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Isolation and characterization of peptides from *Gymnema sylvestre* and their antimicrobial assay against bacterial isolates of diabetic foot ulcer

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Abstract
Diabetic foot ulcers (DFUs) are significant micro vascular diabetes-related lesions that result from a number of predisposing factors, including infections, bone abnormalities, diabetic neuropathy and peripheral arterial disease. If left untreated, these conditions may exacerbate and ultimately result in lower limb amputation. The development of foot ulcers is a significant consequence associated with diabetes. The current global issue is the emergence and spread of antibiotic-resistant microorganisms. Therefore, it is imperative that alternative chemical development proceed immediately. Antimicrobial peptides are referred to as host defense peptides area component of the innate immune system present in every living organism. These small molecules play significant roles in the battle against infection through their wide-spectrum antibacterial action, host immune-modulatory capabilities and other aspects related to wound healing.
The present study was carried out to isolate and characterize peptides having antibacterial activity from leaves of *Gymnema sylvestre* (Gurmar). Crude extract of leaves was prepared in Phosphate Buffer Saline (PBS) and antibacterial activity was checked on Luria Bertani (LB) broth agar plates against several bacteria such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa and Enterococcus facieali*s.
The extract was used for peptide(s) precipitation with different percentage of ammonium sulfate solution, homogeneity and molecular mass of protein fractions was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE). Purification of isolated protein/peptide was done by Gel Filtration Chromatography (GFC) using Sephadex G-50 and characterization was done by Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

Keywords: Antimicrobial peptide, phosphate buffer saline, *Gymnema sylvestre*, *Escherichia coli*, *Staphylococcus aureus*, diabetic foot ulcer, wound, SDS-PAGE

1. Introduction
Diabetes causes alterations to blood vessels and nerves, which contribute to DFU development. Diabetic neuropathy (DN) manifestations vary and rely on the nerve system involved (peripheral sensory/motor or vegetative), resulting in a complicated picture of symptoms and indicators based on the organ. However, peripheral neuropathy is among the most frequent and, according to recent guidelines, could impact over 50% of patients with diabetes mellitus (DM) throughout their lifespan (Pop-Busui *et al*. 2022) [12]. Peripheral neuropathy (PN) is one of the major microvascular consequences of both type 1 (T1DM) and type 2 diabetes mellitus (T2DM). Diabetes-related peripheral neuropathy produces decreased or loss of sensation in the lower limbs. As consequence, any injury or pressure placed on the lower limbs may go unreported due to a lack of sensation. This impairment of the sensory feedback alert system may cause skin injury and wound formation (Galiero *et al*. 2023) [4]. Vascular damage caused by diabetes can also contribute to the production of DFUs (Selvarajah *et al*. 2019) [16].
Currently, the growth and spread of microorganisms immune to conventional antibiotics pose a global danger. In this regard, alternative chemicals are urgently required. Antimicrobial peptides (AMPs) are chemicals in the immune system of animals that fight invading pathogens (Wojciech, 2005) [21]. Peptides are short amino acid (AA) sequences. They are usually <50 AA in length and are often stabilized by disulfide bonds (Hayashi *et al.* 2012) [6]. AMPs are highly effective against a wide range of pathogens, such as viruses, fungi and antibiotic-resistant bacteria and they have anticancer properties with a complex mode of action (Rydlo *et al*. 2006) [14].

They can target the cytoplasmic membrane and disrupt DNA and protein synthesis, protein folding, and cell wall production, thereby interfering with immunomodulatory processes such as inflammation and cicatrization (Teixeira 2023) [17]. Mammalian antimicrobial peptides can be detected in neutrophil granules, epithelial cells in the skin, mucous membranes and protein degradation products. Their use as monotherapy in the treatment of infection, in conjunction with traditional antibiotics for synergistic reasons, immunomodulators and endotoxin neutralizers, has been proposed (Nasseri and Sharifi, 2022) [10]. Medicinal plants are known to have immuno-modulatory characteristics, and these plants are used to treat a variety of disorders involving the host defense mechanism (Arora *et al.* 2021) [1]. The lifetime probability of developing a foot ulcer is thought to be 12–25% for diabetic persons (Miranda *et al*. 2021) [9]. Several antimicrobial peptides have been assessed in DFUs, including nisin, α-helical antimicrobial agents decapeptide KKVVFWVKFK (KSL-W), ubiquicidin 29-41 (UBI 29-41), pexiganan (MSI-78), and beta-defensin-2 (hBD2). However, only pexiganan (MSI-78) is in clinical phase development as a topical cream. Immense necessity of treating drug-resistant bacteria with new generations of antibiotics, antimicrobial peptides are gaining greater attention (Hancock and Sahl, 2006).

Gurmar, also known as *Gymnema sylvestrae* (GS) elongs to family *Asclepiadaceae*, is a herb with widespread use due to its anti-diabetic properties. In India, it is utilised as traditional medicine to treat a variety of ailments (Jamadagni *et al*. 2021) [7]. Moreover, GS is utilised in health supplements, basic tea brew, tea bags, beverages and confections (Tiwari *et al*. 2014) [19]. Due to its inclusion in the medication IME 9, developed by the Central Council for Research in Ayurvedic Sciences and distributed by Kudos Laboratories (Paliwal *et al*. 2009) [11]. It has been found that GS possesses hepatoprotective, antihypercholesterolemic and antibacterial properties. It a blend of triterpene saponins of the oleanane type that are taken from GS's leaves (Thakur *et al*. 2012) [18].

The present study aimed to extract, isolate and characterization of small peptides the leaves of *Gymnema sylvestre* and evaluates their antimicrobial activity against some bacterial isolates of DFU like *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus facieallis and Pseudomonas aeruginosa.*

2. Materials and Methods

2.1 Collection of Plant Material: Plant specimens of *Gymnema sylvestrae* were collected from the various localities of the Kota 25°11'N 75°50'E latitude and 25.18°N 75.83°E longitude Herbarium sheets (Accession No BOT/2019-20/C/MC/03) of the specimens were prepared according to IRBN for the validation and authentication of plants; respective herbarium sheets were assigned taxonomical affiliations by the Department of Botany, B N University and were deposited for future reference.

2.2 Preparation and storage of plant samples: Test samples for antimicrobial activity of *Gymnema sylvestre* were prepared by washing and sun drying the collected leaves. Subsequently, they were ground to fine powder, sieved and stored in airtight container in a refrigerator at 4ºC.

2.3 Procurement of bacterial cultures: Bacterial cultures of *Staphylococcus aureus* (MCC 2043-T), *Pseudomonas aeruginosa* (MCC 2080) *Enterococcus faecalis* (MCC 2409), *Escherichia coli* (MCC 3671) and *Klebsiella pneumonia* (2451) were obtained from National Centre for Microbial Resource Pune. All cultures were sub-cultured at regular intervals on nutrient agar and stored at 4° C as well as at -20°C by making their suspension in 10% glycerol.

2.4 Culture media and inoculum preparation: Trypticase Soy Yeast Extract (TSYE) medium and nutrient broth medium was prepared for revival of bacteria and Nutrient agar and Muller Hinton agar medium was prepared for antibacterial assay. Disc diffusion method was used for antibacterial testing

2.5 Protein extraction from *Gymnema sylvestrae* leaves and their partial purification

Protein extraction from the leaves was carried out by the method described by Rehman and Khanum (2011) [13] with partial modifications. 10 g leaves powder was blended with 100 ml phosphate buffer saline (pH 7.4) in an electric blender and homogenates were frozen and thawed thrice followed by centrifugation at 10,000 rpm/ 20 min/ 4˚C. This supernatant after treatment was used to evaluate antibacterial activity. Further supernatant was precipitated with different volumes of ammonium sulfate solution and the respective solution was centrifuged at 10,000 rpm/ 30 min/ 4˚C and pellets were dissolved in 1 ml of deionized water. The solubilized ammonium sulfate precipitate and supernatant were dialyzed at MWCO 3500 Da against distilled water and protein concentration was procured in crude extract, dissolved ammonium sulfate precipitate and supernatant.

2.6 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE

The dialyzed protein samples of both solubilized ammonium sulfate precipitates and supernatant were first used for SDS-PAGE analysis. Tricine {N-[2-Hydroxy-1, 1-bis (hydroxymethyl) ethyl] glycine} - SDS-PAGE was used for the separation of protein which are less than 30kDa. The protein pellets and supernatant were electrophoresed on 16% Tricine SDS-PAGE gel (Schagger, 2006; Jiang *et al*. 2016).

2.7 Gel filtration chromatography

The protein pellets obtained from SDS-PAGE were purified through gel filtration chromatography. In gel filtration chromatography Sephadex G-50 column was used. The Sephadex G-50 spherical beads were allowed for swollen in buffer overnight. This chromatography buffer was prepared by mixing tris HCl 50mM and NaCl 50Mm in 1:1 ratio. The packed column was allowed to set and washed the column thrice with the same buffer. Now the protein sample added and allowed to move through the column. The fractions of 1ml were collected and stored at 4 °C. The optical density of fractions was calculated at 280 nm.

2.8 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The Reverse Phase High Performance Liquid chromatographic (RP-HPLC) started with equilibrating the C18 silica column with solvent A which was prepared by mixing 999 ml Milli Q and 1 ml Triflouroacetic acid (TFA). The sample was applied to the chromatography column. The column was washed with the above prepared solvent A so that unbound molecules were removed. The gradual decrease in solvent A polarity was obtained by increasing the linear gradient from 100% solvent A to 70% solvent B (solvent B

was prepared by mixing 999 ml Acetonitrile (ACN) which was of HPLC grade and 1ml Triflouroacetic acid) for 30 minutes at a flow rate 0.5 ml/min The bound protein/peptide desorbed from the matrix according to their individual polarity. The absorbance of protein/ peptide was recorded at 214nm and 220 nm. The single peptide/ protein were showed as separate peaks in graph.

2.9 Antibacterial assay

The antimicrobial activity of the solubilized ammonium sulfate precipitates and supernatant of ammonium sulfate precipitates was determined using the disc diffusion method (Bauer *et al*. 1966) [2]. Sterile whatman filter paper discs of 6 mm in diameter were soaked in a 20 µg/ 30 µl test sample and placed on the surface of the agar plate. Chloramphenicol was used as a control. These plates were incubated overnight at 37ºC. The results expressed as the mean±SEM, indicate the standard deviation of the triplicate incubations in millimetre (mm). Excel statistical software was used to analyze the data (Borchardt *et al*. 2008) [3].

Macro dilution assay was performed to establish minimum inhibition concentration (MIC) values of isolated peptides against all bacterial cultures as described by Wang, 2022 [20]. The 108 CFU/ml test cultures were inoculated into LB broth containing 0-200 µg/ml antimicrobial protein preparation.

2.10 Statistical analysis

All the experiments were carried out in triplicate thrice (n=3). The statistical analysis of the data was carried out by analysis of the variance (ANOVA). Results were considered significant when $p < 0.05$.

3. Results

3.1 SDS-PAGE Analysis

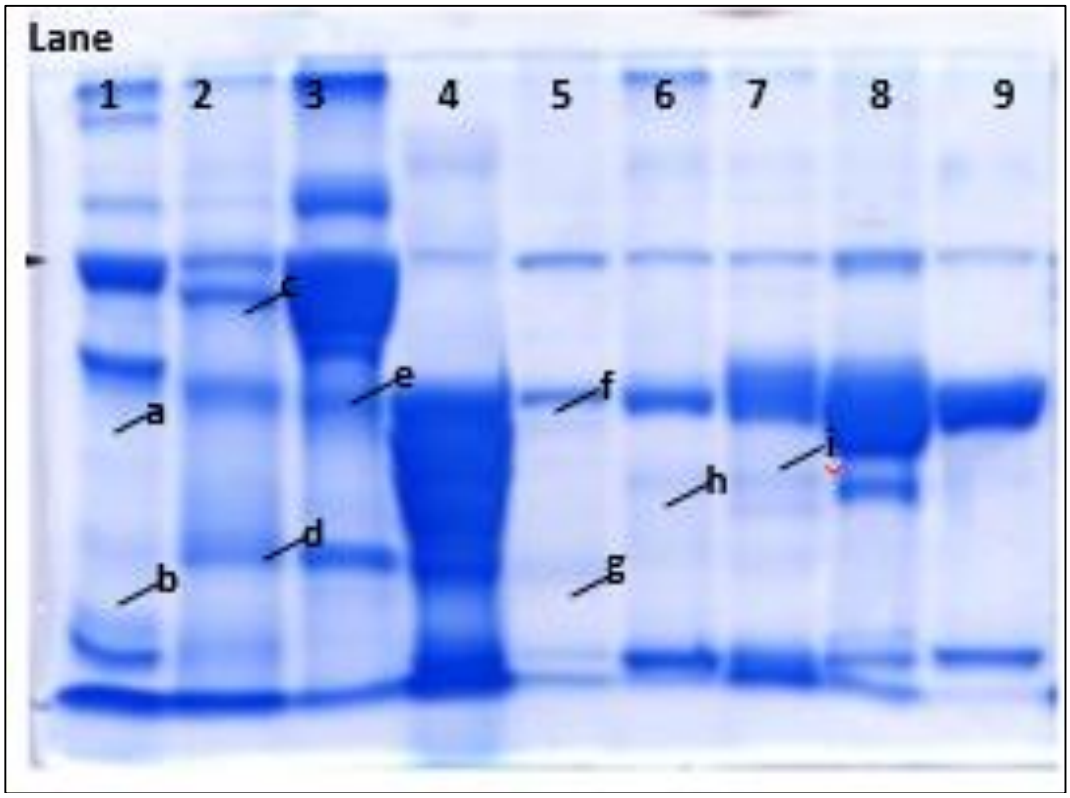


Fig 1: SDS-PAGE analysis of different percentage of precipitated protein pellets of *Gymnema sylvestre* seed extract. Lane 1: (a) 24.12 KDa, (b) 16.32 KDa in 90% protein pellet; Lane 2: (c) 27.26KDa, (d) 17.07 KDa in 50% protein pellet; Lane 3: (e) 22.32 KDa; Lane 4: Marker; Lane 5: (f) 13.13 KDa, (g) 18.14 KDa in 25% protein pellet; Lane 6: (h) 21.28 KDa; Lane 7: (i) 16.63KDa in 75% protein pellet; Lane 8, 9: Supernatant.

3.2-Gel filtration chromatography

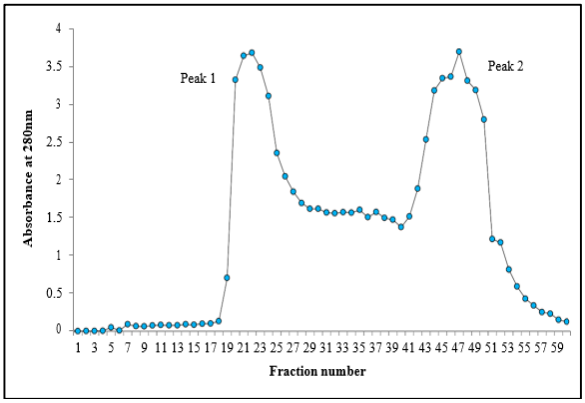


Fig 2: Elution profile of *Gymnema sylvestre* protein pellet from gel filtration chromatography

3.3 Reverse Phase Chromatogram

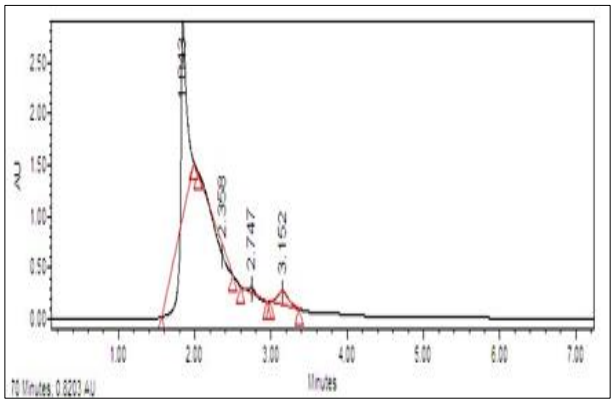


Fig 3: Reverse Phase Chromatogram of *Gymnema sylvestre* protein pellet

3.4 Antimicrobial activity in solubilized and supernatant of ammonium sulfate precipitate

Table 1: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Staphylococcus aureus*

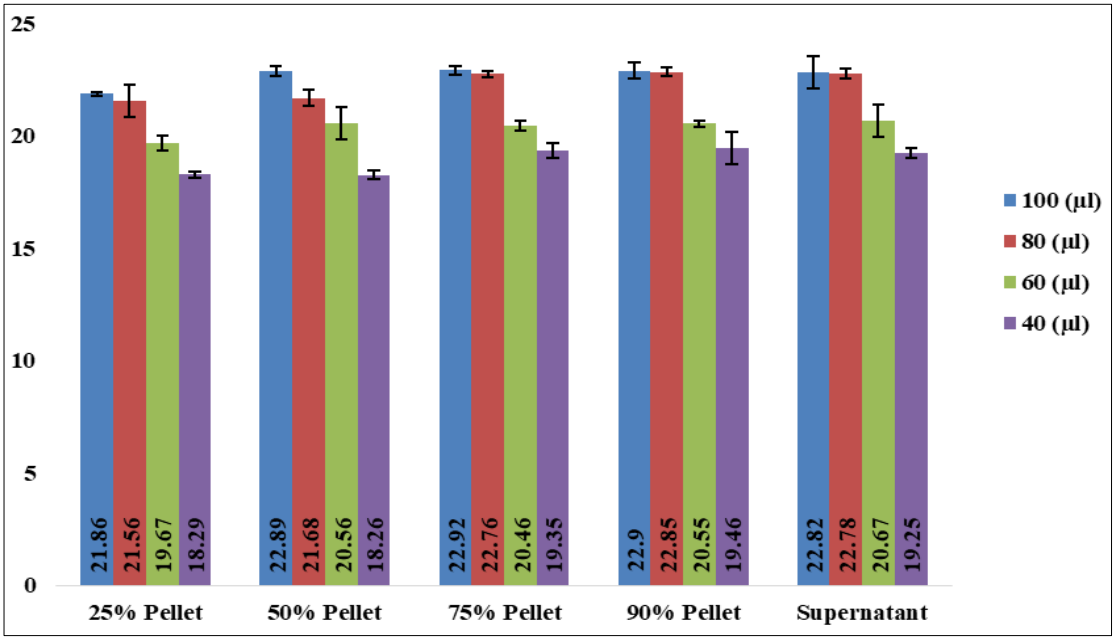
S. No.	Peptide Sample	100 (µl)	80 (µl)	60 (µl)	40 (µl)
1.	25% Pellet	21.86±0.07**	21.56± 0.70**	19.67± 0.35**	18.29± 0.14**
2.	50% Pellet	22.89± 0.21**	21.68± 0.35*	20.56± 0.70**	18.26± 0.21**
3.	75% Pellet	22.92± 0.21**	22.76± 0.14**	20.46± 0.21*	19.35± 0.35**
4.	90% Pellet	22.90± 0.35**	22.85± 0.21**	20.55± 0.14**	19.46± 0.70**
5.	Supernatant	22.82± 0.70**	22.78± 0.21**	20.67± 0.70**	19.25± 0.21**
Mean values±SD(n=3); P≥0.05 (NS), *P<0.1 (S), **P≤0.01 (HS)					

Table 2: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Escherichia coli*

S. No.	Peptide Sample	100 (µl)	80 (µl)	60 (µl)	40 (µl)
1.	25% Pellet	21.89± 0.07**	21.82± 0.70**	19.78± 0.21**	18.67± 0.35**
2.	50% Pellet	21.86± 0.21**	21.80± 0.35**	20.72± 0.14**	18.65± 0.07*
3.	75% Pellet	22.78± 0.35**	22.67± 0.21*	20.66± 0.07**	18.54± 0.21**
4.	90% Pellet	22.72± 0.70**	22.64± 0.35**	20.55± 0.70**	19.46± 0.14**
5.	Supernatant	22.68± 0.35**	21.61± 0.70**	20.58± 0.21**	19.49± 0.70**
Mean values±SD(n=3); P≥0.05 (NS), *P<0.1 (S), **P≤0.01 (HS)					

Table 3: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Klebsiella pneumonia*

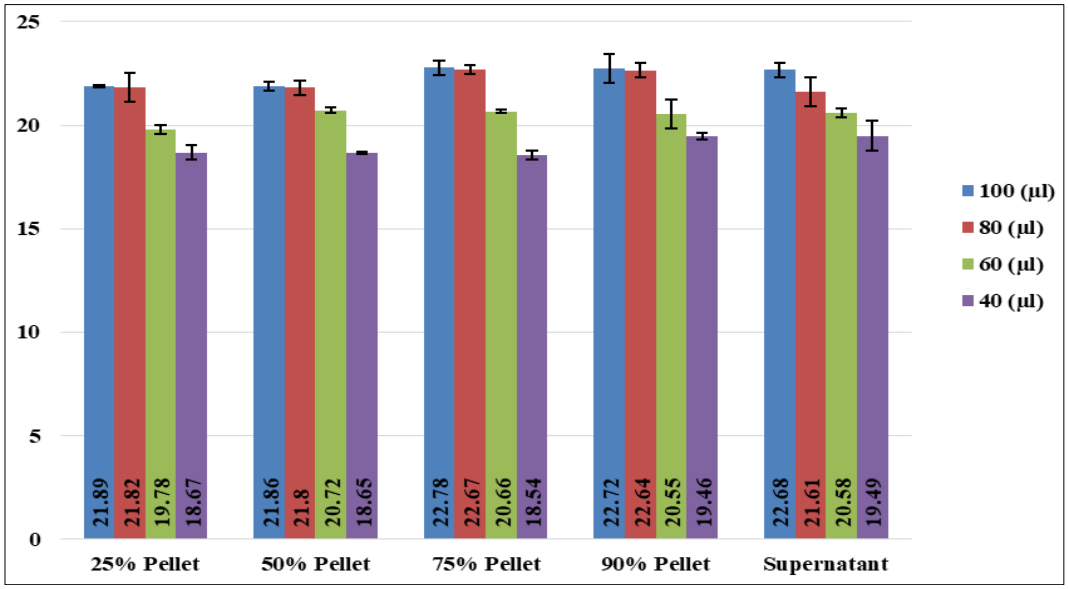
S. No.	Peptide Sample	100 (µl)	80 (µl)	60 (µl)	40 (µl)
1.	25% Pellet	21.89± 0.35**	21.75± 0.21**	20.69± 0.14**	18.61± 0.70**
2.	50% Pellet	22.78± 0.70**	21.77± 0.35**	20.67± 0.21*	18.58± 0.07**
3.	75% Pellet	22.59± 0.35**	21.69± 0.70**	20.65± 0.35**	19.46± 0.35**
4.	90% Pellet	22.69± 0.70**	22.57± 0.35**	20.54± 0.07**	19.40± 0.21**
5.	Supernatant	22.49± 0.35**	21.46± 0.21**	20.52± 0.70**	19.36± 0.14**
Mean values±SD(n=3); P≥0.05 (NS), *P<0.1 (S), **P≤0.01 (HS)					



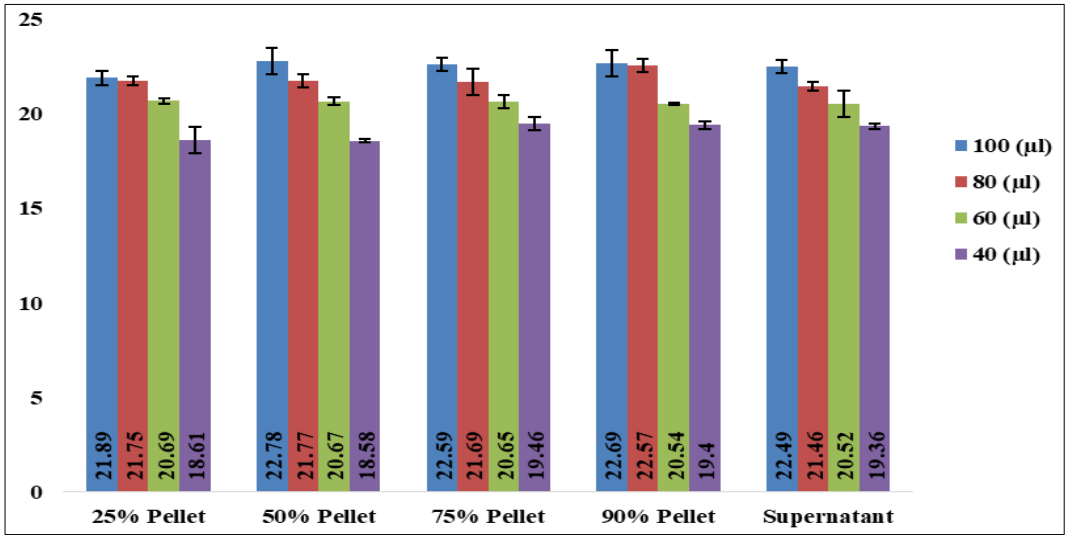
Graph 1: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Staphylococcus aureus*

Table 4: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Pseudomonas aeruginosa*

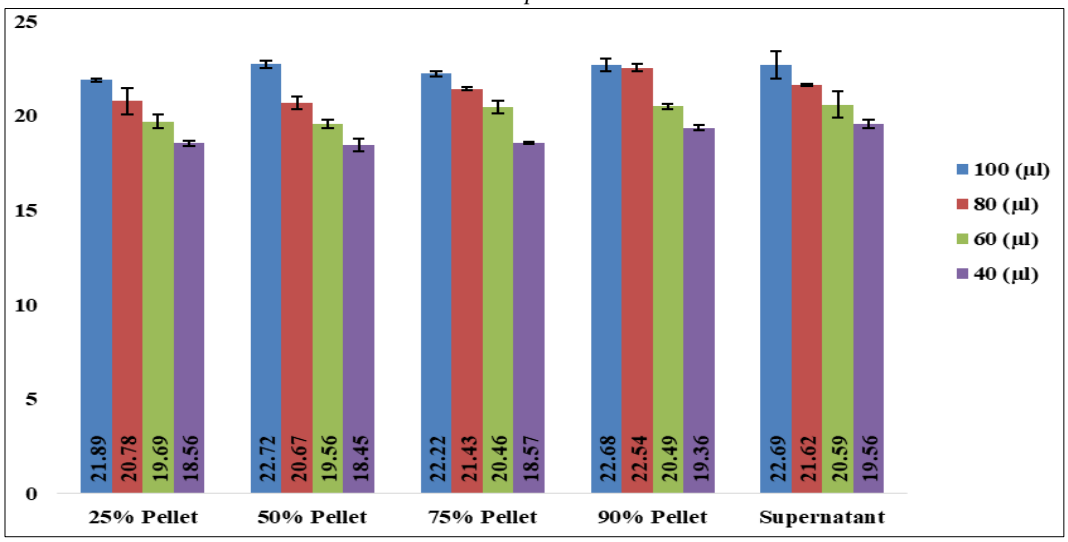
S. No.	Peptide Sample	100 (µl)	80 (µl)	60 (µl)	40 (µl)
1.	25% Pellet	21.89± 0.07**	20.78± 0.70*	19.69± 0.35**	18.56± 0.14**
2.	50% Pellet	22.72± 0.21**	20.67± 0.35**	19.56± 0.21**	18.45± 0.35**
3.	75% Pellet	22.22± 0.14*	21.43± 0.07**	20.46± 0.35**	18.57± 0.07**
4.	90% Pellet	22.68± 0.35**	22.54± 0.21**	20.49± 0.14**	19.36± 0.14**
5.	Supernatant	22.69± 0.70**	21.62± 0.07**	20.59± 0.70**	19.56± 0.21*
Mean values±SD(n=3); P≥0.05 (NS), *P<0.1 (S), **P≤0.01 (HS)					



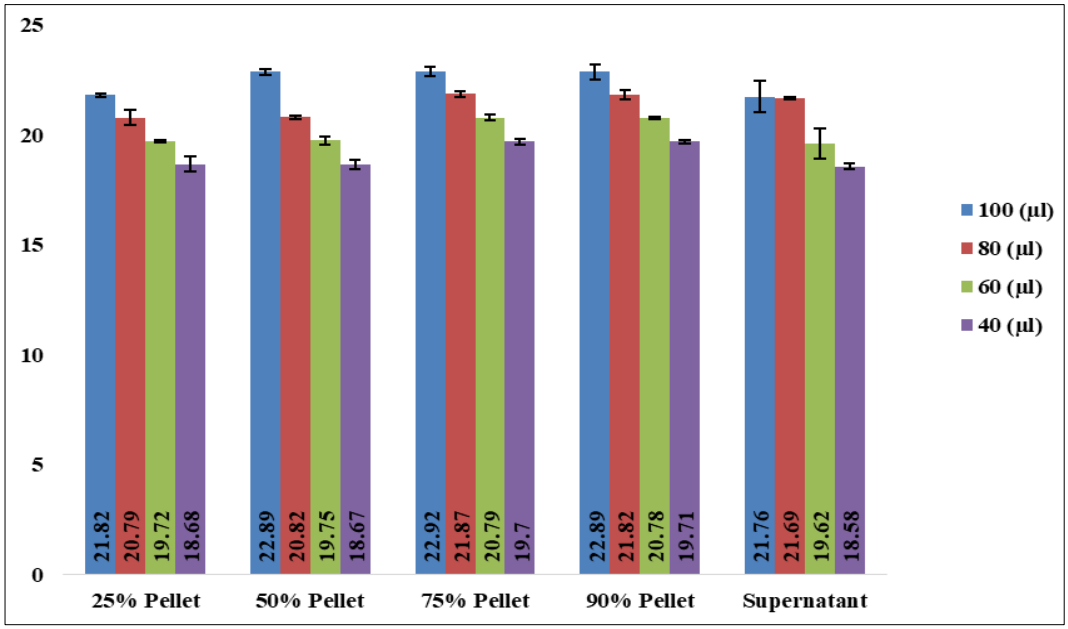
Graph 2: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Escherichia coli*



Graph 3: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Klebsiella pneumoniae*



Graph 4: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of Pseudomonas aeruginosa



Graph 5: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Enterococcus faecalis*

Table 5: Zone of Inhibition (ZOI) of different volumes of peptide pellets isolated from *Gymnema sylvestre* against different volumes of *Enterococcus faecalis*

S. No.	Peptide Sample	100 (µl)	80 (µl)	60 (µl)	40 (µl)
1.	25% Pellet	21.82± 0.07**	20.79± 0.35**	19.72± 0.07**	18.68± 0.35**
2.	50% Pellet	22.89± 0.14**	20.82± 0.07*	19.75± 0.21**	18.67± 0.21**
3.	75% Pellet	22.92± 0.21**	21.87± 0.14**	20.79± 0.14**	19.70± 0.14**
4.	90% Pellet	22.89± 0.35**	21.82± 0.21**	20.78± 0.07**	19.71± 0.07*
5.	Supernatant	21.76± 0.70**	21.69±0.07**	19.62± 0.70**	18.58± 0.14**
Mean values±SD(n=3); P≥0.05 (NS), *P<0.1 (S), **P≤0.01 (HS)					

4. Discussion

Figure 3.1 depicts an SDS-PAGE evaluation of varying percentages of precipitated protein pellets from *Gymnema sylvestre* leaves extract. The chromatogram shows protein bands ranging from 13.13 KDa to 27.26 KDa. It revealed the bands of two major and a few minor proteins. The elution curve (Figure 3.2) of a protein pellet from *Gymnema sylvestre* employing gel filtration chromatography is illustrated in Figure 4.8. The chromatogram displays 2 peaks. Protein fractions showed a peak at 280 nm absorbance. Peak 1 has a fraction number range of 19-25, while Peak 2 has a fraction number range of 45–5. Peak 2 of *Gymnema sylvestre* has higher peptide than Peak 1. Figure 3.3 depicts the reverse phase chromatogram of *Gymnema sylvestre*. Certain significant height values that were well resolved were displayed in the chromatogram. A total of 4 fractions were observed in the chromatogram. The elution time and peak height of the *Gymnema sylvestre* protein pellet samples are as follows: 2 min (1.043 AU), 2.4 min (2.358 AU), 2.9 min (2.747 AU) and 3.2 min (3.152 AU).

The analysis of the extracted protein and supernatant (Table 3.1 and Graph 3.1) from the leaf extract of *Gymnema sylvestre* was conducted to determine the effectiveness of its antibacterial properties. *Staphylococcus aureus* was found to be sensitive to antimicrobial activity in the protein pellet prepared from a 25% ammonium sulphate precipitation of *G. sylvestre* leaf extract, with a maximum inhibition zone of 21.86±0.07 mm at 100 µl and a minimum inhibition zone of 18.29±0.14 mm at 40 µl. The maximum inhibition zone measured in the 50% protein pellet generated from leaf extract was 22.89±0.21mm at 100 µl, whereas the minimum inhibition zone was 18.26±0.21mm at 40 µl.

Table 3.2 and Graph 3.2 demonstrate the susceptibility of *Escherichia coli* towards antibiotic efficacy of *Gymnema sylvestre*. The protein pellet from a 25% ammonium sulphate precipitation leaf extract had a maximum inhibition zone of 21.89±0.07mm at 100 µl and a minimum inhibition zone of 18.67±0.35mm at 40 µl. The 50% protein pellet made from leaf extract had a maximum inhibition zone of 21.86±0.21mm at 100µl and a minimum of 18.65±0.07mm at 40µl.

The protein pellets extracted from *Gymnema sylvestre* leaves were evaluated for their antibacterial properties, which are illustrated in Table 3.3 and Graph 3.3. Evidence of antibacterial action against *Klebsiella pneumoniae* was found in the protein pellet obtained from a 25% ammonium sulphate precipitation leaf extract. The maximal inhibition zone measured at 100 µl was 21.89±0.35mm, while the minimum was 18.61±0.70mm at 40 µl.

Table 3.4 and Graph 3.4 illustrate susceptibility of *Pseudomonas aeroginosa* towards *Gymnema sylvestre*'s antibacterial properties. The protein pellet derived from a leaf extract precipitated with 25% ammonium sulphate exhibited a maximum inhibition zone measuring 21.89±0.07mm at 100 µl and a minimum inhibition zone measuring 18.56±0.14mm at 40 µl. The maximum inhibition zone was 22.72±0.21mm at 100µl and the smallest inhibition zone measured 18.45±0.35mm at 40µl for the 50% protein pellet prepared from leaf extract.

Table 3.5 and Graph 3.5 demonstrate sensitivity of *Enterococcus faecalis* towards antibiotic efficacy of

Gymnema sylvestre. In the protein pellet derived from a 25% ammonium sulphate precipitation leaf extract, the maximum inhibition zone measured 21.82±0.07mm at 100 µl, while the minimum inhibition zone measured 18.68±0.35mm at 40 µl respectively. The 50% protein pellet produced from leaf extract revealed a minimum inhibition zone of 18.67±0.21 mm at 40 µl and a maximum inhibition zone of 22.89±0.14 mm at 100 µl.

5. Conclusion

The present studies involving *Gymnema sylvestre* species and bacterial isolates associated with Diabetic Foot Ulcers (DFUs) reveal promising results. In the chromatogram of *Gymnema sylvestre* leaves extract it showed protein bands ranging from 13.13 KDa to 27.26 KDa. In the elution curve of gas filtration chromatography peak 1 and peak 2 were observed between these peak 2 has higher peptide content. This study has demonstrated that leaf extract of plant species possess antimicrobial peptide which showed antibacterial activity against all tested bacteria associated with diabetic foot infections *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. These findings suggest the potential for antimicrobial peptide based treatments to combat infections in DFUs, potentially offering an alternative to conventional antibiotics. As there are so many bacteria which are multi drug resistant now, antibiotic therapy on such bacteria is not so much effective as well as it has side effects too. Plant antimicrobial peptides play an important role in host defence system similar to human antimicrobial peptides so they can be a better choice because these peptides hinder the growth of microorganisms by creating ion channels in the microbial membranes.

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