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"In Vitro Evaluation of the Antivenom Potential of Methanolic Leaf and Flower Extracts of Clitoria Ternatea against Echis Carinatus Venom"

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Abstract:

Background: Snakebite envenomation remains a major public health concern in tropical countries, particularly India. *Echis carinatus* (Indian saw-scaled viper) is one of the "Big Four" venomous snakes responsible for significant morbidity and mortality. Although polyvalent antivenom is the standard treatment, its limitations, including poor neutralization of local tissue damage and adverse reactions, have prompted the search for plant-derived inhibitors. [1–3]

Objective: To evaluate the in vitro antivenom activity of methanolic leaf and flower extracts of *Clitoria ternatea* against enzymatic components of *Echis carinatus* venom.

Methods: Methanolic extracts of leaves and flowers of *Clitoria ternatea* were prepared and screened qualitatively for phytochemicals. Their inhibitory activity against protease, phosphomonoesterase, phosphodiesterase, hyaluronidase, phospholipase A_2, coagulation, and indirect hemolytic activities of *Echis carinatus* venom was assessed using established biochemical assays.

Results: Phytochemical analysis revealed alkaloids, flavonoids, and saponins in leaf extract, while flower extract additionally contained resins and reducing sugars. Leaf extract demonstrated 85.9% inhibition of protease activity, whereas flower extract produced 84.1% inhibition. Phosphomonoesterase inhibition was higher in leaf extract (47.4%) than flower extract (26.8%). Minimal inhibition of hyaluronidase was observed, while phosphodiesterase, phospholipase A_2, coagulation, and indirect hemolytic activities were not significantly inhibited.

Conclusion: Methanolic extracts of *Clitoria ternatea*, particularly leaf extract, exhibit promising inhibitory effects against selected enzymatic components of *Echis carinatus* venom, especially proteases. These findings support further investigation through phytochemical isolation, mechanistic studies, toxicity evaluation, and in vivo validation to explore their role as adjuncts in snakebite management.

Keywords: *Clitoria ternatea*, *Echis carinatus*, snake venom, antivenom,

protease inhibition, medicinal plants

Introduction

Snakebite envenomation is recognized as a neglected tropical disease and continues to impose a substantial burden on public health, particularly in South Asia, Southeast Asia, sub-Saharan Africa, and Latin America. India reports one of the highest numbers of snakebite-related deaths worldwide, with rural agricultural communities being disproportionately affected. Delayed access to healthcare, limited availability of antivenom, and socioeconomic barriers contribute significantly to mortality and long-term disability.[1,2]

Among the medically important venomous snakes of the Indian subcontinent, *Echis carinatus* (Indian saw-scaled viper) is responsible for numerous cases of severe envenomation. Its venom is a complex mixture of enzymatic and non-enzymatic toxins, including metalloproteinases, serine proteases, phospholipase A_2, hyaluronidase, phosphodiesterases, and phosphomonoesterases. These toxins induce coagulopathy, hemorrhage, tissue necrosis, inflammation, and systemic toxicity.[3]

Currently available polyvalent antivenoms remain the only specific therapy for snakebite envenomation. However, conventional antivenoms exhibit several limitations, including limited effectiveness against local tissue destruction, high production costs, cold-chain requirements, and the potential to induce early or delayed hypersensitivity reactions. Consequently, there is increasing interest in identifying plant-derived compounds capable of neutralizing venom toxins or complementing conventional antivenom therapy.[4]

Clitoria ternatea L. (Fabaceae), commonly known as butterfly pea, has long been employed in traditional medicine for the treatment of neurological disorders, inflammation, diabetes, and snakebite. The plant contains diverse bioactive constituents, including flavonoids, anthocyanins, alkaloids, saponins, triterpenoids, and phenolic compounds, many of which exhibit antioxidant, anti-inflammatory, and enzyme inhibitory properties.[5, 6]

The present study was designed to evaluate the *in vitro* antivenom potential of methanolic leaf and flower extracts of *Clitoria ternatea* against major enzymatic activities of *Echis carinatus* venom. Particular emphasis was placed on inhibition of protease, phosphomonoesterase, phosphodiesterase, hyaluronidase, phospholipase A_2, coagulation, and indirect hemolytic activities.

Materials and Methods

Study Design

The present investigation was an **experimental in vitro study** designed to evaluate the inhibitory potential of methanolic leaf and flower extracts of *Clitoria ternatea* against the major enzymatic and biological activities of *Echis carinatus* venom.

Plant Material

Fresh leaves and flowers of *Clitoria ternatea* L. (Family: Fabaceae) were collected from Bengaluru, Karnataka, India, during March–April 2022. The plant material was thoroughly washed with distilled water to remove dust and foreign matter, shade-dried at room temperature, and pulverized into a fine powder using a mechanical grinder. The powdered material was stored in airtight containers until extraction.

Venom Sample

Crude venom of *Echis carinatus* (Indian saw-scaled viper) was obtained from a healthy adult specimen. The venom was dissolved in sterile 0.9% saline solution and stored at 4°C until further biochemical analysis. Fresh venom preparations were used for all enzymatic inhibition assays.

Preparation of Methanolic Extracts

Twenty grams of dried leaf powder and flower powder were extracted separately with 100 mL of analytical-grade methanol by maceration for 48 hours. The extracts were filtered through Whatman No. 1

filter paper and concentrated by solvent evaporation. The resulting crude extracts were preserved in airtight containers under refrigerated conditions until further analysis.

Preliminary Phytochemical Screening

Qualitative phytochemical screening of the methanolic extracts was performed using standard laboratory procedures to detect the presence of major secondary metabolites, including:

- Alkaloids Flavonoids Saponins Tannins Phenolic compounds
- Glycosides Terpenoids Steroids Proteins Resins
- Reducing sugars

The occurrence of phytochemical constituents was determined based on characteristic colour changes or precipitate formation using standard qualitative tests.[7]

In Vitro Enzyme Inhibition Assays

The inhibitory activity of *Clitoria ternatea* extracts against important venom enzymes was investigated by pre-incubating venom with the plant extracts before enzymatic analysis.

Protease Inhibition Assay

Protease activity was determined using casein as the substrate following the method of Greenberg with appropriate modifications. Venom was incubated with leaf and flower extracts before measurement of proteolytic activity. Protein degradation products were quantified using the Lowry method, and enzyme inhibition was calculated relative to untreated venom controls. [8]

Agar Plate Protease Assay

Proteolytic activity was further confirmed using casein-agarose plates. Venom pre-incubated with different concentrations of plant extracts was loaded into agarose wells and incubated. The diameter of the proteolytic clearance zone was measured, and percentage inhibition was calculated by comparison with control venom.

Phosphomonoesterase Inhibition Assay

Phosphomonoesterase activity was measured spectrophotometrically using p-nitrophenyl phosphate as substrate. Enzyme inhibition was calculated from absorbance values obtained after incubation with plant extracts.[9]

Phosphodiesterase Inhibition Assay

Phosphodiesterase activity was evaluated using sodium p-nitrophenyl phosphate as substrate. Changes in absorbance at the specified wavelength were recorded, and inhibition was expressed relative to untreated venom. [10]

Hyaluronidase Inhibition Assay

Hyaluronidase activity was estimated by the turbidimetric method using hyaluronic acid as substrate. Venom and plant extracts were pre-incubated before enzyme assay, and residual enzymatic activity was determined by measuring absorbance. [11]

Phospholipase A_2 Assay

Phospholipase A_2 activity was evaluated using egg-yolk suspension as substrate. Venom-induced phospholipid hydrolysis was measured spectrophotometrically. An additional agarose plate assay containing egg yolk was performed to confirm inhibition of phospholipase activity by the extracts. [12]

Coagulation Assay

Platelet-poor human plasma was prepared from citrated blood by centrifugation. Coagulation time was recorded after incubation of plasma with crude venom in the presence or absence of methanolic extracts. The clotting time was compared among control and treatment groups to assess neutralization of venom-induced coagulant activity.[13]

Indirect Hemolytic Assay

Indirect hemolytic activity associated with phospholipase A_2 was determined using agarose plates containing human erythrocytes and egg yolk. Venom samples pre-incubated with plant extracts were loaded into wells, incubated overnight and hemolytic halo diameters were measured to determine inhibitory activity.[14]

Statistical Analysis

The data obtained from the in vitro enzyme inhibition assays were analyzed using **descriptive statistics**. enzyme inhibition was expressed as **percentage inhibition (%)**, while coagulant activity was recorded as **clotting time (seconds)**. the results were presented in tables and figures for comparison between the venom control and the methanolic leaf and flower extracts of *clitoria ternatea*. no inferential statistical analysis was performed.

Results

Extraction Yield

Methanolic extraction of *Clitoria ternatea* yielded crude extracts from both leaves and flowers. The percentage yield was **21.8%** for the leaf extract and **2.2%** for the flower extract, indicating a considerably higher recovery of methanol-soluble constituents from leaves than flowers.

Phytochemical Screening

Qualitative phytochemical analysis revealed differences in the chemical composition of leaf and flower extracts. Both extracts contained **alkaloids, flavonoids, and saponins**. In addition, the flower extract tested positive for **resins** and **reducing sugars**, whereas tannins, phenols, glycosides, proteins, amino acids, terpenoids, phytosterols, and steroids were not detected in either extract.

Phytochemical	Leaf Extract	Flower Extract
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Resins	–	+
Reducing sugars	–	+
Tannins	–	–
Phenols	–	–
Glycosides	–	–
Terpenoids	–	–
Phytosterols	–	–
Proteins/Amino acids	–	–

Inhibition of Protease Activity

Among all enzymatic assays performed, the strongest inhibitory effect was observed against protease activity. Pre-incubation of *Echis carinatus* venom with methanolic leaf extract resulted in **85.9% inhibition**, while the flower extract produced **84.1% inhibition**, indicating potent neutralization of venom proteases by both extracts.

In the agar plate assay, protease inhibition increased with increasing concentrations of leaf extract. The leaf extract exhibited **25% inhibition at 100 µg/mL** and **50% inhibition at 200 µg/mL**, whereas the flower extract showed approximately **25% inhibition** across the tested concentrations.

Inhibition of Phosphomonoesterase Activity

The methanolic leaf extract demonstrated **47.4% inhibition** of phosphomonoesterase activity, whereas the flower extract inhibited the enzyme by **26.8%**. These findings suggest that the leaf extract possesses greater inhibitory activity against phosphomonoesterase than the flower extract.

Inhibition of Phosphodiesterase Activity

Neither the leaf nor the flower methanolic extract inhibited phosphodiesterase activity. Enzymatic activity remained comparable to that of untreated venom, indicating that the extracts had no detectable effect on this enzyme under the experimental conditions employed.

Inhibition of Hyaluronidase Activity

Only minimal inhibition of hyaluronidase activity was observed. The leaf extract inhibited enzyme activity by **0.6%**, while the flower extract produced **0.4% inhibition**, indicating negligible neutralization of this spreading factor.

Phospholipase A_2 Activity

Assessment of phospholipase A_2 activity by spectrophotometric and agarose plate methods demonstrated **no detectable inhibition** following treatment with either methanolic leaf or flower extract. The enzyme retained its activity throughout the study, suggesting that the tested extracts do not interfere with venom phospholipase A_2 under the present experimental conditions.

Coagulant Activity

Untreated platelet-poor plasma coagulated in **331 seconds**, whereas crude venom markedly shortened clotting time to **57 seconds**, confirming its potent procoagulant effect. Neither leaf nor flower extract effectively neutralized venom-induced coagulation. Plasma treated with leaf and flower extracts alone showed clotting times of **210 seconds** and **192 seconds**, respectively.

Indirect Hemolytic Activity

No inhibition of indirect hemolytic activity was observed when venom was pre-incubated with either methanolic leaf or flower extract. The absence of reduction in hemolytic halo formation indicates that the extracts were ineffective in neutralizing phospholipase A_2-mediated hemolysis.

Table 2: Inhibitory effects of methanolic extracts of *Clitoria ternatea* against *Echis carinatus* venom

Enzyme/Biological Activity	Leaf Extract	Flower Extract
Protease	85.9%	84.1%
Phosphomonoesterase	47.4%	26.8%
Phosphodiesterase	No inhibition	No inhibition
Hyaluronidase	0.6%	0.4%
Phospholipase A_2	No inhibition	No inhibition
Coagulant activity	No inhibition	No inhibition
Indirect hemolysis	No inhibition	No inhibition

Key findings

The results indicate that methanolic extracts of *Clitoria ternatea* exhibit **selective inhibition** of *Echis carinatus* venom. Protease activity was the most strongly inhibited, followed by moderate inhibition of phosphomonoesterase. In contrast, the extracts showed negligible or no activity against phosphodiesterase, hyaluronidase, phospholipase A_2, venom-induced coagulation, or indirect hemolysis. These findings

suggest that the antivenom potential of *Clitoria ternatea* may be mediated through specific phytoconstituents targeting selected venom enzymes rather than broad-spectrum neutralization.

Discussion

Snakebite envenomation remains a significant public health problem in many tropical and subtropical countries, particularly in South Asia, where *Echis carinatus* is one of the principal causes of venom-induced morbidity and mortality. Although polyvalent antivenom remains the cornerstone of treatment, its inability to effectively neutralize local tissue damage, together with adverse immunological reactions and limited accessibility in rural settings, has prompted continued exploration of plant-derived inhibitors as complementary therapeutic agents.[16,19] In this study, the in vitro antivenom potential of methanolic leaf and flower extracts of *Clitoria ternatea* was investigated against selected enzymatic and biological activities of *Echis carinatus* venom.

Qualitative phytochemical analysis demonstrated the presence of alkaloids, flavonoids, and saponins in both leaf and flower extracts, whereas resins and reducing sugars were detected only in the flower extract. These findings are consistent with previous reports describing *Clitoria ternatea* as a rich source of flavonoids, anthocyanins, triterpenoids, alkaloids, and other bioactive metabolites with antioxidant and anti-inflammatory properties.[20,21] Such phytochemicals are recognized for their ability to interact with proteins and enzymes, making them plausible candidates for venom neutralization.

Among all assays performed, inhibition of protease activity represented the most significant finding. Methanolic leaf extract inhibited protease activity by 85.9%, while flower extract produced 84.1% inhibition. Snake venom proteases, particularly snake venom metalloproteinases (SVMPs), play an essential role in hemorrhage, extracellular matrix degradation, inflammation, and tissue necrosis.[16, 22] Therefore, inhibition of these enzymes may reduce local tissue destruction following envenomation. The high inhibitory activity observed in the present study suggests that constituents of *Clitoria ternatea* may interfere with proteolytic enzymes either through direct binding to catalytic sites or by modifying enzyme conformation.

Leaf extract exhibited greater inhibition of phosphomonoesterase activity than flower extract. Although phosphomonoesterase contributes less to the overall toxicity of *Echis carinatus* venom than metalloproteinases or phospholipase A_2, its inhibition further supports the selective antivenom activity of *Clitoria ternatea*. The superior performance of leaf extract may reflect quantitative differences in bioactive phytoconstituents, particularly flavonoids and alkaloids, which are known to possess enzyme-modulating properties.[24]

In contrast, neither extract significantly inhibited phosphodiesterase activity, phospholipase A_2 activity, venom-induced coagulation, or indirect hemolysis. Hyaluronidase inhibition was also negligible. These observations indicate that the methanolic extracts possess selective rather than broad-spectrum venom-neutralizing activity. Since phospholipase A_2 and coagulant toxins are major contributors to systemic toxicity, additional purification and identification of active compounds will be required to determine whether individual phytochemicals possess stronger inhibitory activity than the crude extracts examined in the present investigation.[25]

The observed differences among enzymatic assays may also reflect the structural diversity of venom proteins. Venom enzymes differ considerably in catalytic mechanisms, substrate specificity, and three-dimensional conformation. Consequently, phytochemicals capable of inhibiting metalloproteinases may not effectively interact with phospholipase A_2 or other venom enzymes. Similar selective inhibition has been reported for several medicinal plants investigated for antivenom activity, suggesting that plant-derived extracts may be most effective as adjuncts rather than replacements for conventional antivenom therapy.[26]

The present findings support the growing body of evidence that medicinal plants constitute valuable sources of bioactive compounds with potential antivenom properties. However, translation of these findings into clinical application requires further investigation. Isolation and characterization of active constituents, elucidation of molecular mechanisms, toxicity assessment, pharmacokinetic studies, and validation in animal models are essential before therapeutic application can be considered. In addition,

studies evaluating synergistic effects between purified phytochemicals and commercial antivenom may identify strategies to improve the management of local tissue damage associated with snakebite.

Study Limitations

The present study has several limitations that should be considered while interpreting the findings:

- The investigation was limited to **in vitro** enzyme inhibition assays.
- Active phytochemical constituents responsible for venom inhibition were not isolated or characterized.
- Statistical analysis was limited in the original study and should be strengthened in future investigations.
- Only a single snake species (*Echis carinatus*) was evaluated.
- Dose-response relationships and IC_{50} values were not determined.
- In vivo efficacy and safety were not assessed.

Future Perspectives

Future research should focus on:

- Bioassay-guided fractionation of *Clitoria ternatea* extracts.
- Identification of active molecules using LC-MS/MS, HPLC, GC-MS, or NMR spectroscopy.
- Molecular docking studies to investigate interactions between phytochemicals and venom enzymes.
- In vivo neutralization studies in experimental animal models.
- Evaluation of combination therapy with commercial antivenom.
- Standardization of extract preparation for reproducibility and potential pharmaceutical development.

Conclusion

The present study demonstrates that methanolic extracts of *Clitoria ternatea* possess selective in vitro inhibitory activity against *Echis carinatus* venom. Among the enzymatic activities evaluated, protease inhibition was the most pronounced, while moderate inhibition of phosphomonoesterase activity was also observed. In contrast, the extracts exhibited minimal or no inhibitory effects against phosphodiesterase, hyaluronidase, phospholipase A_2, coagulant activity, and indirect hemolysis.

These findings indicate that *Clitoria ternatea* is a promising source of bioactive compounds with potential antivenom properties, particularly against venom metalloproteinases responsible for local tissue damage. Nevertheless, the crude methanolic extracts cannot be considered a substitute for conventional antivenom therapy. Comprehensive phytochemical characterization, mechanistic studies, pharmacological evaluation, and in vivo validation are required before clinical application can be considered.

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