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Brief Report

Anti-HIV Activity of Philippine Crocodile (*Crocodylus mindorensis*) Serum on Infected Human Mononuclear Cells

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Abstract: The search for effective inhibitors of HIV-1 replication remains a critical research area of research in virology and immunology. Natural products have emerged as promising candidates for antiviral therapies. In the present study, we assessed the potential inhibitory activity of Philippine crocodile serum at both pre- and post-infection stages of the HIV-1 replication cycle. Freshly collected crocodile serum samples were used in a cell culture-based assay with peripheral blood mononuclear cells. HIV-1 reverse transcriptase activity in the treated cell culture system was assessed using colorimetric enzyme immunoassay. The crocodile serum at 0.5% and 0.25% vol/vol concentrations showed an inhibitory activity against HIV-1 replication both in pre-infection interactions (68.61 ± 1.67% and 69.95 ± 2.24%, respectively) and post-infection interactions (65.68 ± 2.93% and 69.92 ± 0.45%, respective). These findings suggest that Philippine crocodile serum may have potential as a natural inhibitor of HIV-1 replication and warrant further investigation into its therapeutic use.

Keywords: Philippine crocodile serum; anti-HIV activity; peripheral blood mononuclear cells

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1. Introduction

Human immunodeficiency virus (HIV) infection remains a significant global health challenge affecting approximately 38 million people worldwide [1]. Despite the availability of antiretroviral therapy (ART), the emergence of drug resistance and the need for lifelong treatment underscores the urgent need for alternative therapeutic strategies. Natural products have emerged as promising sources of novel anti-HIV therapies, offering potential alternatives to current antiretroviral treatments. These compounds, derived from plants, marine organisms, microbes, and animal sources, possess diverse chemical structures and unique pharmacological properties, which make them attractive candidates for HIV treatment research [2].

Interest in crocodile serum stems from the remarkable immune system of these ancient reptiles, which allows them to survive in pathogen-rich environments and to recover quickly from severe injuries. Crocodile serum, particularly that derived from the Philippine crocodile (*Crocodylus mindorensis*), has shown promise as a potential anti-HIV agent [3,4]. In our previous study, we demonstrated the inhibitory activity of Philippine crocodile serum on HIV-1 reverse transcriptase, using an in vitro model. A remarkable inhibition level of 92.93 ± 0.72%, suggesting that crocodile serum may contain bioactive components capable of combating HIV infection [5]. Further investigations using a more physiologically relevant models are warranted to translate these findings into clinically relevant therapeutic strategies. Therefore, the present study aimed to assess the anti-HIV activity of Philippine crocodile serum by using a cell culture-based assay with human mononuclear cells. Human mononuclear cells, including peripheral blood mononuclear cells (PBMCs), play a crucial

role in the pathogenesis of HIV infection and are commonly used as cell culture-based models for evaluating the efficacy of potential anti-HIV agents [6].

By elucidating the mechanisms underlying the anti-HIV activity of crocodile serum in a cell culture system, this study sought to contribute to the development of novel therapeutic agents for HIV/AIDS treatment. The findings of this study may provide valuable insights into the potential use of crocodile serum as a natural anti-HIV agent and pave the way for further preclinical and clinical investigation.

2. Materials and Methods

2.1. Crocodile Serum Collection

A minimum of 2 mL of serum from one purposively selected adult male *Crocodylus mindorensis* was collected at the Animal Clinic of the Davao Crocodile Park facility, Davao City, Philippines. Freshly collected sera were separated from whole blood samples collected by a veterinarian as part of a crocodile health check. Specifically, 5 mL of whole blood was allowed to clot at room temperature for approximately three hours. The serum was separated by centrifugation at 3000× *g* for 15 min and immediately processed.

2.2. Human Peripheral Blood Mononuclear Cells Preparation

All experiments using human blood cells were carried out with informed consent from the blood donors and following clearance from the Institutional Human Ethical Committee and with strict compliance to the Occupational Safety and Health Administration 1910.1030 Blood-borne Pathogens guidelines (OSHA 1910.1030). Blood (5 mL) was collected from a healthy HIV seronegative donor and peripheral blood mononuclear cells were isolated using the Ficoll density gradient method. Cells (1×10^6) were stimulated for 72 h with the mitogen phytohemagglutinin-P at a final concentration of 5.0 µg/mL (PHA-P; Sigma-Aldrich Inc., Darmstadt, Germany) in the presence of human interleukin 2 (IL-2, 10 ng/mL; Genzyme, Cambridge, MA, USA) before use to promote blast formation and T-cell replication. After stimulation, the cells were washed twice with PBS.

2.3. Micro-Co-Culture Assay

A co-culture of seropositive peripheral blood mononuclear cells (PBMC) and seronegative PHA-stimulated PBMCs is maintained under ideal conditions to allow viral replication in vitro. Most PBMC cultures from HIV-1 seropositive patients yielded detectable HIV-1 antigens using this method [7]. PHA-stimulated seronegative cells (1×10^6) and PBMCs from a seropositive HIV-1 individual were added to duplicate wells of a 24-well tissue culture plate. The final volume was adjusted to 2 mL with growth media containing RPMI1640 with 20% FBS and 10.0 U/mL IL-2. The plates were incubated at 37 °C with 5% CO_2. On days 7 and 14, the cultures were replenished with fresh growth medium containing 5×10^5 PHA-stimulated seronegative cells (feeder cells). The culture continued until day 21 and was terminated. Supernatant fractions from duplicate wells from days 14 and 21 were saved separately and stored at −70 °C until analysis for HIV-1 RT by ELISA. Cultures were considered positive only when day 21 supernatants showed an increase in HIV-1 RT compared to day 14 supernatants.

2.4. Crocodile Serum-Induced Cytotoxicity Assay

The cytotoxicity of crocodile serum was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). Cytotoxicity was assessed after the cells were incubated for 24, 48, and 72 h, with 0.5% and 0.25% (*v*/*v*) crocodile serum.

2.5. Anti-HIV Assay Using Human Peripheral Blood Mononuclear Cells

To evaluate the pre-infection interaction (cell-free HIV), HIV-1 virions from co-cultured cells were first incubated with crocodile serum for 2 h and then infected with PBMCs. A volume (10 µL of HIV-1 virions was added to 10 µL of Philippine crocodile serum

(human serum as a negative control and nevirapine, 1250 μg/mL as a positive control) and incubated for 2 h at 37 °C in a CO_2 incubator. After 2 h of interaction, the experimental moiety–virus suspension was added to 100 μL PHA-stimulated PBMCs and incubated for 2 h in CO_2. After incubation, the cells were washed carefully, and supernatants were aspirated to leave 50 μL/well, after which 100 μL of infected cells were plated into wells and 100 μL of crocodile serum was added. The plates were incubated for 72 h at 37 °C in a CO_2 incubator. The contents of each well were transferred to microfuge tubes, the cells were pelleted at 13,000 RPM for 5 min, and the supernatants were used to determine the HIV-1 RT levels.

For the post-infection interaction (cell-associated HIV), the HIV isolate was first allowed to infect PBMCs, and crocodile serum (0.5 and 0.25% v/v) was added to the suspension after one hour. Briefly, 100 μL of HIV-1 isolate was added to 1 mL of PHA-stimulated PBMCs and incubated for 2 h in a CO_2 incubator to allow the virus to infect the cells. After incubation, the cells were washed twice to remove uninfected HIV. One hundred microliters of both infected cells and Philippine crocodile serum were added to the wells of a 96-well tissue culture plate (human serum as a negative control and nevirapine, 1250 μg/mL, as a positive control). The plates were incubated for 72 h at 37 °C in a CO_2 incubator. The contents of each well were transferred to microfuge tubes, the cells were pelleted at 13,000 RPM for 5 min, and the supernatants were used to determine HIV-1 RT levels.

2.6. HIV-1 Reverse Transcriptase Antigen ELISA

To monitor HIV-1 activity, the level of HIV-1 reverse transcriptase antigen in the supernatant was measured using the ROCHE reverse transcriptase colorimetric assay (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. A standard curve was generated using an HIV-1 reverse transcriptase standard provided by the manufacturer. The unknown concentration of HIV-1 reverse transcriptase antigen in crocodile serum-treated cells was determined by measuring absorbance at 405 nm. The experiments were performed in triplicates.

2.7. Data Analysis

The inhibition of crocodile serum at different concentrations was calculated as a percentage of the negative control (medium, no drug) using GraphPad Prism software (version 9.0). The EC_{50} was calculated using a nonlinear regression curve fit of the dose response curves of each transformed serum concentration and normalized percent inhibition using GraphPad Prism software (version 8.0).

3. Results

3.1. HIV-1 Co-Culture

The Philippine crocodile serum sample from one (1) purposively selected male crocodile collected by a veterinarian was used to assess the potential virucidal activity and post-attachment inhibition. In addition, one HIV-1 seropositive serum sample was processed for peripheral blood mononuclear cell (PBMC) co-culture, from which HIV-1 isolates were obtained. HIV-1 RT activity after 21 d was 0.5928 ± 0.1212 ng/well (Figure 1A). On the other hand, a baseline crocodile serum concentration of 0.5% v/v was used based on a previous study [5], and the cell viability results showed no significant reduction in mononuclear cells after 72 h of incubation (Figure 1B).

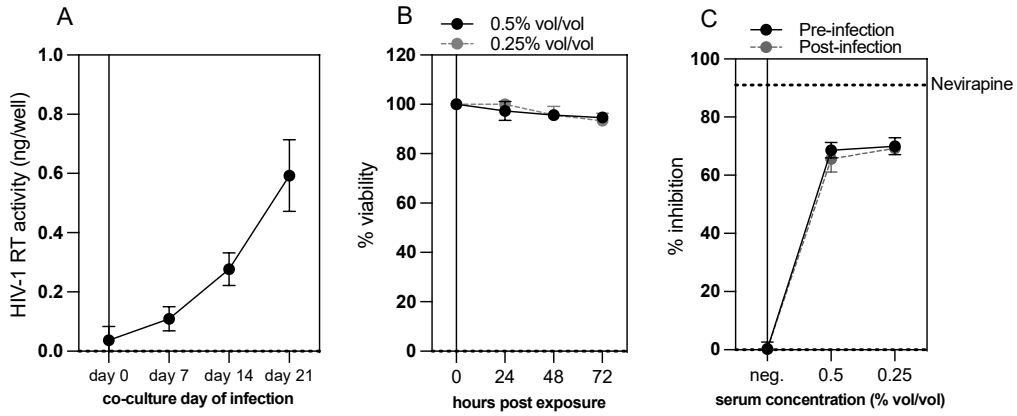

Figure 1. (**A**) The co-culture assay showed relatively increased HIV-1 RT activity at day 21 compared to day 14. (**B**) Viability of PBMCs exposed to different concentrations of crocodile serum showed no significant cytotoxicity. (**C**) The % inhibition of crocodile serum pre- and post-infection of HIV-1 in the PBMC culture system at different serum concentration (0.5 and 0.25% v/v). Nevirapine (1250 µg/mL) was used as a positive control with 91.02 ± 3.45% combined inhibitory activity in both pre- and post-infection.

3.2. Anti-HIV-1 Activity of the Philippines Crocodile Serum

Different concentrations of crocodile serum were tested for inhibitory activity against HIV-1 infection. All tested crocodile serum concentrations showed no significant reduction in mononuclear cells used in the assay (Figure 1B). The inhibitory activity of the crocodile serum in pre-infection interaction at 0.5% and 0.25% (v/v) concentrations provided 68.61 ± 1.67% and 69.95 ± 2.24%, respectively (Figure 1C). Moreover, similar inhibition activity was observed in post-infection interaction with 65.68 ± 2.93% and 69.92 ± 0.45% at 0.5% and 0.25% vol/vol concentrations, respectively (Figure 1C). Moreover, the EC_{50} of both pre- and post-infections was 0.37% (v/v).

4. Discussion

The management of HIV-1 infection has significantly improved with the use of antiretroviral therapy (ART), leading to reduced morbidity and mortality among HIV-infected individuals living with HIV [8]. However, drug resistance remains a critical challenge for both virology and immunology. Despite the effectiveness of the currently available antiretroviral treatments, the emergence of drug-resistant strains compromises therapeutic efficacy. Natural products have emerged as promising candidates for antiviral therapy, owing to their diverse chemical structures and potential therapeutic properties [2]. In this study, we investigated the role of Philippine crocodile serum as a potential inhibitor of HIV-1 replication. The Philippine crocodile (*Crocodylus mindorensis*), a species locally available in the Philippines, has garnered attention for its unique immune system adaptations, which offer potential for the discovery of novel therapeutic agents. In this study, we demonstrated the significant inhibition of HIV-1 replication both pre- and post-interactions with PBMCs.

Notably, the inhibitory effect of crocodile serum was consistent across different concentrations, with both 0.5% and 0.25% (v/v) demonstrating similar levels of inhibition. Even when the serum was diluted twofold, it retained its considerable inhibitory effect, suggesting its potency. This result is in line with the previously reported study that crocodile serum showed inhibition of HIV-1 reverse transcriptase by as high as 92.93 ± 0.72% at 0.5% (v/v) using an in vitro assay [5]. However, the disparity in inhibitory activity could be attributed to the differences in the cell culture systems employed. In vitro conditions offer limited immunity compared with the in vivo environment [9–11], thus providing more favorable conditions for crocodile serum to inhibit HIV-1 activity.

The antiviral activity of Philippine crocodile serum against HIV-1 was remarkable in both pre- and post-infection interactions, suggesting a broad approach to viral inhibition. In

pre-infection interactions, the serum likely interferes with the virus before it enters the host cells, potentially through direct virucidal effects, inhibition of viral attachment, or prevention of membrane fusion. The significant inhibition of HIV-1 reverse transcriptase activity (68–70%) at low serum concentrations (0.5% and 0.25% v/v) in this pre-infection highlights the potency of these effects, suggesting the potential of serum as a prophylactic treatment.

Notably, the serum demonstrates the ability to inhibit HIV-1 replication even after the virus has entered and infected the host cells. This suggests that serum components can interfere with various stages of the viral life cycle in infected cells. The observed inhibition of reverse transcriptase activity (65–70%) in post-infection interactions indicates direct interference with this crucial enzyme, potentially preventing the virus from converting its RNA genome into DNA. Additional mechanisms include protease inhibition, transcriptional regulation, disruption of viral assembly or budding, and possible immunomodulatory effects. The consistency of inhibition across both pre- and post-infection scenarios indicated the presence of a diverse array of antiviral compounds in Philippine crocodile serum. This broad approach to inhibiting HIV-1 replication makes it a particularly promising subject for further research into novel antiviral treatments, potentially offering new avenues for both the prevention and treatment of HIV-1 infection.

Although the specific components responsible for these effects in *C. mindorensis* serum have not been fully characterized, several potential antiviral factors have been identified in other animal sera. These include serum amyloid P component (SAP) [12], complement proteins [13], α-2-macroglobulin [14], and antimicrobial peptides (AMPs) [15]. While the stability of potential antiviral factors in *C. mindorensis* serum is not well characterized, AMPs are generally stable under physiological conditions and can withstand proteolytic degradation to some extent. Moreover, these components can inhibit viruses through various mechanisms, such as binding to viral surface proteins or interfering with virus-host cell interactions. The presence of such factors in *C. mindorensis* serum could contribute to its observed antiviral properties, suggesting that the serum of this endangered species may contain valuable compounds with potential applications in the development of novel antiviral agents, highlighting their importance in both conservation and medical research.

Among the various components of crocodile serum, AMPs have emerged as promising candidates for inhibiting HIV-1 reverse transcriptase (RT), a crucial enzyme for viral replication [5,15]. These 45-amino-acid peptides serve as precursors for immune defense molecules and require proteolytic activation. Notably, even before activation, AMPs can act as antagonists of pathogen-specific enzymes, such as HIV-1 RT. AMPs may inhibit RT through various mechanisms, including direct binding to the enzyme's active site or allosteric inhibition [3,4]. The potential of crocodile serum-derived AMPs as antiviral agents has garnered substantial interest because of their therapeutic implications, particularly their ability to limit HIV-1 replication by targeting multiple stages of the viral life cycle [16–18]. Although other components of crocodile serum may contribute to its overall biological activities, current evidence suggests that AMPs are likely the key active ingredients responsible for the observed anti-HIV-1 effects.

Although the results reported in this study are promising, further research is needed to explore their efficacy, safety, and potential for use as therapeutic agents against HIV-1 in humans. Additionally, other inhibitors present in crocodile serum may contribute to its anti-HIV-1 activity, necessitating further investigation to fully identify and characterize these inhibitors [19–21]. If successful, harnessing the therapeutic potential of crocodile serum could offer new avenues for combating HIV-1 infection and improving patient outcomes.

5. Conclusions

This study provides evidence for the inhibitory activity of Philippine crocodile serum on HIV-1 replication. The significant inhibition observed in both the pre- and post-infection interactions highlights its potential as a natural HIV-1 inhibitor.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of the Immaculate Conception (protocol code EX-12-11-18, 2018). The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Immaculate (protocol code M005, 2018).

Informed Consent Statement: Informed consent was obtained from all the subjects involved in the study.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

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