BIOACTIVITIES OF PEPSIN HYDROLYSATES DERIVED FROM SEED PROTEIN OF MORINGA OLEIFERA

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Abstract: Plant derived source are well known folkculture because of it's medicinal values.Now recent research has showed plant derived source shows less side effects when compared to pharmaceutical drugs. Recent studies has strictly pointed out that plant derived(neutraceutical) drugs are more effective and has less side effects on various life threatening pharmacological targets including cancer, HIV/AIDS, Alzheimer's, hypertension, diabetes, infectious diseases, malaria, and pain than pharmaceutical drugs. This research completely indulged on bioactivity of peptides derived from seed proteins of moringa oleifera which is well known for it's high nutritional values, medicinal properties,cost efficiency and availability. Analysis of the isolated protein and pepsin hydrolysates by SDS-PAGE with Comassie Brilliant Blue staining showed proper protein/peptide bands. Hence, the protein/peptide nature of the material had to be confirmed and estimated by Lowry's methods.The antibacterial activity of crude protein extracts showed the ability of inhibition in pathogens like Pseudomonas,Aeromonas,Kleibsiella and E.coli by varying inhibition at different concentrations.The antibacterial activity of crude protein extracts showed the ability of inhibition towards pathogens like Pseudomonas,Bacillus by varying inhibition diameters with extracted buffer kept as control where as and there is no activity against Klebsiella and E.coli.The results of 50% fractionated protein sample ruled out any chances of these protein to possess antibacterial activity against the above pathogens.Bacillus and Pseudomonas showed the zone of inhibition with a diameter of 13mm for crude protein with a concentration of 500µg/gm and on decreasing concentration it showed decreased diameters.So far analysis well established antibacterial activities of crude seed protein of moringacea,so that it was subjected to study Minimum inhibitory concentration and Minimum bactericidal concentration to check whether the mode of action is bacteriocidal or bacteriostatic.These findings strongly suggest that the moringaoleifera seed proteins have the potential for use in drug formulations for the treatment of microbial infections.
The Antihypertensive or ACE-inhibitory activity of pepsin hydrolysates is generally concentration-dependent. Therefore, protein digests were prepared by pepsin at 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0%, levels (w/w) each, separately and ACE-inhibitory activity was assayed. All the percentage of digests showed the inhibitory activity,the maximum being 68% and 62% for crude and dialysed pepsin digest(4%) respectively .For crude pepsin digest the activity increased steadily from 21% to 68% with 0.5 through 4.0% which then decreased with higher concentrations of the enzyme.like crude dialysed pepsin digest,the activity increased steadily from 49%to 62% with 0.5 through 4.0% then it is decreased.The presence of ACE-inhibitory activity obtained in the in vitro analysis very much correlated with the in silico analysis results since the in silico method has indicated the presence of ACE-inhibitory peptides in moringaoleifera seed proteins. These findings strongly suggest that the hydrolysates of moringa seed proteins have high potential for use in drug formulations for treatment of hypertension and ageing process, and crude protein for the drug formulation against microbial infection. However, more substantial research data have to be generated before getting into any solid conclusions.

Introduction: Plant derived source are well known folkculture because of it's medicinal values. Plant derived medicines and herbal remedies were used in ancient times. Now recent research has showed plant derived source shows less side effects when compared to pharmaceutical drugs. Recent studies has strictly pointed out that plant derived(neutraceutical) drugs are more effective and has less side effects on various "Life Threatening" pharmacological targets including cancer, HIV/AIDS, Alzheimer's, hypertension, diabetes, infectious diseases, malaria, and pain than pharmaceutical drugs.*Moringa oleifera* is nature's miracle tree, known popularly as *drumstick tree*, is a tropical plant grown for its nutritious leafy-greens, flower buds, and mineral-rich green fruit pods. It is a well-recognized member in the *Moringaceae* family of trees, and thought to be originated in the sub-Himalayan forests of the Indian subcontinent. It possesses horse radish-like root and, hence, known to the western world as horseradish tree. Their young, tender seed pods are popular as *murnga* in Tamil, and *malunggay* in Philippines.Moringa is a drought tolerant, medium-sized, evergreen tree that prefers warm, frost-free climates to flourish. Its tender leaves and twigs can be harvested at any time

from a well-established, 1.5 to 2 meters tall plant. Taller plants bear cream-white, small size flowers in clusters throughout the season, which subsequently develop into long slender dark-green, three-sided, edible seedpods with tapering ends. Each pod measures about 6-18 inches in length with constrictions at the seed nodes giving them a typical drumstick-like appearance. Inside, each pod features fleshy pulp and round pea-sized seed encased inside a wing-shaped coat (hull).Fresh tender leaves, flowers, tender pods, and seed-kernels of moringa are edible(Ali *et al*., 2004).*Moringa oleifera* is the most nutritious tree found on the earth. Moringa tree grows in tropical conditions and this tree is native to India and Africa.Moringa tree thrives in dry conditions with very little amount of water. Nowadays Moringa is getting popular world wide as a nutrition supplements because of its very high contents of vitamins and minerals.Moringa tree is cultivated either through its seeds or stem. The benefit of the Moringa tree is that, all the parts of the tree are used and nothing goes as waste.The Moringa tree bears very delicious fruits, commonly known as "Drumsticks" and they form a very important role in Indian cuisines ad they are also very much nutritious. The dried Moringa Pods (drumsticks) bears seeds and these seeds when crushed provide the wonderful Moringa Oil. Moringa Oil is used as a major ingredient in the cosmetic industry. The Moringa seed Powder is a rich source of plant based natural protein.Moringa seeds are large and circular-shaped, and grow inside the lengthy pods of the *Moringa oleifera* tree. The pods can reach well over a foot in length and each pod can provide over a dozen large Moringa seeds. This seeds have two sets of thin flaps extending from the main kernel of the seeds. These flaps serve as wings to carry the seed away from the mother tree, and with the help of the wind, they move across the ground until they find a resting place to germinate.Unlike the fast-growing leaves of the *Moringa oleifera* tree, the seed pods do not grow back every few months. Moringa trees produce seed pods on an annual basis, much like other similar species in the plant kingdom. And as is the case with other healing plants, it is always worth the wait for the trees to produce their seed pods.Moringa trees give off incredible volume of seed pods during their reproduction months. An average-sized tree of fifteen to twenty feet in height can produce hundreds or even thousands of seed pods, yielding countless seeds each and every year. Fresh Moringa seeds are usually quite soft and yield with strong pressure. As Moringa seeds dry out, they harden until they resemble a dried bean or pea.If the moringa seeds are to be used for oil extraction, the seeds are harvested and immediately processed. The fresh, soft seeds are broken into pieces and heated with water, and then they are pressed for oil. Seeds that are cold-pressed can produce up to 40% oil by weight.If moringa seeds are not harvested for oil; they are most likely to be used as food or in cultivating further crops. Moringa seeds are a popular table food in many cultures around the world. The seeds can be steamed or boiled, either in the pod or shelled, much like peas or green beans. They can also be seasoned and roasted as a snack food. They are packed with nutrients, making them as popular as moringa leaves in many household meals and recipes.

*Moringaoleifera*have been reported to possess pharmacological activities such as antidiabetic, antioxidant, anti-ulcer, anti-venom, antibacterial etc. These arebioactivities,and the factor benind bioactivity is bioactive peptides(BAPs). BAPs are the active component of a protein. When separated from the protein, they act as their specific function would normally do if they were still attached to their larger protein. Since BAPs are low in molecular weight and do not carry the excess parts of their larger protein counterpart, they are rapidly absorbed into the bloodstream. From there, depending on their function, they either enter the cell directly or move to their target site or they reside on the cell itself and perform their job on and between the cells. Some act to start a pathway or process; others act as signal mechanisms; and some simply relay or convey information to other parts of a cell. Antimicrobial peptides and proteins (AMPs) are a diverse class of naturally occurring molecules that are produced as a first line of defense by all multicellular organisms. These proteins can have broad activity to directly kill bacteria, yeasts, fungi, viruses and even cancer cells. Insects and plants primarily deploy AMPs as an antibiotic to protect against potential pathogenic microbes, but microbes also produce AMPs to defend their environmental niche. In higher eukaryotic organisms, AMPs can also be referred to as 'host defense peptides', emphasizing their additional immunomodulatory activities. These activities are diverse, specific to the type of AMP, and include a variety of cytokine and growth factor-like effects that are relevant to normal immune homeostasis. In some instances, the inappropriate expression of AMPs can also induce autoimmune diseases, thus further highlighting the importance of understanding these molecules and their complex activities. Many types of antihypertensive peptides that inhibit angiotensin I, angiotensin I converting enzyme (ACE) and Ang II type 1 receptor (AT_1) in the cardiovascular system contribute to the prevention and treatment of hypertension. These inhibitory peptides are derived from many food proteins or artificial synthetic products. Further research examining the bioavailability of ACE inhibitory peptides will lead to the development of more effective ACE inhibitory peptides and foods.There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs.Antioxidants are

compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs; cosmetics and pharmaceuticals.Antioxidants are involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals. In this study we are going to check whether morninga seed protein have the bioactivities such as antihypertensive,antioxidant and antimicrobial.Certain BAPs are correspond to these activities,that are antihypertensive peptides,anti oxidant peptides and anti microbial peptides.

Materials and Methods: *Moringa oleifera* seeds collected from mature *Moringa oleifera* were purchased from local market of Parappanangadi; Malappuram.Goat lung was obtained from the local market of Chelari, Malappuram, Kerala state.

Preparation of Moringa Seed Powder: *Moringa oleifera* seeds were collected and dehulled manually in the order to remove seed coat. seeds were dried in the shade and powdered mechanically.Powdered seed were defatted using the solvent n-Hexane in soxhlet apparatus for 12 hrs. 12.5g of seed powder in 25 ml of n-Hexane (Munirat A. Idris,2013).Protein content in the extracts obtained with different solvents estimated by the standard Lowry's method(Lowry 1951). Moist Test was done using desiccators.The Blue silica gel in the space below the platform is used as the desiccant. The sample was taken in petriplate and measured with and without sample and after 24 hours when sample was taken out. Moist content of *Moringa oleifera* seed meal obtained was 0.1%.

Isolation of Total Protein: The extraction method was standardised by using different solvents such as 0.1 M Phosphate buffer,0.1 M TrisHCl buffer under varying pH and temperature.The bulk extraction method was done by using phosphate buffer, pH 7.3 at room temperature. 10g sample of defatted seed powder was taken in 100 ml of extractant for 12 – 16 hrs.Then centrifuged at 10,000 rpm, 4^{o}C for 10 minutes and the supernatant were collected.The proteins were precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 50% (w/v). The precipitate was obtained after centrifugation (6000 rpm, for 10 minute at 4^{0}C).The precipitated proteins were dissolved in water was dialyzed extensively against distilled water; freeze dried and stored at 4 °C until further use.

Different Solvents Used for Extracting Protein from *Morinaga Oleifera* Seed Powder at Varying pH Values

Sl. No.	Solvents	pH
1	**0.1 M Phosphate buffer**	**7.1, 7.3, 7.7,8.0**
2	**0.1 M Acetate buffer**	**4, 5, 5.6**

Antimicrobial Activity: Antimicrobial activity studied in the Moringa seed protein to understand the activity of protein against common pathogens.

Materials Required: Mullen Hinton Agar,Crude protein sample,Microbial Type Cell Cultures (MTCC) of *E. coli, Pseudomonas* spp*, Klebsiella*spp*., and Bacillus.*

Methods:
Agar Well Diffusion Method: The bacterial culture of each organism spread using sterile cotton swab on a sterile petridish with MH agar.20µl of 500,400,300,200 µg/ml of Moringa seed protein extract were added to each of the 5 wells including phosphate buffer as control.Plates were incubated for 24 hours at 37 degree Celsius.After incubation inhibition of bacterial growth was measured in millimeter.

MBC: This was determined by collecting a loopful of broth from the clear test tubes that were used for MIC determination.Then, it was inoculated on sterile MH agar. The plates were incubated at 37 degree Celsius for 24 hours. After the incubation, the concentration with no visible bacterial growth on the solid agar medium was noted as the minimum bactericidal concentration.

Mode of Action: The mode of action of the processed *Moringa oleifera* seed extract(crude protein) was calculated using the ratio of MBC/MIC as described by (Konate*et al*.,2012) to evaluate if the observed

antibacterial effect was either bactericidal or bacteriostatic. If the ratio of MBC/MIC was less or equal to two, the effect is considered as bactericidal or otherwise bacteriostatic.

MIC: The minimum inhibitory concentration of seed protein was studied by conducting a tube dilution method using Mullen Hinton broth media(Penna*et al*., 2001). This is the lowest dilution of the antibacterial agent that inhibits the growth of microbes, which is judged by lack of turbidity in the tube.

MIC Protocol: Initial concentration of protein 500µg/ml was diluted to 450,400,350,300,250,200,150,100,50µg/ml and added to 4ml of MH broth distributed in each test tubes.All the tubes were inoculated with 40µl of bacterial suspension and incubated for 24hrs at 37 degree Celsius. After incubation, the tubes were examined for any visible trace of growth. The tube in series with no visible growth was taken as the MIC.The results were expressed in microgram per millilitre.

***In Silico* Analysis of *Moringa Oleifera* Protein Sequences:** Available protein sequences of *Moringa oleifera* (globulin, major seed storage protein) were retrieved from the Swiss-Prot and TrEMBL databases at the ExPASy Molecular Biology Server (http://www.expasy.org/sprot) and National Center for Biotechnical Information (NCBI) at the National Library of Medicine protein database (http://www.ncbi.nlm.nih.gov). The sequence entries were then subjected to *in silico* analysis for biological activity using public domain sequence antihypertensive activity analysis tool, BIOPEP (www.uwm.edu.pl/biochemia) which is developed as a strategy suitable for determining the profile of potential protein fragment bioactivities (Dziuba *et al*., 1999).

Preparation of Goat Lung Ace: ACE was extracted from Goat lung acetone powder. One gram of acetone powder was extracted with 10 mL of 0.1 M Tris-HCl buffer(pH 8.2) containing 0.3 M NaCl and 0.5% Triton X at 4 ˚C for 16-18 h.The extract was centrifuged at 10000rpm for 60 min at 4 °C. The supernatant was precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 50% (w/v). The precipitate obtained after centrifugation (6000 rpm, for 10minute at 4^0C). The precipitated enzyme were dialyzed against the same buffer minus Triton X-100 for 24 h and stored at –20˚C until used.

Enzymatic Hydrolysis of Protein: 0.1g enzyme + 1 mL of buffer digested using an enzyme substrate ratio of 1, 2, 4, 6, 8 and 10 % (w/w) at 37 °C for 1 h.The hydrolysis was terminated by heating in a boiling water bath for 5 min.These hydrolysates were used as the source of ACE inhibitor peptides.

SDS-Polyacrylamide Gel Electrophoresis (SDS-Page):

Reagents:

- 30% Acrylamide: Acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved in water (100 mL), filtered and stored in a dark brown bottle at 4°C.
- 4× Separating gel buffer (1.5 M, pH 8.8):Tris (18.15 g), was dissolved in water, the pH of the solution was adjusted to 8.8 with HCl (6N), the volume made up to 100 mL and stored at 4 °C.
- 4× Stacking gel buffer: (0.5 M, pH 6.8):Tris (6 g) was dissolved in water. The pH of the solution was adjusted to 6.8 with HCl (6 N), volume made up to 100 mL with water and stored at 4°C.
- 10% Sodium dodecyl sulfate: SDS (10 g) was dissolved in 100 mL water.
- 10% Ammonium persulfate: was freshly prepared by dissolving 50 mg in 0.5 mL of distilled water.
- 10× Tank buffer: (0.25 M Tris, 1.92 M Glycine): Tris (3.0 g), Glycine (14.41 g), 0.1g SDS were dissolved in 100 mL of water.
- Staining solution:Coomassie brilliant blue R-250 (0.2 g) was dissolved in a mixture of methanol: acetic acid: water (25:15:60 v/v). The reagent was filtered and stored at 25±2°C.
- Destaining solution: Methanol: acetic acid: water (25: 15: 60, v/v).
- 2× Sample buffer: It was prepared in solution C diluted 1:4, containing SDS (4% w/v), β-mercaptoethanol (10% v/v), glycerol (20% v/v) and bromophenol blue (0.1% w/v).

Preparation of Separating and Stacking Gel: The contents of separating gel were mixed, degassed and poured between the assembled glass plates with edges sealed with agar (2% w/v). The gel was layered with 0.5 mL of distilled water and allowed to polymerize at 25 ± 2 °C for 30 min. The contents of stacking gel were mixed and poured above the polymerized separating gel. The gels thus prepared were of the size 10.5 × 9 cm and thickness 0.8 mm. Samples were loaded into the wells immersed in 1×tank buffer (25 mMTris, 192 mM glycine and 0.1% SDS) solution and run at a voltage of 70V for stacking and 90V for separating gel for 1-2 hrs or

until the tracking dye, reached the anode tank buffer.Broad range protein marker (molecular weight of 10-250kDa)ofmerck was used.

Staining: The gels were stained for protein with coomassie brilliant blue for 6 h at 25 ± 2 °C and destained. Polyacrylamide gel electrophoresis under non-denaturing condition was also carried out to evaluate the purity of moringa seed protein. Separating gels was prepared without SDS.

In Vitro Colorimetric Assay of Ace And Ace Inhibitor Activity (Zhang *Et Al.,2009):* A 50 µL aliquot of a sample solution (Enzyme hydrolysate) and 50 µL of ACE solution were added to 50 µL of a 5 mmol/L substrate,hippuryl-histidyl-leucine(HHL) solution in 1 mol/L phosphate buffer at pH 8.3. After incubation at 37 °C for 30 min, the reaction was stopped by adding 150 µL of 1 mol/L HCl.The liberated hippuric acid was extracted with 1 mL of ethyl acetate. The mixture was centrifuged and 0.5mL of the organic phase (ethyl acetate) was transferred to a fresh test tube and evaporated to dryness in a water bath at 100°C. The residue containing hippuric acid was dissolved in 3 mL deionised water and the solution was measured using a UV visible spectrophotometer at 228nm against deionised water as the blank.

DPPH Radical Scavenging Activity (Shimada *et al.*, 1992):
Principle: The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present).

Reagents:
1. 0.2 mM DPPH
2. 80 % Methanol
3. Ascorbic acid (Standard)

Procedure: Various concentrations of plant extracts (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.1 mM. The mixture were shaken vigorously and left to stand for 30 min and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The percentage of DPPH decolourization of the sample was calculated according to the equation:

$$\% \text{ of decolorization} = \frac{1 - \text{Abs sample}}{\text{Abs control}} \times 100$$

The % of decolorization was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC50 (inhibitory concentration 50, µg/ml) which is the amount of sample necessary to decrease by 50 % the absorbance of DPPH.

Result:
***In Silico* Analysis of *Moringa Oleifera* Seed Protein Sequences for Antihypertensive Activity and Antioxidant Activity:** In order to explore the potential existence of encrypted antihypertensive and antioxidantpeptides in the primary sequence of *Moringa oleifera*seed proteins, an *in silico* approach was used. Available protein sequences of moringa were retrieved from the protein databases in NCBI. The retrieved sequences were analyzed with the biological activity analysis tool of BIOPEP (Dziuba *et al.*, 1999) for their potential antihypertensive activity. Below tables shows the moringa seed protein sequences with their antihypertensive di, tri and tetra, penta peptides highlighted as red colour, bold, underline and cross line respectively. The primary sequences of moringa seed protein subunits have potential ACE inhibitory peptides and antioxidant peptides. The *in silico* analysis revealed that there exists several 'hot spots' of potential ACE inhibitory activity and anti oxidant activity because there are several predicted peptides overlap each other.

Insilico Analysis of ACE Inhibitory Peptides in *Moringa Oleifera* Seed Protein:

Accession no.:AAK72809.1

VSTLEKKNLGRIAQII**GPV**LDV**AFP**PGKMPNIYNALVVKGRDTVGQQINVTCEVQQLLGNNRVRAVAMSATDGL
MRGMEVIDMGAPLSVPVGGATLGRIFNVL**GEP**VDN**LGPV**DTRTTSPIHKSAPAFIQLDTKLSIFETGIKVVDL**LAP**
YRRGGKIGLFGGAGVGKTVLIMELINNI**AK**AHGGVSVFGGVGERTREGNDLYMEMKESGVINEQNLAESKVA**LVY**
GQMNEPPGARMRVGLTALTMAEYFRDVNEQDV**LLF**IDNIFRFVQAGSEVSALLG
MPSAVGYQPTLSTEMGSLQERITSTKEGSITSIQAV**YVP**ADDLTD**PAP**ATTFAHLDATTVLSRG**LAA**KGIYPAVDP
LDSTSTM**LQP**RIVGEEHYETAQRVKQTLQRYKELQDIIAILGLDELSEEDRLTVARARKIERFLSQPFFVAEVFTGSP
GKYVGLAETIRGFKLILSGELDSLPEQAFYLVGNIDEATAKATNL

Accession no.:AFQ13429.1

TETKASVGFKAGVKDYKLTYYTPDYETKDTDI**LAA**FRVTPQPG**VPP**EEAGAA**VAA**ESSX**GTW**TTVWTDGL
TSLDRYKGRCYH**IEP**IAGEENQFIAY**VA*YPLDLF***EEGSVTNMFTSIVGNVFGFKALRALRLEDLR**IPP**AY
SKTFQ**GPP**HGIQVERDKLNKY**GRP**LLGCT**IKP**KLGLSAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRW
RDRFLFCAE**AIYK**AQAETGEIKGHYLNATXXXXXXMIKRAVFARELGVPIIMHDYLTGGFTANTSLAHYC
RDNGLLLHIHRAMHAVIDRQKNHGIHFRVLAKALRLSGGDHIHSGTVVGKLEGEREITLGFVDLLRDDFI
EKDRSRGIFFTQDWVS**LPGVLP**VASGGIHVWHMPALTE**IFG**DDSVLQFGGGTLGHPWGNAPGAVANRVAL
EACVQARNEGRDLAREGNEIIREASKWSPE**LAA**ACEVWKEIKFEFPAVDTL

Accession no.: BAP76192.1

MLRIKRVPTVVSNYQKEEGEEGARREGGCGRNCLNKCCIQGAKIPLY**VFK**GLNKTGGSKGVLGHEN**GEP**P
VAFLESLLLGEWEDRSERGLFRYDVTACE***TKVIP***GDYGFIAQLNEGRHLKKRPTEFRVDKV**LQP**FDGNKF
NFTKVGQEEVLFQFEASEDGEVQFFPSAPIDVENSPSVVAIN**VSPIEY**GHVLLIPRVLECL**PQR**IDRDSF
LLALHMAAEAGNPYFRLGYNSLGAFATINHLHFQAYYLAMPFPVEKAPTKK**ITT**TDGGVRISELLNYPVR
SLVFEDGETVQDLSNTVSDACICLQNSN**IPY**NVLIADCGNRVFVFPQCYAEKQALGE**VSP**ELLDTQ**VNP**A
VWEISGHMVLKRKKDYEEASEENAWRLLAEVSLSEERFREVSALIFEAIACSENGEASNEQSSVNKNVHA
IKKSSHSAIVTGTQEC**LVL**Q

Insilico Analysis of Antioxidant Peptides in Moringa Seed Protein:

Accession no.: ADU05538.1
DFEP**VKP**YEVPMTAAGALQSYKLAA**KAI**TRLQSLPSGSIERLCDTMVQEVFELTGYDRVMAYKFHDD**DHG**EVISE
ITKPGLEPYLG**LHY**PATDIPQAARFLFMKNKVRMIVDCHAKHIKVLQDEKLPFDLTLCGSTLRAPHSCHLQYMEN
MNSIASLVMAVVVNEGDEETDGANPVQPQKRKR**LWG**LVVCHNTTPRFVPFPLRYACEFLAQVFAIHVNKELELE
NQIVEKNILRTQTLLCDMLMRDAPLGIVSQIPNIMDLVKCD**GAA**LLYKSK**LWR**LGVTPTDVQ**LHE**IASWLSDYH
MDSTGLSTDSLYDAGFPGALALGDVVSGMAAVRITSKDMLFWFRSHTAAEIRWGGAKHEPGEKDDGRKMHPRS
SFKAFLEVVKTRSLPWKDYEMDAIHSLQLILRNAFKDTKTVEFNTKTIHSKLNDLKLEGMQELEAVTSEMVRLIET
ATVPILAVDVNGLLNGWNTKISELTGLPVDEAIGKHLLTLVEDSSIDTVKKMLYMALQGKEEQDVQFQIKTFGSR
ADAGPISLVVNACASRD**LHE**NVAGVCFVAQDITGQKIVMDKFTRIEGDY**KAI**VQNHNPLIPPIFGADEFGWCTE

Accession no.: ARJ58797.1
MLGDGNEGMSTIPGFNQIQFEGFCRFIDQGLKEELYKFPKMEDTDQEIEFQLFVET**YQL**VEPLIKERDAVYESLTYS
SELYVSAGLIWKPSRNIQEQTIFIGNIPLMNSLGTSIVNGIYRIVINQILQSPG**IYYR**SELDHNGISVYTGTIISDWGGR
LELEIDKKARIWARVSRKQKISILVLSSAMGSNLREILENVCYPEIFLSFLTDKEKKKIGSKENAILEFYQQFSCVGGD
PIFSESLCKELQKKFFHQRCELGRIGRRNLNRRLNLNIPQNNTFLLPRDILAAADHLIGMKFGMGTLDDMNHLKN
KRIRSVADLLQDQFGLALVRLENVVKGTISGAI**RHK**LIPTPQNLVTSTPLTTTYESFFGLHPLSQVLDRTNPLTQIV
HGRKLSYLGPGGLTGRTANFRIRDIHPSHYGRICPIDTSEGINVGLIGSLAIHARIGHWGSLESPFYEIFEKSKKARML
YLSPSRD**EYYM**VAAGNSLALNQGIQEEQVVPARYRQEFLTIAWEEVHLRSIFSFQYFSIGASLIPFIEHNDANRALM
SSNMQRQAVPLSRSEKCIVGTGLERQVALDSGVPVIAEHEGKIIYTDTDKIILSGNGDTLNIPLVMYQRSNKNTCM
HQKPQVRRGKC**IKK**GQFLAD**GAA**TV**GGE**LALGKNVLVAYM**PWE**GYNFEDAVLISERLIYGDIYTSFHIRKYEIQT
HVTSQGPERITNEI**PHL**EGR**LLR**NLDKNGIVMLGSWVETGDILVGKLTPQVAKESSYAPEDR**LLR**AILGIQVSTSK
ETCLKLPIGGRGRVIDV**RWI**QKKGGSSYNPEMIRVYISQKREIKVGDKVAG**RHG**NKGIISKILPRQDMPYLQDGRP
VDMVFNPLGVPSRMNVGQIFECSLGLAGSLLD**RHY**RIAPFDERYEQEASRKLVFSELYEASKQTAN**PWV**FEPEYPG
KSRIFDGRTGDPFEQPVIIGKPYILKLIHQVDDKIHGRSSGHYALVTQQPLRGRSKQGGQRVGEMEVWALEGFGV
AHILQEMLTYKSDHIRARQEVLGTTIIGGTIPKPEDAPESFRLLVRELRSLALELNHFLVSEKNFQINRKEV

Accession no.: AFQ13429.1
TETKASVGFKAGVKDYKL**TYYT**PDYETKDTDILAAFRVTPQPGVPPEEA**GAA**VAAESSXGTWTTVWTDGLTSLDR
YKGRCYHIEPIAGEENQFIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALRALRLEDLRIPPAYSKTFQ**GPPHG**IQ
VERDKLNKYGRPLLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLFCAEAIYKAQAE
TGEIKGHYLNATXXXXXXMIKRAVFARELGVPIIMHDYLTGGFTANTSLAHYCRDNGLL**LHIH**RAMHAVIDRQK
NHGIHFRVLAKALRLSGGD**HIHS**GTVVGKLEGEREITLGFVD**LLR**DDFIEKDRSRGIFFTQDWVSLPGVLPVASGG
IHVWHMPALTEIFGDDSVLQFGGGTLGH**PWG**NAPGAVANRVALEACVQARNEGRDLAREGNEIIREASKWS**PE**
LAAACEVWKEIKFEFPAVDTL

Isolation of Protein: The total proteins of *Moringaoleifera* were isolated from defatted moringa seed flour usingo.1M phosphate buffer pH 7.3 at ambient temperature, since other extract showed less concentration of protein in Lowry's protein estimation method and followed 50% precipitation by ammonium sulfate. The salts in the filterate were later removed by extensive dialysis and this isolated protein was used to check antihypertensive, antioxidant, antimicrobial activity.

Standardisation of Protein Extraction: ***Effect of Extractants:*** The different extractants were used based on the methods reported by various workers have used to identify the extractant which give maximum yield of protein from moringa seed.
Different solvents used and the yield of protein obtained are depicted in Figure no. 4, 5, 6.

Table 4: Effect of Extractant

Sl.No	Extractant	Concentration of protein(µg/gm)
1	**0.1 M Phosphate buffer pH 7.3 at 28°C**	**588**
2	**0.1 M Acetate buffer pH 5.6 at 4°C**	**209**

EFFECT OF pH
As varying yields of protein were obtained with different solvents and under different physical conditions, it was decided to study the effects of conditions such as pH and temperature in detail to standardize the extraction procedure. The results obtained for the effect of pH are summarized in below table**.**Highest concentration of protein obtained at the pH 7.3.

Table 5: Effect of pH on the Protein Extractability by Different Solvents

Sl.No.	Extractant	pH	Concentration of Protein µg/gm
1	**0.1 M Phosphate Buffer**	**7.1** **7.3** **7.7**	**432** **588** **374**
2	**0.1 M Acetate Buffer**	**4** **5** **5.6**	**114** **164** **209**

Effect of Temperature: Effect of temperature on extraction of protein was studied using the above extractants at different temperatures. It was found that maximum protein was obtained at ambient temperature in the case of all solvents as compared to other temperatures.

Table 6: Effectof Temperature

Sl.No.	Extractant	Temperature	Concentration of protein. (µg/gm)
1	**0.1 M Phosphate Buffer**	**4°C** **28°C** **60°C**	**374** **588** **392**
2	**0.1 M Acetate Buffer**	**4°C** **28°C** **60°C**	**209** **200** **188**

Antibacterial Activity Studies of the Moringa Seed Protein: Antimicrobial proteins and protein derived peptides are an evolutionarily conserved component of the innate immune response and are found among all classes of life. Fundamental differences exist between prokaryotic and eukaryotic cells that may represent targets for antimicrobial proteins andpeptides. These crude proteins as well as peptides are potent, broad spectrum antibiotics which demonstrate potential as novel therapeutic agents. Antimicrobial proteins have been demonstrated to kill Gram negative and Gram positive bacteria (including strains that are resistant to conventional antibiotics), mycobacteria (including *Mycobacterium tuberculosis*), enveloped viruses, fungi and even transformed or cancerous cells. Unlike the majority of conventional antibiotics it appears as though antimicrobial protein may also have the ability to enhance immunity by functioning as immune-modulators.

The antimicrobial activity of milk is mainly associated with minor whey proteins, namely lactoferrin. This protein has bacteriostatic and bactericidal properties attributed to its ability to chelate iron or to bind to bacterial surfaces.

The antimicrobial activity of the crude protein of moringa seed were studied using the organisms *Escherichia coli, Pseudomonas spp, Klebsiella spp., Bacillus spp.* by well diffusion method. Antimicrobial effect showed against only *bacillus* and *pseudomonas*. Nevertheless inhibition was not observed against *E.coli* and *klebsiella*. These results ruled out any chances of these proteins to possess antibacterial activity against *E.coli* and *klebsiella.*

Antimicrobial Activity of 50% Protein Fraction: There is no antibacterial activity for protein fraction which could be precipitated by 50% ammonium sulphate.From this result, wecan point out that the antibacterial protein may get precipitated below this percentage.

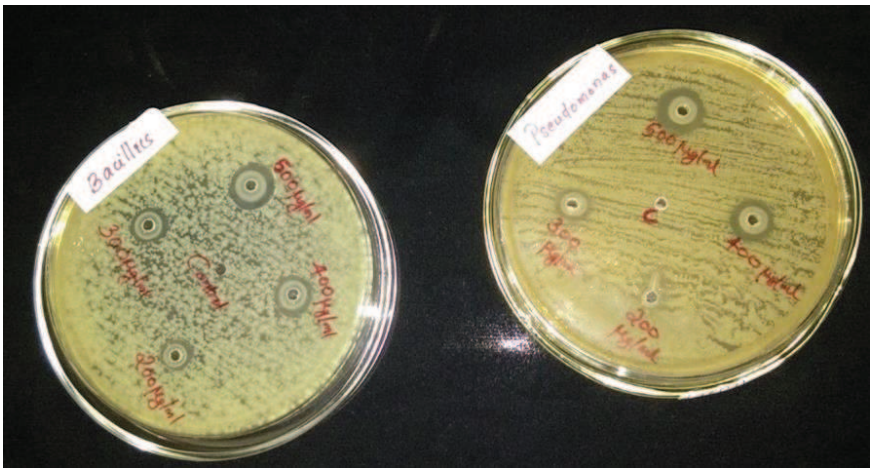

Fig.8: Antimicrobial Activity of Various Concentration of *M.Oleifera* Crude Seed Protein against *Bacilli* And *Pseudomonas* Against *Bacilli* And *Pseudomonas*

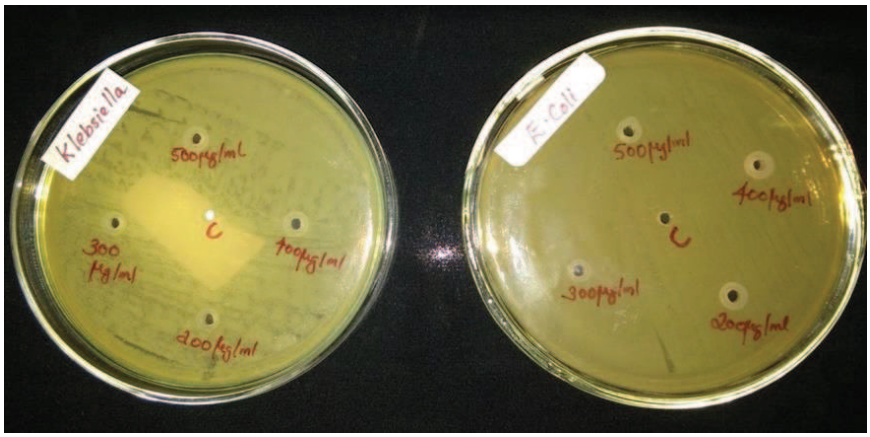

Fig.9: Antimicrobial Activity of Various Concentration of *M.Oleifera* Crude Seed Protein Against *Klebsiella* And *E.Coli*

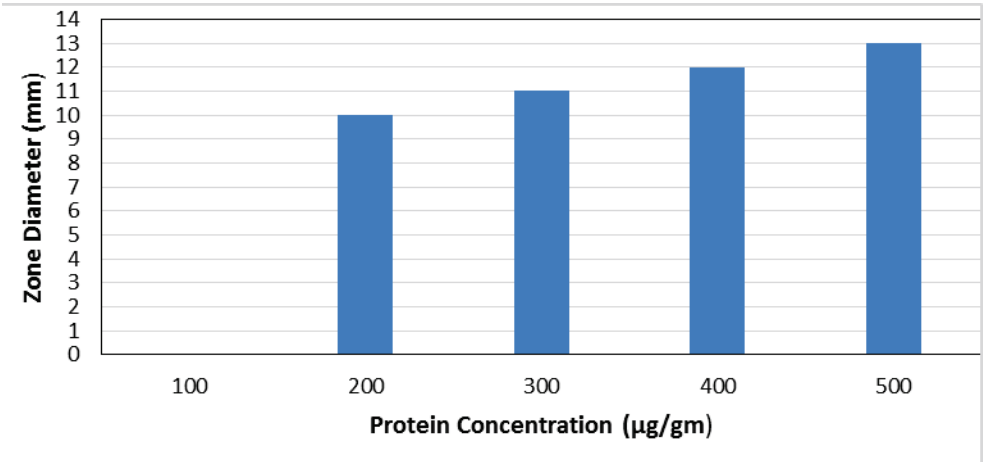

Fig.10: Antimicrobial Activity against *Bacillus spp*

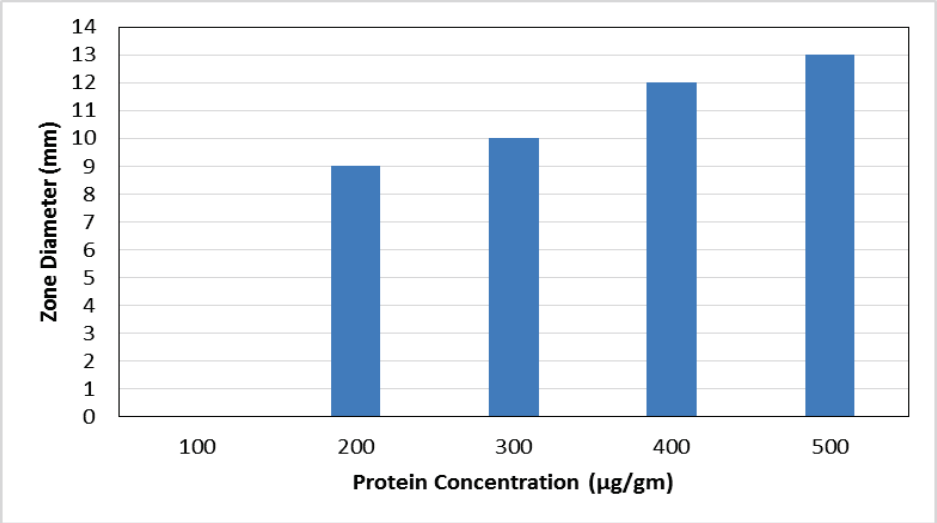

Fig.11: Antimicrobial Activity against *Pseudomonas spp*

Minimum Inhibitory Concentration (MIC): The results of MIC and MBC for the bacterial strains are shown in below table (table 7). The macrobroth dilution assayto determine the antibacterial activity showed that MIC of moringa seed extract was very effective against bacterial strains *bacillus* and *pseudomonas spp.*microbes. The MIC value obtained for *Bacillus s*was 400µg/ml while that of *pseudomonasaeruginosa*was obtained at 500µg/gm. The MBC values obtained for *bacillus* and *pseudomonasaeruginosa* are 500µg/gm and 500µg/gm respectively.

Table 7: Minimum Inhibitory Concentration

Sl.No	Name of Bacterial Strains	MIC(Minimum Inhibitory Concentration)(µg/ml)	MBC(Minimum Bactericidal Concentration)(µg/ml)	MBC/MIC	Action
1	*Bacillus spp.*	400	500	1.25	Bactericidal
2	*Pseudomonas spp.*	500	500	1	Bactericidal

Since the ratio of Minimum inhibitory concentration and Minimum bactericidal concentration of *Bacillus sp* is 1.25 and *pseudomonas* is 1, seed protein is having bactericidal action against both the organisms.

Antihypertensive Activity: ACE inhibitory peptides are present in almost all the amino acid sequences of several food proteins of both plant and animal origin . These bioactive peptides can be released by enzymatic

hydrolysis *in vivo* through gastrointestinal digestion or *in vitro* food processing. In consequence, these food protein derived ACE inhibitors represent natural, physiologically active food grade components, which provide health benefits beyond nutrition. Therefore these food proteins derived peptides as ingredients of functional foods could well contribute to mitigating the risk of cardiovascular and related diseases.

Moringa seeds are a good source of oleic acid, a health-benefiting monounsaturated fat. Moringa, as a high-quality oilseed crop, can be grown alternatively to improve nutrition levels of populations in many drought-prone regions of Africa and Asia.

This lead to took seed protein of moringa as ACE inhibitory source. The total proteins of *Moringa oleifera* seed was isolated and used as the starting material to derive ACE inhibitory peptides.

The total proteins were isolated from defatted moringa seed meal and dialysed seed protein. The total proteins were evaluated by SDS-PAGE. The profile suggested that majority of proteins were found in the low molecular weight region. Further characterizations, including sequencing techniques arerequired for these proteins. This isolated protein was used as the substrate for further hydrolysis.

***Moringaoleifera* Seed Protein Hydrolysis by Pepsin:** The combined action of physiological proteases is expected to release a large number of peptides. Using the gastrointestinal enzyme pepsin to simulate physiological condition, the digestion of moringa seed protein isolated using phosphate buffer. The release of ACE inhibitor peptides from the precursor sequence (moringa seed protein) is a prerequisite to validate the previous *in silico* prediction system. Therefore the ability of gastrointestinal pepsin to release ACE inhibitory peptides from moringa seed protein was studied. Isolated moringa seed protein was digested with pepsin to study the digestion pattern apart from the human gastrointestinal enzymes.Proper digestion pattern was showed by the peptides of protein extracted using phosphate buffer ph 7.3.

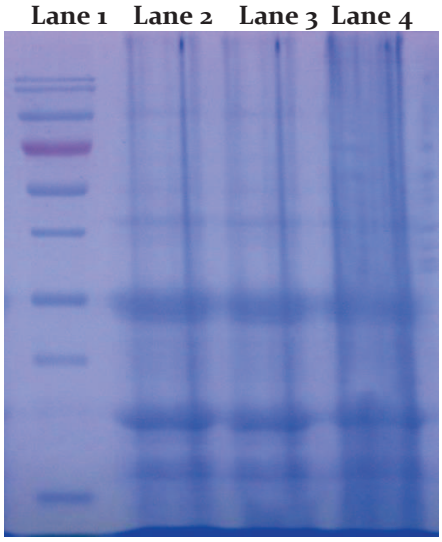

Fig.12 SDS-PAGE profile of moringa seed crude protein extracted in phosphate buffer ph 7.3 (Lane 1 - 10-180 Kd molecular marker, Lane 2 -Crude protein sample, Lane 3&4 - Crude protein sample diluted)

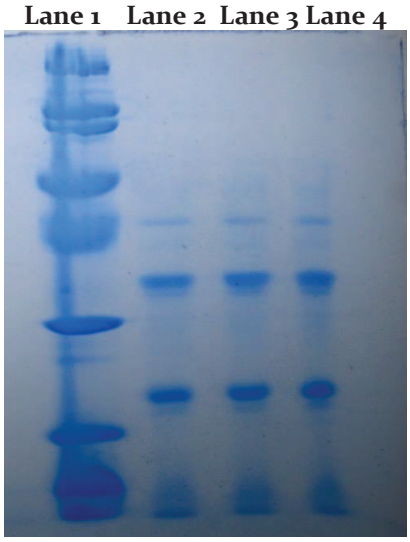

Fig.13 SDS-PAGE profile of moringa seed dialysedproteinextracted in phosphate beffer. (Lane 110-250 KD molecular marker, Lane 2 -dialysed protein sample, Lane 3&4 dialysed protein sample diluted.

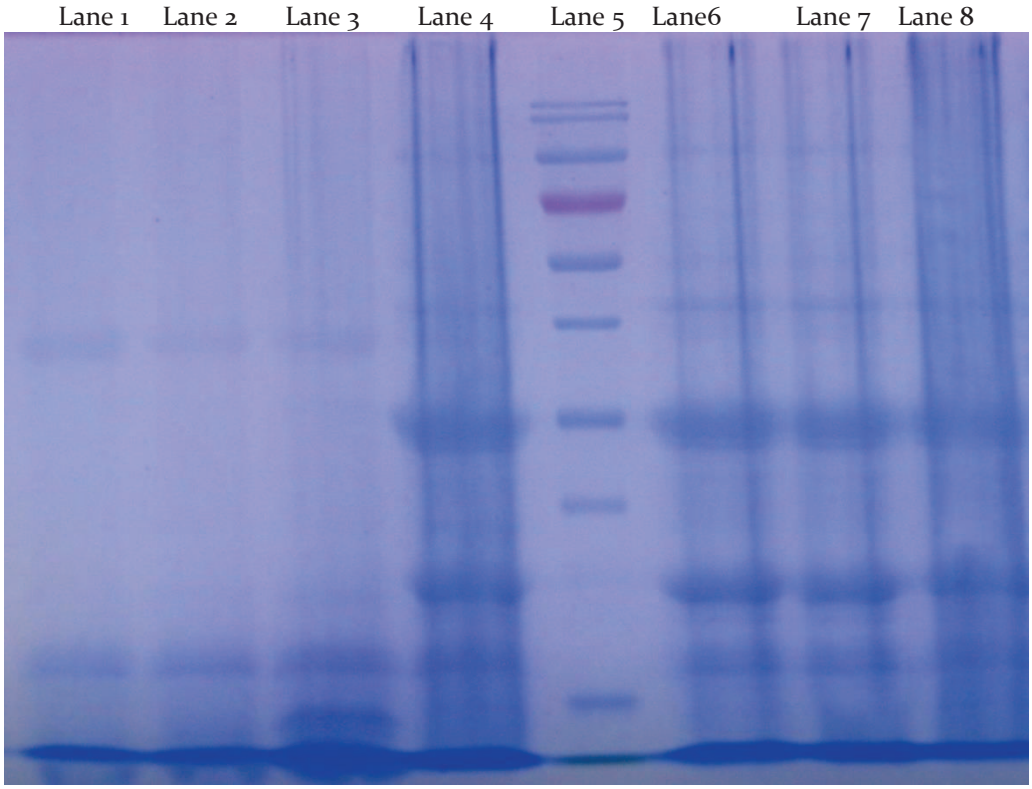

Fig.14 SDS-PAGE profile of moringa seed crude proteinextracted in phosphate befferand its digests with pepsin. (Lane 5 - 10-180 Kd molecular marker, Lane 6 -Crude protein sample,Lane 7&8 - Crude protein sample diluted, Lane 1 to 4- proteindigested with different conc. of pepsin(,8%,6%,4%,2%, respectively)).

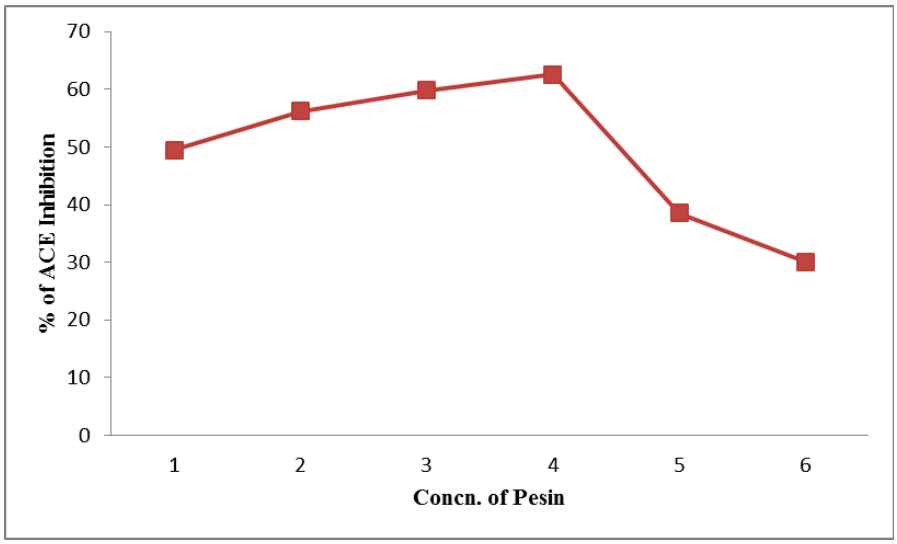

Fig.15 ACE-Inhibitory Activity of Dialysed Protein Hydrolysates of Moringa Seeds Prepared by Different Concentrations of Pepsin

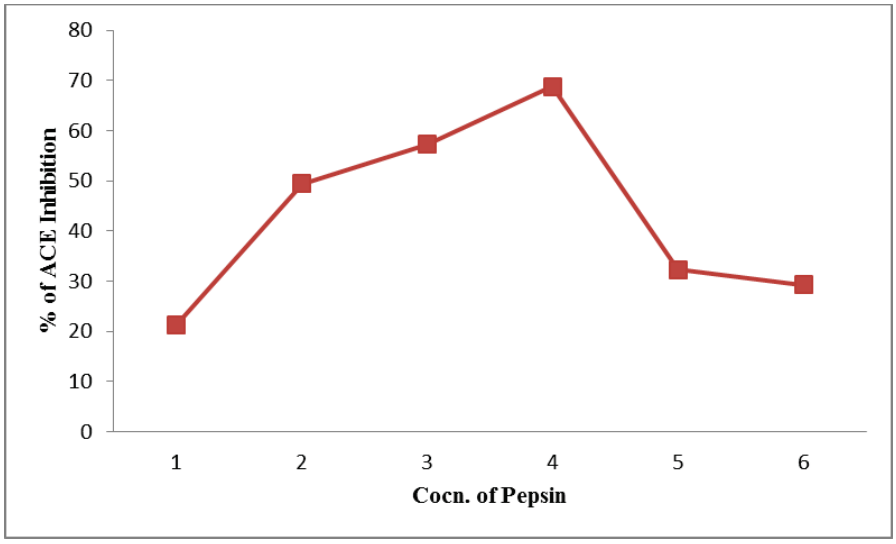

Fig.16 ACE-Inhibitory Activity of Crude Protein Hydrolyzates of Moringa Seeds Prepared by Different Concentrations of Pepsin

Isolated moringa seed protein was digested with enzyme protein to substrate protein ratios of 0.5 through 8% w/w.Seed protein was progressively degraded to smaller fragments when hydrolyzed by pepsin.During digestion, All the percentage of digests showed the inhibitory activity,the maximum being 68% and 62% for crude and dialysed pepsin digest(4%) respectively .For crude pepsin digest the activity increased steadily from 21% to 68% with 0.5 through 4.0% which then decreased with higher concentrations of the enzyme.like crude dialysed pepsin digest,the activity increased steadily from 49%to 62% with 0.5 through 4.0% then it is decreased.The demonstration that digestion with pepsin produces ACE inhibitor definitely shows that such peptides can be produced in vivo following an ingestion of moringa seed protein.

Antioxident Studies of *Moringa Oleifera:* Antioxidative profile of dialysed and crude protein sample of *Moringa oleifera* were studied by DPPH method.Antioxidants are our first line of defense and are critical for maintaining optimum health and well being. Antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules.Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by acting as oxygen scavengers.Antioxidant activities of bioactive peptides are mainly due to the presence of hydrophobic amino acids, some aromatic amino acids . The use of these synthetic antioxidants must be under strict regulation due to potential health hazards. Hence, the search for natural antioxidants as safe alternatives is important in the food industry.

The DPPH radical scavenging test is a sensitive antioxidant assay. DPPH radical is an oil –soluble free radical that becomes a stable product after accepting an electron or hydrogen from an antioxidant.This method is based on reduction of a stable free radical, DPPH to yellow coloureddiphenyl-picryl hydrazine. Any reducing agents that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption at 517 nm.

Protein hydrolysate serves as the main source of bioactive peptides and they become more active after the hydrolysis .Here crude protein hydrolysate of *Moringaoleifera* on treating with 4% pepsin shown maximum of 94.3% antioxidant activity and the least antioxidant activity shown in treating with 0.5 % of pepsin of 94 % and the dialysed protein hydrolysate treated with 10 % pepsin was shown maximum antioxidant activity of 95 % and minimum shown for treating with pepsin 0.5 % was 94.1%

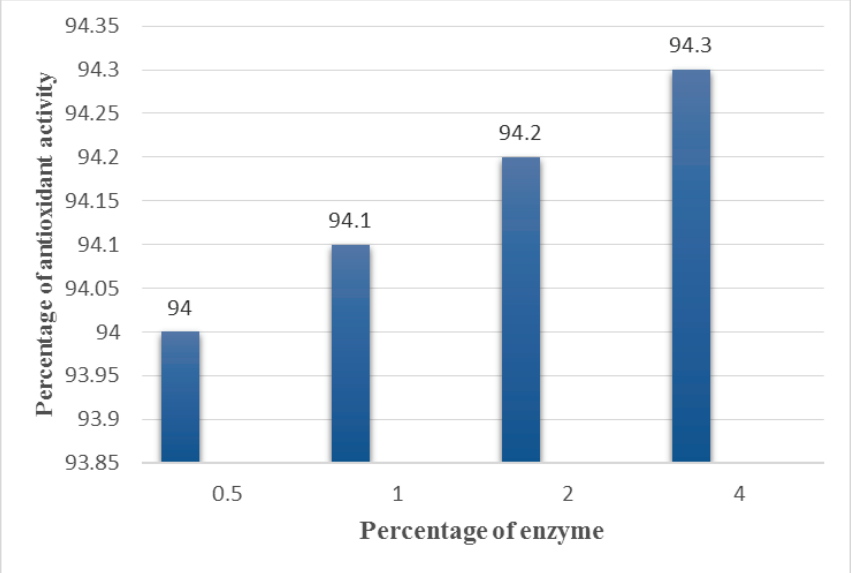

Fig.17Antioxidant Activity of Crude Pepsin Hydrolysate

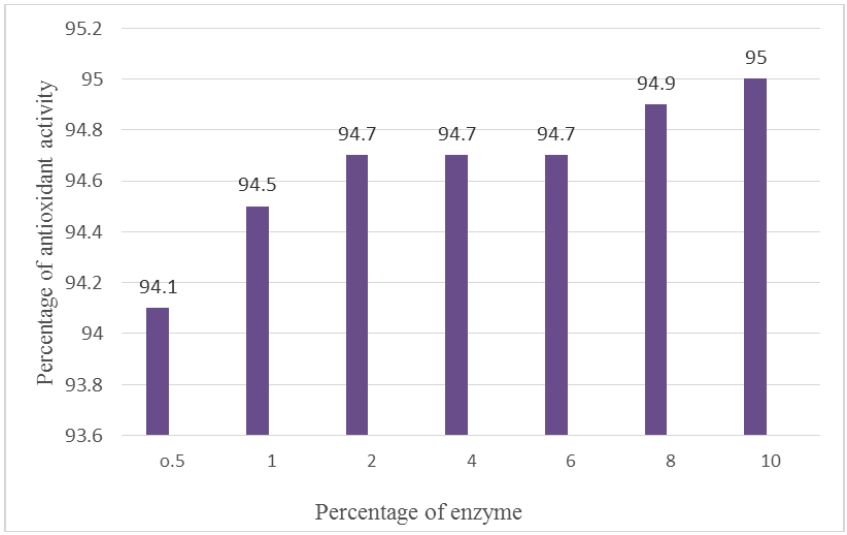

Fig.18: Antioxidant Activity of Dialysed Pepsin Hydrolysates

Discussion: *In silico* analysis of *Moringa oleifera* seed protein was retrieved from the protein databases in the NCBI. The retrieved sequences were analyzed with the biological activity analysis tool of BIOPEP. The one and only available protein sequence of *Moringa oleifera* was Albumin protein where its ACE-inhibitory peptides were reported and antioxidant also. So far no antimicrobial activity has been reported.
The extraction of crude protein of *Moringa oleifera* seed protein was extracted using phosphate buffer, acetate buffer of different temperature and pH conditions. Phosphate buffer pH-7.3 at 28°C gave maximum yield of 588µg per ml.

The antibacterial activity of crude protein extracts showed the ability of inhibition towards pathogens like *Pseudomonas,Bacillus* by varying inhibition diameters with extracted buffer kept as control whereas and there is no activity against *Klebsiella* and *E.coli*.The results of 50% fractionated protein sample ruled out any chances of these protein to possess antibacterial activity against the above pathogens.*Bacillus* and *Pseudomonas* showed the zone of inhibition with a diameter of 13mm for crude protein with a concentration of 500µg/gm and on decreasing concentration it showed decreased diameters.So far analysis well established antibacterial activities of crude seed protein of moringacea,so that it was subjected to study Minimum inhibitory concentration and Minimum bactericidal concentration to check whether the mode of action is bacteriocidal or bacteriostatic.These findings strongly suggest that the *Moringa oleifera* seed proteins have the potential for use in drug formulations for the treatment of microbial infections.

The Antihypertensive or ACE-inhibitory activity of pepsin hydrolysates is generally concentration-dependent. Therefore, protein digests were prepared by pepsin at 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0%, levels (w/w) each, separately and ACE-inhibitory activity was assayed. All the percentage of digests showed the inhibitory activity, the maximum being 68% and 62% for crude and dialysed pepsin digest (4%) respectively .For crude pepsin digest the activity increased steadily from 21% to 68% with 0.5% through 4.0% which then decreased with higher concentrations of the enzyme.Like crude dialysed pepsin digest, the activity increased steadily from 49%to 62% with 0.5 through 4.0% then it is decreased.

The digests of *Moringaoleifera*seed protein was shown a prominent antioxidant effect. Here crude protein hydrolysate of*Moringaoleifera* on treating with 4% pepsin shown maximum of 94.3% antioxidant activity and the least antioxidant activity shown in treating with 0.5 % of pepsin of 94 % and the dialysed protein hydrolysate treated with 10 % pepsin was shown maximum antioxidant activity of 95 % and minimum shown for treating with pepsin 0.5 % was 94.1 %.

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