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RESEARCH ARTICLE

Purification and characterization of a novel anticancer peptide derived from cicer arietinum.

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Abstract

The aim of the study was to identify the novel peptide from Cicer arietinum seeds and investigate its anticancer activities. The Chymotrypsin was applied for enzymatic hydrolysis. Chymotrypsin hydrolyzates, further purified using a Fast-performance liquid chromatography (FPLC) method, which showed cytotoxicity activity on HeLa cancer cells. Finally, a novel anticancer peptide was purified. The peptide from cicer arietinum effectively induced apoptosis on HeLa cancer cells.

INTRODUCTION

The cicer arietinum derived peptide is novel cancer chemopreventive agent. The effects of peptides on the proliferation of HeLa cancer cells were investigated in vitro. These findings suggest cicer arietinum peptides possess anticancer properties and deserve further study as possible chemo preventive agents.

Research has focused on the bioactive peptides which are encrypted within food proteins, utilizing such peptides as functional food ingredients aimed at health maintenance [1]. These functional properties of proteins can be improved by specific enzymatic hydrolysis under controlled conditions [2] The small peptides containing the motif Glu-Asp-Ser which were potential Ras-Raf interaction inhibitors in 1995, these peptides are utilized for a potential or an established use in cancer therapy [3,4]. Anticancer peptides induce cell death with different mechanisms, including apoptosis, affecting the tubulin-microtubule equilibrium, or inhibiting angiogenesis. This finding has increased our knowledge about new potent cytotoxic, and many other properties with novel chemical structures associated to original mechanisms of pharmacological activity. These facts also introduce peptides as a new choice for obtaining lead compounds on biomedical research [5]. Apoptosis as a form of programmed cell death is one of the major mechanisms of cell death in response to cancer therapies [6]. Also, apoptosis is a naturally occurring and evolutionarily conserved pro-cess by which cells that are no longer useful are directed to their deaths [7]. Its deregulation, i.e., either loss of proapoptotic signals or gain of anti-apoptotic signals, can lead to a variety of pathological conditions such as cancer initiation, promotion and progression or result in treatment failures [8, 9]. As apoptosis does not usually trigger inflammatory or immune response, it becomes a prefer-able way of cancer cell death during cancer treatments. As such, modulation of apoptotic pathways and selective induction of apoptosis by chemical agents are likely to be a promising approach for cancer therapy [10–14]. Therefore, developing anticancer peptides that target these molecules has become an important strategy for anticancer therapies.

2.1. Materials

Cicer arietinum seeds was obtained from a Tamil Nadu Agriculture University –Tamil Nadu Coimbatore. Penicillin-streptomycin, Tween-20, chymotrypsin, from Sigma Aldrich Mumbai. MEM was purchased from Hi Media Laboratories, Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai), *HeLa* cell line was obtained from National centre for cell sciences Pune (NCCS).

2.2. Preparation of enzymatic hydrolyzates from Cicer arietinum

Cicer arietinum seeds was lyophilized and pulverized into a powder using a grinder and the enzymatic hydrolyzates were obtained according to the method described by Park et al. [16]. The optimum pH, temperature and characterization of an enzyme are maintained . Buffer solution 100ml were added to 2 g of the dried sample, and then 25 ul (or mg) of enzyme was added after pre-incubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 h to achieve an optimum hydrolytic level, and followed by immediate heating at 100 ° C for 10 min to inactivate the enzyme. Finally, the enzymatic hydrolyzates were rapidly cooled to 20–25 ° C in an ice bath, centrifuged, filtered by a filter paper (Grade 41, Whatman), lyophilized, and stored at −20 ° C until use.

2.3. Purification of anticancer peptides from Cicer arietinum

2.3.1. Fast-performance liquid chromatography (FPLC)

The fraction exhibiting the highest anticancer activity was obtained using - **FPLC** on a C_{10} column (AKTA laboratory scale chromatography system – GE Health care) with a linear gradient of acetonitrile (0–70%) at a flow rate of 2.0 ml/min. The elution peaks were detected at 215 nm (graph-1), concentrated using a rotary evaporator, and lyophilized for 3 days. Five fraction with different mol wt was obtained from the crude extract of Cicer arietinum .Among the five fraction , fifth fraction showing highest anticancer activity on HeLa cells.

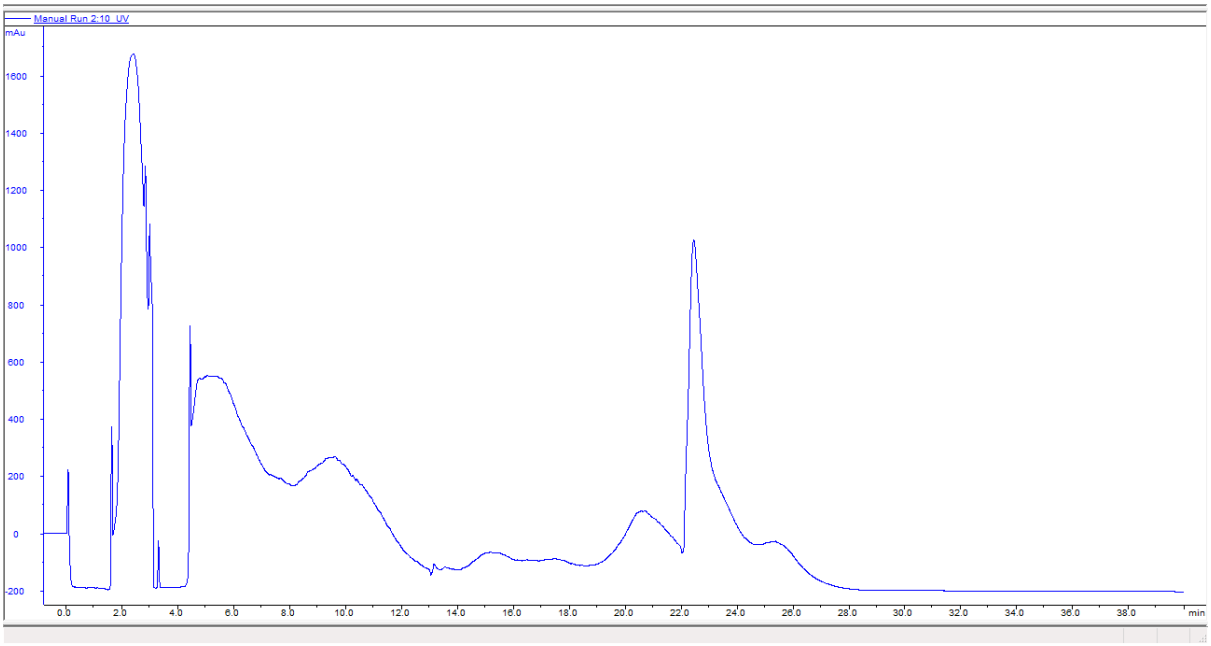
2.4 **Cell line and culture:**
HeLa cell line was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO_2 at 37 °C.

***In Vitro* assay for Anticancer activity (MTT assay) (Mosmann, 1983)**
Cells (1×10^5/well) were plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100μl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank (graph-2). Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

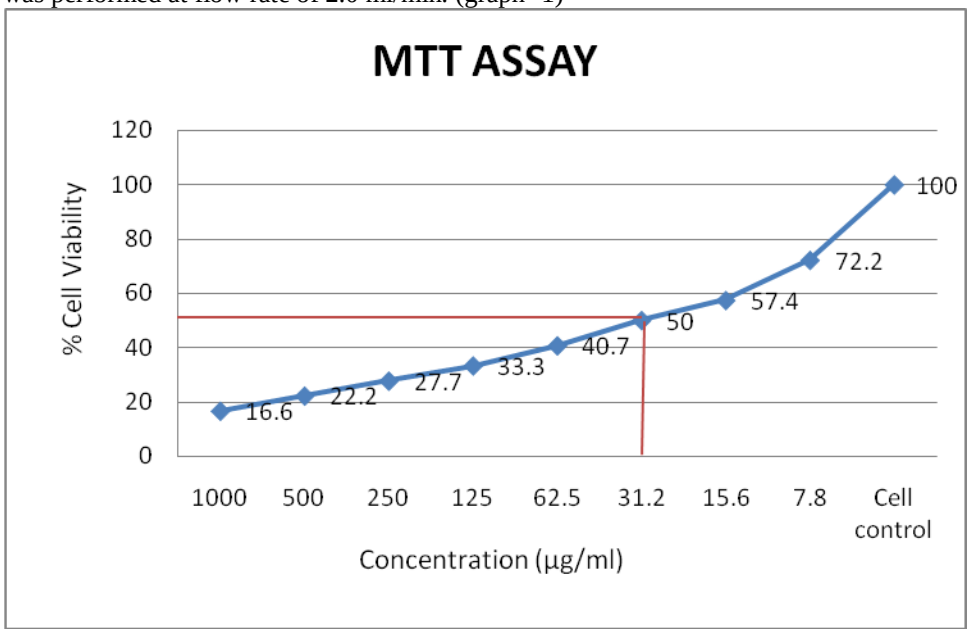
% cell viability = A570 of treated cells / A570 of control cells × 100

3. Results

The chymotrypsin, which preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond is a tyrosine, tryptophan or phenylalanine [18], hydrolyzates from Cicer arietinum showed the highest anticancer activity, resulting in apoptosis rates in HeLa cancer cells with 50% lethal concentra-tions (LC_{50}) values of 31.2 ug/ml (Table-1). Therefore, chymotrypsin hydrolyzates were selected for further study.



Ion-exchange chromatogram by AKTA laboratory scale chromatography system – GE Health care . The elution was performed at flow rate of 2.0 ml/min. (graph -1)



Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.(graph-2)

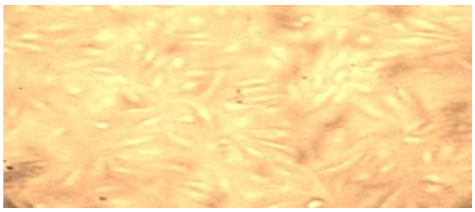
Anticancer effect of Purified Peptide from cicer arietinum Seed extract on *HeLa cell* line (Table-1)

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.09	16.6
2	500	1:1	0.12	22.2
3	250	1:2	0.15	27.7
4	125	1:4	0.18	33.3

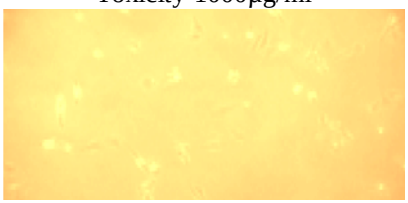
5	**62.5**	**1:8**	**0.22**	**40.7**
6	**31.2**	**1:16**	**0.27**	**50.0**
7	**15.6**	**1:32**	**0.31**	**57.4**
8	**7.8**	**1:64**	**0.39**	**72.2**
9	**Cell control**	**-**	**0.54**	**100**

Anticancer effect of chymotrypsin hydrolyzate Purified Peptide from Cicer arietinum extract on *HeLa cell* line (Fig-1**)**

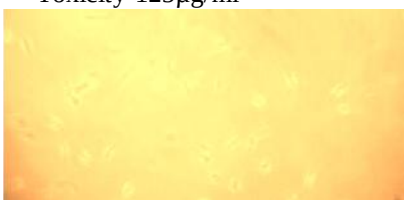
Normal HeLa Cell line



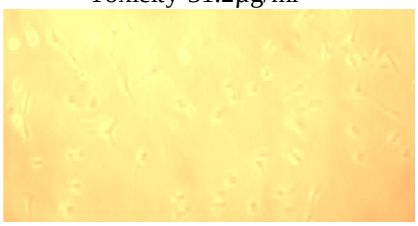
Toxicity-1000µg/ml



Toxicity-125µg/ml



Toxicity-31.2µg/ml



Toxicity-15.6µg/ml

4. Discussion

Biologically active peptides obtained from Cicer arietinum seed are considered to have diverse activities, including immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, and antihypertensive actions. Growing evidence shows that most anticancer peptides with cytotoxicity may trigger apoptosis by targeting many cellular proteins, and the induced apoptotic process involves both intracellular and extracellular pathways [19].

5. Conclusions

In the present study, and we evaluated their anticancer activity from Cicer arietinum as determined by their toxicity capacity. The resulting anticancer peptides were purified using consecutive chromatographic methods. Finally, we obtained an anticancer peptide, In addition, results showed that the novel anticancer peptide efficiently induced cytotoxicity to HeLa cancer cells (Fig-1). However, studies on the structure, amino acid sequence of the and in vivo studies of these bioactive peptides activities need to be further investigated for amino acid sequencing of anticancer peptide .

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