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Evaluation of Cytotoxic Potential of Peptides from Momordica charantia Seed Extracts on Oral Cancer Cells

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ABSTRACT

Abstract: Momordica charantia is a perennial plant reported with many of the health benefits. It is rich in many of the proteins and carbohydrates. Oral cancer which occurs in oral cavity is a form of malignant tumour. The common sites of oral cancer cavity are lip, tongue and floor of mouth. Globally, Oral Cancer ranks sixth among most common type of cancers. The number of new cancer cases have been increased in the past thirty years around the globe. Bioactive peptides are short peptides of amino acids derived from proteins that can exhibit biological activities. Anticancer peptides are small peptides containing amino acid sequences, are selective and toxic to cancer cells. The aim of this study was the evaluation of anticancer activity of extracted peptide against the Oral cancer cells. To check the anticancer activity on the growth and viability of KB-3-1 Oral Cancer cells by in vitro assay in order to detect the cytotoxic activity. The results revealed that the protein at low concentrations exhibiting promising anticancer activity. The molecular weight of protein to be detected 32kDa by SDS-PAGE analysis. The cell viability assay has given best results at low concentration. The cancer cells death may be due to induction of apoptosis. The peptide from Momordica charantia in vitro supporting the potential anticancer property against the oral cancer cell line KB-3-1. Thus it may act as alternative medicine for cancer treatment.

1. Introduction

Cancer is a general term which refers to many different types of cancer diseases that can affect any part of the body.[15] Cancerous cells invade adjoining parts of the body and spread to other organs, this condition is called as metastasis. Tumor is an abnormal tissue that forms when the cells grow and divide more than they should or do not die when they should. Tumors may be benign or malignant. Benign tumors will grow large but not spread into nearby tissues or to any other parts of the body. Malignant tumors will spread and invade nearby as well as other parts of the body.[15] These can also spread to other parts of the body through the blood and lymph called as neoplasms. Cancer is the second leading cause of death after cardiovascular diseases as per statistics from Centre for Disease Control (CDC) and it is expected to emerge as number one position by 2030. In 2010, the total annual economic cost of cancer was estimated at US $ 1.16 trillion. It has an economic impact as well. The traditional approaches such as chemotherapy, radiotherapy, surgery and pharmacotherapeutic treatments showing an adverse side effect in patients. Due to the medical complexities, deleterious effects and high treatment costs creates the need for less harmful and affordable treatments for the patients. Food derived bioactive peptides has scientifically proven as an alternative for cancer treatment. Anticancer peptide treatment can emerge as a new clinical application to decrease new cancer cases and to reduce mortality rate. As per 2020 cancer report, Female breast cancer as the most commonly diagnosed cancer with an estimated 19.3 million cases worldwide. Lung cancer remained the leading cause of cancer death which is followed by colorectal and prostate cancer. There were 10 million cancer deaths in 2020 around the world. GLOBOCAN 2020 will estimate the cancer incidence and mortality rate which is produced by International Agency for Research on Cancer (IARC). As per GLOBOCAN 2020 report it is estimated that 19.3 million new cancer cases and almost 10.0 million cancer deaths worldwide. The most common type of cancer were; Breast cancer (2.26 million), lung cancer (2.21 million cases), colon and rectum cancer (1.93 million), prostate cancer (1.41 million), skin cancer (1.20 million), stomach cancer (1.09 million cases).22 There are more than 200 different types of cancer and they are classified based on their origin. Mainly they are 5 groups;

Carcinoma; this type of cancer occurs in skin tissues mainly epithelial tissues. The subtypes are adenocarcinoma, transitional cell carcinoma, basal cell carcinoma and squamous cell carcinoma.

Sarcoma; the site of origin is connective or supportive tissues like bone, cartilage, muscle, fat tissues.

Leukaemia; this type of cancer begins in the white blood cells. Mainly occurs in bone marrow. The formation of abnormal cells in the blood.

Brain and spinal cord cancers; these are commonly referred as central nervous system cancers.

Lymphoma and myeloma; lymphoma cancer affects the lymphatic system and myeloma begins in the plasma cells. This type of cancer targets the immune system.[21] Diagnosis; There have been different diagnostic methods for early detection of cancer such as; Physical examination for any lumps on the body or any changes in skin colour or

enlargement of organ. Laboratory tests such as urine and blood analysis. For examples in case of leukaemia, the blood test called complete blood count to unusual number of white blood cells. Imaging tests such as Computerized tomography (CT), bone scan, magnetic resonance imaging (MRI), Positron emission tomography (PET) and X-ray to examine bones and internal organs. In many of the severe situation biopsy can be done. During biopsy, a sample collected from the patients and cells are observed under microscope. The normal cells were uniformly organised with similar sizes whereas the cancerous cells less orderly arranged with various sizes.[14] Treatment methods such as; Surgery to remove cancerous cells from the body. Chemotherapy, the use of different drugs to kill the cancer cells. Radiation therapy uses energy beams like X-rays and protons. Through external beam radiation or by placing the machine inside the body known as brachytherapy. Bone marrow transplant commonly called as stem cell therapy, which will replace the diseased bone marrow. Immunotherapy, the use of body's own immune system to target the cancerous cells. Targeted drug therapy which acts upon the specific abnormalities within the cancerous cells.[3] Cancer is not so effectively treated by the use of therapeutic drugs and its resistivity towards drug action, which results in increased side effects such as hair loss, neutropenia, nausea, vomiting and many other.[7] The use of cisplatin, a chemotherapeutic drug which causes damage to kidneys, nerves, liver. Doxorubicin administered intravenously for treatment if lymph and lung cancer which caused the enlargement of heart.[26] The use of peptides against cancer cells can acts as an alternative method for development of anticancer drugs.[7]

Peptides from various sources can be used to treat many types of cancers like lung cancer, colorectal cancer, pancreatic cancer, gastric cancer, prostate cancer and breast cancer at their early stage. Peptides are monomers of amino acids linked by peptide bonds, which will specifically bind to tumor cells and are less toxic to normal tissues.[17] Peptides extracted from different sources can act against human cancer cells through the mechanism of apoptosis, anti-proliferative, cytotoxicity and anti-tubulin activity.

Oral cancer which occurs in oral cavity is a form of malignant tumour. The common sites of oral cancer cavity are lip, tongue and floor of mouth.[9] Globally, Oral Cancer ranks 6th among most common type of cancers. Oral cancer has major socio-economic implications, and it has a major public health concern.[9] The incidence and prevalence rate of Oral cancer vary from region to region across the world. India, Pakistan, and Bangladesh are main countries affected by Oral cancer.[28] The number of new cancer cases have been increased in the past 30 years over the globe.[5] There are around 3,77,713 new oral cavity and lip cancer cases and 1,77,757 deaths in the world as per GLOBOCAN report 2020.[23,24] The causes of oral cancer are the use of tobacco, alcohol consumption and Human Papilloma Virus infections. These are major risk factors among the individuals.[4] The treatment for Oral cancer is very high and it is burden for families and hits the economy of many countries. The countries having larger populations like India and China have an adverse impact on the economy.[2] It is a preventable disease as it involves behavioural and lifestyle factors. Oral Cavity Cancer cases are 2-4 times higher in case of males than females.[28] External beam radiation therapy and Brachytherapy treatment methods employed for early-stage Oral cancer. Chemotherapy combined with radiation therapy and Surgery are the advanced form of treatment methods.[29]

Momordica charantia is a perennial plant which is commonly known as bitter gourd or bitter melon. It is cultivated all over the world and it belongs to Cucurbitaceae family. It is traditionally used a medicine for ulcers, kidney stones, malaria, and type-2 diabetes.[10] Bitter melon it is rich source of carbohydrates, protein, fibres, vitamins and minerals. It is widely grown in India, China, Asia, Africa and South America countries.[8] Bitter gourd is known for its bitter taste and used as common vegetable. It has potential medical applications with its anti-tumour activities, anti-HIV, anti-diabetic properties. Further clinical trials are necessary to verify its medical applications on various tumour cell lines.[11] The antitumor activity of bitter gourd protein named BG-4 has induced apoptosis against colon cancer cell lines HCT-116 and HT-29.[10] The studies showed that 2% of bitter melon extract caused 80% of cell death in breast cancer cell lines.[20]

2. Materials and methods

i. Collection of material.

Bitter gourd was brought from Hubballi Market. Then seeds are taken out and allowed to shade dry for 8-10 days. The seeds were then surface sterilized with 5% sodium hypochlorite and then pulverized into powder with the help of pestle and mortar. The powder mixed with lysis buffer and then centrifuged at 10000 rpm for 10 mins. The supernatant collected for further experimental uses.

ii. Extraction of Protein by Acetone method

Cool the required volume of acetone at -20°C. Place the protein sample in centrifuge tube and add 4 times the sample volume of cold acetone. Vortex and incubate for 60 minutes at - 20°C. Centrifuge at 10,000 rpm for 10 minutes at 4°C.

Decant the supernatant carefully and collect the pellet. Allow the acetone to evaporate. Add appropriate buffer to dissolve protein pellet and used for further analysis.[19]

iii. Estimation of Protein by Biuret method

Biuret reagent; Dissolve 0.3gm of Copper sulphate and 0.9gm of sodium potassium tartarate in 50ml of 0.2M sodium hydroxide and add 0.5gm of potassium iodide and make up volume to 100ml with 0.2M sodium hydroxide. Protein standard; 0.05gm of BSA dissolved in 10ml Distilled water (5mg/ml). Unknown sample: bitter gourd seeds powder and lysis buffer (25mM potassium phosphate,2mM Magnesium choride,2mM EDTA,15% glycerol,0.2% mercaptaethanol and 5% SDS) are mixed and centrifuged at 10,000 rpm for 10 minutes. Then supernatant carefully taken for estimation. Observe the colour change and measure Optical density at 540nm spectrophotometrically.

iv. Ammonium Sulfate Precipitation/Fractionation

The percent ammonium sulfate saturation of the solution in which most of the proteins will precipitate was determined first. Percent saturations used were 0–20%, 20–40%, 40–60%, 60–80% and 80–95%. Then, the protein extract was saturated up to the optimized percent saturation, 40% was gently stirred for 30 min in an ice bath, and was centrifuged at 10,000× g rpm for 30 min at 4 ◦C. After centrifugation, the precipitate was collected while the supernatant was discarded.

v. Enzymatic Protein Digestion

The crude, partially purified and purified protein extracts were hydrolyzed while being submerged in a water bath with shaker at 37 ◦C using two sets of freshly prepared enzymes: Set A consists of pepsin while set B consists of trypsin. For the first part of protein digestion, pepsin was added to the sample and the digestion was allowed to proceed for two hours at pH 2.0. Then, Set B of enzyme was added, and the digestion was allowed to proceed for additional 1, 2, 10 and 22 h at pH 7.0. At every specified time, a 2.0 mL aliquot was obtained from the extracts followed by boiling for 5 min in a boiling water bath to terminate the digestion process. After boiling, the aliquot was stored to freezer prior to activity testing.

vi. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed using the method developed by Laemmli. The run was carried out in a 15% discontinuous denaturing stacking and resolving gels using BIORAD tetracell electrophoresis apparatus. The electrophoretic run was completed for 60 min at 110 V. The gel was stained using staining solution 0.10% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid) for 1 h with shaking and was destained using destaining solution (50% (v/v) methanol, 10% (v/v) acetic acid). The molecular weights of the subunits were estimated using a Benchmark Protein Ladder with a molecular weight (MW) range of 6–250 kDa.

vii. In-vitro cytotoxic effect of Momordica charantia protein extract by MTT assay

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.[1] The cells were trypsinized and aspirated into a 15ml centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM medium, such that 200µl of suspension contained approximately 10,000 cells. To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37^{o}C and 5% CO2 atmosphere for 24 h. After 24 h, the spent medium was aspirated. 200µl of different test concentrations (50, 100, 150, 200 and 250 µg/ml from stock) of test drugs were added to the respective wells. The plate was then incubated at 37^{o}C and 5% CO_2 atmosphere for 24 h. The plate was removed from the incubator and the drug containing media was aspirated. 100µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/ml and the plate was incubated at 37^{o}C and 5% CO_2 atmosphere for 3 h. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a rotary shaker to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) was generated from the dose-response curve for the cell line.

3. Results

I. Estimation of Protein by Biuret method

The biuret test is performed in order to determine the amount of protein present in crude extract of bitter gourd seed protein. The amount of protein present in 0.2 ml of seed extract is 2.125mg calculated by using standard graph.

II. Estimation of Protein by Biuret method

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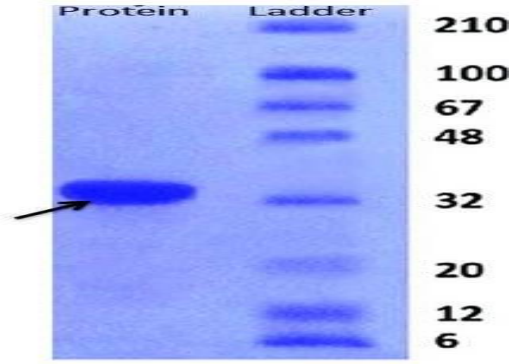

Figure 1; Molecular weight determination by SDS PAGE analysis

III. In-vitro cytotoxic effect of Momordica charantia protein extract by MTT assay

As the concentration of Protein increases, cell viability decreases in dose – dependent manner. The IC50 value is calculated and it was found to be 151.40 µg/ml which indicates effective cytotoxicity of protein against KB-3-1 cell-line. As shown, (figure 2) Bitter gourd peptide extracts of different concentrations dose dependently inhibited the KB-3-1 Oral cancer cells.

Table 1
The IC50 values of the peptide extracts for KB-3-1 cell-line.

	Concentration in (µg/ml)						
%Viability	Untreated	Cisplatin 15 µg/ml	50	100	150	200	250
	100	18.35	75.42	64.52	47.02	37.13	27.59

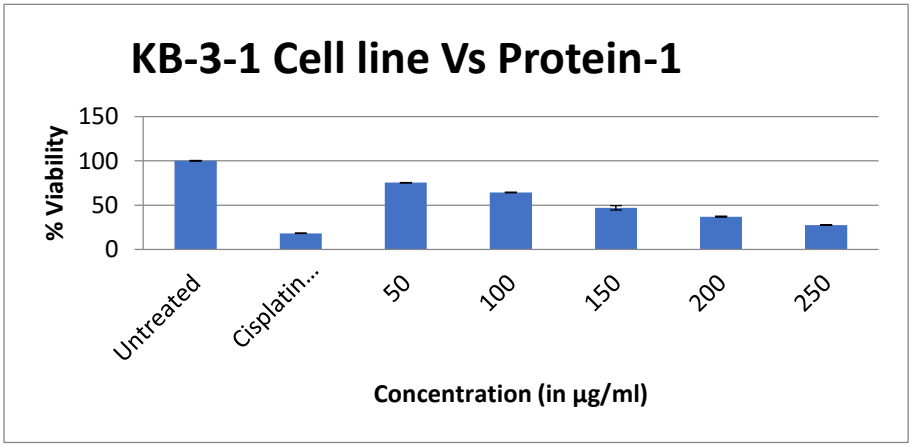

Figure 2: Graph showing In-vitro cytotoxic effect of Momordica charantia peptide extracts against KB-3-1 Oral cancer cell line

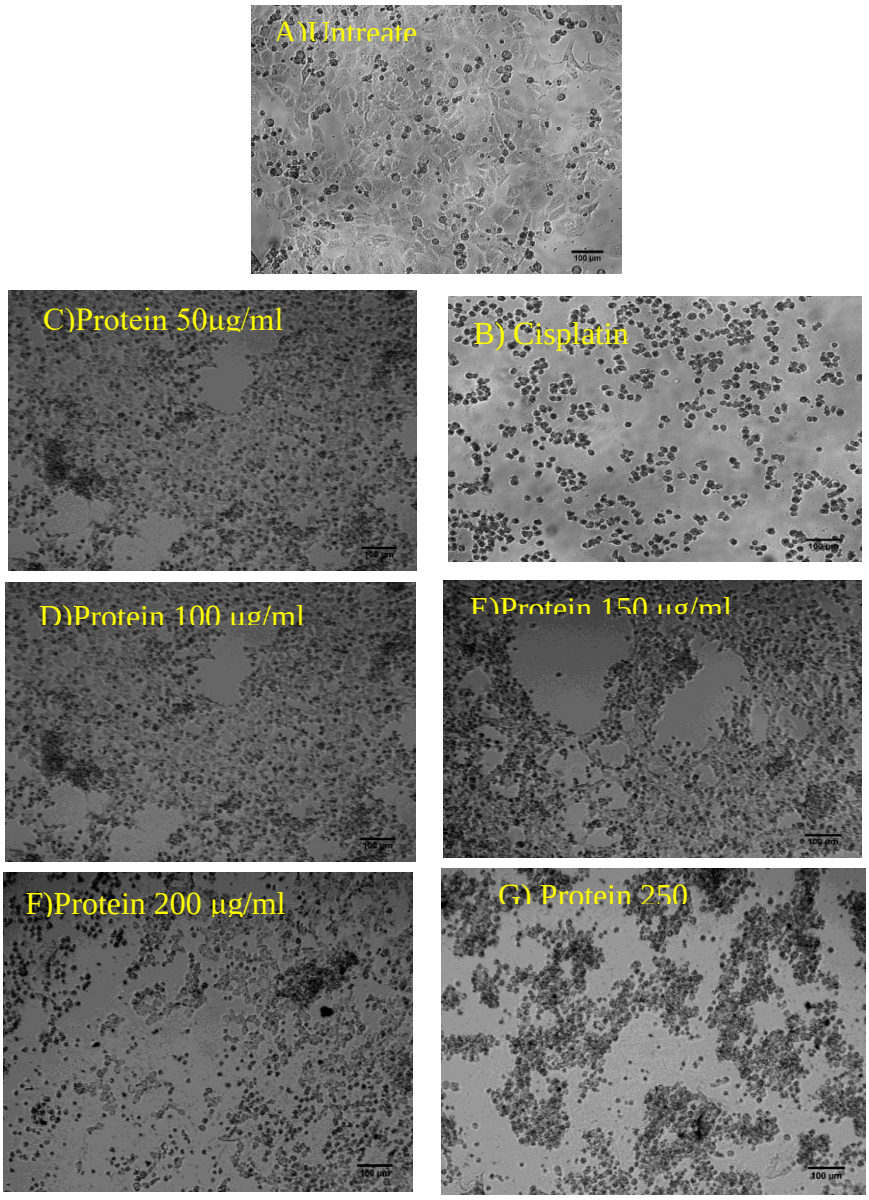

Figure 3: In-vitro cytotoxic effect of Momordica charantia protein extract of different concentrations against KB-3-1 Oral cancer cell-line by MTT assay

4. Discussion

Bioactive peptides which are derived from different sources has shown the antitumor and anticancer activities to certain extent. The use of peptide extracts against the cancer has emerged as a new and alternative treatment method for cancer. Peptides from different sources can be used to treat many types of cancers like lung cancer, colorectal cancer, pancreatic cancer, gastric cancer, prostate cancer and breast cancer at their early stage. Peptides are monomers of amino acids linked by peptide bonds, which will specifically bind to tumor cells and are less toxic to normal tissues.26 Peptides extracted from different sources can act against human cancer cells through the mechanism of apoptosis, anti-proliferative, cytotoxicity and anti-tubulin activity.7 Bitter melon it is rich source of carbohydrates, protein, fibres, vitamins and minerals. It has potential medical applications with its anti-tumour activities, anti-HIV, anti-diabetic properties. The cancer cell death may be due increase in pro-apoptotic bax proteins and decrease in anti-apoptotic bcl2 proteins. The isolated 32kDa peptide dose dependently inhibited the growth of KB-3-1 oral cancer cells. Our isolated 32kDa peptide has given IC50 value of 151.40 µg/ml. Some of the studies have shown that the anti-proliferative effect of soyabean protein against HT-29 colon cancer cells with IC50 value roughly between 319.2 to 386.4 µg/ml. The BG-4 peptide on HT-29 colon cancer cells showed IC50 value 217.249 µg/ml.10 The protein extract was found to be toxic at higher concentration and it showed more cell viability at lower concentration. Bitter gourd peptide treatment after 24-48hrs showed changes in morphology of Oral cancer cells with its cytotoxic activity. The changes in morphology of cells may be associated with process of apoptosis such as shrinkage, blebbing and membrane disintegration. The

mechanism of action involved may be downregulation of antiapoptotic proteins and upregulation of pro-apoptotic proteins and due to the modification of cell cycle proteins p21 and CDK2. Dose dependent concentration of protein extract increases the death rate of KB-3-1 cancer cells. The bitter gourd seed protein has given better results as an anticancer agent against Oral cancer cells. Hence it may be used as a therapeutic agent / drug to treat the Oral cancer and can act as alternative treatment.

Conclusion

The present study carried out on bitter gourd protein successfully demonstrated that isolated peptide exhibited the anticancer property. The results exhibited dose dependent anticancer activity at different concentration of protein extract. Thus, from present study it is concluded that Momordica charantia may be used as potential source in treating Oral cancer. It can be further studied for its Apoptotic or cell death activity in detail. Further the selection of smaller peptides may give the better results. The bitter gourd anticancer peptides may be used as an alternative method for treatment of Oral cancer. The experiment is further extrapolated in order to study the anticancer activity against different cell lines.

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