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In vitro Antifungal and Antiproliferative Evaluation of a Trypsin Inhibitor from Testa of *Citrullus lanatus* Linn.

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ABSTRACT- Medicinal plants contain valuable sources of biological components that are helpful in the control and cure of aging diseases. Protein component present in various parts of the medicinal plants is the rich sources of medicine that contains a permanent cure for several diseases. The studies on edible sources like *Citrullus lanatus* testa (or seed coat) are to be conducted to understand, the better action against human diseases. The purified inhibitor is separated by SDS PAGE and analyzed by MS-MASCOT shown sequence as "MQDVKTYPPAAPVPATPRFGSLAG SLIEINR". The *C. lanatus* testa crude extracts were revealed good antifungal activity against *A. niger* (18mm) and *C. albicans* (13 mm). The *C. lanatus* testa purified extracts were revealed good antifungal activity against *A. niger* (21 mm) and *C. albicans* (20mm). Fluconazole was used as a fungal standard, shown inhibition zone for *A. niger* (14 mm) and *C. albicans* (20 mm). The *C. lanatus* Trypsin Inhibitor (CLTI) extracts from testa at 100µg/ml were shown good activity with *A. niger* acting as antifungal agent compared to standard antibiotic (Fluconazole). The *C. lanatus* testa Trypsin inhibitor, it is also shown good results for anti-proliferative activity. The results were shown good antiproliferation activity with MCF-7 (Breast Cancer) cell line due to gradual decrease in the percentage of cell survival. The IC50 for the standard drug (Tamoxifen) with MCF-7 (Breast Cancer) cell line was shown as 12µg/ml. The IC50of CLTI peptide was shown as 60µg/ml and crude as 190µg/ml. The IC50 for the standard drug (Tamoxifen) with Hep-G2 (Liver Cancer) Cell line is shown as11 µg/ml. The IC50 of CLTI peptide with Hep-G2 (Liver Cancer) Cell line was shown as 41 µg/ml and *C. lanatus* testa crude extract as 144 µg/ml. The experimentation concludes that serine protease inhibitors present in testa of *C.lanatus* shown both antifungal and antiproliferative properties.

Key- Words- *C. lanatus* testa, Trypsin inhibitor, Antifungal activity, Antiproliferative activity, Breast and liver cell lines

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INTRODUCTION

Most of the prominent experts have compiled comprehensive and survey of research in science and allied disciplines [1]. Analyses of the challenges are performing through high quality science research as a main context of the broader information systems community [2]. An advance in genetics, biotechnology, phytochemistry and engineering involves research leading to new manufacturing concepts for the production of medicinal compounds that can lead to a new manufacturing model [3].

Several governments have focused on medicinal plants that provide more sustain study in the subject of drug production [4]. The medicinal plant *C. lanatus* belongs to Cucurbitaceae family is using as fruit and vegetable from the past decades. The fruits and seeds of this plant are helpful in the control of aging diseases like diabetes and cancer. One of the prerequisites in the primary health care is the availability in the use of sustainable drugs from plants that have a common source of medicaments. The plants form traditional preparations are pure, active

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principles from extracts that have actions or uses in therapy [5]. Traditional plants medicines are used throughout the world that clean only depicts that are important in health and economy [6].

The inhibition of proteinase by protease inhibitor is an example of protein-protein interaction, where the special features of tie binding of two partners form complex structures [7]. A characterization of *Helicover paarmigera* contains proteinases and the interaction with proteinase inhibitors using x-ray film contact print technique has been experimented basis on basic method [8].

Enzyme inhibitors combine with enzymes to covalently make them inactivate irreversibly. The irreversible inhibitors are toxic substances and they may be natural or synthetic. Serine proteases like trypsin can be assayed for its activity in the presence of appropriate irreversible inhibitors to study their inhibitory effects of an enzyme activity.

Enzyme activity is influenced by metal ions. The activity depends on the nature of various amino acid residues present at the active site of the enzyme. The effect of several metal ions can be studied by examining the enzyme activity in the presence of metal containing salts.

Plants have well-established class of inhibitors called Serine protease inhibitors that show resistance against microorganisms. Protease inhibitors will able to disruption cell damage and enhance the cell's life-span [9]. Plants produce various peptides and proteins that are extracted and purified which act as antimicrobials [10]. A novel method can be used for development of novel antimicrobial agents. Homologous inhibitor show molecular modelling and dynamics studies that are measured with *Escherichia coli* trypsin and chymotrypsin proteins. The interactions are provided based on inhibitor–enzyme docking studies [11].

Protease inhibitors ALLN and ALLM are of calpain and cathepsin proteases inhibitor that shows antiproliferative activity [12]. The action of Bowman-Birk protease inhibitor from leguminous family was shown antitumor and antiproliferative properties [13]. Aprotinin, pepstatin, and soybean trypsin inhibitor exhibit anticancer properties [14]. *L. acutangula* (var) Amara annual herb is belonging to the family, Cucurbitaceae contains ribosome-inactivating proteins, monocotyledon mannose-binding lectins, amaranthins and Cucurbitaceae phloem lectins act as antiproliferative activity [15]. Calf serum (FCS), Dulbecco's Modified Eagle medium (DMEM), paclitaxel (PTX), EDTA, trypsin, penicillin, amphotericin exhibit a powerful antiproliferation [16]. Protein inhibitors of trypsin from the seeds of cucurbitaceae plants have shown a good antiproliferative activity [17].

MATERIALS AND METHODS

Collection of *C. Lanatus* fruits

In the present experiment, *C. lanatus* fruits were collected from the fields of Visakhapatnam district, AP, India during March to June 2011. The plants are authenticated by Dr. P.V. Arjun Rao, Ethanobotanist, Dept. of Botany, Phyto-pharma Technology Laboratory, Visakhapatnam (No. Res/2 dated 21-09-2010). *C. lanatus*., on comparison with the details given in "FLORA OF THE PRESIDENCY OF MADRAS" by J.S. Gamble, Volume i, Page no. 534-536, Bishen Singh Mahendra Pal Singh publishers, India (2004) and "Flowering plants from Chittoor district, Andhra Pradesh, India" by K. Madhava Chetty, K. Sivaji and K. TulasiRao, First edition published by students offset printers, Thirupathi, India, pp. 137-39 (2008).

Preparation of Crude Extract

The seeds present in the fruits are collected and dried for two days. The testa is separated and crushed to a fine powder using motar and pestle. The fine testa powder is selected for the present experimentation.

The testa powder was depigmented, dehydrated, and defatted by washing with acetone for several times, followed by hexane and Folch's mixture (chloroform: methanol, 2:1) and with 1% PVP. The solvents were

removed by filtration and the powder air-dried. Testa powder was homogenized in 100 ml of 0.1M phosphate buffer pH 7.0 and the extract was prepared in 500 ml conical flask. The homogenate was mixed by incubating the extract in a rotary shaker at 120 rpm for 30 minutes at room temperature. Then the cell debris was removed by the slurry filtered through cheesecloth. The filtrate was collected and centrifuged at 10,000 rpm for 15 minutes at 4°C [18] . The crude extract appears as a clear supernatant was selected for further precipitation of inhibitor by Ammonium sulphate precipitation.

Ammonium sulphate precipitation and dialysis

Citrullus lanatus testa protein was precipitated by the Ammonium sulfate precipitation method. The protein isolation was done based on the method done by Englard and Seifter, 1990 [19] . The unwanted proteins are removed by ammonium sulphate precipitation and at the same time the protein of interest could be concentrated. Varying concentrations of ammonium sulphate (30%, 50%, 70% and 90%) to the crude extract was kept at 4˚C for about one day precipitation to optimize the selected protease inhibitor. The precipitated protease inhibitor was collected by centrifugation of extract at 10,000 rpm at 4˚C for 15 minutes. The precipitated protein was further dialyzed against 0.01M phosphate buffer (pH 7.0) to remove the ammonium sulphate present in the precipitate as details given below.

The dialysis tube (Sigma-Aldrich) was washed in running water for about 3-4 hrs. The tube was rinsed with the 0.3% *(w/v)* solution sodium sulfide at 80°C for 1 minute. After washing with hot water (60°C) for 2 minutes, the solution was acidified with 0.2% sulphuric acid (v */v)* and rinsed with hot water (60°C). The process is done to build the pores of the tube more clear. Tube will be opened, then pack the sample solution and this packed solution was keep in 0.01M phosphate buffer. This method has helped to removal of salts in the sample solution. Finally the protein was lyophilized and subjected to various analytical techniques and also used in the further purification.

Purification of Trypsin Inhibitor

Ion Exchange Chromatography

The active protease inhibitor fraction that was attained after the process of dialysis by ammonium sulphate precipitation was purified by Ion exchange chromatography using an anion exchanger called DEAE cellulose. Proteins obtained due to surface charge will bind to ion exchangers. The reversibly adsorbed proteins were eluted out by using either through a salt gradient or pH.

Activation of DEAE Cellulose

The DEAE cellulose 10g was soaked in double distilled water, allowed to settle, and the fine particle was removed by decanting. It was then suspended in 0.1M HCl for overnight. Remove the hydrochloric acid and wash with double distilled water, then add 0.1M NaOH incubate for overnight. Decanted the sodium hydroxide solution and washed several times with distilled water in a sintered glass funnel using vacuum filtration, until the pH of the washings became neutral. Equilibration of the resin in appropriate buffer by repeated washings with the same buffer was conducted.

Purification Using DEAE Cellulose Column

DEAE cellulose activated as carefully packed up (1.5X30cm) column and was equilibrated with phosphate buffer pH 7.0. A protein content of 4.1 mg/ml from 30 ml of the dialyzed sample was used in the pre-equilibrated DEAE cellulose column. The complete addition of sample into pre-equilibrated DEAE cellulose column was connected to the reservoir that contains the phosphate buffer that adjusted with a flow rate of 2 ml per minute.

The unbound proteins obtained were washed out until absorbance reached near to zero at 280 nm. An elution was completed at a flow rate of 2ml per minute using gradients stepwise with sodium chloride that ranges from 0.1 to 0.5M that was prepared in 0.01M phosphate buffer at 7.0 pH. About 2 ml of fractions from columns were collected and

the protein content of each fraction was being estimated by measuring the OD at 280 nm. The peak fractions from the column were then pooled and were again dialyzed against the 0.01M phosphate buffer at pH 7.0. The dialyzed fractions from testa of *C. lanatus* were assayed for protease inhibitory activity, protein content and specific activity.

Gel Filtration on Sephadex G -50

Sephadex G-50 activated as carefully packed up (1.5X50 cm) column without any air bubble and the column was equilibrated with 0.1M phosphate buffer pH 7.0. The samples were dissolved in 0.1M phosphate buffer pH 7.0, that samples were loaded on sephadex G-50 column. The column was previously equilibrated with 0.1M phosphate buffer pH7.0 and the sample was eluted in the same buffer with a flow rate of 2 ml/minute, 2 ml fractions were collected at a flow rate of 120ml per hour and protein absorbance was measured at 280 nm.

Calculation of yield of protein or protease inhibitor activity of each fraction during purification is the percent activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract. Fold of purification in each step was calculated by dividing the specific activity of the respective fraction with that of the crude extract.

$$\text{Yield of protein} = \frac{\text{Total protein content of the purified fraction}}{\text{Total protein content of the crude extract}} \times 100$$

$$\text{Fold of purification} = \frac{\text{Specific activity of the purified fraction}}{\text{Specific activity of the crude extract}} \times 100$$

$$\text{Yield of activity} = \frac{\text{Total activiaty of the purified fraction}}{\text{Total activity of the crude extract}} \times 100$$

The purified inhibitor is further purified by SDS PAGE and analysed by MASCOT.

Antifungal Activity

The antifungal activity was conducted based on zone method.

Microorganisms

Microbes from ATCC (American Type Culture Collection), USA have been used in the present study. The fungi used in the present experimentation are *Aspergillus niger* (ATCC *6275) and Candida albicans* (ATCC 10231).

Antifungal Activity Using Zone Method

Antifungal tests were carried out by agar well diffusion method [20]. About 8 mm wells on inoculated Sabouraud dextrose agar plates were filled with 10, 25, 50, 100 µg/ml of crude and Purified proteins respectively that is made in 10% DMSO. The Flucanozole used (10 µg/ml) as positive reference standard. The agar plates were incubated for 48 hours at 25˚ C. The antifungal activity was assessing quantitatively by the absence or presence of inhibition zones and zone diameters.

Antiproliferative Activity

As the crude and isolated protein extracts has shown efficient antifungal activities, a preliminary investigation has been made for finding antiproliferative effects of crude and isolated samples from testa of *C. lanatus* on MCF-7 (Breast Cancer) and Hep–G2 (Liver Cancer) cell lines. The cell lines were procured from National Centre for Cell Science, Pune. The whole cells have been grown in Minimal essential medium (MEM, GIBCO) was being supplemented with 4.5 g/L glucose, 5% fetal bovine serum (FBS) (growth medium) and 2 mM L-glutamine and at 37°C in 5% CO_2 incubator.

By using MTT assay, can determine the inhibitory effects of sample compounds on cell growth *in vitro*, these assay developed by Mosmann and was modified has been used. The T-25 flask is a 96-well flat-bottomed tissue culture plate. In the culture plate each well seeded with trypsinized cells. Each well density of cell culture was maintained at $5x10^3$ cells/ well in the growth medium and cultured at 37°C in 5% CO_2 to adhere. Culture cells were incubated upto 48 hrs, after the Incubation, the supernatant was discarded.

The cells were mixed with various concentrations of sample compounds (6.25, 12.5, 25, 50, 100 and 200 µg/ml) used to bring about a final volume of 100 µl, before that cells were pretreated with growth medium and then cell were cultured for 48 hours. The sample compound was equipped as 1.0 mg/ml stock solutions in PBS. In this assay used controls are Solvent and culture medium. Each well contains 5 µl of fresh MTT, add about 0.5mg/ml in PBS and then incubate for 2 hoursr at 37°C. The supernatant contains growth medium, supernatant removed from the wells and for solubilize the colored formazan product added 100 µl of DMSO. Incubate upto 30 min, by using ELISA reader (Anthos 2020 spectrophotometer) colored culture product absorbance read (OD) at wavelength of 572 nm.

RESULTS AND DISCUSSION

The peptide sequenced in MALDI was selected for in vitro analysis. The sequence of the peptide sequence shown by the Mascot report was shown below:

>gi|296399226|gb|ADH10401.1| photosystem I subunit IX [Selaginella moellendorffii]

MQDVKTYPPAAPVPATPRFGSLAGSLIEINRLSPDAP VSPPA

The plant extract and purified C. lanatus Trypsin Inhibitor (CLTI) peptide was analyzed for antifungal activity against various test microorganisms. All the prepared extracts were shown good antifungal activity. The antifungal activity results are represented in Table 1 and 2.

The *C. lanatus* testa isolated extracts was revealed good antifungal activity against *A. niger* (21 mm) and *C. albicans* (20 mm). The *C. lanatus* testa crude extracts was revealed good antifungal activity against *A. niger* (18 mm) and *C. albicans* (13 mm). Fluconazole has used for fungal standard shown inhibition zone for *A. niger* (14 mm) and *C. albicans* (20 mm) (Table 1 and 2). The protein and *C. lanatus* Trypsin Inhibitor (CLTI) extracts from testa at 100 µg/ml was shown good antifungal activities compared to standard antibiotic.

Table 1: Antifungal activity of Crude protein extract from Test of *C. lanatus*

Fungi	Zone of Inhibition in mm					
	Crude protein extract				DMSO (Control)	Fluconazole (Standard)
	10µg/ml	25µg/ml	50µg/ml	100µg/ml		
Candida albicans	–	–	10	13	–	20
Aspergillus niger	11	12	14	18	–	14

The potential and active compounds to develop the antimicrobial compounds from medicinal plants appeared worthy which leads to the improvement of phytomedicine used against microbes. Due to fewer side effects that are frequently associated with synthetic antimicrobials, the Plant-based-antimicrobials have huge therapeutic potential [21].

Table 2: Antifungal activity of Trypsin inhibitor from *C. lanatus* testa purified

Fungi	Zone of Inhibition in mm					
	C. lanatus purified testa extract				DMSO (Control)	Fluconazole (Standard)
	10µg/ml	25µg/ml	50µg/ml	100µg/ml		
Candida albicans	–	–	18	20	–	20
Aspergillus niger	–	12	20	21	–	14

The *C. lanatus* testa crude and Trypsin inhibitor has also shown good results for anti-proliferative activity.

Table 3 is shows the dose response of *C. lanatus* testa crude, Trypsin inhibitor and standard (Tamoxifen) against MCF-7 (Breast Cancer) cell line. The results were shown good cancer inhibition due to gradual decrease in the percentage of cell survival. Fig. 1 is shows Standard IC_{50} was shown as12 µg/ml. The IC_{50} of CLTI was shown as 60 µg/ml and crude as 190 µg/ml. Fig. 2 shows antiproliferative effects of *C. lanatus* testa isolated extract on MCF-7 (Breast Cancer) cell line.

Table 3: Dose Response of *C. lanatus* testa crude and Trypsin inhibitor MCF-7 (Breast Cancer) cell line

Conc. (in µg /ml)	Tamoxifen		Crude extract		Isolated extract	
	Cell survival (%)	Cell inhibition (%)	Cell survival (%)	Cell inhibition (%)	Cell survival (%)	Cel inhibition (%)
6.25	82.3	17.7	97.8	2.2	93.5	6.5
12.5	45.5	54.5	84.1	15.9	89.5	10.5
50	30.9	69.1	76.7	23.3	56.4	43.6
100	16	84	58.5	41.5	32.3	67.7
200	4.9	95.1	49	51	21.2	78.8
250	0.4	99.6	30	70	13.4	86.6

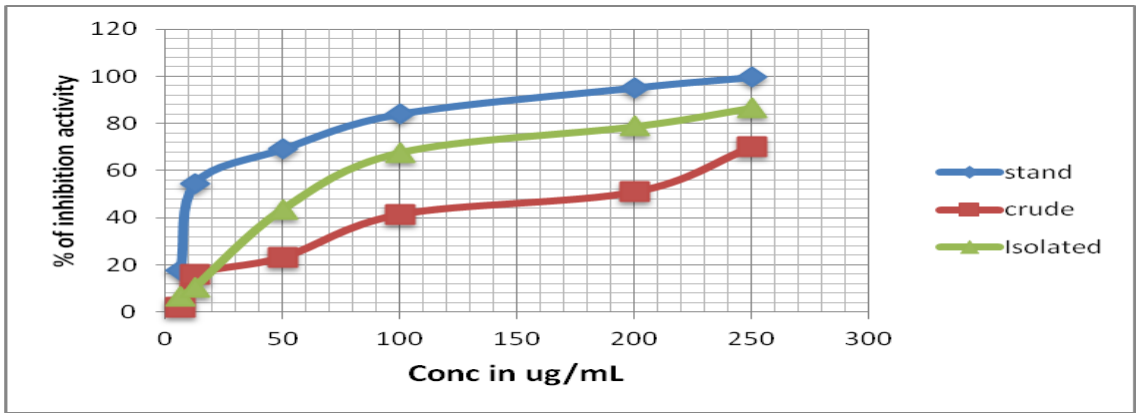

Fig. 1: Graphical representation for Antiproliferative activity of *C. lanatus* testa crude and Trypsin inhibitor on MCF-7 (Breast cancer) cell line

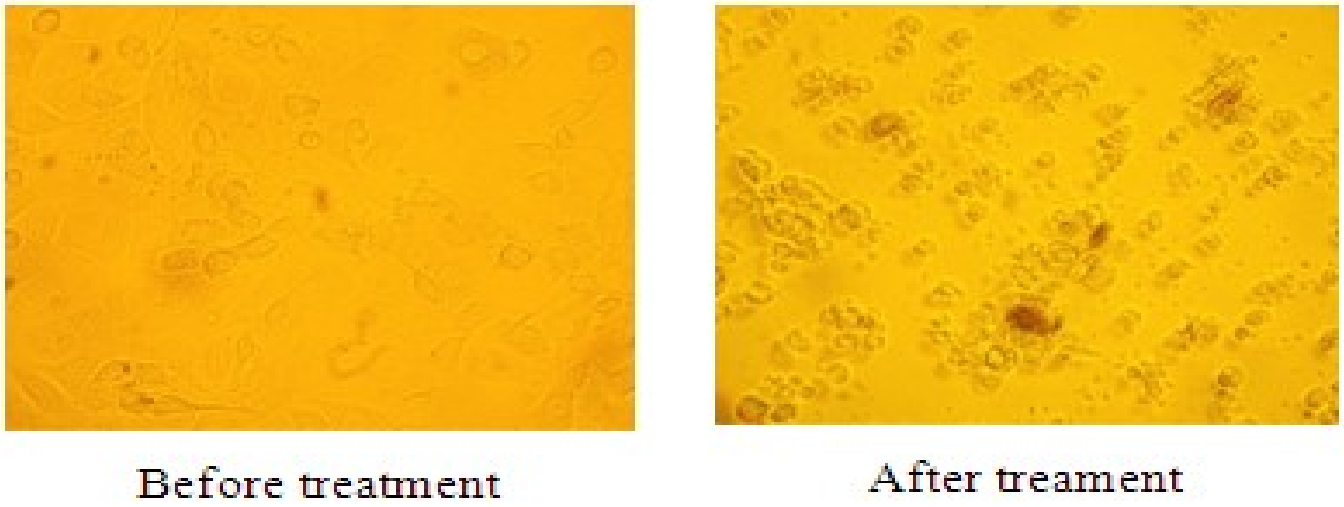

Fig. 2: Antiproliferative activity of Trypsin inhibitor from *C. lanatus* testa on MCF-7 (Breast Cancer) cell line before and after treatment

Table 4 was shown the dose response of *C. lanatus* testa crude, Trypsin inhibitor and standard (Tamoxifen) against Hep –G2 (Liver Cancer) Cell line. The results were shown good cancer inhibition due to gradual decrease in the percentage of cell survival. Fig. 3 shows Standard IC_{50} as 11 µg/ml, the IC_{50} of CLTI peptide as 41 µg/ml and crude as 144 µg/ml. Fig. 4 shows antiproliferative effects of *C. lanatus* testa isolated extract on Hep –G2 (Liver Cancer) Cell line.

Table 4: Dose Response of *C. lanatus* testa crude and Trypsin inhibitor on Hep –G2 (Liver Cancer) Cell line

Conc. (in µg /ml)	Tamoxifen		Crude extract		Isolated extract	
	Cell survival (%)	Cell inhibition (%)	Cell survival (%)	Cell inhibition (%)	Cell survival (%)	Cell inhibition (%)
6.25	82.3	17.7	94	6	89.7	10.3
12.5	45.5	54.5	87.9	12.1	65.8	34.2
50	37.4	62.6	70.1	29.9	45.5	54.5
100	29.2	70.8	58.4	41.6	38.6	61.4
200	21	79	39.7	60.3	31.5	68.5
250	11.2	88.8	22.2	77.8	13.6	86.4

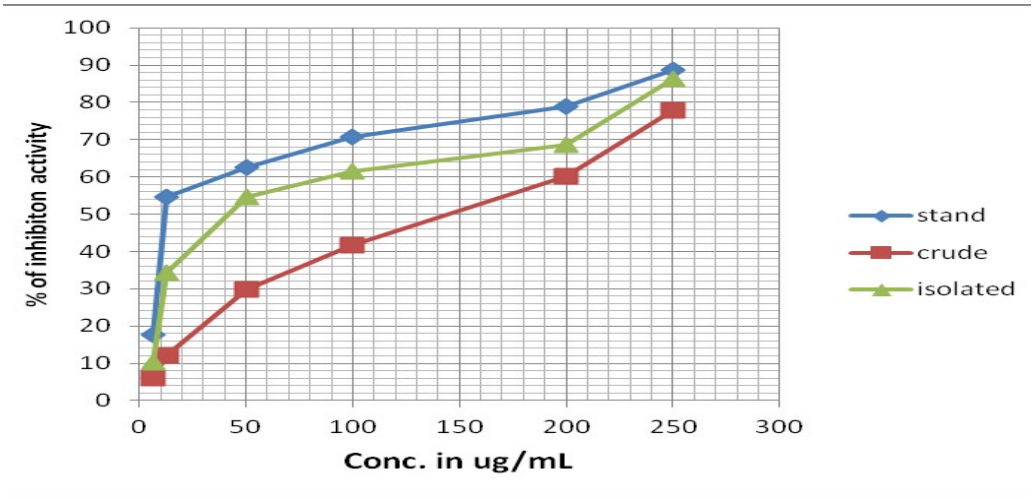

Fig. 3: Graphical representation of antiproliferative activity of *C. lanatus testa* crude and Trypsin inhibitor on Hep –G2 cell line

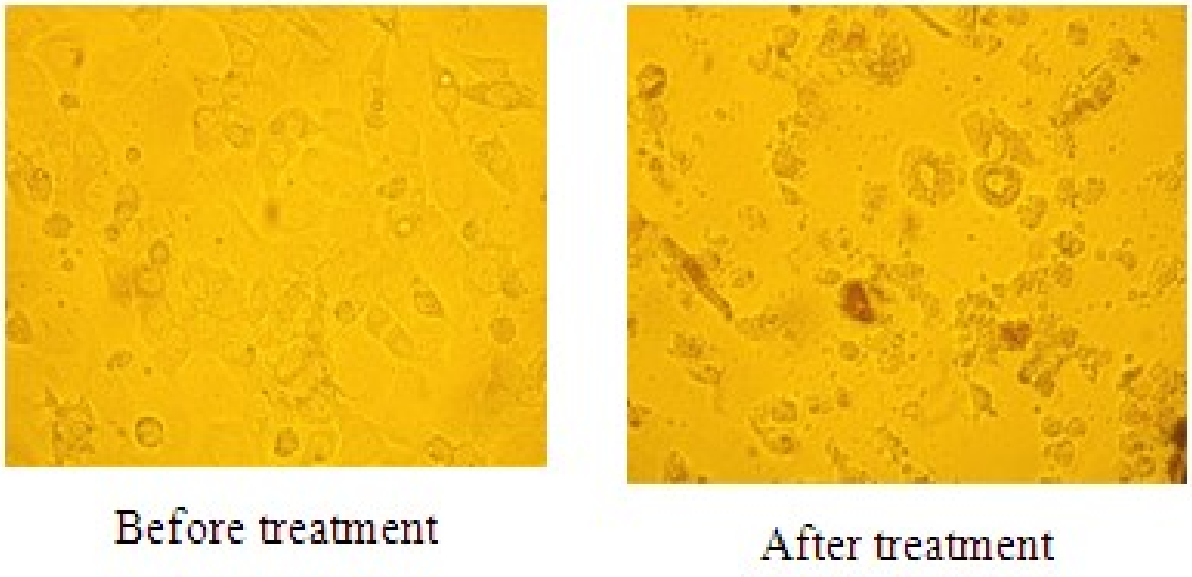

Fig. 4: Antiproliferative activity of Trypsin inhibitor from C. *lanatus* testa on Hep –G2 cell line

Gram-positive and Gram-negative bacteria were capable to bind with rMjSerp1 through the reaction estimated by the microorganism binding assay. The rMjSerp1 acts as a microbial serine protease inhibitor such as subtilisin A and proteinase K [22]. Various external secretions consists serine proteases that are inhibited by endogenous inhibitors like Antileukoprotease (ALP), or secretory leukocyte proteinase inhibitor. Antileukoprotease (ALP) comprises two homologous domains, one of the domains contain proteinase inhibitory activities that are located in the COOH-terminal domain, and the NH2-terminal domain function is unknown. The *E. coli* or *S. aureus* was incubated with intact ALP or its isolated first domain and resulted in killing of these bacteria [23]. Antimicrobial peptides (AMPs) have central role in infection and inflammation [24]. The protozoan parasite *Toxoplasma gondii* asexual development were affected by serine protease inhibitors like 3,4-dichloroisocoumarin and 4-(2-aminoethyl)-benzenesulfonyl fluoride and these were prevented invasion of the host cells [25].

Plants protease inhibitors can potently inhibited the growth of bacterial and fungal pathogenic strains. PIs are excellent agents for development of novel antimicrobial agents [26]. Potato tuber has antifungal protein (AFP-J), AFP-J was purified and strongly inhibited yeast fungal strains like *Candida albicans*, *Trichosporon beigelii* and *Saccharomyces cerevisiae*, AFP-J could not inhibited the crop fungal pathogens [27]. The *Momordica cochinchinensis* (MCo) squash seeds consist three trypsin inhibitors (TIs), these inhibitors have been isolated and purified using gel filtration and used as anti fungal agents [28].

CONCLUSIONS

The present experimentation was shown good PIs of *C. lanatus* testa having antifungal and antiproliferative activities. Hence Trypsin inhibitor from testa of *Citrullus lanatus* has good biological activities.

CONFLICT OF INTERESTS

The author declares that there is no conflict of interests regarding the publication of this paper.

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