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Cysteine Protease Inhibition and Anti-Proliferative Activity of the Protein Fraction Isolated from *Justicia Wynaadensis*

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ABSTRACT

Protease inhibitors of natural origin are gaining importance in cancer drug development. Various in vitro and in vivo studies have shown that protease inhibitors reduce tumorigenicity. Protease inhibitors are ubiquitously present in plant kingdom. In the present study, An important medicinal plant,Justicia wynaadensis was selected due to its traditional health food practices and consumption as pro-health supplement. The plant was subjected for protein extraction. The protein fraction was analyzed for cysteine protease inhibition activity which showed around 40% inhibition. The protein fraction was partially purified and its antiproliferation activity was performed using yeast cells. Further, at molecular level, the protein fraction could affect fragmentation of DNA as analyzed by agarose gel electrophoresis by which the protein fraction from the plant can be attributable to have pro-apoptotic activity in actively dividing cells.

KEY WORDS: *Justicia wynaadensis, protein fraction, cysteine protease, protease inhibition, antiproliferation, DNA fragmentation*

INTRODUCTION

The development of cancer is a multistep process. The progression from benign to metastatic nature of the disease depends on a multitude of enzyme activities. Studies have revealed that proteases play important role in the progression and metastatic spreading of tumor cells from localized to the one that has the ability to invade and metastasize to secondary sites. There is a positive correlation between the aggressiveness of a tumor and the secretion of various proteases. Proteases are implicated during increased malignancy passing through stages of angiogenesis induction, degradation of vascular basement membrane, dissolution of cell-cell junctions, remodeling of extracellular matrix allowing cancer cells to be released from primary tumor mass. Proteolysis is involved during intravasation and exravasation of cancer cells. Proteases may co-operatively mediate these steps with individual ones having distinct roles [1-3]. Therefore, targeting proteases and inhibition of their activity might be one of the means to combat the development of cancer.

In view of the above, identification of protease inhibitor from medicinal plants offers an approach for selective inhibition of proteases or their application to further investigate the role of proteases in cancer development. The protease inhibitor could be used for reprogramming the uncontrolled proliferation of cancer cell growth towards controlled growth and homeostasis. Medicinal plants are the most important source and play a major role in the discovery of new therapeutic agents for drug development. Medicinal plants have been widely used for the treatment of diseases in traditional way for several years. An interaction between ancient medicine and biotechnological tools has to be established towards newer drug development.

Justicia wynaadensis, is an important medicinal plant of the region of Kodagu. The plant is locally called as *Aati soppu* or *Maddu soppu* by the local populace of the Kodagu, India, The plant is traditionally believed to imbibe major medicinal properties during the traditional celebration on Aati padinema (18th day of the Hindu calendar month of Kataka or Adi) on which day the plant is harvested. The plant is boiled in water and deep purple coloured extract is used for preparing a unique sweet dish which is consumed on the day. This is a traditional seasonal health food practice in the region that is believed to keep the people healthy throughout the year. ***Justicia wynaadensis*** belonging to Acanthaceae is endemic to the rainforest region of the Western Ghats. The plant is said to possess catalase and peroxidase activity, polyphenols and flavonoids have been identified and estimated [4]. GC-MS analysis of phytocomponents in the methanolic extract of *Justicia wynaadensis* has revealed around 30 components that have been identified out of which the the major constituents are Dihydrocoumarin, Phytol and Palmitic acid. Significant quantities of Linoleic acid, Stearic acid, Squalene and phytosterols such as Campesterol and Stigmasterol have been reported [5, 6]. The plant is also investigated to possess cellular cholesterol and cholesteryl ester lowering properties and inhibitory effect on the uptake of oxidized-LDL by human macrophage cell line [7]. With this available literature, this study was undertaken to check whether this plant could bring any effect of protease inhibition and antiproliferative activity in yeast cells.

Protease inhibitors are ubiquitous in plants; generally acts as storage proteins and wound-induced defence responses of plants against herbivores and pathogens [8]. Protease inhibitors play essential role in biological systems regulating proteolytic processes and in defence mechanisms against insects, and other pathogenic microorganisms. Therefore, the present study was undertaken to extract protein fraction, with its partial purification from *Justicia wynaadensis and* to analyze the cysteine protease inhibition activity. The partially purified fraction was further estimated for anti-proliferative effect and DNA fragmentation in proliferating yeast cells, which renders it to be pro-apoptotic that can be attributable to its anti-cancer activity.

MATERIALS AND METHODOLOGY

Collection of plant material and isolation of crude protein fraction:

Justicia wynaadensis was collected during the month of July-August, from the region of Kodagu, India. About 100 g of fresh leaves were weighed and washed thoroughly with distilled water and air dried. The fresh leaves were crushed using 0.01M phosphate buffer of pH 7.6 by using pestle and motor. The crude extract was collected in a conical flask and filtered by using muslin cloth and kept in rotary shaker for 2 hr. Centrifuged at 10000rpm for 15 min at 4ºC in cooling centrifuge. The supernatant was collected for further purification and assay.

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Isolation of protein fraction from *Justicia wynaadensis:*

a) Ammonium sulphate precipitation:

The centrifuged supernatant about 50 ml was taken and pre-chilled to 4^{0}C. The proteins were precipitated by gradual addition of ammonium sulphate to obtain 60% saturation. The solution was stirred at 4ºC for 1 hr and centrifuged at 9000 g for 30 min at 4^{0}C. The pellet was re-dissolved in 0.01M phosphate buffer of pH 7.6. The precipitated fraction was dialyzed against 0.01M buffer of pH 7.6, for 72 hr.

Estimation of Protein concentration:

The protein content was estimated by Lowry method using the sample obtained from the crude extract, and using dialyzed sample. The absorbance was read at 660 nm.

Proteolytic Activity of Cysteine protease:

The reaction mixture containing 0.10 ml Cysteine protease enzyme, 0.30 ml of buffer and 0.10 ml of activating agent (40 mM cysteine, 20 mM EDTA disodium salts) was added and incubated at 37^{o}C for 5 min. The reaction was initiated by adding 0.50 ml of 1% (w/v) Casein solution. After 30 min, 1.50 ml of 5% cold Trichloroacetic acid (TCA) was added to terminate the reaction. The supernatant of the mixture was separated by centrifugation at 9000 g for 20 min. The absorbance was measured at 280 nm.

Assay for protease inhibition activity:

Reaction mixture containing 0.10 ml of Cysteine protease was taken and 0.30 ml of phosphate buffer and 0.10 ml of activating agent (40 mM cysteine and 20 mM EDTA disodium salts) was added and incubated at 37^{0}C for 5 min. 0.1ml of dialysed protein extract was added prior to the addition of substrate. The reaction mixture was incubated for 30 min. Then 0.50ml of 1% Casein was added and further incubated for 30 min at 37^{0}C. 1.50 ml of 5% cold TCA was added to terminate the reaction. The supernatant of the mixture was separated by centrifugation at 9000 g for 20 min. The absorbance was measured at 280 nm.

SDS-PAGE:

Polyacrylamide gel with 12.5 % pore size was prepared using acrylamide and bis-acrylamide along with denaturing agent SDS. The protein profile of the extract was analysed by loading the crude protein extract, Ammonium sulphate precipitated fraction and dialyzed extract along with a protein molecular weight. The gel was run at a voltage of 50 volt initially for half an hour and the voltage was increased to100 v for separation of protein bands. The gel was stained overnight using Comassie brilliant blue staining solution.

De-staining of gel:

The gel was placed in de-staining solution (Methanol: Glacial Acetic acid: distilled water (1:1: 8)). The de-staining solution was changed at 2 hr interval for complete de-staining. After the complete removal of the stain, the gel was documented.

Antiproliferative effect of *Justicia wynaadensis* protein fraction:

i) Preparation of yeast inoculum:

Saccharomyces cerevisiae was inoculated into a 100 ml sterilized Sabouraud Dextrose broth (SD) and incubated at 37°C for 24 hr and maintained as seeded broth.

ii) Yeast suspension preparation:

The yeast culture from seeded broth, about 0.5 ml was taken and transferred to 2.5 ml of SD broth containing 0.2 ml of phosphate buffer and incubated at 37ºC for different time intervals for 2 hr, 4hr and 6hr. and 8 hr. This set up was used to observe the proliferative rate of the yeast cells. The test sample was prepared along by taking 0.5 ml yeast seeded culture transferred to 2.5 ml of SD broth. The protease inhibitor 0.2 ml in phosphate buffer was added and incubated for different time intervals as above.

iii) Cell Viability count:

The yeasts cells after treating with or without protein fraction were counted using haemocytometer after staining with trypan blue dye. The viable cells were transparent, unstained and were counted against the dead cells which had taken the dye and appeared dark coloured and opaque cells. In brief, 10 µl of trypan blue dye was added to a volume of 990 µl phosphate buffer containing 10 µl cell suspension taken after with or without treating with *Justicia wynaadensis p*rotein fraction. The cell viability counts were performed for each time interval of incubation. The mean was calculated. The cells per ml and percentage of cell viability were calculated by following formula. Percentage of cell viability = Total viable cells / Total cells x 100.

Yeast DNA isolation:

The yeast cell culture with or without treating with the protein fraction was used for DNA isolation. The Yeast DNA isolation was performed according to Hoffman and Winston, method [9]. In brief, the culture was centrifuged at 2000 g for 5 min and the pellet was re-suspended in 0.2 ml of detergent lysis buffer (2% Triton X 100, 1% SDS, 100 mM Nacl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA), 0.2 ml of phenol, chloroform and isoamyl alcohol (25:24:1), and 0.3 g of acid washed glass beads were added. The cells were disrupted by vortexing for 3-4 min. Spun at 13000 rpm for 5 min and transferred the aqueous top layer to a fresh tube. The DNA was precipitated by adding 1.0 ml of 100% ethanol and inverted the tube to mix. Further, the vials were spun for 2 min in a microcentrifuge. The pellet was re-suspended in 0.4 ml TE plus 30 µg RNase A, followed by incubation for 30 min at 37^{o}C. Further, 10 µl 4M Ammonium acetate and 1.0 ml 100% ethanol were added and the tubes were inverted for mixing and placed on ice for 10 min. The tubes were spun for 2 min in a microcentrifuge, the obtained DNA pellet was air dried and re-suspended in 50 µl Tris-EDTA buffer pH 8.0. The purity of isolated DNA was checked by taking 5µl of DNA sample in a 995 µl of TE buffer. Absorbance was read at 260 and 280 nm.

DNA fragmentation analysis:

The DNA isolated from yeast cells treated with or without the protein fraction of *Justicia wynaadensis* was analyzed on 1.0 % agaraose gel electrophoresis that had been incorporated with 5 µl of 10 µg/ml ethidium bromide and recorded under UV-transilluminator.

RESULTS AND DISCUSSION

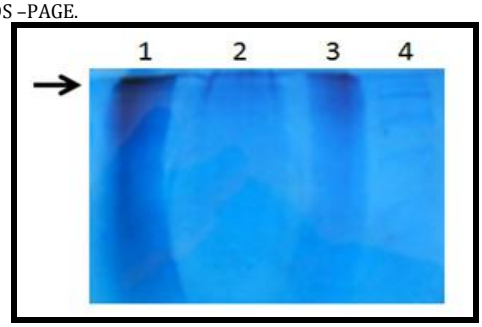
Proteases play important role in mammalian physiology. They are involved in the pathophysiological events in diseases, such as cancer. Proteases are secreted by pathogenic microorganisms during their mode of entry and infections into the host. Considering their involvement in such pathological processes, their inhibition by various inhibitors can provide useful targets for drug discovery [10, 11]. Plant sources offer more stable and potential lead molecules. Therefore the present study could demonstrate the extraction of protein fraction from *Justicia wynaadensis* which showed cysteine protease inhibition activity when assayed for enzyme activity.

Table No 1: Cysteine protease inhibition by *Justicia wynaadensis* protein extract.

Sl. No.	Volume enzyme (ml)	Volume of buffer (ml)	Volume of activating agent (ml)	Incubated at room temperature for 5 min	Volume of Protein fraction (ml)	Volume of substrate (ml)	Incubated at 37^{o}C for 30 min	Volume of TCA (ml)	Centrifuge at 9000 rpm for 30 min	Absorbance at 280 nm
1.	0.1	0.3	0.1		_	0.5		1.5		0.3203
2.	0.1	0.3	0.1		_	_		1.5		0.0533
3.	_	0.3	0.1		_	0.5		1.5		0.2309
4.	0.1	0.3	0.1		0.1	0.5		1.5		0.0225

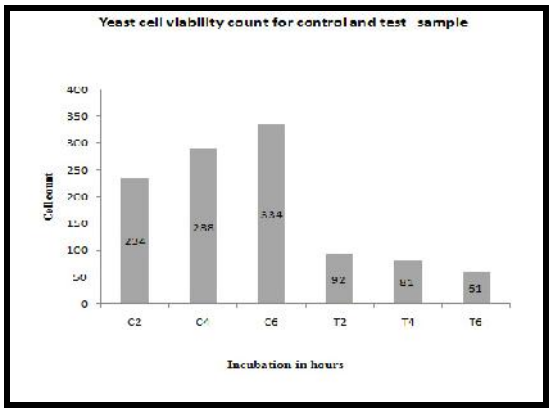
The absorbance value is the average of three independent experiments. The activity of cysteine protease estimated as above was found to be inhibited by the protein fraction extracted from *Justicia wynaadensis* as shown in the Table No. 1. The average percentage inhibition of around 40 % was calculated. Further, the crude extract was precipitated using ammonium sulphate salt and dialyzed against phosphate buffer. To examine the protein profile of *Justicia wynaadensis*l that is having cysteine

protease inhibiton was subjected to SDS-PAGE for protein separation. As shown in the Figure 1, the crude extract, ammonium sulphate precipitated and dialysed protein sample were separated by SDS –PAGE.



1: Crude protein fraction, 2: Ammonium sulphate precipitated protein fraction, 3: dialyzed protein fraction, 4: Molecular weight marker

Fig. 1: SDS-PAGE profile of the *Justicia wynaadensis* protein fraction



Graph 1: Antiproliferative effect of *Justicia wynaadensis* protein fraction on yeast cell proliferation

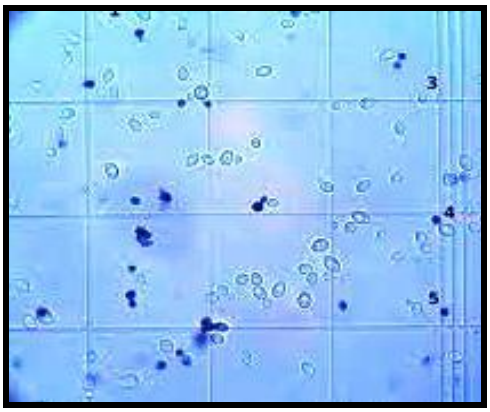


Fig. 2: Stained and unstained yeast cells

The mean percentage of cell viability were determined for each treatment of 2 hr, 4 hr and 6 hr and recorded as 39 %, 28 % and 18 % respectively. Further, to understand the effect of the protein fraction at the molecular level, its effect on yeast DNA was analyzed as follows. DNA Fragmentation is considered an hallmark event in the cells undergoing programmed cell death, However to check the effect of the protein fraction on yeast cell DNA, which is affecting in a way that resulted in the decreased cell number, its effect on Yeast DNA after treating with the protein fraction showed DNA fragmentation at six hour incubation time as shown in the Figure 3. This implies that the protein fraction obtained from *Justicia wynaadensis* is having a pro-apoptotic activity in actively dividing cells. Thus antiproliferative activity could be attributable by the protein fraction in actively dividing cells.

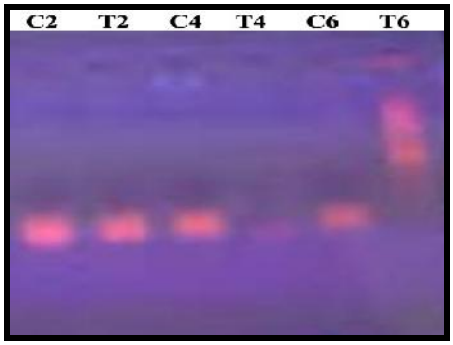


Fig. 3: DNA fragmentation in yeast cells upon treating with *Justicia wynaadensis* protein fraction

Studies on increased expression,elevated activity and mislocalization of certain enzymes have indicated that members of the cysteine proteases have been implicated in cancer progression. The proteolytic process is associated both with early tumor development affecting tumor cell proliferation and angiogenesis, and with dissemination of malignant cells from primary tumors. Therefore inhibitors of proteases are most intensively studied [16].

Henceforth, in our studies, we have shown that the protein fraction extacted from *Justicia wynaadensis* showed cysteine protease inhibition when assayed using papain-cysteine proteases and substrate casein. The protein fraction was subjected to partial purification and bioassayed for antiproliferative activity against the yeast cells that resulted in the reduction of yeast cells upon treating with the *Justicia wynaadensis* protein fraction. The protein fraction further was checked for its affect on DNA fragmentation in yeast cells, attributing to its pro-apoptotic activity in proliferating cells, and it could demonstrate DNA fragmentation of yeast cells, after 6 hour of treating with the *Justicia wynaadensis* protein fraction. Thus, this work substantiates cysteine protease inhibition and antiproliferative activity of the protein fraction from *Justicia wynaadensis.*

CONCLUSION

Traditional health food practices of certain indigenous community using plants and other resources have immense medicinal potential to scientifically explore certain pharmacological effects. With the available scientific literature on this medicinal plant *Justicia wynaadensis,* we could demonstrate cysteine protease inhibition and antiproliferative activities with pro-apoptotic activity Further this work requires more mechanistic based approach for further studies and characterization of protein fraction from *Justicia wynaadensis.*

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