversion of prothrombin to thrombin depends on FXa. Any drug that inhibits FXa or any of the serine proteases that are involved in the formation of FXa will cause an increase in the PT.

aPTT was increased with CPUFL and heparin treatments. Thromboplastin is a complex enzyme that is found in the
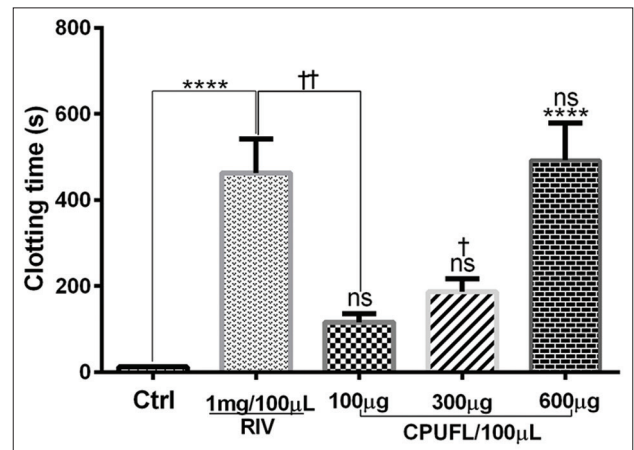
Figure 4: The effect of *Carica papaya* unripe fruit latex (CPUFL) and rivaroxaban on *in vitro* clotting time. Values plotted are means±standard error of mean; (n=7). ****p≤0.0001; *p>0.05 compared to control (one-way ANOVA followed by Sidak's multiple comparisons *post-hoc* test). ††p≤0.01; †p≤0.05 comparison between rivaroxaban and the doses of CPUFL.
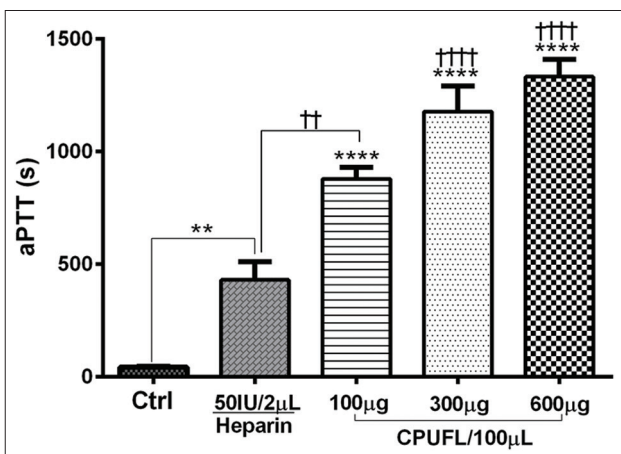
Figure 3: The effect of *Carica papaya* unripe fruit latex (CPUFL) and heparin on activated partial thromboplastin time. Values plotted are means±standard error of mean; (n=7), ****p≤0.0001; **p≤0.01 compared to control (one-way ANOVA followed by Sidak's multiple comparisons *post-hoc* test). ††††p≤0.0001; ††p≤0.01 comparison between the reference anticoagulant and the concentration of CPUFL.
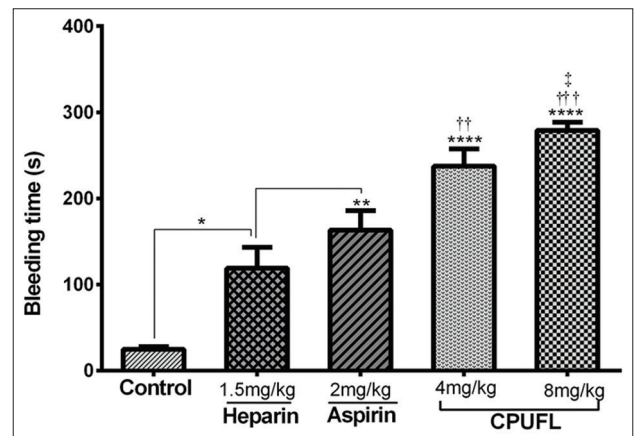
Figure 5: The effect of *Carica papaya* unripe fruit latex, aspirin, and heparin on the bleeding time in New Zealand White rabbits. Values plotted are means±standard error of mean; (n=5). ****p≤0.0001; **p≤0.01, *p≤0.05 compared to control (one-way ANOVA followed by Sidak's multiple comparisons *post-hoc* test). †††p≤0.001; ††p≤0.01 comparison between the heparin and the various doses of the CPUFL. ‡p≤0.05 comparison between aspirin and various concentration of the CPUFL.

brain, lung, and other tissues and especially in blood platelets and functions in the conversion of prothrombin to thrombin in the clotting of blood.[9] When manipulated, a derivative is created called partial thromboplastin. Partial thromboplastin is used to measure the intrinsic pathway as it monitors factors XII, XI, IX, VIII, V, II, I, prekallikrein, and high molecular weight kininogen. The aPTT is utilized to detect congenital and acquired abnormalities of the intrinsic coagulation pathway and to monitor patients receiving heparin.[18] Heparin shows the anticoagulant effect by inactivating thrombin and activated factor X (Xa) through an AT-dependent mechanism.[2] It acts as a catalyst to markedly accelerate the rate at which AT III neutralizes thrombin and activated coagulation factor X (Xa).[2] AT III generally neutralizes these activated coagulation factors slowly and irreversibly; however, in the presence of heparin it neutralizes these factors almost instantaneously.[19]

The clotting time of whole blood was affected significantly by CPUFL at 600 µg/100 µL. This effect was similar to the 1 mg/100 µL Rivaroxaban treatment. Clotting time test is a very significant indicator of blood clotting and clot retraction. Combining findings from PT, aPTT, clotting time tests, it is suggestive that CPUFL has components that affect the blood clotting cascade by possibly inhibiting clotting factors in the intrinsic clotting pathway or by increasing AT III activity.

The antiplatelet activity of CPUFL was revealed as it showed a significant increase in bleeding time *in vivo,* comparable to aspirin. In bleeding the animals, blood vessels were broken/damaged which needed to be plugged to stop the bleeding. During clot formation, platelets adhere at the site of vascular injury, become activated and release chemicals like thromboxane A2, adenosine diphosphate (ADP), p-selectin, 5-HT, etc. This leads to platelet aggregation and activation of the blood coagulation mechanism to form the plug. Activation of platelets by collagen is a multistep event. In fact, after an initial attachment to platelets through second messenger pathways, collagen stimulates the release of thromboxane and ADP, which are important platelet agonists that induce aggregation.[20] Prolongation of bleeding time when a blood vessel is injured involves inhibition of platelet activation and aggregation (primary hemostasis) even, therefore, inhibition of the coagulation cascade (including extrinsic, intrinsic or common pathways) as established for heparin. Aspirin, a reference antiplatelet drug, blocks production of thromboxane A_2 by acetylating a serine residue near the active site of platelet cyclooxygenase-1 (COX-1), the enzyme that produces the cyclic endoperoxide precursor of thromboxane A_2 or dissolution of fibrin clot formed.[21] The extract significantly prolonged the bleeding time as compared to aspirin and heparin suggesting a possible antiplatelet activity and confirming anticoagulant activity.

Although the precise mechanism by which the CPUFL inhibit blood coagulation cannot be elucidated from this study, widespread research on natural products shows that plant constituents such as alkaloids, flavonoids, glycosides, terpenes, and many other secondary metabolites may exhibit both anticoagulant and antiplatelet effects. The effects of the plant constituents may be inhibition of one or more of the following; platelet plug formation (platelet activation and aggregation), conversion of the serine proteases (zymogens) into active forms, activated forms of the clotting factors (such as Xa and thrombin), calcium release from the cytosol or enhancing the effects of the natural anticoagulants such as thrombin-activatable fibrinolysis inhibitor, AT III, and protein C.

The phytochemical analysis of CPUFL showed that the latex contained flavonoids, saponins, condensed tannins, cardiac glycosides, alkaloids, and terpenoids, which could have conferred anticoagulant activity to the latex. Studies have showed that flavonoids inhibited platelet aggregation *in vitro*.[22-24] Guglielmone et al.,[25] found that quercetin 3-acetyl-7,3',4'-trisulfate and quercetin 3,7,3',4'-tetrasulfate obtained from *Flaveria bidentis* shows significant prolongation of the aPTT. It has been suggested that the effect of flavonoids on platelet aggregation is dependent on the inhibition of the COX pathway, resulting from the inhibitory effect of flavonoids on the platelet formation of thromboxane A_2, a potent aggregating and vasoconstricting agent.[22-24] In a study by Akiyama et al.,[26] on antibacterial action of several tannins against *Staphylococcus aureus*, indicated that tannins exhibit anticoagulant effect as a result of decrease in ionic calcium concentration, inhibition of enzyme production, and hindrance of enzymatic reactions. Another study by Zhang et al.,[27] showed the effect of saponins on blood coagulation. Total steroidal saponins extracted from the rhizome of *Dioscorea zingiberensis* inhibited platelet aggregation, prolonged aPTT, inhibited factor VIII activities in rats, and increased the protection rate in mice in a dose-dependent manner. They speculated that the saponin may execute anti-thrombotic activity through inhibiting factor VIII activities and platelet aggregation. Alkaloids also have an anticoagulant effect. A study by Singh and Singh on an alkaloid of tobacco, (nicotine) affected the clot-formation property of thrombin, on fibrinogen (thrombin time). Higher concentrations of nicotine retarded the clot-formation property of thrombin. From this experimental evidence, it is suggested that nicotine being an alkaloid does alter the clot-forming properties of thrombin on fibrinogen.[28]

CONCLUSION

Latex from the unripe fruit of *C. papaya* L. has anticoagulant and antiplatelet properties and would, therefore, be useful as adjunctive therapy in the management of blood coagulation disorders.

with exceptional luck that one gets the opportunity to work with such individuals who are so dedicated to their work.

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