EXTRACTION AND PARTIAL PURIFICATION OF PHYTOCYSTATIN FROM *Calotropis procera*

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ABSTRACT

Phytocystatins are cysteine protease inhibitors (CPIs) that are known for their several applications in biotechnology and medicine. Various extraction media which include, Sodium Chloride, Sodium Hydroxide, Hydrochloric acid, Sodium phosphate buffer and distilled Water were used to evaluate Leave, root, flower, latex and stem bark of Calotropis *procera* for cysteine protease inhibitory activity against Papain enzyme. Sodium phosphate buffer extract from latex of *C. procera* with higher CPI activity were concentrated by cold acetone precipitation and further subjected to purification using ammonium sulphate fractionation and Phenyl sepharose column. The fifty percent inhibitory concentration (IC_{50}) for crude latex extract was 1.35mg/ml. Electrophoretic analysis of partially purified protein showed a band with an estimated molecular weight of 10.23 kDa. Cysteine protease inhibitor investigated in this work could serve as template in biotechnology of traditional medicine and transgenic crops to arrest the negative pathogenic expressions of cysteine protease.

Keywords: Phytocystatins, cysteine, protease, inhibitory activity

INTRODUCTION

Phytocystatins are plant proteins that specifically inhibit cysteine proteinase and belong to papain family. Unlike cystatins of animal origin (except stefins), plant derived cystatins have no di-sulfide bond and putative glycosylation sit They are plant proteins cysteine protease inhibitors that constitute a powerful regulatory system for endogenous proteases which may otherwise cause excessive proteolysis and cell damages (Karthik *et al.*, 2014). Cysteine proteases are expressed throughout the animal and plant kingdoms as well as in microorganism. These enzymes have been implicated in many adaptation mechanisms for surviva in the host, including modulation of th host immune system, invasion an destruction of host tissues, parasi dissemination, and acquisition essential nutrients that assure surviv and proliferation to sustain the infecti (Pirta *et al.*, 2016).

These enzymes has drawn spec attention as drug target for cure several diseases such as osteoporo arthritis, cancer and many paras infections (Rosenthal *et al.*, 1999).' interaction between cysteine protea and inhibitor is a necessary in aver unwanted potentially destruc proteolysis. These plant derived inhibitors are gaining consideration as potential candidate which can be exploited in the chemotherapy and in the engineering of transgenic crops.

Calotropis procera is a plant of *Asclepiadaceous* family and are is widely distributed in West Africa. The plant has received special attention because of its use in folk medicine (Lawal *et al.*, 2015). Previous reports have confirmed that this plant possesses various pharmacological properties that may be used for the treatment of inflammatory disorders such as arthritis, cancer and sepsis (Kumar *et el,* 2014). Furthermore, numerous bioactive proteins were also isolated from this plant and their therapeutic effects documented (Al-snafi, 2015).

MATERIALS AND METHODS

The plant parts was collected between periods of November to January in Samaru, Zaria (Latitude 11.15^{0}N and Longitude 7.65^{0}E). The plants were identified in the Department of Biological Sciences, Ahmadu Bello University, Zaria with voucher number 900219.

Extraction of Phytocystain

Fresh parts (25g each of); leaves, flowers, latex, root and stem barks of *C.procera* were blended with 100 mL each of sodium chloride 15% (w/v) , sodium hydroxide 0.2% (w/v), hydrochloric acid 0.05M, phosphate buffer 0.1 M (pH 7) and distilled water as described by (Wu and Whitaker, 1990). The clear supernatant obtained after centrifugation (10,000 rpm, 15 min, 4^{0}C) represent the crude extract, and was assayed for CPI activity and protein content. The phosphate buffer extract with highest activity was used for further studies.

Acetone Precipitation

Four volumes of cold acetone was added with stirring to the crude extract. After 20 min stirring, the precipitate was collected by suction filtration, washed with cold acetone. The precipitate was air dried and stored at 4°C under desiccation. This was suspended in 100mM phosphate buffer, pH 7.6 and was designated as the crude protein extract (Rao *et al.*, 1983).

Inhibitory Effect of Acetone Precipitate on Papain Activity

Different concentrations of protein precipitate (ranging from 0.1-3.0mg/ml) were incubated with 1mg/ml papain. Activity of inhibitor was assayed as described below.

Protease Inhibitor Assay

Protease inhibitor activity was assayed according to the method of Wannapa et al., (2006). Assays were performed by adding 290µL of 50mM Tris–HCl (pH 7.6) and 200µL of 1.25mM BApNA solution to the previously preincubated cysteine protease (1mg/ml papain prepared in 100mM phosphate buffer, pH 6.8, containing 0.3mM EDTA and 2 mM cysteine-HCl) with inhibitor extract for 10min, at 250 C. After 30 min at 370C, the reaction was stopped by adding 150µL of acetic acid (30%). The resulting color was measured spectrophotometrically at 410nm. Protein Estimation

Protein content was determined according to the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard.

Purification of Crude Extracts from *C.procera*

Crude protein extract was precipitated different percentages of ammonium sulphate saturation (10-80%)

precipitated proteins were separated by centrifugation at 10,000 rpm at 4°C for 20 min. Active protein pellets was dialyzed against the buffer. The dialyzed sample was used for Hydrophobic interaction chromatography on a Phenyl Sepharose column (Gupta *et al* .,2005).The column (1 cm×6.5 cm) was equilibrated with 100mM sodium phosphate buffer, pH7.2 containing 1M ammonium sulphate . The bound proteins were then eluted from the column using 0.1M sodium phosphate buffer, pH 7.2 containing a decreasing step gradient of ammonium sulphate (0.5 M, 0.2 M, 0.1 M). The final fraction was eluted with 0.1M sodium phosphate buffer, pH 7.2 containing no ammonium sulphate.

Electrophoresis on SDS-PAGE

This was carried out as described by Laemmli (1970) to determine the Molecular weight.

RESULTS

The Latex of *plant* showed higher protein contents compared to other parts of the plants (Figure1). The results for inhibitory activity of the different parts of the plant extracts with papain enzyme (Figures 2) revealed that 0.1M phosphate buffer soluble protein extracts from latex possessed higher inhibitory activity. The Phosphate buffer extracts had higher specific activity (Figure 3) when compared to other parts of the plant and solvents used.

Figure 4 showed dose dependent inhibitory activity of different concentrations of acetone precipitated protein for latex of the plant. The fifty percent inhibitory concentration (IC_{50}) for crude latex extract was 1.35mg/ml.Crude acetone precipitated proteins showed maximal specific protease inhibition between 20-60% saturation of ammonium sulphate with maximum activity recorded at 50% saturation (Figure 5).

The activity yield and protein purificatior fold of cysteine protease inhibito extracted from the *C.procera* latex summarized in Tables 2. The fin purification fold of 3.95 and activity yie of, 48.72 percent respectively w observed for CPI. SDS-PAGE analysis partially purified protein peak shov prominent band with an estima molecular weight of 10.23 kDa (Figure

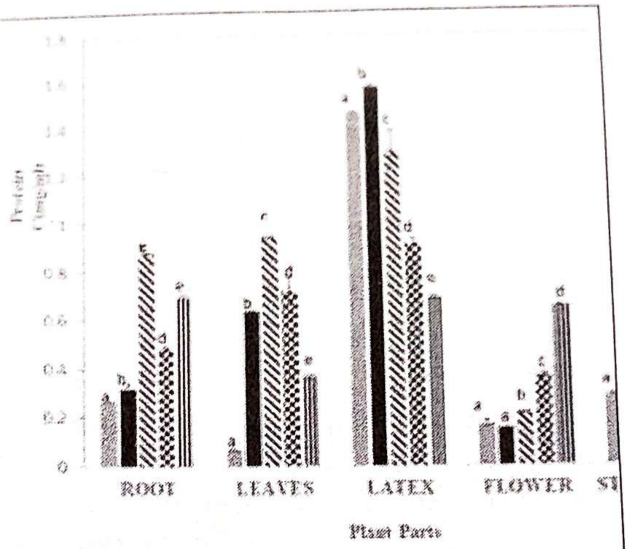


Figure 1: Protein Concentration in Extracts of *C. procera* Parts using Different Solvents.

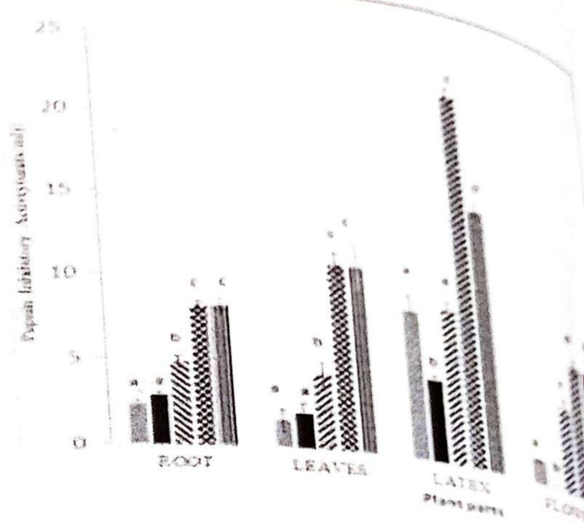


Figure 2: Protease Inhibitor Activity in Extrac
C. procera Part using Different Solvents.
Values are Mean ± SD. (n=3)

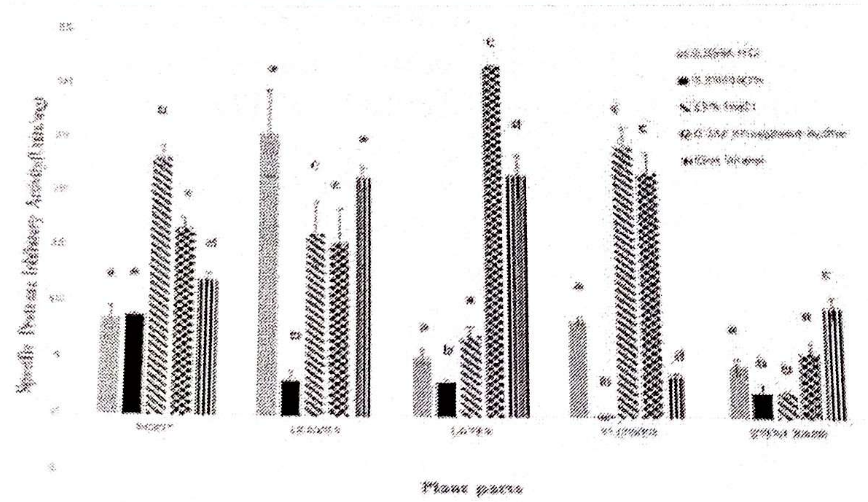


Figure 3: Specific Protease Inhibitory Activity in Extracts of *C.procera* using Different Solvents.
Values are Mean ± SD (n=3)

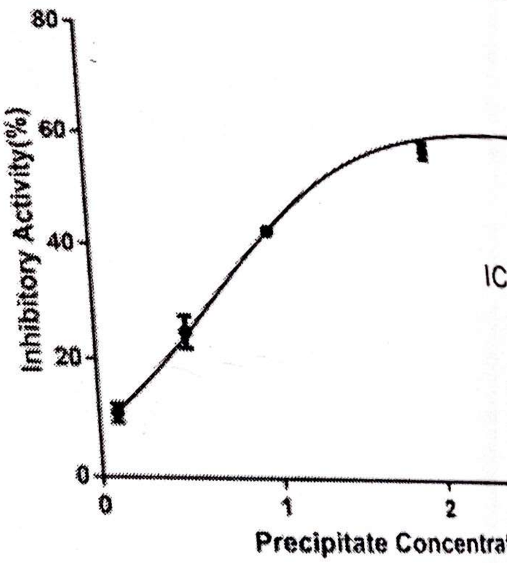
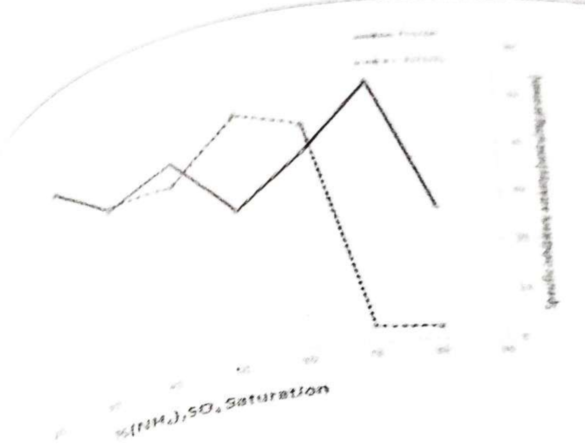


Figure 4: Protease Inhibitory Activity o
Extracts of *C. procera* Latex.
Values are Mean ± SD (n=3).



5: Ammonium sulphate Fractionation Profile of Protease tor from Extracts of *C. procera* Latex

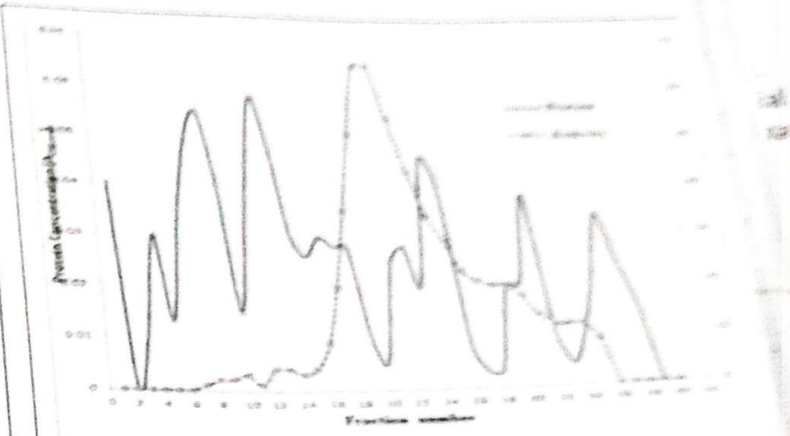


Figure 6: An Elution Profile for the Hydrophobic Interaction Chromato graph of *C.procera* Latex Inhibitor on phenyl-Sepharose Column.

	Specific Protease inhibitor activity (Units)/mg protein	Purification Fold	Yield (%)
rude extract	19.29	1	100
cetone recipitation	39.66	2.05	73.40
$NH_4)_2SO_4$ raction (20-60)	35.84	1.85	58.29
ydrophobic nteraction hromatography	76.33	3.95	48.72

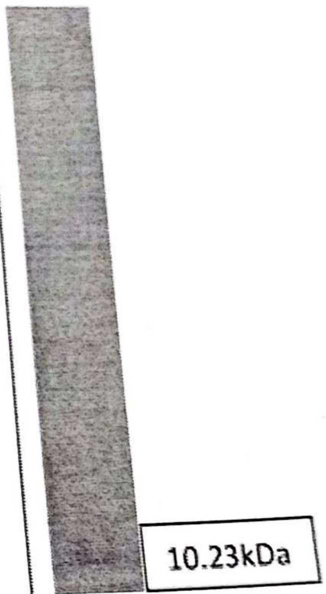


Figure7:Electrophoregram of Partially Purified In

DISCUSSION

The central role of cysteine proteases in the pathogenesis and survival parasites in the host erythrocytes makes them attractive drug targets (Rosenthal, 2011). Inhibition of these proteases by natural proteins/peptides that will not have off target binding could be useful in the treatment of these parasitic dise (Karthik *et al.*, 2014; Rosenthal, 2011)

In the present investigation, significantly higher specific cysteine protease inhibition was exhibited by phosphate buffer extract compared to other solvents, thus, phosphate buffer was selected as the effective extraction medium for maximal extraction of protease inhibitor without any loss in activity. Saline extract, which contain proteins from the latex of *C.procera,* was reported to have anticancer activities (Oliveira *et al.,* 2010). Other researchers used 0.01MNaOH (Marconi *et al.,* 1993), distilled water (Jung *et al.,* 2012) and 0.1 M phosphate buffer, pH 7.5 (Bijina *et al.,* 2011) as extraction media for various therapeutic proteins.

The crude acetone protein precipitate exhibited dose dependent inhibitory activity with papain enzyme with an IC_{50} value of 1350 µg/ml C.procera. These findings are more than hundred fold those reported by Abd El-latif (2015) with an IC_{50} value of 21.04 µg/ml on papain by inhibitor extracted from *Callosobruchus maculatus.*

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Phenyl-Sepharose as an adsorbent for hydrophobic interaction chromatography has been reported to be effective for the purification of some proteases and with purification factors in the range of 2.9 - 60 and activity yields in excess of 88% were obtained (Gupta *et al.,* 2005). The increase in activity of up to 48.7% in this investigation is an indicative of exclusions of endogenous protease and an improved stability of the inhibitors. Karlsson *et al.* (1985) successfully separated calpain and their inhibitors in one single step on phenyl-sepharose column.

SDS-PAGE analysis of the inhibitors under reducing conditions gave an estimated molecular weight of 10.23kDa for *C.procera* CPI.. Other researcher had earlier reported small peptide and proteins of molecular weight between 5 - 15kDa, with cysteine protease inhibitory activity (Soares *et al* 2015).

CONCLUSION

Cysteine protease inhibitors were isolated and purified from *C.procera* latex, with in *vitro* inhibitory activity against papain enzyme. This inhibitor could serve as potential candidate in biotechnology of transgenic crops and chemotherapy.

ACKNOWLEDGEMENTS

We thank University Board of Research (UBR) of Federal University of Technology, Minna for funding part of this work.

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