



Full Length Article

Antidiabetic, Antibacterial and Antioxidant Potential of Bioactive Peptides Extracted from the Germinated Seeds of *Poncirus trifoliata*

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Abstract

The seeds proteins are converted into the peptides during the germination through peptidases. These bioactive peptides are fractionated through the enzymes. The bioactive peptides are reported with the number of therapeutic effects like antioxidant, antibacterial, antidiabetic, anti-cancerous and antihypertensive etc. Synthetic drugs have number of adverse effects along with the therapeutic effects, but natural sources are safer and free from this problem. In the present research, *Poncirus trifoliata* seedling peptides extracts were used to determine antibacterial, antioxidant and anti-diabetic activities. The peptides extract showed substantial growth of inhibition against pathogenic strains including *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* having the superior effect as compared to amoxicillin antibiotic as a standard drug. The IC₅₀ value of *P. trifoliata* seeds peptides extract was 32.91 µg mL⁻¹ as compared with standard (IC₅₀) value 25.52 µg mL⁻¹ of ascorbic acid showed good potential as antioxidant activity. The extracted germinated seeds peptides showed considerable antidiabetic activity in controlling the blood glucose level of alloxan induced diabetic rabbits to compare with standard drugs like glibenclamide. Thus, this study confirmed the antibacterial, antioxidant and antidiabetic effects of *P. trifoliata* seeds peptides is significantly higher as compared to pure drugs. Therefore, further complete separation and detailed investigation study is needed to isolate target peptides and revealed mechanism of action of bioactive peptides and formulation as a drug to replace the synthetics drugs with safe one. © 2021 Friends Science Publishers

Keywords: Alloxan induced diabetes; Antibacterial activity; Antioxidant; Bioactive peptides; Seedling; *Poncirus trifoliata*

Introduction

Proteins are vital ingredients present in foods and give a widespread variety of biological, nutritional and functional properties (Mazorra-Manzano *et al.* 2018). Peptides and bioactive peptides are differentiated on the bases of the therapeutic or regulatory effect. The peptides that have therapeutic and regulatory effects in the body are classified as the bioactive peptides. Bioactive peptides are defined as specific fragments of protein having an optimistic effect on function and condition of body (Walther and Sieber 2011; Kaur *et al.* 2020). These are biologically active organic substances having length of 2 to 10 amino acids joined together by peptide or amide bond, which is covalent in nature while proteins are composed of polypeptides having larger molecular weight (Moller *et al.* 2008; Fields *et al.* 2009). The sequence and composition of amino acids regulates the peptide activity when they are released after proteolytic action on proteins (Naveed *et al.* 2020). Proteins and their peptides play a significant role

in metabolism of living creatures and subsequently in human well-being. They show drug like and hormonal actions and further classification of these peptides based on their activities as antithrombotic, opioid, antimicrobial, antioxidative, anticancer, antihypertensive, and immunomodulatory agents (Fields *et al.* 2009). Now, in 'BIOPEP-UMB' database 4031 bioactive peptides have been reported (Minkiewicz *et al.* 2019).

Natural sources like plants, microbes, animals and their releasing products encompass innumerable proteins in them. In this era, Global scenario is moving towards utilization of renewable and natural resources material into value added commodities (Nadeem *et al.* 2019). *Poncirus trifoliata* belongs to the family of Rutaceae. Its native range is Central and Northern China. It is well-branched, deciduous shrub or small tree which typically grows 8–15' (less frequently to 20') tall. Three-lobed (trifoliolate as per the species name) leaves emerge yellowish-green in spring, turn glossy dark green in summer and fade to yellow in autumn (Tundis *et al.* 2016; Hall *et al.* 2018). Now in this work,

seeds of *P. trifoliata* were selected because at germinating stages genome expresses and produces a maximum amount of protein/peptides. Research was planned to access the pharmacological potential of these peptides isolated from germinating seeds.

Materials and Methods

Chemicals and collection of seeds

In this work, all chemicals were used as an analytical grade. The seeds of ripened fruit *P. trifoliata* were collected from District Sargodha (also known as the Kino capital of the World) Pakistan as shown in (Fig. 1). All seeds were rinsed with the 0.1 M sodium hypochlorite for 5 min and then washed with distilled water to remove the debris and dust particles.

Seed's germination

Poncirus trifoliata seeds take a long time for germination so for reduction of their germination time we removed seeds coating. Then soaked them in water for 5 to 6 h and then transferred them on the sterilized paper. *P. trifoliata* seeds were taken 10 to 15 days for growth at 25–30°C.

Extract preparation

Poncirus trifoliata seedling was started on the 10th day when the seedling became prominent. On the 12th day all seeds were collected and referred to the grinding using chilled pestle and mortar. Peptides were extracted by using tris HCl 0.1 Molar and 0.5 molar NaCl (pH 8) for 2 h and then supernatant was collected. The seeds by weight and volume of molar solution were used by 1:4 ratios. Then this supernatant was subjected to 12000 rpm for 20 min at 4°C and again the supernatant was collected for further processing (Costa *et al.* 2001).

Total Protein/Peptides content determination

To check the protein/peptides content Bovine serum albumin (BSA) was used as a standard by spectrophotometric method (Ali *et al.* 2016).

Antibacterial activity

The disc diffusion method was used to check the antibacterial activity against the pathogenic strains including *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Khan *et al.* 2017).

Microbial growth

The pure culture of microbial strains *E. coli*, *S. aureus* and *B. subtilis* were taken from Department of Food

University of Sargodha, Pakistan. The fresh colonies were prepared by using nutrient Agar medium. The composition of NA medium was as follow (g/100): peptone: 0.5, Yeast extract: 0.3, NaCl: 0.5 and Agar: 1.5. It was autoclaved at 120°C for sterilization purpose for 30 min (Mehmood *et al.* 2019). Stock was preserved at 4°C on slants of nutrient agar medium (Muruganatham *et al.* 2016).

Sample preparation

A stock solution has peptides concentration of 12.8 mg mL⁻¹. The four types of dilution solution were used to evaluate the antibacterial activity. The original Conc used and two-folds diluted with distilled water (6.4 mg mL⁻¹). Three-folds (4.266 mg mL⁻¹) of the diluted solution of peptides extract was prepared adding distilled water and also used four-folds (3.2 mg mL⁻¹) of diluted solution of peptides extract was prepared by adding distilled water and the standard solution of the amoxicillin (10 mg mL⁻¹).

Disc diffusion method

The antibacterial activity of peptides was checked by disc diffusion method. Whatman filter paper No.1 having 6 mm in diameter was used to form the paper discs. These paper discs were impregnated with the standard drug amoxicillin (10 mg mL⁻¹, 100 µg/disc used) and the sample dilutions to be tested. These impregnated discs were placed on the culture media to compare the antibacterial activity. These plates were incubated at 37°C for 48 h. The diameter of zone of growth inhibition around the impregnated discs was measured to check the antibacterial activity of the extract in relation to the standard drug amoxicillin (Salman *et al.* 2016).

Antioxidant Assay

Antioxidant activity was done according to the methodology adopted by Brand-Williams *et al.* (1995) with alterations. In this method, the stable compound α , α -biphenyl- β -picrylhydrazyl (DPPH; C₁₈H₁₂N₅O₆, M = 394.33) which is simply known as the 2, 2'-diphenylpicryl-1-hydrazyl DPPH was used. This assay depends upon the scavenging activity of the antioxidant which is shown towards the DPPH. The reduction of the odd electrons of hydrazine nitrogen occurred by receiving the hydrogen from the antioxidants. The activity of radical scavenger was uttered in terms of the volume of antioxidants essential to reduce the initial absorbance of DPPH by 50% (IC₅₀). Different concentrations of peptides extract *i.e.*, 8, 16, 25.6, 32, 42.6, 64, 106, 128 (µg mL⁻¹) were prepared and the 5 mL reagent of DPPH (4 mg 100 mL⁻¹) was added in each extract solution. The test tubes were covered by the aluminum foil to avoid the effect of light. The prepared solution will reagent vigorously and incubate for 30 min at

room temperature. The absorbance was taken at 517 nm and methanol was taken as the blank. Ascorbic acid was taken as the positive control. The equation given below was used to calculate the percentage free radical scavenging activity.

$$\% \text{Antiradical activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Antidiabetic assay

Male and female rabbits (*Oryctolagus cuniculus*) having weight of 1 to 1.2 kg respectively were obtained from University of Veterinary and Animal Sciences Lahore, Pakistan. The animals were saved in huge commodious cages in the animal stock of Department of Pharmacy, University of Sargodha, and were retained according to "Principals of Laboratory Animal Care" (NIH publication 85-23, reviewed in 1985) (Health 1985). Rabbits were retained under standard conditions of temperature ($23 \pm 12^\circ\text{C}$) and humidity ($55 \pm 15\%$) with 12 h light and dark cycle (Iranloye *et al.* 2011). Experimental rabbits facilitated the food and abundant water.

Sample preparation

Rabbits (*Oryctolagus cuniculus*) were divided into three groups. Group 1 was served with 20 mL solution of 2% gum tragacanth which serves as a control group. Group 2 was alloxan induced diabetic (Type 1) group. To induce diabetes animals were fastened overnight and injected 150 mg kg⁻¹ dose of the alloxan through the ear vein with the help of 25-gauge catheter for easiness. Induction of diabetes was confirmed after the 72 h by testing the blood glucose level. The rabbits which have shown blood glucose in the range of 250–300 mg dL⁻¹ were considered as the diabetic and were employed to check the anti-diabetic activity of the extract (Tafesse *et al.* 2017). Group 3 was also the diabetic group of rabbits to which the standard drug glibenclamide 600 µg kg⁻¹ was given. The glucose level of all groups of rabbits was measured on day 0, 15 and 30.

Statistical analysis

All the trial was performed in triplicates. Means and standard errors of means (Mean \pm S.E.) were calculated for each trail and used to draw the tables and Figures where pertinent (Ayatollahi *et al.* 2019).

Results

Protein/peptides estimation

Bradford assay was used to check protein/peptide contents of *P. trifoliata* germinating seeds. Results declared that the concentration of the protein/peptides in germinating seeds extract solution is 12.8 mg mL⁻¹.

Antibacterial activity

A stock solution has peptides concentration of 12.8 mg mL⁻¹. The four types of dilution were used to evaluate the antibacterial activity. Take 1 mL of stock solution of peptides extract was added in test tube and make volume up to 10 mL (1.28 mg mL⁻¹). Then prepare two-folds (0.64 mg mL⁻¹) of diluted solution of peptides extract by (1.28/2 mg mL⁻¹). Three-folds (0.4266 mg mL⁻¹) of the diluted solution of peptides extract was prepared by 1.28/3 mg mL⁻¹ and four folds (0.32 mg mL⁻¹) of diluted solution of peptides extract was prepared by 1.28/4 mg mL⁻¹ and also dissolve the standard solution of the amoxicillin (1 mg mL⁻¹) drug.

Antioxidant potential

The antioxidant activity of *P. trifoliata* seeds peptides/protein extract was evaluated by DPPH assay. Results showed when concentration of peptides extract increases absorbance of peptides extract decreases it shows that when peptides extract concentration increases more peptides bind with free radicals and color of DPPH reagent decreases it indicates more free radical scavenging activity (Table 1, 2). DPPH assay aims for measuring hydrogen atom or electron giving capacity of the extracts to the DPPH stable radical formed in resultant solution. Results were presented by inhibitory concentration (IC₅₀) defined as those concentration where sample caused 50% decreased as compared to initial concentration of DPPH•, O₂•-, HO• and ABTS• and (IC₅₀) was calculated by linear relationship between sample concentration and free radical scavenging activity in (Fig. 2). (IC₅₀) value of *P. trifoliata* seeds peptides extract was 32.91 µg mL⁻¹ when compared with standard (IC₅₀) value 25.51 µg/mL of ascorbic acid. Results showed the good antioxidant activity of peptides extracted from *P. trifoliata* seeds which is nearly equal to the ascorbic acid as a standard free radical scavenging activity.

Antidiabetic potential

Antidiabetic activity of *P. trifoliata* seeds peptides extract was assessed by alloxan induced method. Experiment was performed on three groups. Results presented in (Fig. 3) in which alloxan induced diabetic rat of group 2 were treated with 51.2 mg kg⁻¹ dose of peptides extract. The blood glucose level was recorded on day 0, 15 and 30. Results of group 2 came near with group 3 blood glucose level which was diabetic rabbits and treated with standard drugs like glibenclamide 600 µg kg⁻¹. The *P. trifoliata* seeds peptides extract showed convincing antidiabetic activity in controlling the glucose level of blood. Naturally sources can play a significant character in preparing the cells for insulin and activates the β-cells of pancreas to raise the production of insulin. The considerable antidiabetic activity of *P. trifoliata* germinating seeds peptides extract provides us with a natural remedy against the diabetes mellitus.

Table 1: Zone of inhibition of *P. trifoliata* seeds peptides extract in comparison of antibiotic amoxicillin used as a standard

S. No.	Antibacterial solutions	Growth inhibition Zone in mm		
		<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
1	Amoxicillin (1 mg mL ⁻¹)	17	14	15
2	One-fold peptides extract (1.28 mg mL ⁻¹)	18	15	16
3	Two-fold peptides extract (0.64 mg mL ⁻¹)	12	10	12
4	Three-fold peptides extract (0.4266 mg mL ⁻¹)	5	4	5

Table 2: Percentage inhibition activity of *P. trifoliata* seeds peptides extract

S. No.	Concentration of peptide extract (µg mL ⁻¹)	Absorbance of extract (517 nm)	Inhibition % Ctrl-abs/ctrl×100
1	8	0.1498	42.4228
2	16	0.1424	45.2728
3	25.6	0.1354	47.9631
4	32	0.1302	49.9615
5	42.6	0.1226	52.8823
6	64	0.1052	59.5695
7	106	0.0791	69.6003
8	128	0.0565	78.2859

Ctrl = absorbance of control, Control value = 0.2602, Abs = absorbance of extracts



Fig. 1: *Poncirus trifoliata* ripened fruit and seeds

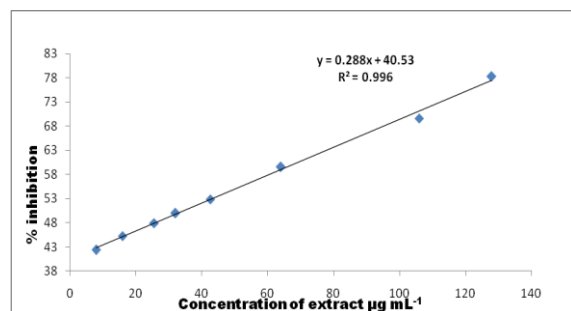


Fig. 2: Percentage inhibition of free radicals by different concentration of *P. trifoliata* seeds peptides extract

Discussion

Based on previous literature reported by Kumar *et al.* (2016) evaluation of different parameters was done on non-

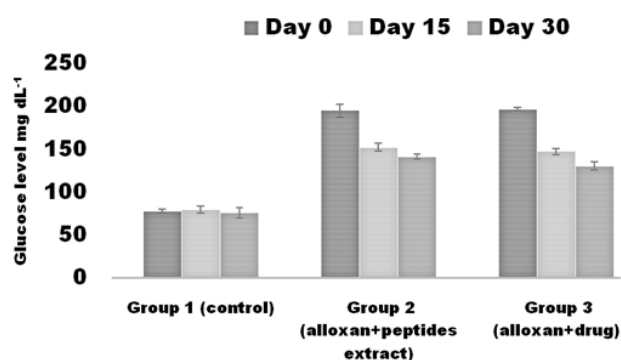


Fig. 3: Antidiabetic activity of *P. trifoliata* seeds peptides extract

germinating and germinating seeds of *Cucurbita maxima*. Results revealed that non-germinating seeds contained high amounts of reducing, non-reducing sugars, lipids and calorific value while germinating seeds showed high amounts of proteins, DNA and phytochemicals. Germination of seed is a critical phase in growth of plants and can be recognized as a basis for productivity of plants. It initiates with water (H₂O) imbibition, food reserve mobilization, significance radicle protrusion and synthesis of protein. To maintain a worthy seedling growth, seed stocks a food reserve mainly as proteins, carbohydrates and lipids. Proteins and lubricant bodies are core reserves in oilseed which embody as a source of energy in the form of nitrogen and carbon throughout seedling formation (Abuin *et al.* 2002). After the genome starts expressing stored proteins undergo hydrolysis and releases free amino acids, for protein synthesis in embryo and endosperm for proceeding the process of germination (Ali and Elozeiri 2017).

Our results show similarity with already reported data in which hydrolysis of casein was done by cysteine proteases from *Jacaratiaco rumbensis*. After 2 h hydrolysis, the release product was energetic against bacteria like *B. subtilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*,

Klebsiella pneumonia, *E. coli* and *S. aureus* (Arruda *et al.* 2012). Mohanty *et al.* (2016) reported whey protein release peptides having antibacterial activity against *E. coli*, *Clostridium* sp., *B. stearothermophilus*, *Vibrio cholerae*, *Legionella pneumophila*, *B. subtilis*, *Haemophilus influenzae* and *Streptococcus mutans*. Bromelain was capable of liberating antibacterial peptides from *Meuschenia* species protein of leather jackets. This release of hydrolysate showed antimicrobial against *B. cereus* and *S. aureus* (Salampessy *et al.* 2010). The bacteria showed resistance against artificial antibacterial agents so their usage is limited to preserve in foodstuff. Antibacterial peptides obtained from natural sources have a great potential to substitute artificial antibacterial agents owing to their less toxic nature and fewer ecological risk. Antibacterial peptides produced by food proteins hydrolysis catalyzed by proteases present in plants have grown significantly.

Our findings show similarity with Grancieri *et al.* (2019) who reported that *Salvia hispanica* L. (chia seed) are a good source of antioxidants. It inhibits NF- κ B transcription factor activation *in vitro* along with reducing carcinogenic and inflammatory processes and provides protection against the nitrogen and reactive oxygen species (ROS). Kooti and Daraei (2017) reported celery (*Apium graveolens*) seeds, leaves and essential oils have good antioxidant properties because of presence of these compounds like ferulic acid, luteolin, caffeic acid, tannin, *p*-coumaric acid, apigenin, kaempferol and saponin while diverse complexes of this plant with different concentration having different therapeutic effects. It has been reported by Derakhshan *et al.* (2018) that after experimental study on pomegranate seeds, juice, and peels it was observed that all parts of pomegranate showed good antioxidant activity but more activity was obtained by pomegranate peels. Medical herbs have rare side effects because of presence of phenolic compounds and flavonoids which are widely distributed in plants along with antioxidant activities than chemical drugs and their antioxidant features decrease the drugs toxicity. Nowadays herbal drugs are used as a substitute to chemical drugs because of their lower side effects as compared to chemical drugs.

Our findings show similarity with Payamalle *et al.* (2017) who reported that *Garcinia xanthochymus* seeds extract shows antioxidant, anti-diabetic and biochemical activities in alloxan induced diabetic rats. High pressure liquid chromatography (HPLC) study ensured the existence of phenolic complexes specially gallic acid, tannic acid and 4-hydroxybenzoic acid which ensure an imperative role as an anti-diabetic action. Tafesse *et al.* (2017) who reported that *Ajuga remotabenth* leaves extract shows good antidiabetic activity by reducing blood glucose levels probably because of the occurrence of antioxidant ingredients like flavonoids. It was reported by Ayatollahi *et al.* (2019) that seed extract of date in alloxan induced diabetic rat's shows anti-hyperglycemic and anti-lipidemic effects so results proved that date seed extract recognized as a

harmless and effective candidate in fighting against diabetes. Diabetes mellitus is a syndrome distressing the worldwide public fitness in both developing and underdeveloped states. It was reported by IDF that in 2015, 415 million adolescents are affected by DM and it is expected that this ratio is augmented up to 642 million by 2040 (Payamalle *et al.* 2017). Now, several man-made drugs have been utilized for the cure of DM. But they have severe side effects including weight gain, drug resistance and dropsy. So, innumerable medicinal peptides/plants have shown impending anti-diabetic outcomes with fewer side effects.

Conclusion

The current work exposed that *P. trifoliata* seeds peptides extract were scrutinized for antibacterial, antioxidant and antidiabetic activity. The outcomes of the present study revealed that seeds extracted from *P. trifoliata* have significantly bioactive peptides that showed higher potential as an antibacterial, antioxidative and antidiabetic effect. Therefore, *P. trifoliata* seeds extract can be recognized as a harmless and capable for utilized as antibacterial, antidiabetic agent and an imperative source for herbal drugs synthesis and lessen the problem of multiple drug resistance particularly in case of pathogenic bacterial strains.

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Author Contributions

TM and RWA conceived and planned the experiments. RWA carried out the experiments. TM, FM, SS, HM and SI contributed to the interpretation of the results. TM, FN and SS took the lead in writing the manuscript. All authors provided critical feedback and helped to shape the research, analysis and writing manuscript.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethical Approval

The animals were saved animal house at Department of Pharmacy, University of Sargodha, Pakistan, according to "Principals of Laboratory Animal Care" (NIH publication 85-23, reviewed in 1985).

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