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Extraction of biologically active protein from ayapana (*Ayapana triplinervis* Vahl) and evaluation of antimicrobial activity

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Abstract
Peptide therapy is an emerging technology in medicine. Plant-based peptide drug discovery would be an effective and target-specific therapeutic agent which overcomes the traditional drawback. The study aims to explore and characterise the potent protein candidate from fresh leaves of *Ayapana triplinervis Vahl*, to study the efficiency against various clinical pathogens. The proteins have been extracted, precipitated, and characterised. Three methods have been used to extract proteins, and the EB TA (Extraction buffer - TCA acetone) method favours a good yield of protein. The extracted protein was completely solubilised in Tris-Cl (pH 7.4) buffer than other solubilizing buffers. The molecular weight of the extracted proteins was determined by plotting a graph of log Molecular weight with relative migration distance for standards. The proteins separated were less than 30 kDa by all three methods. The proteins extracted by using the EB-TA method elicited antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Streptococcus* species and MRSA (Methicillin-Resistant Staphylococcus aureus) *strains* isolated from clinical wounds. The above results suggest that the protein extract of the fresh leaves of *Ayapana triplinerve Vahl* has potent antibacterial activity.

Keywords: *Ayapana triplinerve Vahl*, protein, peptides, log Molecular weight, antibacterial activity, solubilization

1. Introduction
Plants have been used for therapeutic applications traditionally since ancient times. They are rich sources of secondary metabolites, proteins and peptides, which show an array of properties used for various medical applications. Among them, proteins/peptides that are produced in plants which act as host defense mechanisms and are low molecular weight, cysteine-rich, can escape drug resistance mechanisms, exert dynamic interchange in their structure and topology and are Nontoxic. Peptide promiscuity is their special property making them a novel drug candidate for use in treating various diseases. Metabolic instability, shorter half-life, low immunogenicity and cost of production are its major drawbacks which makes them not popular in the market. Even though the peptides are used in cosmetics, food supplements, health drinks and pharmaceuticals which are synthesized chemically or it is obtained by enzymatic digestion of animal proteins, they are not accepted by consumers because of the risk associated with them. These could be overcome by usage of the compatible drug carrier molecules along with the peptide. On the other hand, Plant peptides are safe and effective because of their specificity in their function and are also capable of interacting with a huge number of macromolecules. A new emerging trend was the use of plants based bioactive peptides for therapeutics which could be a replacement for traditional drugs in the market for various medical conditions [1]. *Ayapana triplinervis* Vahl (otherwise called *Eupatorium triplinervis* Vahl) is a medicinal shrub commonly called Ayapana which was traditionally used by the tribal community for chronic wounds, and hemorrhage and is used as a skincare herb in East Kalimantan [2]. Also, the research findings report that various phyto-constituents extracted from the *Ayapana triplinervis* Vahl were known to show antimicrobial [3], (Carlos, Sobrinho, Maia De Morais, *et al*., 2017), anti-helminthic [5], anti-oxidant [6], Anti-nociceptive [7], analgesic activity (Vahl, 2016), and anti-inflammatory properties (Parimala *et al*., 2012). The traditional medical treatment uses the raw fresh leaves of *Ayapana triplinerve* Vahl for treating the scars and piles (Murugesan *et al*., 2023). This fascinates us to explore the proteins and peptides fSrom *Ayapana triplinerve* Vahl fresh leaves that have potent medicinal properties. The current study aims to isolate and characterize the potent Proteins and peptides from fresh

leaves of *Ayapana triplinerve* Vahl and validate its antibacterial activity on specific clinical pathogens.

2. Materials and Methods

All the chemicals used were purchased from Hi-media Chemicals. 8-16% of Gradient markers are purchased from Genie, India. The bacterial cultures were obtained from the department of microbiology, PSG Institute of Medical Science and Research (PSGIMSR), Coimbatore, India.

2.1 Plant collection, propagation and Authentication

The *Ayapana triplinerve* Vahl plant saplings were collected from the nursery at the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, Tamil Nadu. The samplings were propagated in the medicinal plant garden, at PSG College of Arts & Science, Coimbatore, Tamil Nadu for collecting the fresh leaves. The plant was authenticated by the Botanical Survey of India, TNAU, Coimbatore, Tamil Nadu, India as *Ayapana triplinervis Vahl* (*Ayapana triplinervis*) belonging to the Asteracea family.

2.2 Optimisation of extraction method

The fresh leaves were collected, washed using deionised water and spread in Whatmann filter paper to remove excess water. Then the leaves were plunged in liquid nitrogen and ground in pre-cooled mortar. The ground powder was weighed 10 g, and stored at -80°C until use. Total proteins were extracted by three different methods (Table 1).

Table 1: Optimization of Total protein extraction methods.

Method I: Direct extraction of Protein using TCA Acetone method (TA method)(Wang *et al*., 2016), [12], (Méchin *et al*., 2007)	**Method II: Extraction using the Extraction buffer mix and precipitation by TCA Acetone (EB - TA method) [14, 15]**	**Method III: Extraction using TCA Acetone and precipitation by water saturated Phenol (TA - WSP method) [16, 17]**
Ten grams of the powdered leaves were added to the pre-chilled 15% trichloroacetic acid (TCA) in acetone using a mortar and pestle. To the lysate, 100 µl of 100 mM phenyl methyl sulfonyl fluoride (PMSF), 25 mg polyvinyl pyrrolidone (PVP), and 100 µl of 40 mM Dithiothreitol (DTT) were added. The volume was brought to 50 ml with 15% TCA in acetone and vortexed briefly for 5 seconds. The mixture was incubated at -20 °C for 2 hours, followed by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 90% ice-cold acetone, with repeated vortexing and centrifugation until the precipitate became pale white. The final pellet was air-dried and stored at -20 °C	Ten grams of the powdered leaves were mixed with an extraction buffer consisting of 1M Tris-HCl (pH 7.4), 1M EDTA, 1M $MgCl_2$, 100 mM PMSF, 40 mM DTT, and 0.1% Triton X-100. The lysate was transferred to a centrifuge tube, the volume adjusted to 50 ml with the extraction buffer, and vortexed for 5 seconds. The mixture was incubated at -20 °C for 2 hours and then centrifuged at 10,000 g for 15 minutes at 4 °C. The supernatant was collected, and the pellet was discaede 2-3 times with the extraction buffer. The supernatant was combined with an equal volume of 15% TCA in acetone and incubated at -20 °C overnight for protein precipitation. After incubation, the protein precipitate was centrifuged at 10,000 g for 20 minutes at 4°C and the pellet was washed thrice with 90% ice-cold acetone. The resulting air-dried pellet was stored at -20 °C.	Ten grams of the powdered leaves were added with pre-chilled 10% TCA in acetone. To the lysate, 100 µl of 100 mM PMSF, 25 mg PVP, and 100 µl of 40 mM DTT were added, and the volume was adjusted to 50 ml with 10% TCA in acetone. After vortexing for 5 seconds, the mixture was incubated at -20 °C for 2 hours for protein extraction. The mixture was then centrifuged at 16,000 g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 0.1 M ammonium acetate in 80% methanol and centrifuged at 10,000 g for 30 minutes (4°C). Followed by washing with 90% ice-cold acetone and centrifugation at 10,000 g for 30 minutes (4°C). The pellets were air-dried and resuspended in 50 ml of 1:1 water-saturated phenol: SDS-PAGE lysis buffer, vortexed, and centrifuged at 16,000 g for 5 minutes at 4°C. The upper phenol phase was removed and mixed with an equal volume of 0.1 M ammonium acetate in 80% methanol, then incubated overnight at -20 °C. The next day, the mixture was centrifuged at 16,000 g for 5 minutes at 4°C. The final pellet was washed once with 90% ice-cold acetone and 100% methanol, air-dried and stored at -20 °C.

2.3 Effect of buffer on protein solubilisation and quantification

The final protein pellet was reconstituted with various buffers Tris-HCl (pH 6.8), Tris-HCl (pH 7.4) and Urea buffer. Thus, the effect of pH on the solubilization of proteins with different buffers was studied. The reconstituted protein sample was estimated by Direct UV (Nanodrop). BSA was used as a standard protein to determine the unknown concentration of test samples.

2.4 SDS - PAGE analysis of protein extract

The extracted protein was electrophoresed in 8-16% gradient SDS - PAGE (Lamelli *et al*.,1970) in mini slab gel (16cm X 20cm). 4X sample loading dye was added to all the samples and markers. Then the gel was stained using a CBB G250 staining solution. The image of the gel was captured using the Gel Documentation system (UVITEC, Cambridge). The molecular weight for the separated bands was calculated by plotting a graph of log MW (Molecular weight) vs. relative migration distance (R_F) for standard markers [18].

2.5 Antimicrobial activity of the protein extract

The bacterial strains used for these studies are clinical isolates of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Serratia marcescens, Klebsiella pneumonia, Proteus vulgaris, Streptococcus mutans, MRSA, Enterobacter aerogenes.* These strains are the common pathogens that manifest in the wound. The cultures were procured from the Department of Microbiology, PSG Institute of Medical Science and Research, Coimbatore, India. The antimicrobial tests for the clinical bacterial isolates were done by the approved standard method of the National Committee for

Clinical Laboratory Standards (NCCLS) [19]
The microdilution method was carried out in 96 wells; Column 11 serves as a negative control (only with bacterial culture) and Column 12 as a control (Media alone). Serial dilution of the protein extracts was made in 96-well plates to get 0.4 - 200 µg/ml concentrations, and the antibiotics Amikacin, Streptomycin and Ciprofloxacin (positive control) were used. The samples were loaded in triplicate for statistical analysis. 5 µl of bacterial culture (10^4 - 10^5 CFU/ml of inoculum) was loaded into all the wells except the control and incubated overnight at 37 °C. The turbidity in the plates was read in an ELISA plate reader (iMARK BIO-RAD) at 600nm. The minimal inhibitory concentration was calculated.

3. Results

3.1. Plant collection, propagation, Authentication of Plant

The plant *Ayapana triplinervis* Vahl was collected from the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu and the same was authenticated as *Ayapana triplinervis* Vahl R. M. King & H. Rob (Syn. *Ayapana triplinervis* Blume, *Ayapana triplinervis* Vahl, *Ayapana ayapana* vent) belonging to family Compositae (Asteraceae) with the authentication No.773/FECC/IDI/IFGTB/2018. The Plant specimen with ACC.No.24248 is deposited in the Institute's Fischer Herbarium (Acronym: FRC)
The authentication was conducted by the Botanical Survey of India (BSI) after the blooming of the Flower. The specimen was authenticated as *Ayapana triplinervis* (Vahl) R.M. King & H. Rob - (authentication number BSI/SRC/5/23/2019/tech/249).

Fig 1: Aerial view of *Ayapana triplinervis* Vahl.

3.2 Effect of buffer on protein solubilisation and quantification

Among all three methods, extraction potentiality, solubility of buffers (pH) were the core properties to consider to get a biologically active protein for various medicinal applications. The proteins extracted with three methods were solubilized in three different buffers (Fig. 2) and estimated by the nanodrop technique. The EB-TA method has shown the highest quantity of protein as 1650 mg/g, 720 mg/g, and 890 mg/g in Tris-HCl (pH 7.4), Tris-HCl (pH 6.8) and urea buffer, respectively. However, the protein yields were as high as 120 mg/g; 355 mg/g; and 320 mg/g; in Tris-HCl (pH 7.4), Tris-HCl (pH 6.8) and urea buffer by TA - TA-WSP method, respectively. The TA method showed a very low concentration of protein, which revealed the poor extraction by TCA.

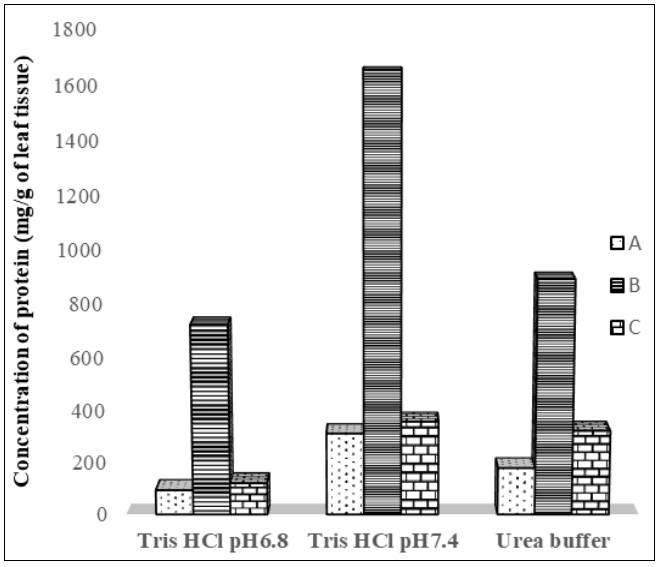

Fig 2: Comparison of protein concentration extracted by TA method (A); EB - TA method (B); TA - WSP method (C) and solubilised in various buffers: Tris-HCl (pH 6.8); Tris-HCl (pH 7.4); and Urea buffer)

3.3 Characterization of Proteins by Sodium Dodecyl Sulphate (SDS) Polyacrylamide gel

The protein extracted by various methods was solubilized in different buffers and analyzed in 8 - 20% gradient SDS polyacrylamide gel (Fig. 3). The observation of bands indicated that TA - WSP method gave more no of protein bands in all the buffers used for solubilization. Tris-HCl (pH 7.4) solubilized proteins from EB - TA method gave some good quality bands compared with Tris-HCl (pH 6.8) and urea buffer solubilization. TA method does not show any significant bands. The results of protein concentration and SDS - PAGE analysis revealed that the proteins extracted in Tris-HCl (pH 7.4) buffer showed quality protein compared with other solubilization buffers.

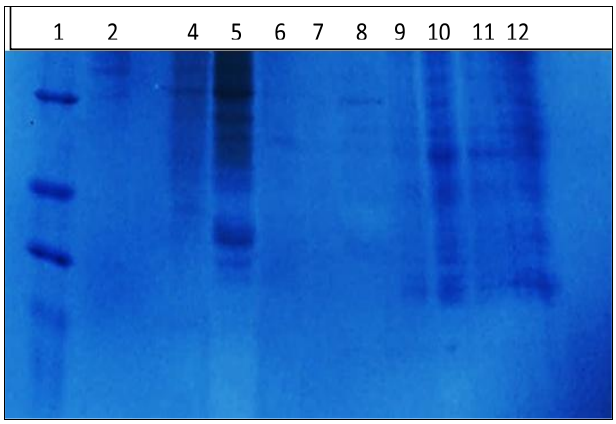

Fig 3: Effect of buffers in solubilizing the proteins was assessed using SDS - PAGE

1 - Marker; 2 - Std BSA; 4 - EB - TA method extracted protein in Tris HCl (pH 6.8); 5 - EB - TA method extracted protein in Tris HCl (pH 7.4); 6 - EB - TA method extracted protein in urea buffer: 7 - TA method extracted protein solubilized in Tris HCl (pH 6.8); 8 - TA method extracted protein in Tris HCl pH (7.4); 9 - TA method extracted protein in urea buffer; 10 - TA - WSP method of protein extracted in Tris HCl (pH 6.8); 11 - TA - WSP method of protein extracted in Tris HCl (pH 7.4); 12 - TA - WSP method extracted protein in urea buffer.

3.4 Determination of protein molecular weight by Gradient SDS PAGE (8-16%)

The Tris-HCl (pH7.4) solubilized proteins of all three methods were separated by gradient SDS-PAGE (8-16%) and molecular weight was determined. The EB - TA method resolves more protein bands with molecular weight ranging from 119 - 9 KDa (Lane 2) compared with other methods. Around 10 high-intensity bands were determined in the EB - TA method (Fig 4). TA method (Lane 3) shows very low intensified bands due to poor solubility of proteins. TA - WSP method (Lane 4) has five bands but the quality of the band was poor which may be due to phenols. The molecular weight for the separated bands was calculated by plotting a graph of log MW (Molecular weight) vs. relative migration distance (R_f) for standards. The Molecular weight of the protein extracted from the TA method, EB - TA method, and TA - WSP method of *Ayapana triplinervis* Vahl was tabulated (Table 2). Figure 4 shows the protein bands of different methods separated in SDS PAGE.

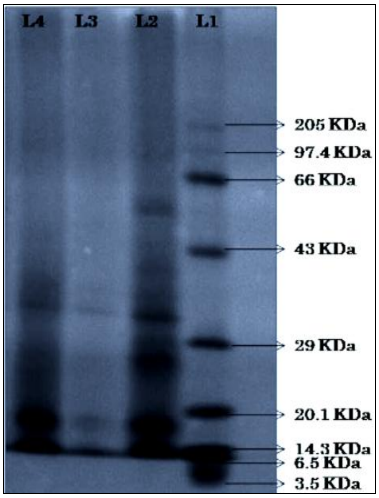

Fig 4: Gradient SDS PAGE (8-16%) showing the separated protein band of the TA method, EB - TA method, TA - WSP method from fresh leaves of *Ayapana triplinervis* Vahl

Table 2: The molecular weight of Protein is calculated by plotting a graph of log MW (Molecular weight) vs. relative migration distance (R_f) for standards

S. No.	Lane 2 (EB - TA method extracted protein)		Lane 3 (TA method extracted protein)		Lane 4 (TA - WSP method extracted protein)	
	Migration Distance (mm)	M.W. (KDa)	Migration Distance (mm)	M.W. (KDa)	Migration Distance (mm)	M.W. (KDa)
1.	4.4	118.74	-	-	-	-
2.	6.2	71.91	-	-	-	-
3.	7	57.54	-	-	-	-
4.	7.9	44.78	-	-	-	-
5.	8.9	33.89	-	-	8.5	37.89
6.	9.7	27.12	9.3	30.32	9.3	30.32
7.	10.4	22.32	-	-	10.9	19.41
8.	11	18.88	11	18.88	-	-
9.	12.8	11.43	12.8	11.43	12.8	11.43
10.	13.6	9.15	13.6	9.15	13.6	9.15

3.5 Antibacterial activity of protein extract by Micro dilution technique

The antibacterial activity of the protein, extracted from fresh leaves of *Ayapana triplinervis* Vahl, was assessed by the microdilution technique (Fig. 5), and the minimum inhibitory concentration (MIC) was calculated (Table 3). From Fig. 6, the results revealed that the protein extract showed the highest growth inhibition at a Minimum Inhibitory Concentration of 0.26 µg/ml against *Escherichia coli*, 1.05 µg/ml for *Staphylococcus aureus* and MRSA, and 1.13 µg/ml for *Streptococcus species* which was compared against the antibiotic standards Amikacin, Ciprofloxacin and Streptomycin. Compared with the standard antibiotics, the protein samples of the EB -TA method exhibited a potent antibacterial activity.

The protein samples extracted from leaves of *Ayapana triplinervis* Vahl exhibited antibacterial activity against *Staphylococcus aureus* (MIC of 1.05µg/ml) which was better compared with the reports of Mamun [20] who assessed antibacterial activity for seed kernel of *Ricinus communis* against *Staphylococcus aureus* (MIC of 62.5µg/ml). The phytochemical extracts from the leaves of *Ayapana triplinervis* Vahl were also known to exhibit antibacterial properties. The best antibacterial activity result was obtained against *Vibrio* (MIC 125µg/ml) and antifungal properties against *Colletotrichium corchori* (MIC 62.5µg/ml) compared with the other organisms used in the study [21]. The hydroalcoholic extract possesses antibacterial activity against gram-negative bacteria (MIC 94µg/ml). The crude protein extract of *Actinobacterium* elicited antibacterial activity against *Salmonella typhimurium*. Comparing the standard antibiotics and previous research reports, it was evident that the plant proteins extracted from fresh leaves of *Ayapana triplinervis* Vahl showed a good antimicrobial activity with a minimum inhibitory concentration.

Table 3: Minimum Inhibitory Concentration of *Ayapana triplinervis* plant protein extract of EB-TA Method with the known antibiotics against various Bacteria isolated from clinical wound

Test Organisms			*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus mutans*	*Klebsiella pneumoniae*	*Serratia marcescens*	MRSA	*Enterobacter aerogenes*	*Proteus vulgaris*	*Pseudomonas aeruginosa*
Concentration (µg/mL)	Antibiotics	Amikacin	255	255	258	260	263	269	258	255	253
		ciprofloxacin	500	510	500	500	521	521	510	500	500
		streptomycin	25	25	25	25	26	27	25	26	25
	Protein sample from EB-TB Method		0.26	1.05	1.13	117	7.96	1.05	19.27	4.11	12.5

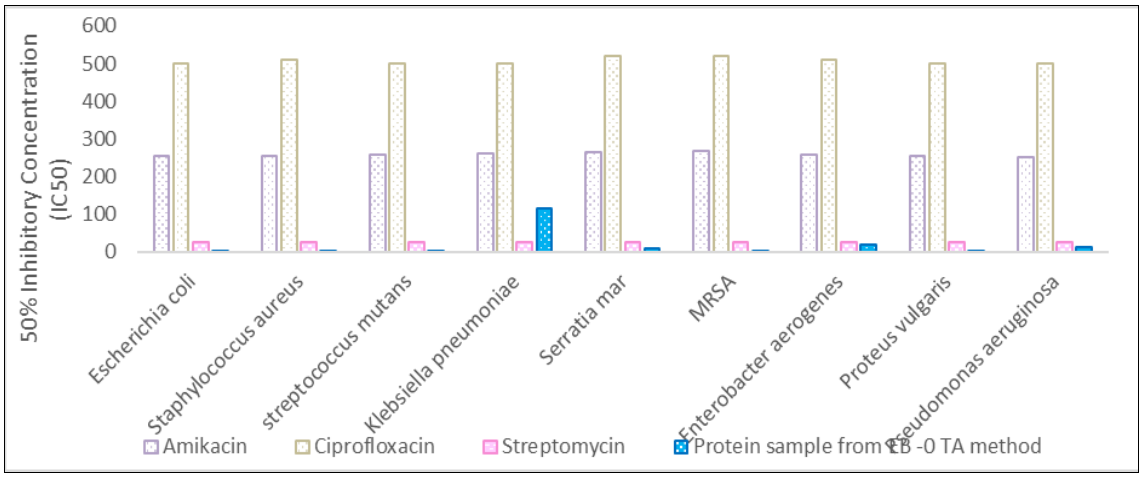

Fig 5: Comparison of MIC values of the protein samples with the standard Antibiotics

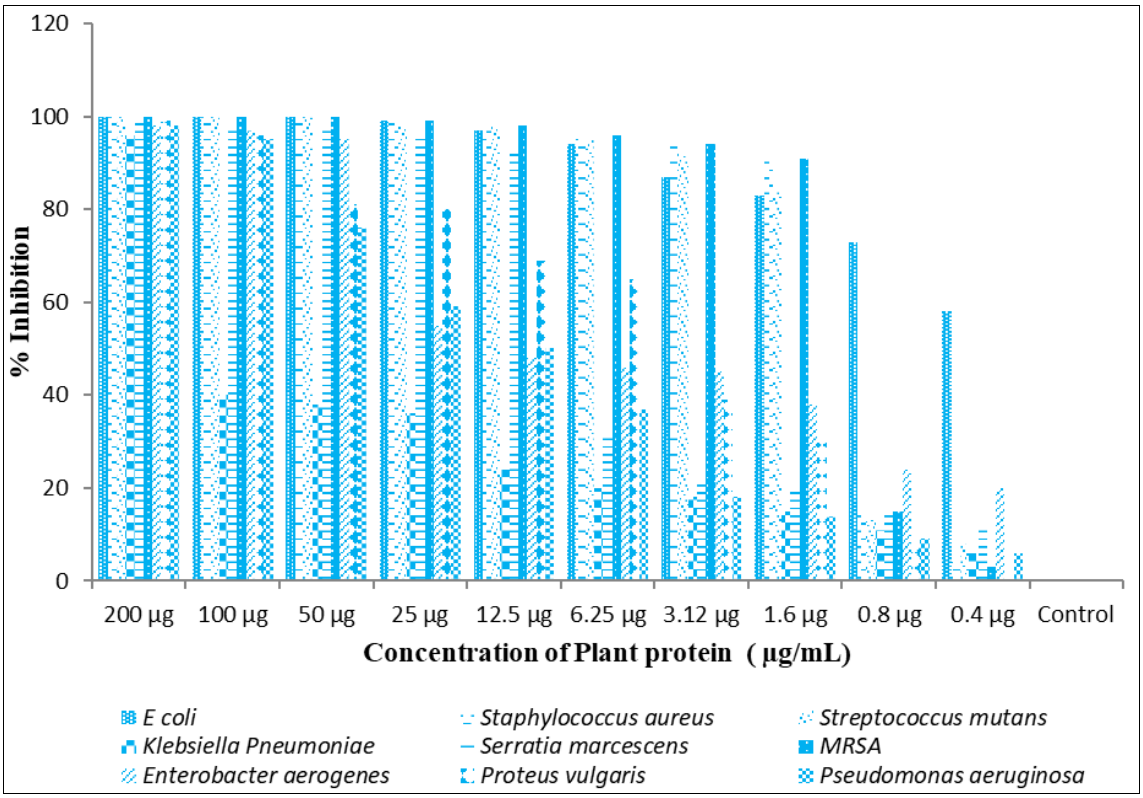

Fig 6: Growth Inhibitory activity of the plant protein extract against the bacterial cultures.

4. Discussion

In traditional medicine, *Ayapana triplinervis* Vahl was used to treat cuts, wounds, scrapes, ulcers, coughs, colds, and sore throats. Studies revealed that the leaves of *Ayapana triplinervis* Vahl were known to exhibit antibacterial (Regina Matos Lopes *et al*. 2015; Carlos *et al*. 2017), antinociceptive (Cheriyan *et al*., 2017), antineoplastic [25], antiulcer [26], analgesic [8] and anti-inflammatory properties(Derbel *et al*., 2023), [9]. The methanolic extract was known to have hepatoprotective and antioxidant activity in rats induced with hepatitis [28]. In the research report by Ventola [29] frequent usage of antibiotics was the lead cause for the bacteria to become resistant to antibiotics. The current study explores the bioactive protein/ peptides from fresh leaves of *Ayapana triplinervis* Vahl which could be an alternative to antibiotics. A different extraction method was used to extract a good quality bioactive protein from fresh leaves of *Ayapana triplinervis* Vahl. The major difference was the usage of Tris HCl (pH 7.4) buffer in the EB- TA method to extract hydrophilic proteins followed by TCA-Acetone precipitation [30]. Tris HCl preserves protein from denaturing and also extracts the cytoplasmic protein by cleaving the cell membrane [31]. In the TA method lyophilized plant leaves were subjected to TCA in acetone which extracts denaturing protein under acidic /hydrophobic conditions. TCA removes the other contaminant which makes protein purification to be easier. But TCA tends to disrupt the Hydrogen bonding between the protein structures causing denaturation. It also dehydrates the protein sample making the protein pellet so hard to rehydrate for future use [32]. In TA - WSP method the protein was extracted by TCA Acetone and precipitated using water-saturated phenol with SDS lysis buffer. The use of phenol removes the non-protein component making a good recovery of hydrophobic protein and the SDS lysis buffer denatures the proteins [11, 12]. Among different extraction methods, the EB -TA method gave a high concentration of protein on solubilizing with Tris HCl pH 7.4 when estimated by direct Nanodrop technique. The optimum pH for solubilizing the protein was 7.2 - 7.8. The Tris HCl pH 7.4 buffer gave the best solubilization compared with other

buffers in all three extraction methods. The results of protein concentration were compared with the reports of [14, 33] who extracted the total protein from *Azadirachita indica* (Neem) [34, 35] compared the extraction method for *Ficus deltoidea* (Mistletoe). The EB-TA method gave more proteins while resolving them in gradient SDS PAGE (8- 16%). The extraction buffer used in this method maintained a good quality protein. The combination of TCA-acetone with Phenol precipitation also gave a good yield of protein, but the number of proteins resolved in the gradient SDS page (8- 16%) was poor. The TA method extracted protein showed poor quality and a low number of protein bands in gradient SDS PAGE. The TCA makes the protein pellet so hard to reconstitute in rehydration buffers. Most antimicrobial peptides were short oligomeric sequences (2KDa - 20KDa) with cationic charge and amphipathic. In plants, the level of antimicrobial peptides is elevated during the host defence mechanism. So, the extracted protein was tested for its antimicrobial activity, which showed growth inhibition against various bacteria, which was compared with the antibiotics and the previous research reports. The crude proteins from seeds of different plants *Foeniculum vulgare, Cucumis sativus, Ammi majus, Allium ascolinicum, Cichorium intybus* and *Rumex vesicarius*, were assessed for their antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Pseudomonas vulgaris and Staphylococcus aureus* [36]. Among these *Ammi majus* and *Allium ascolinicum have* shown antibacterial activity against *E coli* and *Proteus vulgaris. Foeniculum vulgare* exhibited a potent antibacterial activity against all the species used in the study. The proteins/ peptides, especially the cationic peptides, react with the negatively charged membrane proteins and destroy the bacteria.

5. Conclusion

The plant *Ayapana triplinervis* Vahl had a good medicinal value. Identifying antimicrobial proteins/peptides will be an alternative to antibiotics, which are effective against antibiotic-resistant bacteria. In this study, we have optimized the extraction technique for the extraction of biologically active protein/peptide from fresh leaves of the plant. The extracted protein showed good antibacterial activity against various pathogens isolated from clinical samples. This study concludes that the EB-TA method would be the best method to give a good yield and quality proteins/peptides and shows antibacterial activity against specific bacteria.

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