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In Vitro Protease Inhibition, Modulation of PLA2 Activity and Protein Interaction Studies of *Calotropis gigantea*

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Abstract

Plant protection strategies have been proven to be the most promising approach on selection of protease inhibitors. Protease inhibition and PLA2 activity of white and violet varieties of *Calotropis gigantea* L., has been studied. Comparative protease inhibition studies on white and violet varieties have shown small variation in protease inhibition. In trypsin inhibition, white variety has not shown inhibition but the violet variety has shown the inhibition at 10 µl concentration. In Protease K inhibition, white variety has not shown inhibition but the violet variety has shown the inhibition at 10 µl and 5 µl concentrations respectively. In Chymotrypsin inhibition, both white and violet varieties have not shown any protease inhibition. The plant has also exhibited PLA2 inhibition activity in blood agar containing egg yolk. Protein interaction network profile has shown interactions with circulatory, neural and immune system components that can modulate and simulate the mechanism in the system approach.

Keywords: *Calotropis gigantea*n; Protease inhibition; PLA2 activity; Protein interaction

Introduction

Plant cells contain different types of proteases essential for cellular activities and regulatory processes [1]. The function of proteases is repressed by molecules like Protease inhibitors, permitted the functions of the cellular processes like antigen production and the degradation of membrane or regulatory proteins [2].

Several natural products showing protease inhibitors are proteins that are important inferences for diagnosis and therapy [3]. Proteases are ubiquitous present in many living systems that are physiologically necessary for cellular processes [4]. When cells are disrupted, enzymatic proteases releases and quickly degrade proteins that show severe disruption of cellular and protein metabolism [5,6]. Proteases that are harmful to the cells can be inhibited by protease inhibitors. This can lead in protecting the protein of interest from degradation [7].

Many plant protection strategies have been established by various researchers and the most promising approach that is focused on selection of the natural protease inhibitors (PIs) [8] and applicable in diagnosis and therapy. Hydrolytic cleavages of proteolytic enzymes catalyzing specific peptide bonds in target proteins are called as proteases [9]. Proteases play key roles in various biological processes in higher organisms that serve as mediators of signal transduction in many of the cellular events such as apoptosis, inflammation, hormone processing and blood clotting pathways.

Calotropis gigantia is used as a traditional medicinal plant since ancient times with unique properties [10,11]. Traditionally Calotropis is used with other medicinal or alone to treat common disease such as rheumatism, asthma, elephantiasis, fevers, diarrhea, indigestion, cough, cold, eczema, nausea, and vomiting. *Calotropis gigantea* L. is used traditionally in India to treat snake bite patients.

Naja naja is one of the most dangerous snake species causing high number of human deaths in both tropical and subtropical countries. Structural and Biological characterization of venom toxins has been conducting from the past to understand biochemical nature of venom toxin components [12-14]. Results of previous research showed that *Naja naja* cobra venom contains several proteins and peptides with enzymatic and non-enzymatic activities that belong to different groups including phospholipases A2 (PLA2), cardiotoxins and neurotoxins [15-17].

Indian cobra (*Naja naja*) also known as Asian cobra or spectacled cobra is a species of the genus Naja found in the Indian subcontinent and a member of the "big four", the four species which inflict the most snake bites in India. A powerful post-synaptic neurotoxin and cardiotoxin present in Indian cobra's venom acts on the synaptic gaps of nerves, paralyzing the muscles, leading to failure of respiratory system or cardiac arrest. Enzymes present in the venom components like hyaluronidase cause lysis and increase the spread of the venom [18-22].

To evaluate the protein activity, leaves of pink and white varieties has been collected and tested for protease inhibition and anti-venom activity.

Materials and Methods

Protease inhibition

Calotropis gigantea for the study were washed thoroughly in distilled water and air-dried. Buffer extract is prepared in about 500 ml conical flask by homogenizing 25 g of plant leaves in 100 ml of 0.1M phosphate buffer with pH 7.0 in an electrical blender. The homogenate was further mixed thoroughly by incubating the contents at room temperature in a rotary shaker for 30 minutes at 150 rpm. The slurry was then filtered through cheese cloth and the filtrate was centrifuged at 10,000 rpm for 15 minutes at 4°C for removing any cell debris that remains in the preparation. The clear supernatant obtained represented

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the crude extract, and was assayed for protease inhibitor activity.

Approximately 10 µl of plant isolate is mixed with 10 µl of protease (0.5 mg/ml) and spotted on to a strip of X-ray film. About 3 µl of protease was mixed with 10 µl phosphate buffer 0.l M (pH 7.0) as the control and spotted on to the X-ray film. Approximately 10 µl of plant isolate is mixed with 10 µl of protease (0.5 mg/ml) and 10 µl of PLA2 is also spotted on to a strip of X-ray film. The X-ray film is incubated for 10 minutes at 37°C. The film should be washed under tap water and dry the film so that the zone of gelatin hydrolysis by protease is to be visualized. If zone is not visualized, protein inhibition may be present in the tested sample.

PLA2 activity

In order to evaluate the phospholipase A2 activity, the indirect hemolytic activity was assayed. Phospholipase A2 enzyme of the *Naja naja* kaouthia (Indian cobra) venom were bought from Sigma Chemical Co. in lyophilized form and is used in the present experimentation. To isolate PLA2, C. m. Venom (0.9 g) was reconstituted in starting buffer (0.05 mol/1 Tris-HCl, 0.1 mol/l KOI, pH 8.3). Approximately 300 µl of packed sheep erythrocytes (1.2%) washed for four times with saline solution, containing 300 µl of 1:3 egg yolk solution in saline as a source of lecithin (1.2%) and 250 µl of 0.01 M $CaCl_2$ (10 mM) solution were added to 25 ml of 1% (w/v) of agar at 50°C dissolved in 0.8% PBS pH 7.2. The mixture is to be applied to Petri plates (135×80 mm) and allowed to gel. Then, 4 mm diameter wells are to be made with gel puncture and the wells should be filled with 10 µl of samples. Experiment was carried out in triplicate. After 20 h of incubation at 37°C, the diameters of hemolytic halos were measured. In contrast, PBS solution did not induce haemolysis. When egg yolk was not added to the gels there was no hemolysis, indicating that hemolysis was only of the indirect type, i.e. due to PLA2 activity in this venom.

Protein-protein interaction

Protein interaction studies have been conducted by submitting PLA2 protein to string, a system protein network server. Input for the protein is provided as PLA2. The server is predicted with proteins of *Oryctolagus cuniculus* appears to be matched. The studies related to this protein networks is been conducted in the present experimentation.

Results and Discussion

Protease inhibition activity of white and violet varieties of *Calotropis gigantea* Linn., has been presented. Comparative studies on white and violet varieties have shown small variation. In trypsin inhibition, white variety has not shown inhibition but the violet variety has shown the inhibition at 10 µl concentration. In Protease K inhibition, white variety has not shown inhibition but the violet variety has shown the inhibition at 10 µl and 5 µl concentrations. In Chymotrypsin inhibition, white and violet varieties have not shown any inhibition (Figure 1).

Figure 2 shows the protein inhibition activity of *N. naja* combines with plant (white) variety. Both the venom and the combination of venom and plant protein showed protein inhibition.

Figure 3 has shown the activity of the selected plants with PLA2 protein. The protein component has shown good result (halo) with

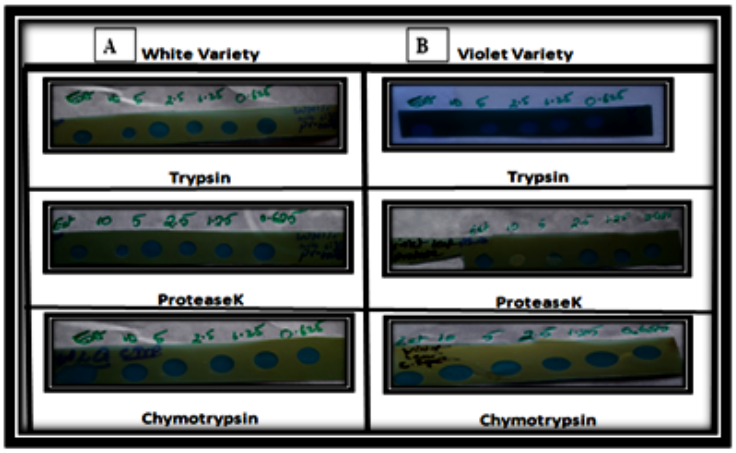


Figure 1: Protease inhibition activity of *Calotropis gigantean.*

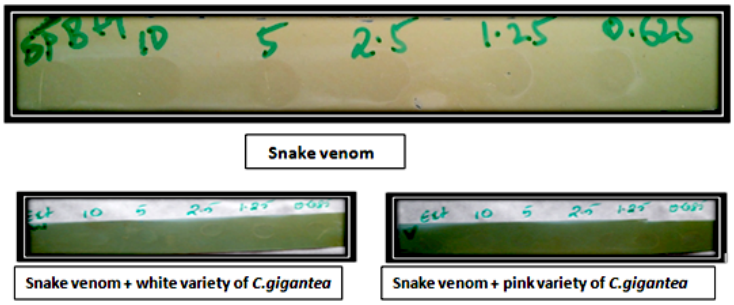
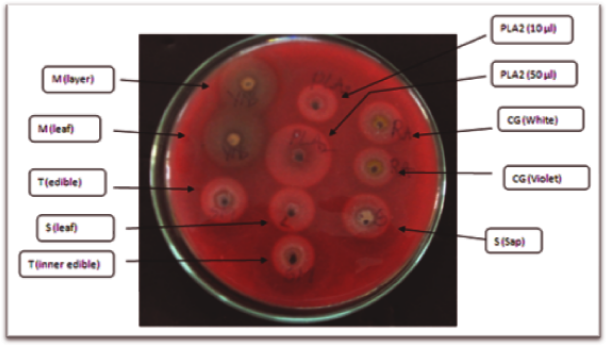


Figure 2: Protease inhibition activity of *N. naja* with plant extract.



Note: PLA2: Phospholipases A2; CG: *Calotropis gigantea*; S: *Pouteria sapota*; M: *Borassus flabellifer* (Fruit); T: *Borassus flabellifer* (Sprouts)

Figure 3: PLA2 activity of *N. naja* with plant extracts.

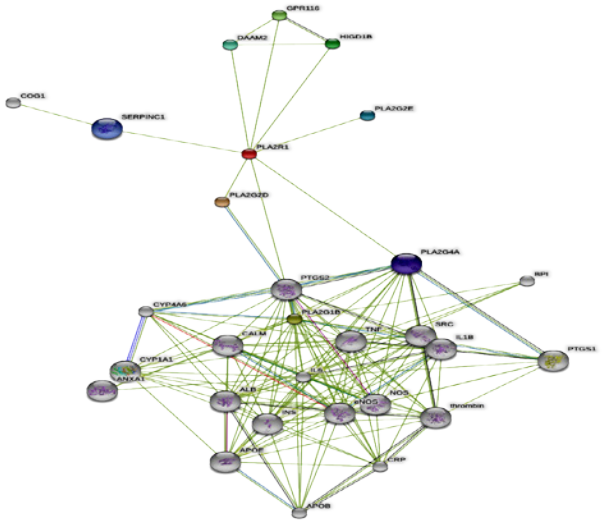


Figure 4: Protein interaction networks of PLA2 with other proteins.

Sample	Zone of inhibition in mm (including well size of 4 mm)
PLA2 (10 µl)	6 mm
PLA2 (50 µl)	14 mm
***Calotropis gigantea* (white)**	6 mm
***Calotropis gigantea* (Violet)**	6 mm
***Pouteria sapota* (Sap)**	8 mm
***Pouteria sapota* (Leaf)**	7 mm
***Borassus flabellifer* (Sprouts inner edible)**	8 mm
***Borassus flabellifer* (Sprouts edible)**	7 mm
***Borassus flabellifer* (fruit sap)**	12 mm
***Borassus flabellifer* (fruit coat)**	12 mm

Table 1: Zone of inhibition.

lowest zone formation in *C. gigantea*. The other plant extracts has not shown any activity with PLA2.

Borassus flabellifer shows different zone of inhibition based on the selected samples (7 mm to 12 mm) and not formed any haloes. About 10 µg/ml of PLA2 produced 6 mm diameter haemolytic halo. This shows that *Naja naja* venoms have the enzymes (phospholipase A2) that has the ability to lyse sheep RBC's. Calotropis leaf extract (CLE) was capable of inhibiting phospholipase A2 dependant hemolysis of sheep. RBC's induced by PLA2 in a dose dependant manner. *Pouteria sapota* leaf shown 7 mm and sap shown 8 mm of zone of inhibition and not formed any halo. *Borassus flabellifer* fruits and sprouts are also not shown any halo. This shows that *Pouteria sapota* and *Borassus flabellifer* has not shown any activity against snake venom. It is also confirmed that *C. gigantea* has shown good inhibition activity with PLA2. The plant *C. gigantea* extract has shown halos (PLA2 activity) and so the experiment has found good PLA2 activity against *N. naja* venom (Table 1).

Figure 4 has shown the protein-protein interaction with "PLA2". The protein submitted to string, a functional protein association networks server with PLA2 as input has predicted link with many components associated with neurons. Most are dependent with network of proteins like GPR116 (G protein-coupled receptor), HIGD1B (HIG1 hypoxia inducible domain family, member 1B), DAAM2 (dishevelled associated activator of morphogenesis 2), PLA2G2E (phospholipase A2, group IIE), SERPINC1 (serpin peptidase inhibitor, clade C (antithrombin), member 1), PLA2G4A (Cytosolic phospholipase A2 (cPLA2) (Phospholipase A2 group IVA)), Tumor necrosis factor, Precursor (TNF-alpha), Calmodulin (CaM), Apolipoprotein E Precursor (Apo-E), Insulin Precursor [Contains Insulin B chain;Insulin A chain], Thrombin Fragment, etc. These are relevant to circulatory and nervous system.

Plant genome contains set of sequences that codes many proteases which specify unrelated families showing similar properties. The biological roles of such proteases are mostly mysterious that expresses different diseased genes that have abnormal function [23]. Gene silencing, over expression and mutant alleles studies provided phenotypes for a growing number of proteases [24]. Proteases play a key role as regulators of a striking variety of biological processes like gametophyte survival, meiosis, embryogenesis, cuticle deposition, seed coat formation, stomata development, chloroplast biogenesis, epidermal cell fate, and systemic and local defense responses [25].

The six major classes of enzymes that show protease family are aspartic, cysteine, glutamic, metalloproteases, threonine, and serine, which are implicated in disease mechanism.

Protease inhibitors have great interest as possible targets for the development of novel therapies [26]. The present plant sample has shown protein inhibition based on experimental studies. Studies on phospholipid-hydrolysing enzyme, PLA2, have been conducting from the past few decades to understand the pathological and physiological importance.

PLA2 of *Naja naja* is also be inhibited forming hemolytic halo in blood agar containing egg yolk. Protein interaction studies of PLA2 have shown networks linked to components involved in circulatory and nervous system.

Conclusion

Calotropis gigantea acts as protease inhibitors containing plant compounds, applicable as targets in molecular therapies. Both white and violet verities of *Calotropis gigantea* have also shown good PLA2 inhibition activity. Most of the PLA2 proteins has network link with proteins involved in circulatory and nervous system.

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