M.Sc. PROJECT AND DISSERTATION

ON

EXTRACTION OF BIOACTIVE PEPTIDES FROM PUMPKIN SEED AND THEIR APPLICATION

DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY

SCHOOL OF AGRICULTURE

LOVELY PROFESSIONAL UNIVERSITY

INDIA

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PROJECT AND DISSERTATION PLAN PROPOSAL

Of the proposed Research Project for the degree of

MASTER'S OF SCIENCE

IN

Food Science and Technology

Name of the Research Scholar: Harjeet Kaur

Registration No: 11705950

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Name of the Supervisor: Er. Poorva Sharma

Title of Research**: Extraction of Bioactive Peptides from Pumpkin Seed and their Application**

Signature of the Research Scholar**:**

Approved by Coordinator

CERTIFICATE

This is to certify that Harjeet Kaur (Registration $N_{O.}$ 11705950) has personally completed M.Sc. Pre-dissertation entitled 'EXTRACTION OF BIOACTIVE PEPTIDES FROM PUMPKIN SEED AND THEIR APPLICATIONA' under my guidance and supervision. To the best of my knowledge, the present work is the result of hid original investigation and study. No part of dissertation-1 has ever been submitted for any other purpose at the university.

The project report is appropriate for the submission and the partial fulfillment of the conditions for evaluation leading to the award of Master of Food Science and Technology.

Date: May,2018Signature of Supervisor

Er: Poorva Sharma

Assistant Professor

School of Agriculture

Lovely Professional University

Phagwara, Punjab, India

DECLARATION

I hereby declare that the work presented in the pre-dissertation report entitled 'EXTRACTION OF BIOACTIVE PEPTIDES FROM PUMPKIN SEED AND THEIR APPLICATIONA' is my own and original. The work has been carried out by me at school of Agriculture, Lovely Professional University, Phagwara, Punjab, India; under the guidance of Ms. Poorva Sharma, Assistant Professor (Food Science and Technology) at school of Agriculture, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree of Master of Food Science and Technology.

Date: May, 2018Harjreet Kaur (11705950)

I certified that the above statement made by the student is correct to the best of my knowledge and belief.

Date: May, 2018, Er. Poorva Sharma

Assistant Professor

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Table of Content

Introduction CHAPTER-1

Bioactive peptide

Bioactive peptides are the protein particles which show biological activity and give constructive effect on body function eventually impact health (Kitts & Weiler, 2003). It is formed by amino acids when joining with the help of peptide bond. Bioactive peptides are derived from various food sources for their benefits and these sources includes peanut, pumpkin seed ,sesame seed, milk, and marine sources. The beneficial desirable effects of bioactive peptides are attributed to different properties found in peptides such as antimicrobial (Reddy et al., 2004; Rajanbabu and Chen 2011), antioxidant (Sarmadi and Ismail, 2010), antithrombotic (Wang and Ng, 1999), anti-hypertensive (Erdmann et al., 2008), immunomodulatory activities (Georgiev, 1990; Gauthier et al., 2006), and probiotic potentials among others. The consumption of processed foods with chemical preservatives has led to increased consumer concern and the demand for more natural and minimally processed foods. As a result, researchers have shown a growing interest in natural antimicrobial agents such as certain peptides. The growing interest in BP has motivated the scientific community and the food industry to researching and exploring the development of new food additives and functional products based on these peptides. Bioactive peptides extract from the pumpkin seed as compared to the whole pumpkin is more beneficial due to more protein content. Although some BP exists free as a natural source, majority of the known BP are encrypted in the structure of the parent proteins and are released mainly by enzymatic reactions. Some BP 5/11/2018has been synthesized chemically.

To extract bioactive peptides from food with probioactive microorganisms is helps to increases the beneficial components which influences on health. Probioactive strains like as Lactobacillus helveticus, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus brevis used in the study for the extraction of bioactive components. Our main aim is extraction of bioactive peptides and checks their applications.

Review literature CHAPTER-2

Bioactive peptides are proteins particles combined in the cell in the evolution of immense prepropeptides, which are again split and alter to give active outcomes. As signaling molecules, the bioactive peptides play important roles in physiological functions and pathogenesis (INT.J BIOAUTOMATION, 2011). Bioactive peptides are ubiquitous biomolecules widely abundant and easily obtained from food protein. There are so many bioactive peptides are obtained from single food protein. The role of protein derived active substances in the diet is increased. In recent year it has found that dietary proteins provide a rich source of biologically active peptides.

Peptides have been studied for over 100 year. In 1902 a substance secreted by the intestine stimulated the pancreas to secrete digestive enzyme. This substance was named secretin and the class of signaling molecules carried by the blood stream was named hormones. Once secretin was purified and found to be a peptide, its amino acid sequence was determined. Many other peptide hormones were discovered using a similar approach in which a bioactive substance was purified to homogeneity and sequenced and several of these discoveries resulted in Nobel Prizes for the novel groundbreaking scientific insights how the body communicates internally locally between cells and especially via the blood stream between distant cells and entire organ systems. Currently more than 1500 different BP have been reported in a database named 'Biopep' (Singh et al., 2014).

Bioactive peptides made from long chain of amino acids. There are 20 amino acids are there. When peptide linked together then form the chain and which helps to cure the disease. The amino acid composition and sequence determines the activity of the peptides once that they are released from the precursor protein where they are encrypted. There are various types of bioactive peptides which are antimicrobial, antithrombotic, antihypertensive, opioid, immune modulatory, mineral binding and antioxidative. Bioactive peptide derived by enzymatic hydrolysis and chemical process. Bioactive peptides have numerous properties antimicrobial, antioxidative, anticancer, antihypertensive, mineral binding, and immunomodulatory.

There are various food sources from where we extract bioactive peptides like milk, peanut, pumpkin, peas, soya, sprouted beans, wheat germ, cheese, guava, yogurt, cocoa power, oats, corn, quinoa, edamama, whey protein. Fruits seed and vegetables seed contains high amount of

bioactive peptide compared there whole product. Pumpkin seed contains 30.2gm compared whole pumpkin.

Bioactive peptide from pumpkin seed

The seeds of the pumpkin are sometimes referred to as pepitas, Spanish for "little seed of squash." The seeds of pumpkin (Cucurbita sp.) are generally considered to be agro-industrial wastes and discarded. In some parts of the world, the seeds are consumed raw, roasted or cooked, but only at the domestic scale. With the discovery of their richness in protein, fibres, minerals, polyunsaturated fatty acids and phytosterols, they are being regarded valuable for the food industry. Pumpkin seeds hold excessive quantity of protein, fatty acids, and significant number of micronutrients like P, K, Mg, Mn and Ca (Sohini Roy and Santa Datta, 2016). Pumpkin seed extracts and oils have been found useful in the treatment of diseases such as cancer, diabetes, obesity, hypertension, and immunomodulatory. Extraction of bioactive peptides from the seed shows good results to inhabit the agents which cause the disease. So the study more focuses on the potential benefits of bioactive peptides extract from the pumpkin seed.

Description of the plant

Pumpkin plant has large leaves, sprawling veins with coiled, modified leaves called tendrils. The leaves are simple, alternate and shallowly to deeply lobed. The root near the surface, stem would be square, flowers will be bright yellow and the fruit is more fibrous and less sweet than winter squash. Seed vary in size based on diversity and type (Fruh Writh & Hermetter - 2007). Pumpkin seed have a real defensive skin called the hull. The pumpkin plant has been grown since the earliest history of mankind (Brucher - 1989).

Taxonomy

Division:	Spermatophyta
Sub – Division:	Angiospermae
Class:	Dicotyledonae
Sub – class:	Poly patellae

Series: Caliciflorae

Order: Passiflorales

Family: Cucurbitaceae

Genus: Cucurbita

Species: Maximus

Pumpkin is a squash that an ordinary weighs from 4 to 8 kilograms with the huge of the species have the ability of to extend a weight of 34 kilograms (Pumpkins, 2003). They vary greatly in shape, ranging from oblate through oblong. Fruits are usually orange, yellow, white, and red or grey (Pumpkin, 2003). Pumpkins are monoecius having both male and female flowers

Bioactive peptide	**Source**	**Reference**
Antimicrobial peptide, Antithrombotic peptide, Antihypertensive peptide, Immune modulatory peptide, Mineral binding and Antioxidative peptide.	Pumpkin	Ting Zhou et al,2007
Antimicrobial peptide, Antioxidative peptide, Immunomodulatory peptide, Mineral- carrying peptides	Milk	Korhonen and Pihlanto et al,2007
Antioxidative peptide, Anticancer peptide, immunomodulatory peptide.	Peanut	Dias et al. 2015
Antioxidative peptide, Antimicrobial peptide, Antihypertensive peptide,	Soybean	Agyei D et al,2015

Anticancer and Immunomodulatory peptide.		
Antihypertensive Peptides, Antioxidant Peptides, Antimicrobial and Antiproliferative Peptides,	Meat and Meat products	Joseph Thomas Ryan et al,2011

Pumpkin

Pumpkin is one of the most important crops of family Cucurbitaceae. Cucurbitaceae used as vegetable and medicine throughout the world. Cucurbita maxima (C. maxima) are an extremely diverse species. This species originated in South America, over 4000 years ago. India, Bangladesh and Myanmar are considered to be secondary centers of diversity for Cucurbita maxima. Other most important verities of pumpkin Cucurbita pepo and Cucurbita moschata. There are many names of pumpkin in different language: Pumpkin(English), Kumbra(Bengali), Kohlu (Gujarati), Kaddu (Hindi), Kumbala (Kannada), Paarimal(Kashmiri), Mathan or Chakkara kumbalanga (Malayalam), Lal bhopla,(Marathi), Kakharu (Oriya), Sitaphal (Punjabi), Purangikkai or Pooshanikai (Tamil), Gummadi kayi (Telugu), Dangaree (Sanskrit) (Gopalan et al., 2011).

Pumpkin comes in different shapes like oval, small, large, spherical, obviates, longitudinally grooved and non grooved (Harry S. Pairs 1988). Color of pumpkin varies from light green to dark green. The color of pumpkins is due to the carotenoid pigments, beta-cryptoxanthin, alpha and beta carotene, which is the precursor of vitamin A. It is sweet in test. Pumpkin produce at worldwide. India is the first in the production of pumpkin. The word pumpkin derived from the word pepon, which is Greek for "large melon". The French alter this name to pompon, which then British modify to pumpion and to adjust American colonists became admitted as pumpkin.

In numerous areas of the world, like North America and the United Kingdom, pumpkin traditionally cite to only some round, orange varieties of winter squash,

predominantly obtained from Cucurbita pepo, while in Australian English, pumpkin name as winter squash of whichever reflection.

Pumpkins are produced in all over the world for several of reasons from agricultural needs (such as animal feed) to commercial. The large international manufacture of pumpkins like as the United States, Canada, Mexico, India, and China.

Pumpkins are a warm-weather crop that is generally grows in advance July. The particular things required for producing pumpkins such as soil temperatures 8˚ (3 in) deep in earth. Pumpkin crop deteriorate due to less water availability or because of cold temperatures (in this case, below 18 °C or 65 °F; frost can be detrimental), and sandy soil with less water retention power. Pumpkins are hardy, and even if more leaves and portions of the vine are taken away, the plant can very frequently re-grow into secondary vines to replace what was removed.

Production of pumpkin in different countries-

Country	**Production in tons**	**Area in ha.**	**Yield, h/ha**
India	3500000	360000	97222
China	6315000	328000	192530
USA	864180	39500	218780
Germany	83100	2200	377727
Japan	237000	17000	139412
Spain	315000	7500	420000
South Africa	95000	12000	79166
Pakistan	255000	26000	98076
Mexico	17000	5500	212727

Columbia	60000	3650	164384

(Anju K. Dhiman et al., 2009)

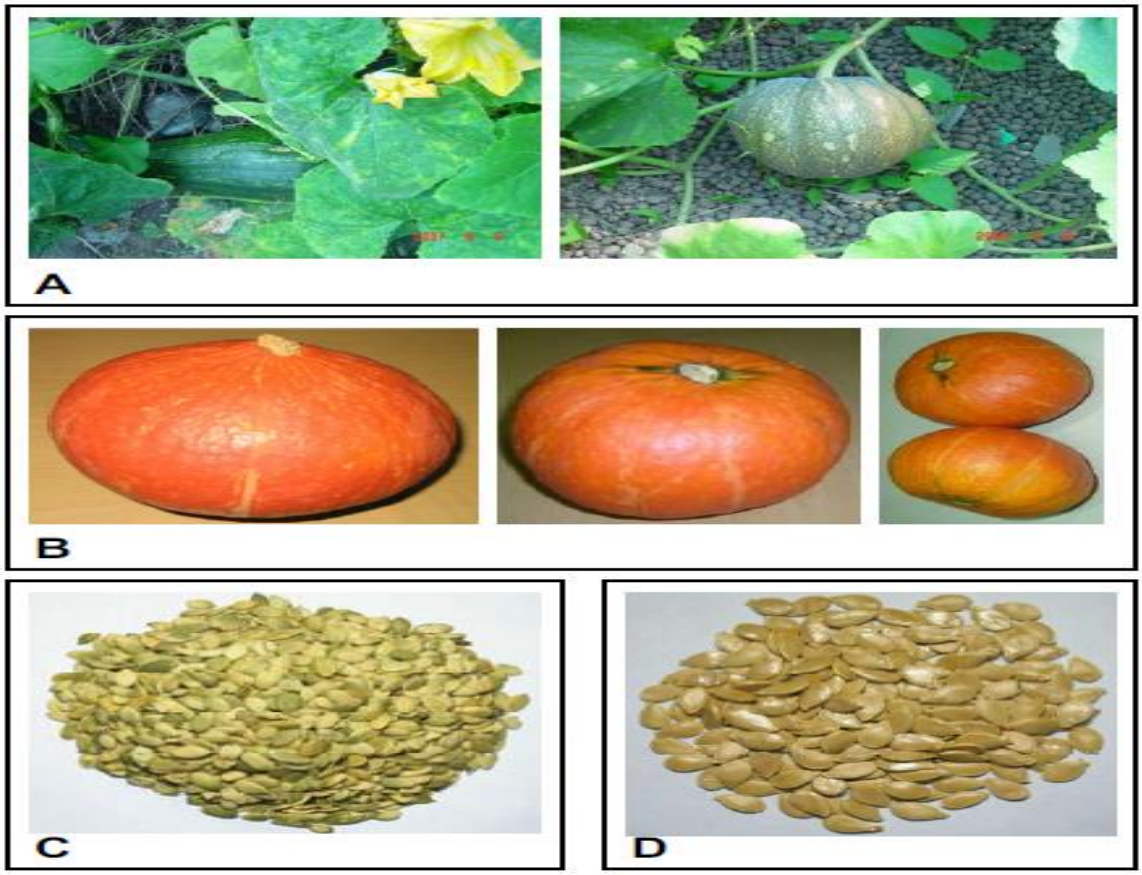
Long-time period preservation of pumpkins gives decision that the pile of lutein and β-carotene with a minor reduction in zeaxanthin. These chemicals vary between different cultivars and seasons: β-carotene from 175mg to 693mg/100g; lutein 20mg to 59mg/100g; and zeaxanthin not detected to 3mg/100g. Zhang et al. (2014) suggest that accumulation may be due to "the increase likely resulted from a continuous biosynthesis with a possibly reduced turnover and/or enhanced sequestration, suggesting a complex regulation of carotenoid accumulation during fruit storage.

Pumpkin rise iron bioavailability and antioxidant action (Dias et al. 2015). Gibson & Hotz (2001) suggest that a sauce made from pumpkin leaves would increase bioavailability of zinc and iron over a sauce produced from sweet potato.

Pumpkin contain varies kinds of minerals like P, Ca, Fe, Mg, Zn, Na and many more. It contains varies kinds of amino acids like Arginine, Aspartic acid, Glutamic acid, Alanine, Glycine, Histidine, Leucine, Isoleucine and many more.

Nutritional content in pumpkin is good. As compared to whole pumpkin, pumpkin seed contain more nutrients which are enhancing the immune system.

Pumpkin and Pumpkin seed



(Global Science Book)

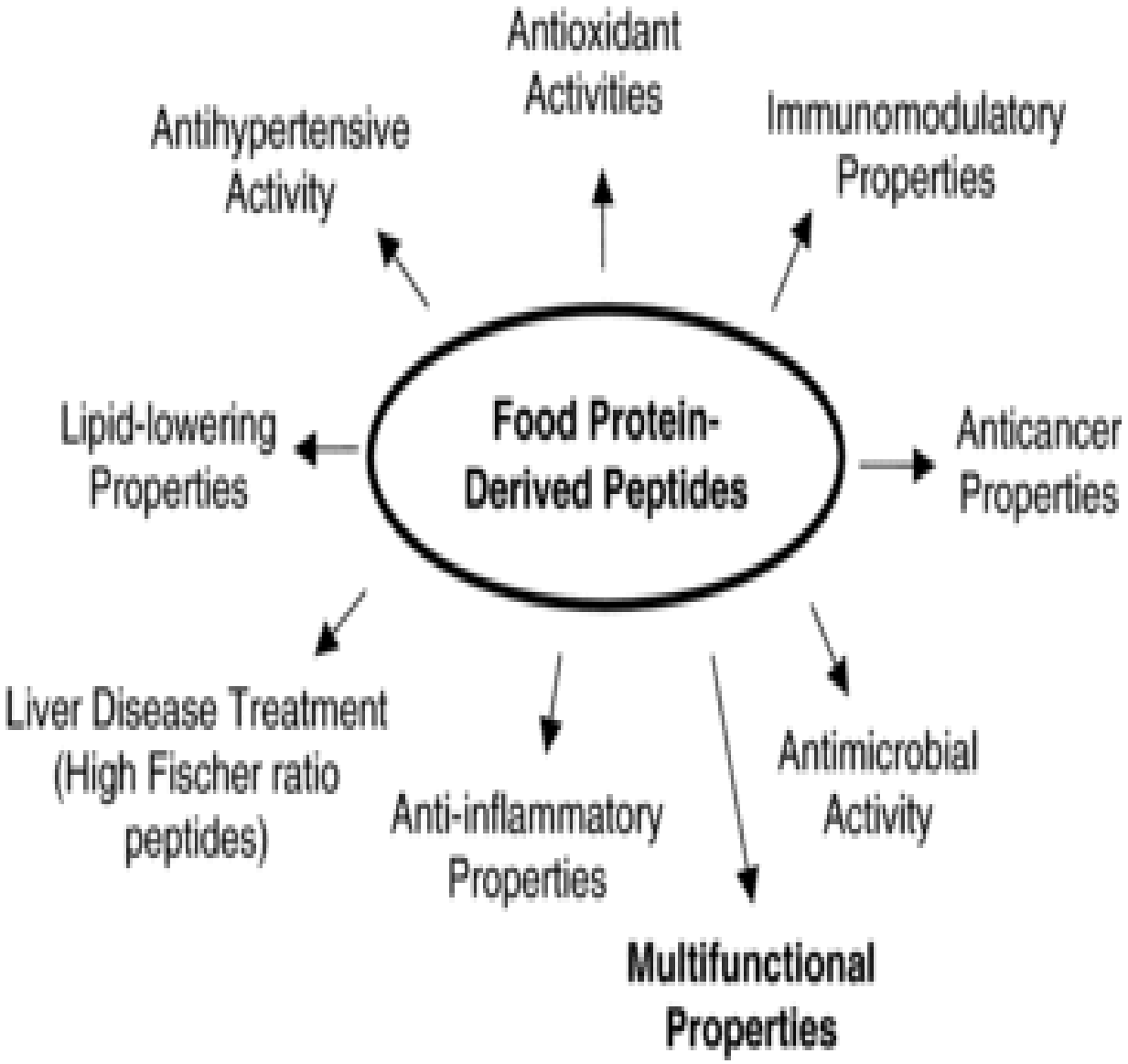
Chemical composition of pumpkin

Variety of pumpkin	Moisture content	carbohydrates	protein	lipids	fibre	Ash	Reference
Cucurbita maxima	88.50%	77.33%	-	-	8.55%	7%	Arun mohan et al.
Cucurbita pepo	-	66%	3%	2.3%	11.46%	16%	Manu dose et al.
Cucurbita moschata	79%	1.90%	.97-1.41%	.07-.16%	.56-1.56%	.57-.89%	Chandu chopra at el.

Chemical composition of pumpkin seed

Variety of seed	**Moisture content**	**carbohydrates**	**protein**	**lipids**	**Fibre**	**Ash**	**Reference**
Cucurbita maxima seed	4.06%	1.08%	34.56%	36.7%	2.91%	3.8%	Amit et al.
Cucurbita pepo seed	5.20 ± 0.28%	25.19 ± 3.3%	25.40 ± 0.61%	41.5±2.7%	2.49 ± 0.11%	5.34± .04%	Gohari Ardabili et al.
Cucurbita moschata seed	51.79 ± 6.04g/kg	140.19 ± 7.60g/kg	298.11±14.75g/kg	456.76±11.66g/kg	108.51± 8.36g/kg	53.15± .20g/kg	Santosh et al.

Bioactive peptides properties



(Chibuike C.Udenigwe et.al2011)

Bioactive peptides content depends upon the protein concentration present in food which is different in different food. Major sources of protein in the diet in developed and developing countries (USDA, 1993)

source	Developing %	Developed %
Cereals	58.8	29.1
Meat	8.6	26.4
Pulses	7.4	1.7
Milk and dairy	5.6	16.7
Fish, seafood	4.1	7.3
Oil crops	3.8	1.9
Vegetables	3.5	3.5
Starchy roots	3.1	3.2
Eggs	1.6	4.3
Offals	1.2	2.2
Fruits	1.0	1.1

(J. Agric. Food Chem., Vol. 44, No. 1, 1996**)**

Medicine properties of pumpkin seed

Anti cancer activity: Cancer is a disease caused by uncontrolled division of abnormal cells in a part of body. Today cancer is the most predominant reason for the death of the person in all over the world. . Cancer cells spread in any parts of the body and from one cell at the end it becomes trillions cells so we call that it is metastasis means uncontrollable which spread by blood and through the lymph node. It is a one of the most danger chronic disease which grows at a fast rate. Traditionally to prevent chronic disease a person gone towards the expensive treatments like chemotherapy and radiations which leads to side effect but now various alternatives are find out. Researchers found out consumption of food which is rich of bioactive components extract from pumpkin seed heaving ability to fight against the cancer cell such as gastric, breast, lung and colorectal cancers.

Anti-diabetic activity: Diabetes is a type of diseases in which a person suffering from high blood glucose level because of the power of the body impaired to produce or respond to the hormone insulin which is made by pancreas, helps to use glucose from food to get into cells of the body to produce energy. There is two types of diabetes is there. Type 1diabetes is occurred according to the study in 5% population in which children, adolescents are there. In this type body produce little or no insulin. Therefore in this condition childrens go for the daily treatment for their survival. In case of type 2dibetes a person able to produce insulin but they does not do its job properly. In occur in 95% people which belong to above 40 year age. It can be cure by medicine and by precautions.

Antihypertension activity: Hypertension is basically a condition in which the blood flow rate against the artery walls is too high. Another name of the hypertension is called high blood pressure. It increase the risk of heart diseases, stroke. It is increases by excess salt, body weight, smoking, alcohol. Consumption of nutraceutiticals and functional foods heaving ability to inhabit this kind of chronic disease. These foods contain some active components which are responsible for the curing of this disease. Extraction of bioactive components from protein which derived from various plants and animal sources having power to prevent hypertension.

Antimicrobial activity: It refers to the process of killing or inhabiting the disease causing microbes. Antimicrobial peptides grouped according to the microorganisms they act primarily against like as antibacterial. It is a small molecular weight protein with broad spectrum

antimicrobial activity against bacteria, fungi, viruses. They are generally present between 12-50 amino acids (Brij Pal Singh).

Pumpkin seeds for depression: The World Health Organization (WHO) defines depression as a common mental disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self- worth, disturbed sleep or appetite, low energy and poor concentration (WHO, 2006). In some reports, the amino acids tryptophan, phenylalanine tyrosine, and methionine are frequently practical in deal with countless mood infection, including depression (McLean, 2004). Eagles (1990) and Axford et al. (1991) wrote on the mental health benefits of pumpkin seed; Kim et al. (2016) wrote on β-carotene advantages in Sweetme sweet Pumpkin (baked Cucurbita moschata), which is use to deal with patients with depression in Korea.

Probiotic

It is substance which helping to spark the progress of microorganisms, particularly those which heaving useful effects on intestinal flora. In other term we can say that it is a food for probiotics. Probiotic term discovered by Lilly and Stillwell. in 1965 Probiotics means “let good microbes work for you in different fields get their benefits and take a rest”. It helps to maintain the digestive system’s pH.

Characteristics of Effective Probiotic

- Able to survive the crossing through the digestive system.
- Able to couple to the intestinal epithelia and colonies.
- Able to balance the good viability.
- Able to utilize the nutrients and components from normal food.
- Non pathogenic and non toxigenic
- Able of expend a good impact on the host.
- Stability of required characteristics throughout processing, storage and transportation.
- Anti-inflammatory, antimutagenic, immunostimulatory.

Probiotic strains

Lactobacillus species

- L. acidophilus
- L. plantarum
- L. caseisubspecies rhamnosus
- L. brevis
- L. delbreuckiisubspecies bulgaricus

Bifidobacterium species

- B. bifidum
- B. longum
- B. infantis
- B. breve

- B. adolescentis

Others

- Streptococcus salivarius ssp. Thermophilus
- Lactococcus lactis ssp. Lactis
- Lactococcus lactis s ssp. Cremoris
- Enterococcus faecium
- Leuconostoc mesenteroides ssp.Dextranicum
- Propionibacterium freudenreichii
- Pediococcus acidilactici
- Saccharomyces boulardii

Probiotics Food

Yogurt- we use culture Streptococcus thermophilus and either Lactobacillus acidophilus or Lactobacillus bulgaricus.

Chocolate & Granola bars- we use culture Lactobacillus acidophilus.

Yakult Dairy Drink-Lactobacillus Casei shirota.

Health benefits of probiotics

They are able to compete with the bad microbes and colonize our digestive system.

They are able to ferment our food to simpler byproducts and could promote our health by many different mechanisms.

Probiotics promote health by removing toxic microbes, supply the useful microbes, reduces the work of our digestive system and the amount of food needed by our bodies due to the correct digestion and metabolism of any amount of food.(Salminen et ai,1998)

Statement problems CHAPTER-3

Probiotics are good for digestion system, immune system, gastrointestinal track and it prevent the various diseases like colon cancer. On the other hand bioactive peptides of pumpkin seed reduce obesity, cholesterol, infection and it acts as an antioxidant.

In order to combine the properties of these two components, selective probiotic microorganisms will be inoculated to extract the bioactive peptides to enhance the nutritional status of the individuals.

Objective CHAPTER-4

1. To optimize the extraction of milk from pumpkin seeds.

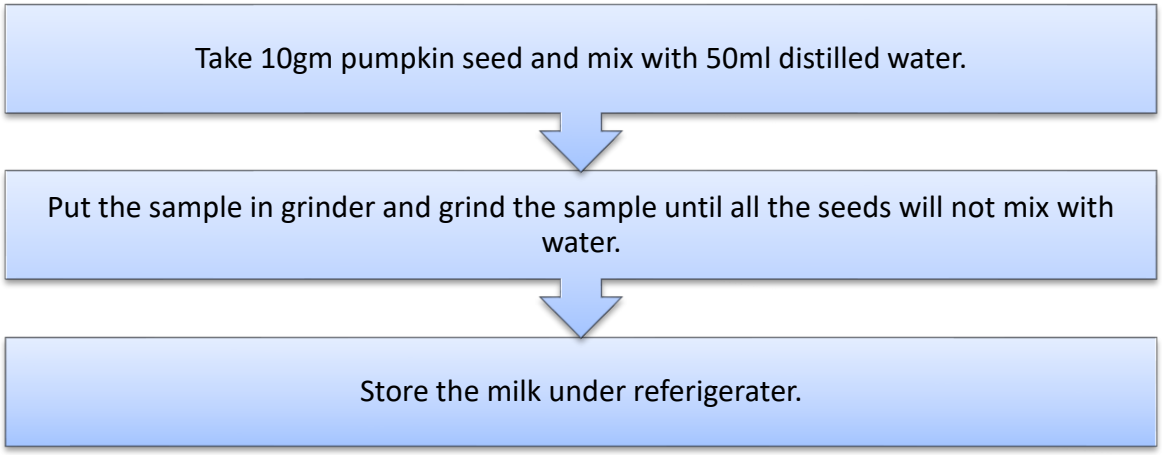
2. To determine the proteolytic activity of probioactive strains.

3. To analysis the Physico – chemical properties of extracted pumpkin seed milk.

4. To determine the applications of extracted bioactive peptides.

Research methodology CHAPTER-5

Preparation of pumpkin seed milk:



Nutritional analysis of pumpkin seed milk:

Fat estimation: Fat of pumpkin seed milk will be estimated by Gerber method.

Reagent required: Con Sulfuric acid

: Iso–Amyl Alcohol

: Pumpkin seed

Procedure: pumpkin seed milk extract mix with sulfuric acid and iso- amyl alcohol in a special Gerber tube. Tube is centrifuged and the fat climbs into the calibrated place of the tube which will measure as a percentage of the fat amount of the milk protein sample.

Moisture:

Moisture content was determined by the AOAC, (2000) procedure. Accurately weighed 5g milk sample were taken in petri plate and heated at 110±2 ^{0}C for 4 h in hot air oven. The plates were removed, cooled to room temperature over silica gel in a desiccator and weighed. The plates were heated again at 110 ± 2^{0}C cooled and weighed. The process of heating, cooling and weighing was continued till the difference in 2 successive reading was less than 1 mg. the moisture content was calculated from loss in weight of the sample on heating at 110 ^{0}C as indicated below.

Moisture content %= W_2-W_1/W×100

Where W_1 and W_2=weights of petri plates along with the sample before and after drying respectively and W=weight of sample.

Total ash:

Total ash was determined by the AOAC, (2000).

Procedure:

Dry ash was carried out by incineration of food samples at a very high temperature (550 ^{0}C) in a muffle furnace. Ash is equivalent to the mineral content of the food sample. Accurately weighed samples 3g were taken in a tarred silica dish and ignited over a low flame to char organic matter. After complete charring, the dishes were placed in a muffle furnace and heated at 550 ^{0}C for 3-4 h, till grayish to off white color ash was obtained. The silica dish containing ash

was cooled in desiccator and weighed. Percentage of total ash calculated as follows:-

Ash % = wt. of ash /wt. of sample ×100

Titratable Acidity:

Titratable acidity will be estimated by titrating a known volume of the sample against standard 0.1N NaOH solution by using phenolphthalein as an indicator up to the end point (pink color).

% Lactic Acidity = ml of .1NaOH used × .009 × 100/Wt. Of sample

pH:

pH will be measured with the help of ELTOP-3030 pH meter. Prior to pH measurement, the instrument will be standardized with the buffer solutions of pH 4, 7 and 9. The pH of the samples would be estimated directly.

Extraction of bioactive peptides using selective probiotic microorganisms:

Selective probiotic micro-organisms such as Lactobacillus helveticus, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus brevis used in the study for the extraction of bioactive components will

be acquired from Microbial Type Culture Collection (MTCC), Chandigarh, India. All procured strains will be kept at frozen condition in MRS agar broth with glycerol (15%).

The proteolytic potential of the above mentioned LAB strains will be examined by the methods applied by Beganovic et al., 2013 using skim milk agar for the assay. The well of the skim milk agar will be inoculated with a viable culture of probiotic strains and incubated at a temperature of 37°C for a period of 4h. The presence or absence of a clear zone around the inoculated agar well after the incubation period will be checked and recorded.

The dual plate assay method as described by Pescuma et al., (2010) will be used to check the compatibility of the bacterial strains with the proteolytic activity. The LAB strains with the highest proteolytic activities which are used for the extraction of bioactive peptides.

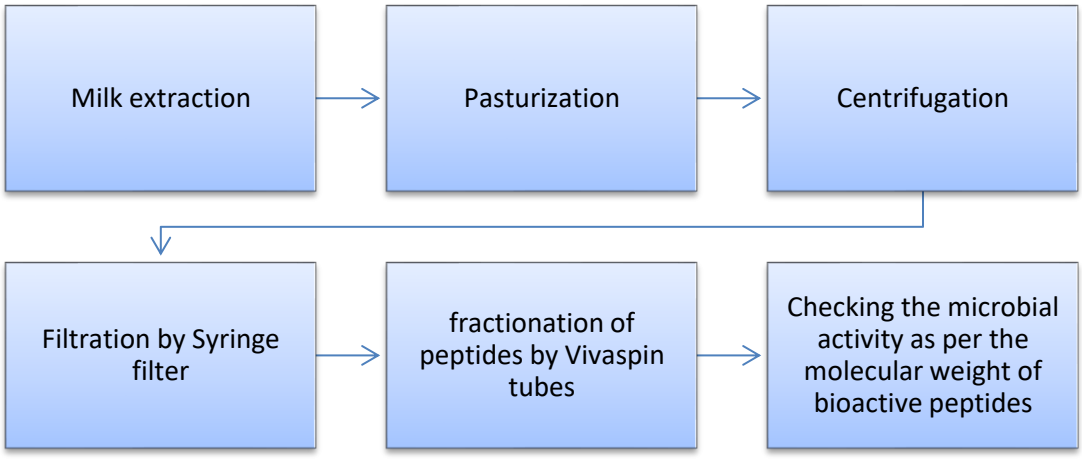
Pumpkin seed milk fermentation:

LAB culture mix with in equal volume of pumpkin seed milk and incubated at 37˚C for fermentation to take place.. during fermentation, at every 2h time interval, the pH, proteolytic activity, titrable acidity and total soluble solids will be checked.

Purification

After fermentation, the solution will be centrifuged at 10,000rpm for 10 mins and the supernatant passed through a syringe filter for purification.

Extraction of bioactive peptides will be done as per the method given by Singh et al., 2015.



Method for the determination of different potential activities from the extract bioactive peptides:

Method for the detection of antimicrobial activity: Antimicrobial activity of extracted peptide will be checked by using Agar Well Diffusion Method (Singh et al., 2015)

Nutrient agar 15-20ml place in petri plate and allow to be solidify under laminar air flow.

⇊

Nutrient agar overlaid with 7ml of soft agar inoculated with 100ul of overnight active culture of indicator strains (Pathogens).

⇊

Soft agar allows to be solidifying. Several wells were punched on the agar plate with sterile borer. Then plate refrigerated at 4˚C for 1 hour. Wells filled with extract bioactive peptide sample at a concentration of 100ul.

⇊

Plate need to be again refrigerated at 4˚C for 3-4 hours and then incubated at 37˚C for 24-48 hours.

⇊

The diameter around the wells measured and the clear zone of 1mm or more was considered positive inhibition.

Antioxidant activity:

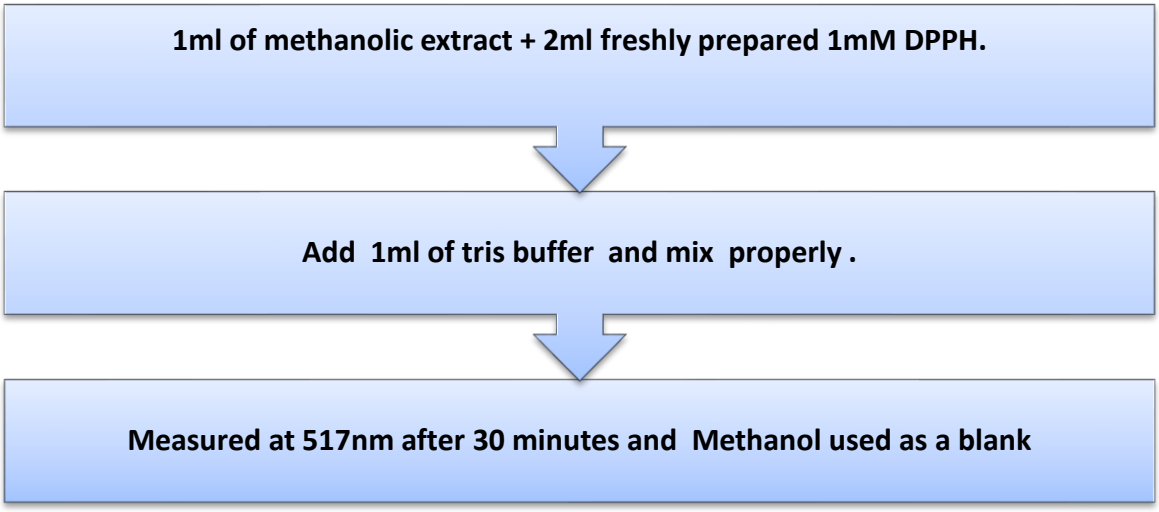
Take 5g sample and extract with 50ml of 80% methanol by refluxing it at 50-60˚C for 2 hours and filter the extract.

⇊

The residual re-extracted with an additional 50ml of 80% methanol for 1 hour.

⇊

Filter the extract. Collect the filtrate and make volume 100ml with 70% methanol.



DPPH radical scavenging activity (%) = $(A_0 - A_s)/A_0 \times 100$

A_0 - Absorbance of control at 517nm

A_s - Absorbance of sample at 517nm

Antihypertensive activity: ACE inhibitory activity will be checked by spectrophotometric (228 nm) method described by El-Fattah et al., 2016.

Take 50 ul extract and mix with equal volume of ACE solution.

⇓

The mixtures were pre-incubated at 37 C for 10 min in a heating block.

⇓

A 150ul of 4.15 mM substrate (i.e. hippuryl-histidyl-leucine in borate buffer containing 0.3 M NaCl, pH 8.3) was then added into the sample and incubated at 37˚C for 30 min. The reaction was subsequently stopped by adding 500ul of 1 M HCl.

⇓

Ethyl acetate (1.5 ml) was then added to extract the hippuric acid. The resulting mixture was vortex for 1 min and stood for 5 min.

⇓

A 800ul of ethyl acetate layer was then transferred into 2 ml microcentrifuge tube and vacuum dried in a vacuum concentrator at 45˚C for 30 min. After drying, 1 ml of deionised distilled water was added and vortex until the residual was fully dissolved.

⇊

The concentration of hippuric acid was determined using spectrophotometer at 228 nm.

Inhibition (%) = $(A_{Control} - A_{sample})/A_{Control} \times 100$

Control sample was prepared by replacing test sample with distilled water.

Antidiabetic activity: Antidiabetic activity will be checked as per the method described by Eckert et al., 2015.

The test sample (25 ul) was mix with an equal volume of the substrate Gly-Pro–p-nitroaniline (1.6 mM) at 37˚C for 10 min.

⇊

50uL mix sample mix with 0.1 M Tris–HCln buffer, pH 8.0 then incubated at 37˚C for 60 min.

⇊

The reaction was stopped by the addition of 100 ml of 1 M sodium acetate buffer, pH 4.0. The released p-nitroaniline as a hydrolysis product was measured at 405 nm.

= % inhibition of α-glucosidase and Dipeptidyl-peptidase-IV (DPP- IV) will be checked.

Results

Fat Content: Fat content estimated by Gerber method and the expected outcome is = 5.5%

Titratable Acidity:

The titratable acidity would be expressed as percent malic/citric acid (AOAC, 2004).

% Lactic Acidity = ml of .1NaOH used × .009 × 100 / Wt. Of sample

= 1.9 × .009 × 100 / 10

= .171%

Moisture Content :

Moisture content was determined by the AOAC, (2000) procedure.

Moisture content %= W_2-W_1/W×100

Where W_1 and W_2=weights of petri plates along with the sample before and after drying respectively and W=weight of sample.

S.No.	Initial Weight	After 1hour	After 30 min.	After 30 min.	Final weight	Weight of sample	% Moisture content
1.	41.37g	37.24g	37.22g	37.22g	4.15g	4.7g	88.29
2.	38.80g	34.40g	34.37g	34.36g	4.44g	5.03g	88.27

Ash Content: Total ash was determined by the AOAC,

Ash % = wt. of ash /wt. of sample ×100

S. No.	Initial Weight of Crucible (gm)	Weight of Sample	Final Weight of Crucible with Ash(gm)	Loss in Weight	% Ash
1.	22.57	5	**22.73**	.16	3.2
2.	21.34	5	21.51	.17	3.4

Protein Content: protein content determine by Kjeldahl method.

Volume used for the titration of blank = 1.3ml

Volume used for the titration of sample = 4.4 ml

Titer volume of sample = 4.4 – 1.3 = 3.1ml

Volume taken for digestion = .5ml

Total volume made for the digestion of sample = 50ml

Nitrogen content present in 50ml of digested sample = 3.1 × .00014 × 50 / .5

% Nitrogen content = 3.1 × .00014 × 50 × 100 / .5 = 4.34%

% Protein content in sample = 6.25 × 3.1 × .00014 × 50 × 100 / .5 = 27.125%

pH: pH will be measured with the help of ELTOP-3030 pH meter. Prior to pH measurement, the instrument will be standardized with the buffer solutions of pH 4, 7 and 9. The pH of the samples would be estimated directly.

ph: 5.01

Expected outcome CHAPTER-6

Extraction of bioactive peptides by probiotic microorganisms from pumpkin seed and check their applications to enhance their nutritional value.

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