



ANTITHROMBOTIC AND ANTICOAGULANT PROPERTIES OF *PENICILLIUM CHRYSOGENUM* (PCL 501) CULTURE EXTRACTS

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ABSTRACT

Penicillin and related β -lactam antibiotics inhibit platelet aggregation and can cause bleeding diathesis; their potential antithrombotic and anticoagulation effects are being investigated. An *in vitro* study was carried out to determine the effect of culture extract of *Penicillium chrysogenum* (PCL501) on thrombin and whole blood clotting time. Visual and spectrophotometric measurements showed that the extract significantly ($P < 0.05$) lengthened thrombin and blood clotting time, and inhibited blood clot formation. Delay in thrombin and blood clotting time was proportionate to the extract concentration; a threshold level was reached when it totally inhibited clot formation. The reference drug, commercial benzyl penicillin, exhibited a similar inhibitory pattern. The culture extract and reference drug, at 6.0 and 5.0 mg/ml concentration respectively, prevented clot formation initiation by thrombin. No blood clot was observed at 30.0 and 20.0 mg/ml concentration respectively of the extract and reference drug. This study demonstrates that the culture extract of *P. chrysogenum* (PCL501) has *in vitro* antithrombotic and anticoagulant properties. It also suggests that inhibition of thrombin activity could be the major mechanism through which β -lactam antibiotics impair platelet functions and impede blood clotting process. The strain of *P. chrysogenum* can be exploited for low-cost antithrombotic and anticoagulation therapy.

Keywords: *Penicillium chrysogenum*, thrombin, blood clotting, antithrombotic effect, anticoagulant

INTRODUCTION

Penicillin and related β -lactam antibiotics exert antibacterial activity by acylation of serine residues at the active site of enzymes involved in the biosynthesis of bacterial cell walls;^{1,2} β -lactam ring is the major structural component required for recognizing and binding to the target enzymes.^{3,4} These antibiotics also inhibit the serine enzymes of viruses and fungi, and affect a wide array of mammalian enzymes with nucleophilic serine residues including thrombin, human leukocyte elastase, human cytomegalovirus protein, prostate specific antigen, co-enzyme A independent transacylase, γ -aminobutyric acid aminotransferase, and human cytosolic phospholipase A2.⁵ Mammalian serine proteases are involved in a wide range of cellular activities which include blood clotting, immunity, and inflammation.^{6,7} Thrombin, an activation product of prothrombin in the natural coagulation cascade, is largely recognized for its ability to convert fibrinogen into fibrin. It participates in the normal recovery from injury through its roles in blood clotting, clot lyses and tissue repair, and provides a strong stimulus for platelet activation and aggregation. Besides its central role in the coagulation cascade, thrombin is also implicated in thrombosis, an inflammatory disorder that often has fatal outcome. Thrombotic occlusion due to unregulated thrombin production in an inappropriate location is a frequently encountered problem. Thrombin has also been linked to the aetiology and development of several pathological events such as oedema, cell enlargement, lyses and release of cellular products.⁸ Arterial or venous thrombotic events cause significant morbidities including paralysis from non-fatal thrombotic stroke, cardiac disability, loss of vision due to retinal vascular thrombosis, fetal wastage syndrome from placental vascular thrombosis, stasis ulcers and other manifestations of post-phlebotic syndrome from recurrent

deep venous thrombosis.⁹ Thromboses in the venous circulation and the embolization of venous clots have long been recognized as important contributors to these pathological states. Whole blood clotting time and thrombin time are among the tests used to identify patients suffering from blood clotting defects. Thrombin inhibitors have been recommended for such conditions. More recently, the problem of clot formation in the arterial circulation has led to the development of both biochemical and mechanical interventions to disrupt clots that cause myocardial infarction and stroke.¹⁰ Excessive bleeding and increased bleeding time have been reported for patients and volunteers on high doses of penicillin and related β -lactam antibiotics.¹¹ The inhibitory effects of the antibiotics on blood coagulation may not be unconnected with their coating of platelet surface and impairment of platelet activation.¹² *Penicillium chrysogenum* PCL 501 produces β -lactam antibiotics with antibacterial activities comparable to commercial benzyl penicillin.¹³ Improved penicillin production was achieved by UV mutation of the fungus.¹⁴ In this work, thrombin time and whole blood clotting time assays were used to evaluate the *in vitro* antithrombotic and anticoagulation effects of culture extract of *P. chrysogenum* (PCL 501) grown on sugarcane pulp, an agro-industrial waste. This is with a view to assessing the potentials of the fungal extract as a low-cost source for antithrombotic and anticoagulation therapy.

MATERIALS AND METHODS

Organism

The strain of *P. chrysogenum*, PCL501, was isolated from a wood-waste dump in Lagos, Nigeria and identified as described previously.¹⁵ The organism was maintained on PDA slant at 4°C. The fungus was sub-cultured on fresh PDA plates and incubated at 30°C.

Production of Culture Extract of *P. chrysogenum* (PCL501)

Spores of *P. chrysogenum* (PCL501) were obtained from a 5-day culture on PDA plate and washed into a sterile beaker with 0.1 % Tween 80 (pH 7.0), in 0.1M potassium phosphate buffer.¹⁶ The spore suspension was standardized as described by Oyegeme-Okerenta *et al.*¹³; the optical density (530 nm) was 0.48 for 1 in 10 dilutions. Five milliliter (5 ml) of the spore suspension was used to inoculate 50 ml of a sterile basal media containing sugarcane pulp as sole carbon source. The basal media contained (per litre of distilled water): 6.0 g Ammonium acetate, 0.5 g NaSO₃, 0.02 g ZnSO₄.7H₂O, 0.25 g MgSO₄.7H₂O, 6.0 g KH₂PO₄, 0.02 g FeSO₄.7H₂O and 10.0 g sugarcane pulp powder (pH 5.6). The culture was placed in 250 ml Erlenmeyer flask and incubated at 30°C with continuous agitation at 100 rpm using Graffin Flask shaker for 10 days. The culture was centrifuged at 6000 x g for 15 minutes at 4°C using an ultra centrifuge (Super-speed RC-B, USA); the supernatant was collected and pH was adjusted to 2.5 with chloroform: 10 % phosphate buffer (20:1) solution. The culture extract was precipitated with 1 % phosphate buffer (pH 6.0) and freeze dried to complete dryness.

Standard Reference Drug

Benzyl penicillin (Retarpen, Sandoz, Austria) was purchased from the University pharmacy at the College of Medicine, University of Lagos. The drug was dissolved in 0.1M potassium phosphate buffer (pH 7.0) and used as standard reference drug for the *in-vitro* tests.

Whole Blood Clotting Time

Blood sample (0.2 ml) was aseptically transferred to a plain test tube of 8 mm internal diameter and placed in a water bath at 37°C. The stop clock simultaneously started. This was examined at intervals of 30 seconds for clotting by gently tilting the tubes. The timer was stopped at the appearance of a clot. The test was done in quadruplicates. The average clotting time was calculated and recorded as the normal blood clotting time. The test was repeated with the blood sample containing 1 – 10 mg/ml of the culture extract and reference drug. The test for a given concentration was carried out in quadruplicates and the average time was recorded.

Determination of Thrombin Time

A modified method of Cazenave *et al.* (2008) was used for the kinetic study. Blood samples were aseptically transferred into a 3.2 % buffered sodium citrate tubes, centrifuged and used for assays. Plasma (0.2 ml) was placed into pre-warmed test tubes at 37°C, and incubated for 3 minutes; 0.1 ml of reconstituted Bovine thrombin reagent was added and a stop watch was simultaneously started. The timer was stopped at the appearance of a fine mesh of insoluble fibrin polymers; that signalled the end point in thrombin clotting assays. The test was repeated with the blood sample containing 1 – 50 mg/ml of the culture extract and benzyl penicillin (reference drug). The test was carried out in triplicates and the mean was calculated and reported to the nearest 0.1 seconds.

Effect of thrombin concentration on rate of clot formation

One millilitre (1.0 ml) of citrated plasma was placed in a test tube at 25°C and 0.2 ml bovine thrombin was added. Absorbance at 400 nm was measured with a spectrophotometer at 3-second intervals for 15 seconds. The test was repeated using different concentrations (0.4 mg/ml – 1.0 mg/ml) of bovine thrombin.

Effect of culture extract concentration on thrombin activity

The effect of varying concentrations of the culture extract of *P. chrysogenum* (PCL 301) and benzyl penicillin (reference drug) on thrombin activity was determined by monitoring the absorbance at 400 nm with a spectrophotometer at 25°C. Citrated plasma (1.0 ml) was placed in a test tube and 0.1 ml of bovine thrombin was added and the absorbance at 400 nm was measured with a spectrophotometer after 30 seconds. The test was repeated with the plasma containing 1 – 50 mg/ml of the culture extract and benzyl penicillin.

RESULTS

The effect of varying concentrations of the culture extract of *P. chrysogenum* (PCL501) and benzyl penicillin (reference drug) on blood clotting time is shown in Figure 1. The normal blood clotting time was 6.3 ± 0.2 minutes. Addition of the extract and reference drug caused a significant increase in clotting time; this was concentration dependent. Blood clotting time was increased by 8.3 minutes (130.5 %) and 26.0 minutes (410.6 %) respectively by 1.0 and 5.0 mg/ml concentration of the extract. Clot formation was not observed at 6.0 mg/ml of the culture extract. Benzyl penicillin caused 96.5 % increase in blood clotting time at 1.0 mg/ml and achieved a total inhibition of clot formation at 5.0 mg/ml concentration. Figure 2 shows the effect of varying concentrations of the culture extract and benzyl penicillin (reference drug) on thrombin time. The normal thrombin time was 12.6 ± 0.4 seconds at 37°C. There was a significant increase in thrombin time when the plasma samples were incubated with the fungal extract and reference drug; the increase was concentration dependant. At 1.0, 5.0, 10.0 and 20.0 mg/ml concentration of the extract, the thrombin time was increased by 14.8, 113.1, 208.9 and 286.2 seconds respectively. Benzyl penicillin increased thrombin time by 21.9, 126.1 and 235.9 seconds respectively at 1.0, 5.0 and 10.0 mg/ml concentration. There was no visible clot formation at 30.0 and 20.0 mg/ml concentration of the extract and reference drug respectively. The effect of varying concentrations of bovine thrombin on citrated plasma is shown in Figure 3. Absorbance at the different incubation time increased proportionately with the increase in thrombin concentration, showing that the speed of coagulation is dependent on the dose of thrombin. Figure 4 shows the effect of varying concentrations of the Reference drug and culture extract on thrombin. There was a significant decrease ($p < 0.05$) in absorbance with every increase in the concentration of the culture extract and reference drug. This indicates that the inhibition of clot formation by the extract and reference drug was concentration dependent. The spectrophotometric measurement collaborate the visual observation (Figure 2).

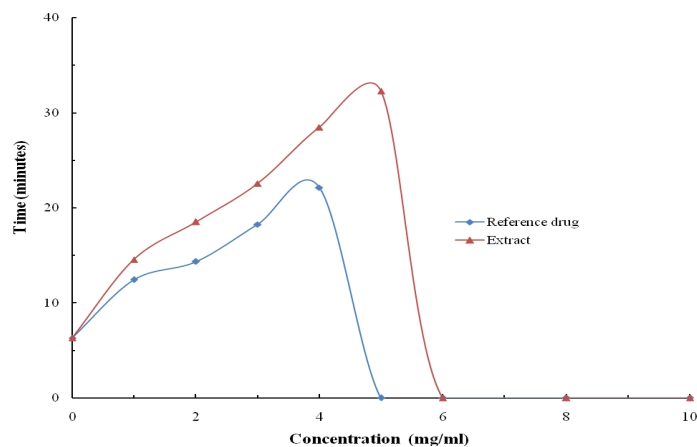


Figure 1: Inhibitory effects of different concentrations of culture extract of *P. chrysogenum* (PCL 301) and reference drug (benzyl penicillin) on whole blood clotting time

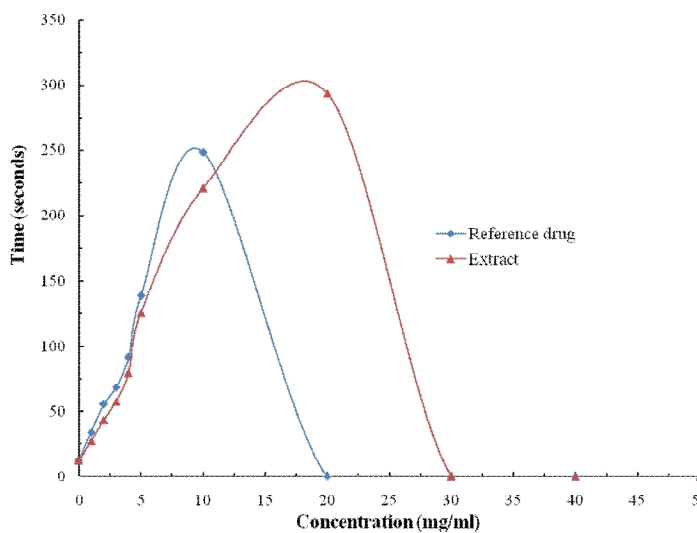


Figure 2: Inhibitory effects of different concentrations of culture extract of *P. chrysogenum* (PCL 301) and reference drug (benzyl penicillin) on Thrombin Time

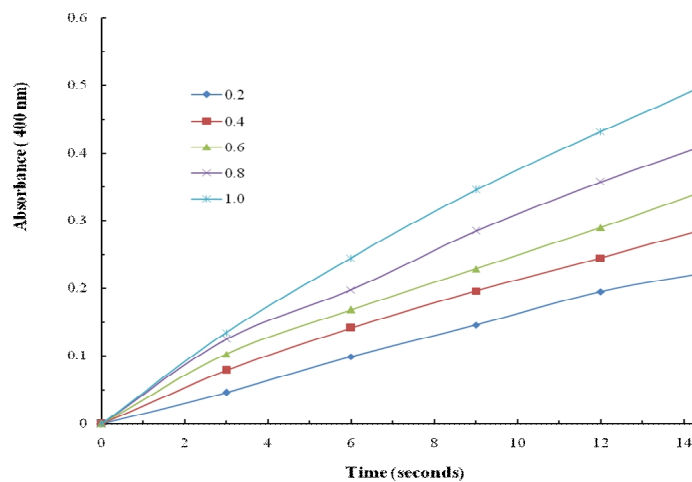


Figure 3: Effect of different concentrations of bovine thrombin on initial rate of clot formation at 25°C

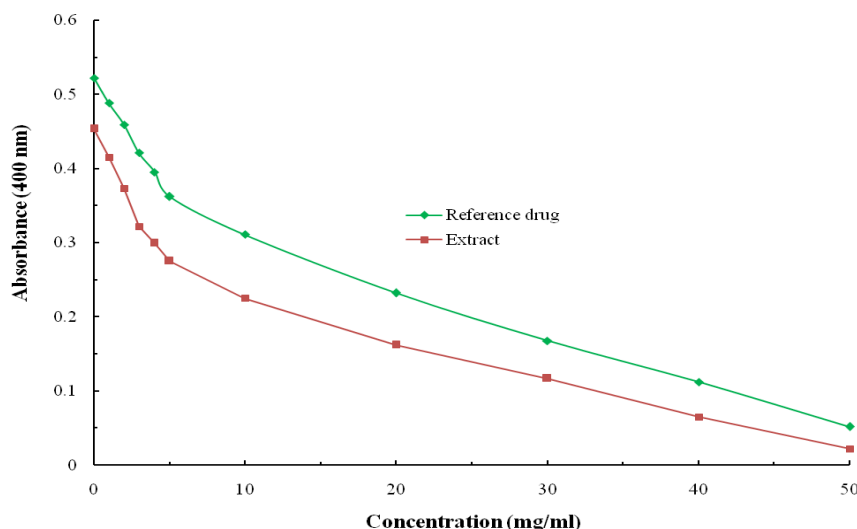


Figure 4: Inhibitory effects of different concentrations of culture extract of *P. chrysogenum* (PCL 301) and reference drug (benzyl penicillin) on thrombin activity (Absorbance was monitored at 25°C)

DISCUSSION

The visual study (Figure 1) showed that the extract of *P. chrysogenum* (PCL501) and reference drug interfered with normal blood clotting process; they caused significant delay in blood clotting time. The inhibitory effect on clot formation was directly proportional to their concentration. Total inhibition of blood coagulation occurred at 6.0 and 5.0 mg/ml concentration of the extract and benzyl penicillin respectively. Penicillin G and some other antibiotics have been found to exert a dose-dependent anticoagulation and antithrombotic effects.^{8,17} Large doses of penicillin and related antibiotics have been reported to cause excessive bleeding by impairing platelet aggregation.^{11,12,18} The bleeding could be attributed to the anticoagulation effects of the antibiotics. Such high doses may not, however, be required for patients on antibacterial therapy. *Penicillium chrysogenum* (PCL501) is known to produce penicillin antibiotics with antibacterial effects similar to the reference drug, benzyl penicillin.^{13,14} This explains the similarity in their pattern of the anticoagulation effects. The culture extract of *P. chrysogenum* PCL501 and reference drug significantly affected the thrombin time (Figure 2). The time proportionately increased with higher concentration of the extract and reference drug. Total inhibition of thrombin activity and clot formation was achieved at a high concentration 30.0 and 20 mg/ml concentration of the extract and benzyl penicillin respectively. The inhibitory effects of the extract and benzyl penicillin on thrombin and blood clot formation is collaborated by the significant decrease in absorbance of the whole blood incubated with varying concentrations of the extract and reference drug (Figure 4). It is not surprising that the absorbance (at 400 nm wavelength), which is a function of the amount of clot formed, decreased steadily with increasing concentration of the antibiotics. Penicillin and related antibiotics have been reported to exert a dose-dependent inhibition of thrombin.^{8,17} Several workers have tried to explain the mechanism for platelet impairment which largely accounts for the anticoagulant properties of β -lactam antibiotics. Rao *et al.* reported that penicillin inhibited platelet aggregation response and release reaction without blocking the ability of these platelets to convert arachidonic


acid to thromboxane.¹⁸ *In vitro* and *in vivo* exposure to beta-lactam antibiotics was also reported to cause irreversible platelet functional and biochemical abnormalities¹². Shattil *et al.* also suggested that the impairment of the interaction of several agonists with their specific receptors on the platelet surface membrane could be a possible mechanism for the hemorrhagic as well as the potential antithrombotic effects of penicillin and related antibiotics if their inhibition of platelet function *in vitro* operates *in vivo*.¹¹ Our result shows that thrombin triggers the formation of blood clot in a dose dependent manner (Figure 3). Absorbance at the different incubation time increased proportionately with the increase in thrombin concentration suggesting a dose dependant increase of blood coagulation by thrombin. Thrombin time assay is based on the ability of thrombin to catalyze the polymerization of fibrinogen into a fibrin clot. It is a simple test used to screen for conditions that can interfere with conversion of fibrinogen to fibrin. The test specifically focuses at the final stage of the coagulation pathway, the conversion of fibrinogen to fibrin. The mechanism of thrombin activity and clot formation involves the cleavage of peptides (fibrino-peptides A and B) from the amino-terminal ends of the alpha and beta chains of fibrinogen by the proteolytic action of thrombin. The resulting fibrin monomer molecules ultimately polymerize into a fibrin clot. Thrombin is degraded by protease inhibitors such as anti-thrombin III, heparin cofactor II, and α -2-macroglobulin. An abnormally long thrombin time shows a deficiency of factor 1 (Fibrinogen). Our findings agree with the report that β -Lactam derivatives inhibit a range of other enzymes with nucleophilic serine residues, including mammalian serine proteases by acylation of serine residues at the active site of enzymes.⁵ The centrality of thrombin to this system makes it an extremely important enzyme in coagulation and as such the most suitable enzyme to target for anticoagulant drug therapy; hence, to control the action of thrombin would mean to control the entire coagulation system.¹⁹ Thrombin provides a powerful stimulus for platelet activation and aggregation. It is a potent platelet agonist and thus constitutes an interesting target for drugs that would prevent the formation of fibrin- and platelet-rich thrombi induced by thrombin.⁵ This study

therefore corroborates other reports indicating that thrombin inhibition largely account for anticoagulation effects of penicillin and related antibiotics. Thrombin is degraded by protease inhibitors such as antithrombin III, heparin cofactor II, and alpha-2 macroglobulin. Since it is the main target for antithrombotic intervention, interest in direct thrombin inhibitors is rising owing to the limitations of other anticoagulant drugs. The β -lactam antibiotics specifically bind to thrombin, thereby inhibiting its ability to cleave fibrinogen. They can inhibit thrombin that is tied to a thrombus and restrict further thrombus formation when the thrombin is clot-bound or when thrombolysis takes place. Carbenicillin and ticarcillin have been reported to exert dose-dependent inhibition of experimental arterial thrombosis.¹⁷ These studies demonstrate that both *in vitro* and *in vivo* routes penicillin induced temporary impairment of platelet function. Thrombin has continued to elicit the interest of researchers due to its numerous functions and potency, particularly as a pro-coagulant and pro-inflammatory mediator. The present study demonstrates that the culture extract of *P. chrysogenum* PCL501 has *in vitro* anticoagulation and antithrombotic effects akin to commercial benzyl penicillin. Besides its prospect as a potent antibacterial agent¹³, this low cost natural fungal extract also has the potential of serving as an antithrombotic and anticoagulating agent in therapeutic medicine.

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