



ISOLATION AND PAPAINE DIGESTION OF NOVEL ANTIFUNGAL PEPTIDES FROM RED RADISH (*RAPHANUS RAPHANISTRUM SUBSP SATIVUS*) AND ANALYSIS OF SELECTIVE CYTOTOXICITY FOR CANCER TREATMENT

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ABSTRACT

Treating cancer with chemotherapy is accompanied with side effects and drug resistance by cancer cells which call for alternative anticancer agents such as cationic antimicrobial peptides. In specific, antifungal peptides were chosen for cytotoxicity analysis due to their deteriorating effects on fungi (eukaryotic cells). Novel peptides were isolated and analysed from red radish seeds. Water soaked seeds were ground with phosphate buffer (pH 7) and the antifungal assay was carried out and an inhibition zone of 0.5 ± 0.2 mm was noticed for 50 μ l of the extract at 1 mg/ml concentration. The study was extended further, by digestion with papain, trypsin and pepsin with appropriate buffer and pH of which papain digests of red radish seeds produced a zone of inhibition of 1.00 ± 0.2 mm at 500 μ g/ml concentration, approximately. This sample was processed with a 3 kDa cut off membrane and the filtrate was confirmed for its antifungal activity (MIC 32 μ g/ml). Further, for size confirmation, the sample was subjected to MALDI- TOF-MS analysis, and a peak was obtained at 1680 Da (1.68 kDa). To check the anticancer activity of the antifungal peptides obtained, the sample was tested on HeLa cell lines by method of MTT Assay and significant cell death was obtained at MIC 12.5 μ g/ml which increased with higher concentrations of the sample. Thus, it is confirmed that red radish seed extract digested with papain is effective in inhibiting the growth of both fungal and cancer cell lines making them a good therapeutic agent against cancer.

Keywords: Papain digest, Red radish, MALDI TOF MS, HeLa cell lines, Anticancer, Antifungal peptides

INTRODUCTION

Cancer is described as a group of diseases involving abnormal cell growth which as tumours, colonize the surrounding areas and begin to invade other parts of the body (World Health Organization, 2017; National Cancer Institute, 2017). The severity depends on where and how intense the cancer cells develop and proliferate. Currently, there are a multitude of cancer treatment processes. Many dietary requirements are considered to prevent cancer, but the evidence provided is not very definitive (Kushi et al., 2012; Wicki et al., 2011). Under controlled conditions, it is possible to prevent and treat cancer with drugs such as NSAIDs, COX₂ inhibitors etc. but their administration could cause harm to the body (Rostom et al., 2007; Rothwell et al., 2011; Cooper et al., 2010; Thomsen et al., 2008). When it comes to the treatment of cancer, surgery and radiation therapy is opted, which is proven to be effective. But, in the initial stages, the usual treatment of choice is considered to be chemotherapy (Espinosa et al., 2003). This, as known, is accompanied by various deleterious side effects (Cassidy et al., 2002; Kalyanaraman et al., 2002). Also, the cells become resistant to chemotherapeutic agents by altering their mechanisms such as increasing their ability to repair DNA damage etc., especially; slowly proliferating cancerous cells do not get affected by drugs that act on their DNA (Naumov et al., 2003; Gatti et al., 2005). Thus, even while drugs are available, there is a great interest developing for new anticancer agents such as antimicrobial peptides (Mathew et al., 2012) which do not have side effects on the body (Jamie et al., 2006; Reddy et al., 2004). Antimicrobial peptides (AMPs) are host defense peptides that are present in all living organisms ranging from single celled to multicellular organisms. They kill many strains of bacteria, fungi and other microorganisms and also cancerous /abnormal cells (cytotoxicity). Plant antimicrobial peptides (2-9 kDa, 10-50 AA) are promising antibiotic compounds (David et al., 1778; Robert et al., 2014). Of the various plant AMPs, defensins inhibit the growth of a wide range of fungi and in a lesser extent are toxic to mammalian cells or plants (Nicole et al., 2013). One important plant defensin is the one obtained from Radish seeds namely Rs AFP1 and Rs AFP2 and of the two classes of proteins obtained, proteins from the first class have shown sequence homology to pea pod proteins containing antifungal

activity and the second class of proteins (characterized 2s seed storage albumins) have also exhibited potential antifungal activity (Patricia et al., 2008; Terras et al., 1992; Thevissen et al., 2004; Vriens et al., 2014). Considering their mode of action on fungi which could be further extended to other eukaryotic cells, this study is aimed at analysing the anticancer activity of antifungal peptides obtained from red radish seeds, hence categorising them as potential anticancer agents.

MATERIALS AND METHODS

Collection and Germination of seeds (Hexiang Wang et al., 2001; Rupert W. Osborn et al., 1995)

Seeds of *Raphanus raphanistrum subsp. sativus* – of both red and white varieties and *capsicum annum* were collected, washed and soaked in double distilled water twice with an interval of 2-4 hours (done to remove the soil particles and pesticides present on them). These were allowed to germinate by roll towel method. The seeds were incubated at room temperature for 12 hours. The beginning phase of germination was chosen for preparing the extract rather than the dried seeds/completely germinated seeds because it is during the growth of roots from the seeds that several proteins involved in protection of the seed and its development gets activated by water and comes out.

Preparation of Extract

The slightly germinated seeds of *R. sativus* and the chilli flakes were ground in a mortal pestle, separately with cold phosphate buffer of pH 8.0 at 0 °C. The concentration of the extract prepared was 1 g/ml. The extract obtained was centrifuged at 10000 rpm for 10 minutes and the supernatant obtained was discarded. The pellet contains all the cellular proteins. The pellet obtained was stored in Eppendorf tubes at 4 °C.

Crude Protein Assay (Hexiang Wang et al., 2001; Rupert W. Osborn et al., 1995)

The antifungal activity of red radish, white radish and chilly flake extract was checked on the organism *Aspergillus fumigators* (test organism chosen) which were streaked on Potato Dextrose Agar medium plates. Different concentrations of the crude protein ranging 25-1000 µg/ml were tested for antifungal activity with Clotrimazole used as control. The inoculated plates were incubated at 37 °C for 24 hours and examined for zone of inhibition.

Enzyme digestion (Lijun You et al., 2011)

Protein digestion with three different enzymes was carried out for the red radish extract viz., Pepsin, papain and trypsin used as digestive enzyme.

- 1) Pepsin (pH 8.0; Tris-HCl Buffer) - 5 µg/µl
- 2) Papain (pH 2.0; Acetate Buffer) - 50 µg/µl
- 3) Trypsin (pH 6.8; Phosphate buffer) - 50 µg/µl

The enzyme digestion was done using their respective pH with standard buffers. Three extract samples were taken, to each of which each of the enzyme solution were added and incubated at 37 °C for 18 hours and the enzymes were deactivated by neutralizing the pH of the solution and by boiling it for 20 minutes at 99 °C.

Antifungal assay (Hexiang Wang et al., 2001; Rupert W. Osborn et al., 1995)

The antifungal activity of the protein extract after digestion, containing peptides of our interest was checked on the same test organism on Potato Dextrose Agar (PDA) plates. 100 µl of the digested protein extracted was added to a well created in the plates. The inoculated plates were incubated at 37 °C for 48 hours and examined for zone of inhibition. Based on the results obtained, papain digested samples were added in different concentrations ranging from 50 to 500 µg/ml and the zone of inhibition was recorded.

Protein purification -Ammonium Sulphate Precipitation (McQueen-Mason et al., 1992)

To the digested protein extract of 25 ml that showed high antifungal activity, 4.4 g of ammonium sulphate was added to bring a 30% peptide precipitation. The solution was stored at 4 °C for 6 hours.

After 6 hours incubation, the solution was centrifuged at 10000 rpm for 10 minutes. The pellet containing proteins and peptides of 30% precipitation was stored at 4 °C and the supernatant was subjected to 60% peptide precipitation. The above steps were followed again and finally 85% precipitation was also performed and the protein obtained after each centrifugation was labelled and stored at 4 °C.

Peptide activity assay (Hexiang Wang et al., 2001; Rupert W. Osborn et al., 1995)

Antifungal activity of peptides (100 µl) obtained at various saturation 30%, 60% and 85% was tested on the test organism on PDA plates and examined for zone of inhibition.

3 kDa filtration-dialysis and antimicrobial assay (Hexiang Wang et al., 2001; Rupert W. Osborn et al., 1995)

The precipitate that showed the antifungal activity was dissolved in 5 ml of Phosphate buffer of pH 8.0 and then equal volume of acetone was added to this solution and stored at 4 °C. This extract was thawed and subjected to filtration via 3 kDa cut off membrane (Merck, Germany). The crude sample was centrifuged (12,000 rpm, 10 min) until the peptides got segregated size wise, and the filtered peptides (<3kDa) were checked for antifungal activity in which positive results (zone of inhibition) were obtained.

Quantitative analysis

To check the concentration of peptides in the sample obtained, the peptides were subjected to quantitative analysis. 10 µl of appropriate concentrations of the sample was taken and made up to 1 ml by addition of 990 µl of distilled water. 3ml of Coomassie Brilliant Blue reagent was added to it and vortexed. This was checked for absorbance in UV spectrophotometer at 595 nm. The OD obtained was 0.6 and further calculations showed that the concentration of peptides was 4 µg/ml sample (<3 kDa).

Matrix-assisted Laser Desorption/ionization (MALDI-TOF MS) analysis

The 20 µl of gel eluted sample was used for the MALDI-TOF MS analysis for exact mass determination, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) time-of-flight spectrometer, with a pulsed nitrogen laser (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive high-mass mode. A saturated solution of α-cyano-4-

hydroxycinnamic acid in a 1:1 mixture of acetone and water along with 0.1% trifluoroacetic acid was used for obtaining the mass spectra.

Cytotoxicity activity against on Cancer cells- MTT ((3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay (Farkhondeh Nemati et al., 2013)

Preparation of HeLa cell suspension

A subculture of HeLa in Dulbecco's Modified Eagle's Medium (DMEM) was subjected to trypsinisation (standard ATCC protocol). The culture medium with floating cells was removed. The cell layer was rinsed briefly with with 1 ml of Phosphate Buffer Saline (twice) to remove all traces of serum, which contains trypsin inhibitor. 750µl of Trypsin-EDTA solution was added to the flask and cells were observed under an inverted microscope until cell layer was dispersed (within 3 minutes). 6.0 ml of complete growth medium DMEM (with 11% FBS) was added and cells were aspirated by gentle pipetting.

To remove trypsin-EDTA solution, cell suspension was transferred to the centrifuge tube with the medium and spun at approximately 1100 rpm for 10 minutes. The supernatant was discarded and cells were suspended in fresh growth medium. Appropriate amount of aliquots of cell suspension were added to new culture vessels and incubated in 5% CO₂ at 37°C.

Cytotoxicity assay

800 µl (around 1,20,000 cells) of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentration of tested sample (peptide) (0 to 300 µg/ml) and incubated at 37 °C in a humidified CO₂ incubator with 5% CO₂. After 48 hrs incubation the cells were observed under an inverted tissue culture microscope and then 10 µl MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent was added to the wells and incubated for 3 hr in room temperature until the purple precipitate was visible. All wells were removed off the content using pipette and 100 µl SDS in DMSO were added to dissolve the formazan crystals, absorbance were read in Lark LIPR-9608 micro plate reader at 540 nm.

RESULTS AND DISCUSSION

Table 1 shows the zone of inhibition obtained for different concentrations of the crude protein extract of red radish seeds, confirming their effective antifungal activity. The antifungal activity of various extracts obtained was analysed. Of all those extracts, the crude protein extract of seeds of Red Radish showed significant antifungal activity. Hence, different concentrations (25, 50, 100, 200, 400, 800, 1000 µg/ml) of this extract was tested against the model organism *Aspergillus fumigatus*. The antifungal activities of the extract were insignificant in low concentrations but increased linearly with increase in concentration of extract (µg/ml). In both the trials carried out significant zone of inhibition was noticed from 200 µg/ml concentration of the extract. For 50 µl of the extract at 1 mg/ml concentration, an inhibition zone of 0.5±0.2 mm was noticed. Different studies (Haluk caglar kaymak et al., 2018; Jeries Jadoun et al., 2016) have shown different intensities of activity for the extracts of Red radish seeds and the variability could be attributed to the extraction methods (solvents) employed. As the main aim was to check only the basic antifungal activity (qualitative analysis), primitive extraction method was followed and the activity was confirmed.

Table 1 Antifungal activity for different concentrations of the crude protein (red radish seed extract -trial 1 and trial 2)

Concentration of the crude protein (µg/ml)		Zone of inhibition (mm)	
Trial 1	Trial 2	Trial 1	Trial 2
25	25	negligible	Negligible
50	50	negligible	Negligible
100	100	negligible	Negligible
200	200	0.1±0.05	0.2±0.01
400	400	0.2±0.02	0.2±0.1
800	800	0.3±0.1	0.4±0.1
1000	1000	0.5±0.1	0.5±0.3

Table 2 shows the different zone of inhibitions obtained for the different concentrations of papain digests employed. While using three enzymes digests to check the activity of specific peptides, papain digested extracts showed significant activity. The major antifungal peptides from seeds of red radish had reduced activity (or were completely inhibited) when treated with enzymes (proteases) such as trypsin. Also, due to its broad specificity papain was used as one of the testing enzymes and different concentrations ranging from 50 to 500 µg/ml of this extract

were tested of which, starting from 200 µg/ml the antifungal activity was evident. At, 500 µg/ml concentration a zone of inhibition of 1.00±0.2 mm was obtained. The sample was further subjected to filtration by a 3kDa cut-off membrane and the minimum inhibitory concentration was found to be 12.5 µg/ml

Table 2 Antifungal activity for different concentrations of the papain digested protein (trial 1 and trial 2)

Concentration of the papain digested protein (µg/ml)		Zone of inhibition (mm)	
Trial 1	Trial 2	Trial 1	Trial 2
50	50	negligible	negligible
100	100	0.3±0.1	negligible
200	200	0.5±0.1	0.3±0.1
300	300	0.6±0.05	0.7±0.05
400	400	0.8±0.01	0.8±0.05
500	500	0.9±0.2	1.1±0.1

Figure 1 illustrates the graphical representation of the distribution of peptides with different molecular weights present in the papain digested sample obtained from MALDI-TOF analysis. This analysis was carried out to determine the accurate molecular weight of the peptides responsible for the significant antifungal activity obtained. The intensity was maintained in low values, ranging from 111 AU to 11225AU for 100-1600 m/z ratios, whereas the intensity was found to be to 31055.7AU for the m/z ratio of 1681.857599. Also, the intensity decreased drastically for higher mass to charge ratios. Hence, this result suggests that the size of the peptide of interest corresponds to 1680Da (1.68kDa).

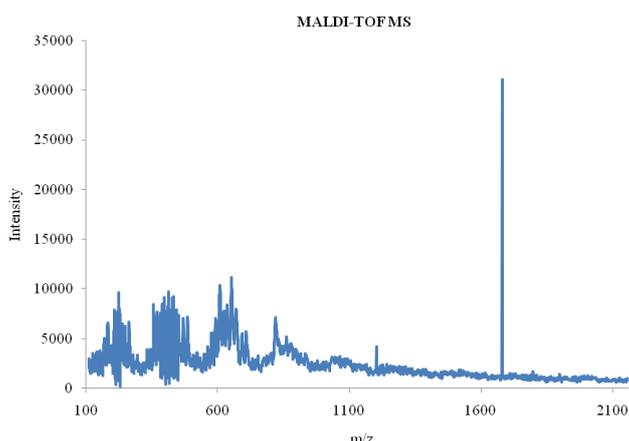


Figure 1 MALDI-TOF MS analysis of SDS-PAGE eluted peptide sample

Figure 2 shows the subculture of HeLa cell lines obtained. The cell lines obtained were cultured in DMEM medium at 37°C in 5% CO₂ and were trypsinised when they were reached 80% confluent. The papain digested peptide extracts were tested for their anticancer activity against HeLa cell lines (Figure 3 and 4). The cytotoxic effects of the peptide sample against cancer cell lines were checked with MTT assay after an incubation time of 48 hours. In Figure 3, the control is shown which contains about 1, 20,000 cells per well. In 12.5 µg/ml concentration of the peptide a 10% decrease in the number of cells was noticed, whereas in 100 µg/ml concentration of the sample the cell viability was around 51.57%. The cell viability corresponding to 75 µg/ml concentration of the sample was 57% which shows almost half the population of cells were destroyed in concentrations ranging from 75-100 µg/ml of the peptide sample. From the graphical data obtained, it was noticed that in low concentrations of the peptide the survival rate of cells was high whereas from 50 µg/ml concentration almost half the number of cells failed to survive. IC₅₀ value for the papain digested peptides was found to be 100 µg/ml and for higher values of peptide concentration, the rate of cell viability gradually decreased (almost linearly). At peptide concentration 300 µg/ml almost 80% of the cells were killed. This proves the effect of antifungal peptides from red radish seeds to be effective in killing cancer cells. This study could be further confirmed by testing their specificity for various cancer cell lines to design appropriate drugs.

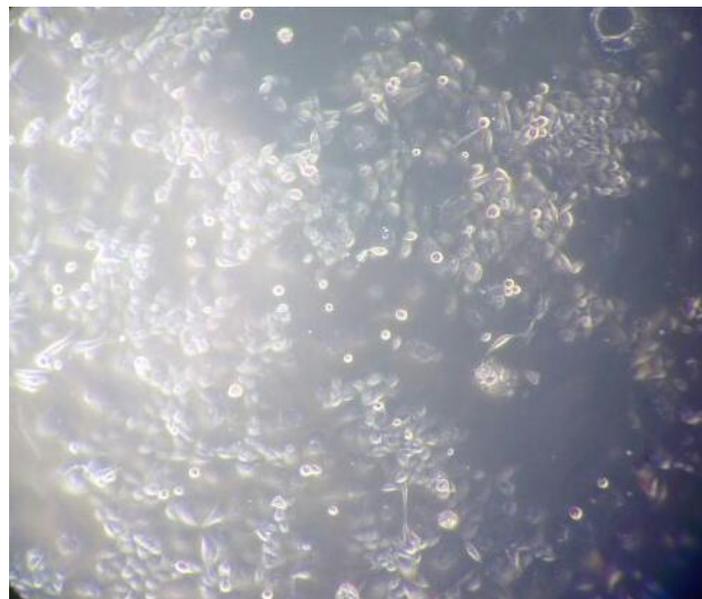


Figure 2 Subculture of the HeLa cell lines obtained

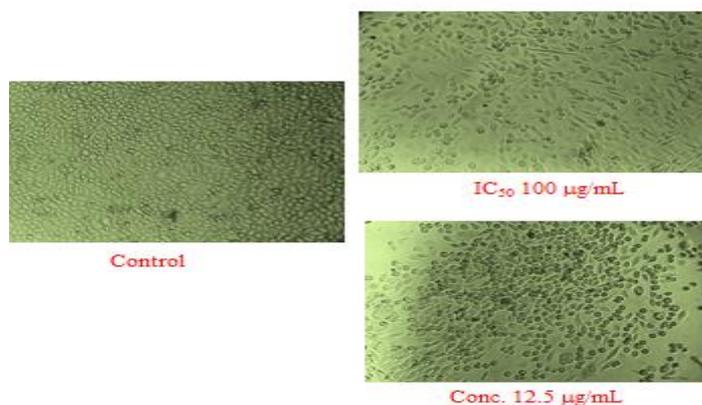


Figure 3 Cytotoxicity analysis of peptide from red radish papain digest against HeLa cell lines

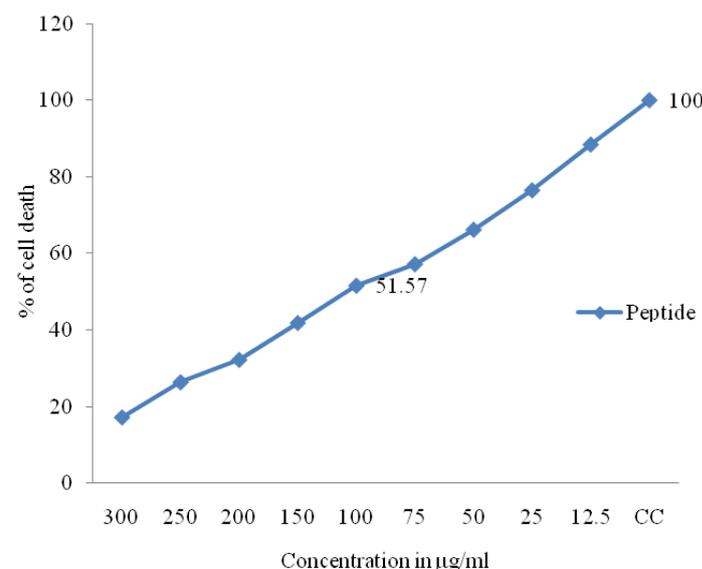


Figure 4 Cytotoxicity analysis of peptide from red radish papain digest against HeLa cell lines

CONCLUSION

Though many treatment methods are available for cancer therapy such as radiation, drugs etc., they are associated with their own side effects. Just like how microorganisms develop resistance against antibiotics, the cancer cells develop

resistance against the drugs used (especially cancer stem cells). Thus there is a need for identification of new therapeutic agents. Here, the potential of antifungal peptides was employed and it was found that among the chosen sources of antifungal peptides, red radish seeds had notable effects of growth inhibition of cancer cell lines.

Experiments were conducted to check the extent of antifungal activity of peptides from Red Radish (*Raphanus raphanistrum subsp sativus*) and further, these were analysed for their anticancer activity by testing them on HeLa cell lines. The molecular weight of papain digested peptides was found to be 1.68 kDa by MALDI-TOF analysis. These peptides showed significant activity from MIC 12.5 µg/ml and their activity increased with higher concentrations. 50% of cells were killed in the concentration of 100 µg/ml.

Also, among the various enzymes used for digestion, papain digested peptides showed good anticancer activity. This idea could be further extended for the production of anticancer drugs by digesting radish extracts with papain (both are naturally available which makes them safe for use). To further confirm their effects, they could be tested on various other cancer cell lines under appropriate conditions.

Thus, from the experiments performed it is concluded that antifungal peptides from red radish if used in appropriate concentrations could act as potential therapeutic agents for cancer treatment.

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