Toxicology International, *30(1) : 111-119; 2023. DOI: 10.18311/ti/2023/v30i1/31892*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Validating the Antimicrobial Potentiality of Peptides from Pods of *Acacia nilotica* Willd.ex Delile: A Spotlight on Bacterial Fauna

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Abstract

Contagious infections cause over 17 million human deaths in a year among which diseases caused by bacteria are the most prominent ones. Additionally, in the last few years, the haphazard usage of antibiotics has provoked the condition of multifarious defiance in the bacterial strains against these chemical drugs thus contributing to an upsurge towards severe economic and communal welfare-related uncertainties. Thus, to annihilate such situations there is a compelling demand to explore novel substitutes to overcome these concerns. Hence, such problematic situations bring up the limelight towards plant-derived proteins/peptides possessing antimicrobial activity which prove to be excellent alternatives against bacterial pathogens causing serious maladies in humans. Therefore, in this context the current study investigated the antibacterial potential of the Ethanol, Methanol and Chloroform peptides/protein isolate obtained from pods of *Acacia nilotica* by Disc-Diffusion Assay and MIC (Minimum Inhibitory Concentration) against *E. coli* and *B. subtilis* bacterial strains. The antibacterial activity of all three solvent peptide isolates was found to be exhibiting antimicrobial activity at a concentration of 100µg/ml against *B. subtilis* with Disc-Diffusion as well as MIC with Chloroform isolate displaying the highest activity against gram-positive Bacillus species. Therefore, such plant-derived antibacterial proteins/peptides in the future can be used as a therapeutic drug to combat various severe bacterial maladies thus providing a better alternative than antibiotics both in terms of safety against unwanted secondary effects as well as will also help in bringing down the graphs of Antimicrobial Resistance (AMR) posing a serious threat to the human health. As a result, the study concludes that peptide isolates obtained from *Acacia nilotica* pods are effective against gram-positive Bacillus species, making them an important antibacterial compound.

Keywords: *Acacia nilotica,* Antimicrobial Resistance, Plant-Derived Antimicrobial Peptides

1. Introduction

With context to the contemporary medicinal scenario invention of antibiotics was one of the dominant innovations that the human world had encountered so far. But in the past couple of years, the progression of some maladies that were not treatable with the use of the available antibiotics due to increased (AMR) Antibiotic Resistance (a condition in which microbial population develops resistance towards substances that they had endured beforehand and hence stops any kind of reaction towards it) of disparate microbial pathogens had been seen to gain immense progression. Around 70% of bacterial strains responsible for causing infections are found to be relatively resistant to at least one

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Article Received on: 08.11.2022 **Revised on:** 21.11.2022 **Accepted on:** 02.01.2023

class of antibiotics. The fact that different disorders are related to health protection, cases linked with resistance to various medications had been observed frequently in various bacterial pathogens universally. Apart from difficulties related to fitness-related issues repercussions of Antimicrobial Resistance (AMR) also pose a high level of impact on the communal as well as remunerative affairs of a nation. Antimicrobial resistance majorly consists of 2 types of resistance i.e., intrinsic and adaptive type of resistance. The ability of microbes to resist the effect of antimicrobial compounds due to their natural functioning attributes is termed intrinsic resistance, while the capacity of the microbial fauna to sustain situations of distress by rapidly modifying their transcripts according to the surroundings is called adaptive resistance[1]. In the interim of the last 40 years, around 50 disease-causing organisms have been discovered out of which around 10% were bacteria[2]. Observational research considering animal studies validates the action of various bacterial strains in cancer development. However, the entire responsible mechanism behind the condition was observed to be quite complicated and implicate interactions among persistent inflammation, bacterial by-products that influence stem cell stability, cell biology and cell-signalling[3]. In addition to this, bacterial contaminations are examined to be crucial biological determinants for causing infertility in the case of males Bacterial strains including *Escherichiacoli, Ureaplasma, Mycoplasma, Staphylococcus aureus* and *Chlamydia trachomatis* adversely disturb the mechanism of sperm formation (spermatogenesis), sperm activity as well as also affect imperative parts of the male reproductive system including the urethra, seminal tracts and prostate and hence elevate complications related to infertility in men[4]. Apart from this, various gram-negative bacterial strains for example *Citrobacter spp., Pseudomonas aeruginosa, Proteus vulgaris* and *Escherichia coli* are found to be responsible for causing complicated urinary tract infections and are seen to be gaining defiance against various antibiotics hence giving rise to social health-related issues[5].

Hence, the ferocious accumulation of intransigence may create the benefits related to antibiotics counter-productive and hence may give rise to a post-antibiotic scheme[6]. Thus, such problematic scenarios related to disease conditions caused by bacteria and increasing rates of antimicrobial resistance on a universal level have brought up many adverse effects on developing countries and hence have directed the investigation towards Antimicrobial Peptides (AMPs) (that are essential gene-coded proteinaceous entities that fall under the category of modern antimicrobials and are widely efficient and less probable to cause resistance in microbes). AMPs are tiny substances (<10kDa) with undeviating anti-infective functions. These essential peptides are a distinct class of native, defence-providing substances and their complete form generally consists of 12-100 amino acid units with an amphipathic organization carrying overall plus charge[7] (usually varying from +2 to +11). AMPs have effective and wide-range functionality in opposition to various groups of fungi, bacteria, parasites, viruses and yeasts and are formed by all living individuals as crucial and pivotal elements of their intrinsic defence structure, working as a primary source of immunity against pathogenic interventions in Eukaryotes or are generated to confine the development of additional microbial species in case of Prokaryotes. In addition to this, the significance of AMPs has currently expanded at the time of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic to investigate an advanced virus-inhibiting substance to prevent COVID-19. AMPs were first identified in 1939 by Rene Dubos, a microbiologist who procured an anti-pathogenic molecule gramicidin which was established to prevent pneumococcal disease in mice from a *Bacillus* strain found in soil. Eventually, various AMPs have been detected in Prokaryotes as well as Eukaryotes which include compounds like Tyrocidine and Purothionin which works against bacteria as well as fungi and are formed by *Bacillus brevis* (Bacteria) and *Triticumaestivum* (Plant) respectively. So far around 3,000 AMPs have been identified, defined and elucidated in the AMP database (APD3) knowing the fact that the epidermis area of the frog itself consists approx 300 distinct AMPs. In the case of plants, AMPs are vital molecules for providing quick protection against fungal and bacterial diseases and in addition, also play a significant role in cell-signalling processes. Around ten groups of plant, AMPs have been reported out of which snakins, defensins and thionins fall into the category of groups that are studied extensively so far. Various bioactive AMPs have been taken out from seeds, roots, flowers, leaves, stems as well as fruits as described in the case of Avocado and *Capsicum* and were reported to be having antimicrobial action against diseases caused by *Streptococcus aureus* and *Escherichia coli*[8]. Although a large number of plant species have been studied for various AMPs found in them, information on some of the most therapeutically relevant plant species in terms of proteomic data to elucidate peptides/proteins available in them that can exhibit properties and activities similar to AMPs has yet to be studied. One such plant species that is well known for its therapeutic benefits but is mainly

investigated for the various secondary metabolites present in it but lacks any information regarding its proteomic data is *Acacia nilotica* (L.) Willd.ex Delile (Mimocaceae) (*A. nilotica*) commonly known as "Prickly *acacia*" in Australia is an important versatile tropical and subtropical plant with extensive distribution in America, Asia, Australia and Africa. It is a 5-20 m high tree, with its stems and branches commonly dark to black coloured with thin, straight, light, grey spines and with flowers usually golden to yellow. The pods (fruits) of *Acacia* are greatly compressed, thick, white to grey coloured and hairy in appearance. The plant is found to be an affluent source of various amino acids like methionine, lysine, tryptophan, and cystine and also contains some amounts of manganese, iron, potassium and phosphorus. The plant *Acacia* is found to be largely valuable for the treatment of a variety of diseases for instance bronchitis, dysentery, bleeding piles etc. It also exhibits antidiabetic, anti-hypertensive, as well as antispasmodic properties. Besides this, various experimental investigations carried out by the usage of extracts made by various plant parts of *Acacia* have shown its efficacy as an anti-microbial, anti-fungal, antiviral and molluscicidal agent[9]. Chemical investigation to evaluate the quantity of different nutritious peculiarities from the fruits (pods) of *Acacia nilotica* species has revealed that the pods of *Acacia* contain about 21.4% crude protein, 24.77% fats, as well as 11.67% ash content[10]. Hence, the major aim of the present study is to determine the antimicrobial activity of the proteins/peptide isolates from the pods (fruits) of *Acacia nilotica* (L.) Willd. ex Delile through major antimicrobial assays like the disc-diffusion method and Minimum Inhibitory Concentration Determination method (MIC) to investigate the role of the peptide/protein isolates in the inhibition of disease-causing bacteria like *Escherichia coli* and *Bacillus subtilis* and confer the role of *A. nilotica* as a medicinally significant plant species by establishing the observation that the proteins/peptides present in the plant species exhibit antimicrobial activity and thus can further be utilized as a pivotal alternative therapy in place of antibiotics which can contribute to human welfare by bringing a declining phenomenon in cases related to microbial resistance due to enormous antibiotic usage and contagious disease conditions.

2. Materials and Methods

2.1 Materialsand Microbial Strains

Pods of *A. nilotica* were collected from forests of *Acacia* located in remote areas of Lucknow, Uttar Pradesh, India. The bacterial strains gram-negative *Escherichia coli* (MTCC 82) and gram-positive *Bacillussubtilis* (MTCC 1305) were obtained from the Council of Scientific and Industrial Research (CSIR-Institute of Microbial Technology), Chandigarh, India. Muller-Hington Agar (MHA) and Muller Hington Broth (MHB) were obtained from Himedia Laboratories.

2.2 Preparation and Extraction of Plant Materials

The pods of *A. nilotica* were dried and then turned into powdered form with the help of a grinding technique. The powder obtained was then subjected to the homogenization buffer consisting of (Tris-HCl, NaCl, Polyvinylpolypyrrolidone (PVPP), and Phynylmethylsulfonyl fluoride (PMSF)) for extraction of proteins from the pod powder. The homogenization solution was then taken to undergo acetone precipitation after incubation for 24-36 hours at normal room temperature ranges. The protein isolate obtained in the form of a pellet was then taken up for quantification and profiling through Bradford protein assay and sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) respectively. Finally, the protein/peptide isolate was taken up for Liquid Chromatography-Electrospray-ionization-Mass Spectrometry (LC/MS-ESI) to evaluate the number and types of proteins/peptides present in the isolate obtained from the pods (fruits) of *Acacia nilotica*.

2.3 Disc Diffusion Assay

The antibacterial activity of the protein/peptide isolate procured from the pods of *Acacia nilotica* was tested against two bacterial strains i.e., Gram-negative; *Escherichia coli*(MTCC 82) and Gram-positive; *Bacillus subtilis*(MTCC 1305)by applying disc-diffusion method. The stock solutions of both bacterial stains were maintained at -20° C. About 200µL of stock solution was inoculated in freshly prepared sterile nutrient agar solutions prepared for both the bacterial strains separately to prepare pure cultures of the strains. The solutions were then maintained at 37° C for both *E. coli* as well as *B. subtilis* with the agitation of 180 rpm till the stationary phase was obtained, about 24hrs *(E.coli)* and 34 hrs*(B.subtilis)*. The Disc diffusion method was performed according to the modified[11] methods. Muller-Hington agar (MHA) was prepared by dissolving 38g/l in distilled water. The freshly prepared, sterile MHA was then poured into sterile Petri plates and was left to set in. The inoculants were then swabbed on MHA with a sterile cotton swab and were left to dry out. Sterile discs (5 mm) were saturated

with different dilutions of peptide/protein isolates prepared as 500,100,50 and 10 µg/ml concentrations in solvents like chloroform, methanol and ethanol for both the bacterial strains separately. 10µg/ml BSA (Bovine Serum Albumin) is taken as the negative control and antibiotic Gentamycin 20µg/ml is taken as a positive control. Plates were then stored at 37° C inside an incubator overnight in the upright position for the growth of bacterial strains and to evaluate the activity of the protein/peptide isolate sample obtained from the pod of the concerned plant species. The discs were tested in triplicates. The diameters of inhibition zones were then calculated and results were analyzed accordingly.

2.4 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the protein/peptide isolated and procured from the pods/fruits of *Acacia nilotica* was tested using a broth dilution method. The test was applied against Gram-negative; *Escherichia coli* (MTCC 82) and Gram-positive; *Bacillus subtilis* (MTCC1305). The stock solutions of both the bacterial strains were maintained at -20° C. Muller-Hington Broth (MHB) was prepared by dissolving 21g/l in distilled water. About 200µL of stock solution was inoculated in freshly prepared sterile Muller-Hington Broth to prepare pure cultures of both bacterial strains individually. The solutions were maintained at 37° C for both the bacterial strains with agitation till the stationary phase was obtained. The Minimum Inhibitory Concentration (MIC) was performed according to the modified approach[12]. Different dilution concentrations of the peptide/protein isolates were prepared as 500, 100, 50 and 10 µg/ml with different solvents like ethanol, methanol and chloroform. 100µl of the respective peptide/protein dilutions were added to the test tubes for each solvent dilution along with inoculation of 100µl of bacterial samples. Bacterial tubes were then incubated for 24 hrs at 37° C. Finally, the optical densities were analysed at 600nm and results were analysed. Antibiotic Gentamycin 20µg/ml was taken as positive control while Bovine Serum Albumin (BSA) 10µg/ml was taken as a negative control.

2.5 Statistical Analysis

Tests and examinations were done in triplicates. The mean values±Standard deviation (SD) of triplicates was calculated and analysed. The values were considered significant when $p<0.05$.

3. Results

3.1 Antibacterial Activity Determination by Disc-Diffusion Assay

The antibacterial activity of the protein/peptide isolate obtained from the pods of *Acacia nilotica* is displayed in (Table 1 and Figure 3). The activity of the peptide/protein isolate in the case of Chloroform, Ethanol and Methanol isolates was observed in the case of gram-positive *B. Subtilis*bacterial strain with chloroform isolate exhibiting the highest inhibition zone with a mean inhibition zone of 1.933±1.429 mm at a concentration of 100µg/ml of peptide/protein isolate, while in the case of Ethanol and methanol, the

Table 1. Antimicrobial activity of *Acacia nilotica* pod protein/peptide isolate by disc-diffusion Method

Microorganisms	Ethanol Extract (µg/ml)						Methanol Extract (µg/ml)		
	Pod Isolate Concentration (µg/ml)	Trial 1	Trial 2	Trial 3	Average (mm)	Trial 1	Trial 2	Trial 3	Average (mm)
Escherichia coli (Gram-negative)	500	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0
Bacillus subtilis (Gram-positive)	500	1.2	1.2	0	0.8±0.092	0	0	0	0
	100	1.2	0.9	1.3	1.13±0.208	0.3	1.25	0.2	0.583±0.579
	50	1.2	0.4	0	0.533±0.611	0	0	0	0
	10	1.2	0.9	0.5	0.866±0.351	0	0	0	0

Microorganisms	Pod Isolate Concentration (µg/ml)	Chloroform Extract (µg/ml)			
		Trial 1	Trial 2	Trial 3	Average (mm)
Escherichia coli (Gram-negative)	500	0	0	0	0
	100	0.7	0	0	0.233±0.404
	50	0	0	0	0
	10	0	0	0	0
Bacillus subtilis (Gram-positive)	500	2.2	1.05	0	1.083±1.100
	100	3.5	1.6	0.7	1.933±1.429
	50	1.9	1.7	0.85	1.483±0.557
	10	2.05	1.8	0.3	1.383±0.946

mean inhibition zones were 1.13±0.028 mm and 0.583±0.579 mm respectively at a concentration of 100µg/ml which was highest among all the other considered concentrations of the isolates taken to check the inhibition activity against the bacterial strains.

However, zero or very little amount of inhibition activity was observed in the case of gram-negative *E.coli* in the case of Ethanol as well as Methanol while a mean inhibition zone of about 0.233±0.404 mm at a concentration of 100µg/ml was observed in the case of Chloroform peptide/protein isolate, The mean inhibition zone of Antibiotic Gentamycin was 7.0±1.0 mm (*B. subtilis*) and 5.0± 2.0 mm (*E. Coli*) while mean inhibition zone of BSA (Negative control) was observed to be 0 in the case of both bacterial strains. Figure

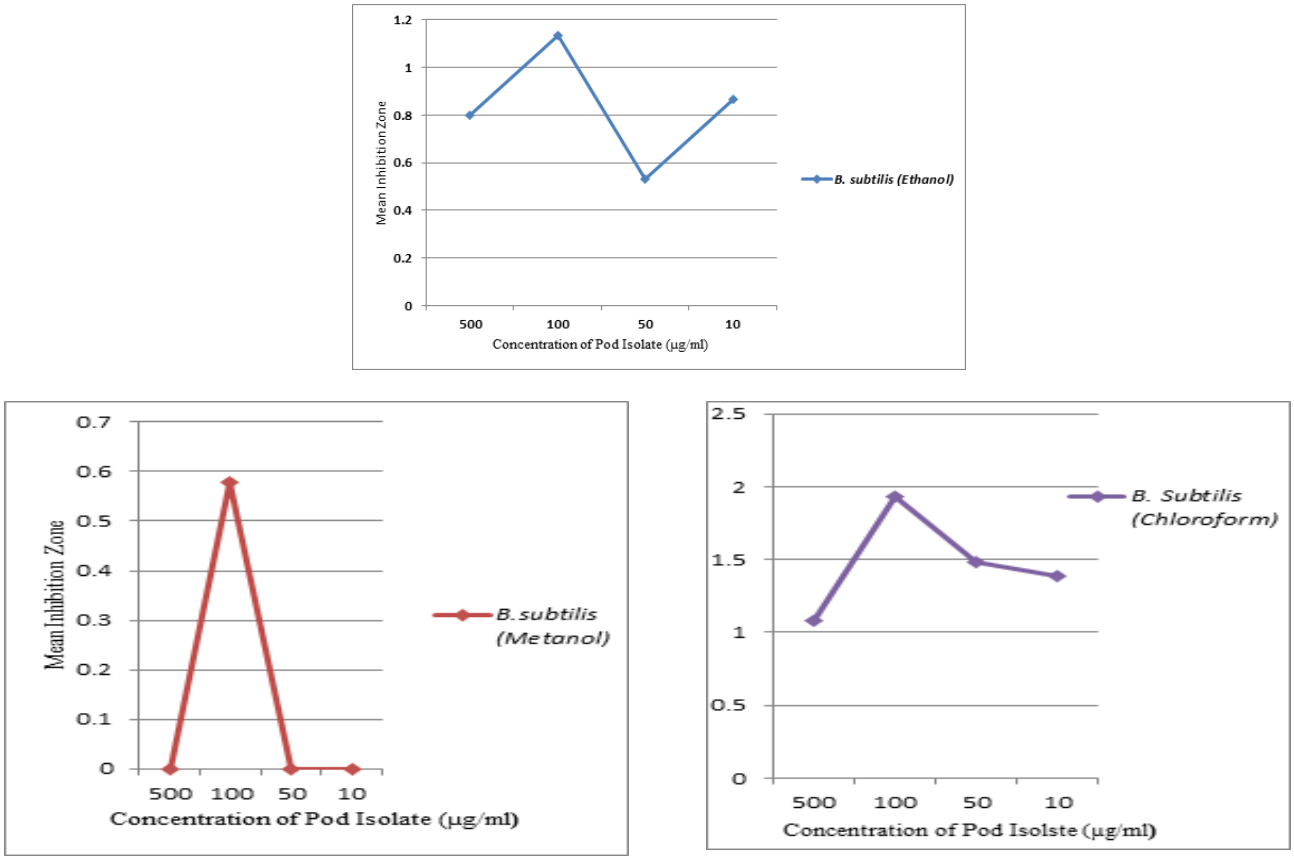


Figure 1. Showing graphical representation of the antimicrobial activity of *Acacia nilotica* pods protein/peptide isolate against *Bacillus subtilis* by Disc-Diffusion Method.

1 explained through line graphs displays mean inhibition zones between the Ethanol, Methanol and Chloroform protein/peptide pod isolate at different concentrations against gram-positive *B. subtilis*.

3.2 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the peptide/protein isolate obtained from the pods of *Acacia nilotica* was investigated to examine the minimum concentration that could entirely constrain the detectable growth of bacterial strains. The antibacterial activity of the peptide/protein isolate in consideration of MIC is displayed in (Table 2). The antibacterial activity of Ethanol, Methanol and Chloroform peptide/protein isolates were seen to be displaying inhibition potential towards gram-positive *B. subtilis* with chloroform isolate showing the highest mean value of 0.24±0.08 at a concentration of 100µg/ml. On the other hand, the mean inhibition value of Ethanol and Methanol towards *B. Subtilis* was observed to be 0.15±0.04 and 0.14±0.023 respectively at a concentration of 100µg/ml. The mean values obtained in the case of gram-negative *E.coli* in consideration of Ethanol, Methanol and Chloroform Isolate were found to be non-significant. Figure 2 explained through line graphs displays mean OD (600nm) in case Ethanol, Methanol and Chloroform protein/peptide pod isolate at different concentrations against gram-positive *B. subtilis*

Figure 2.

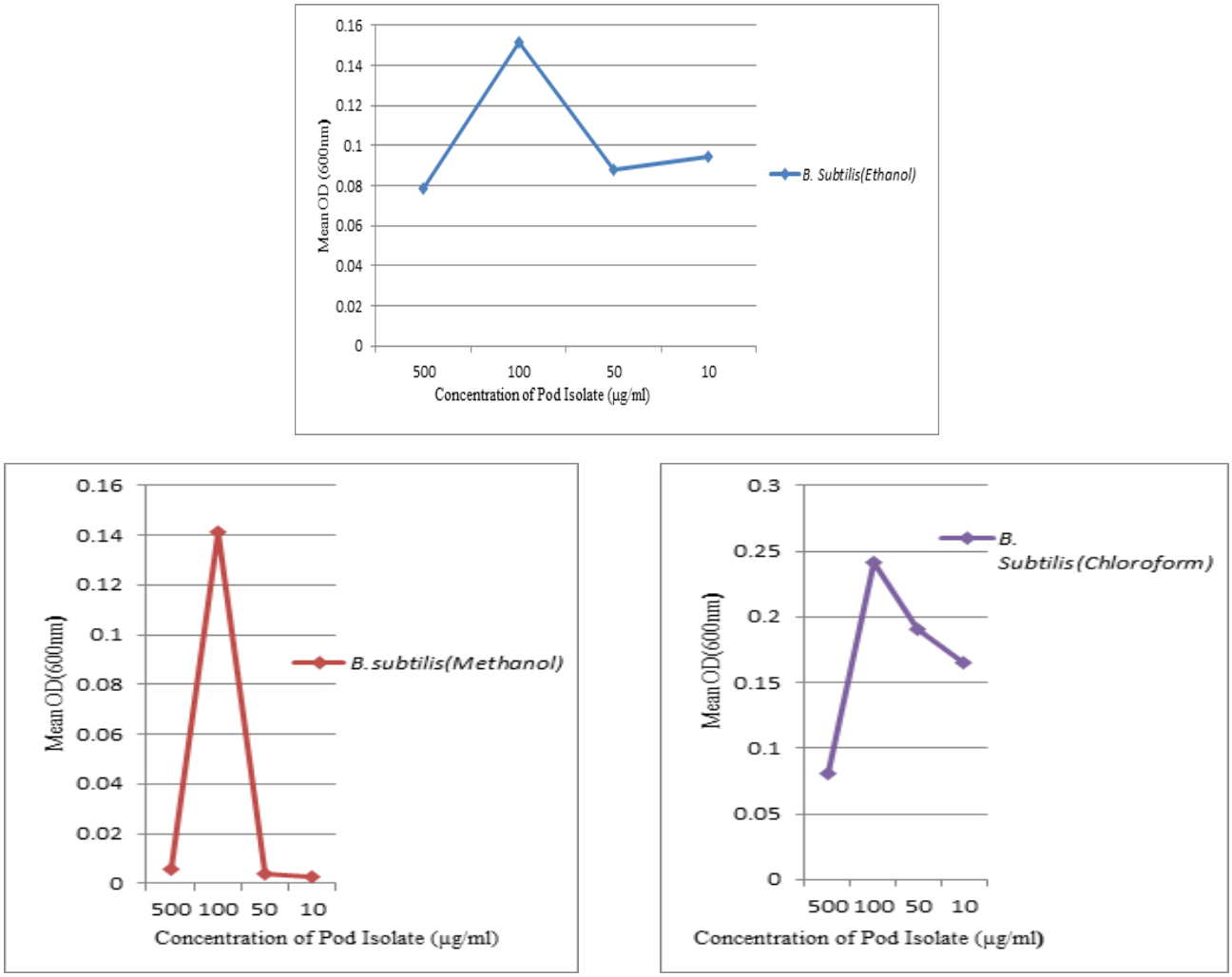


Figure 2. Showing graphical representation of Antimicrobial activity of *Acacia nilotica* pods protein/peptide isolate against *Bacillus subtilis* by Minimum Inhibitory Concentration that was reported to be at (100µg/ml) in the case of gram-positive *Bacillus subtilis* bacteria.

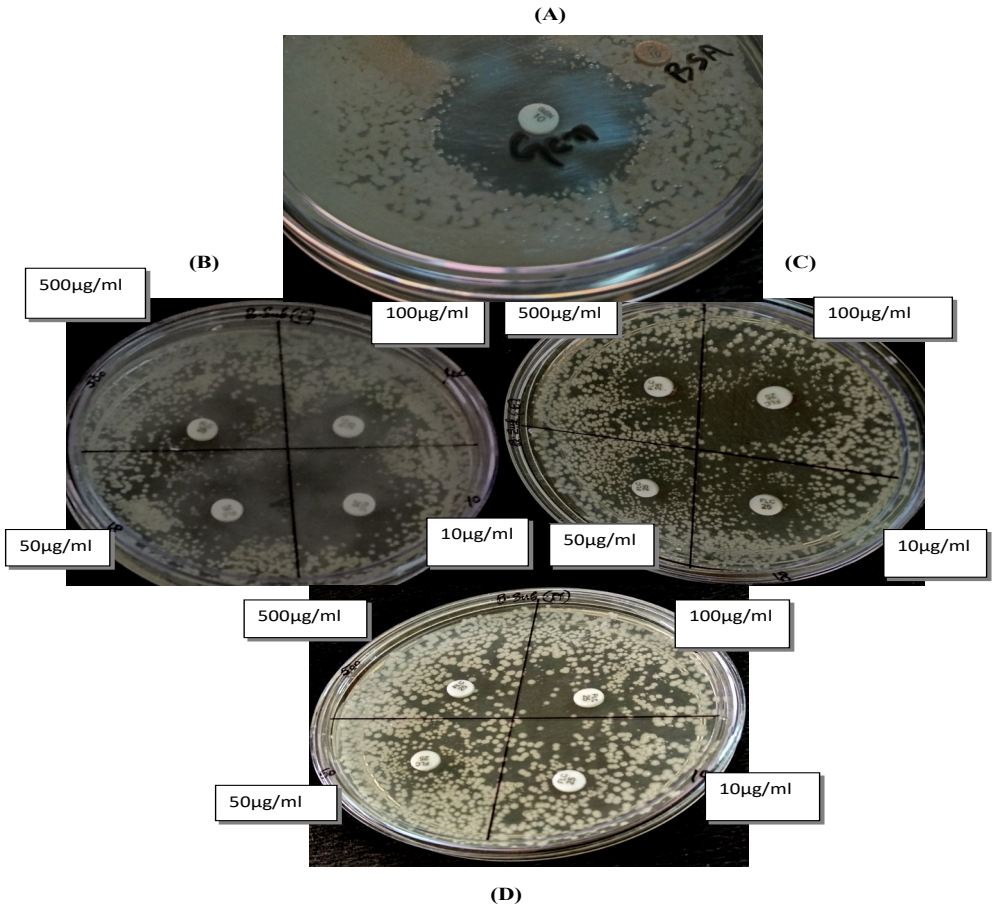


Figure 3. Showing Antibacterial resistance of *Acacia nilotica* pod protein/peptide isolate against *Bacillus subtilis* by disc-diffusion method. **(A)** (10µg/ml) BSA was taken as negative control while the antibiotic Gentamycin (20µg/ml) was taken as a positive control. **(B)** Chloroform isolate. **(C)** Ethanol isolate. **(D)** Methanol isolate

Table 2. Displaying Minimum Inhibitory Concentrations (MIC) (Mean±SD) of *Acacia nilotica* pod peptide/protein isolate against the bacterial pathogens

Bacterial Strains	Positive Control Gentamycin (µg/ml) (mm)	Negative Control Bovine Serum Albumin (µg/ml) (mm)	Pod Isolate Concentration (µg/ml)	Ethanol (µg/ml) (mm)	Methanol (µg/ml) (mm)	Chloroform (µg/ml) (mm)
Escherichia coli (Gram-negative)	5.3±0.5	0	500 100 50 10	0.0019±0.0007 0.004±0.0005 0.001±0.0005 0.0008±0.0003	0.004±0.0006 0.006±0.001 0.0034±0.0013 0.0023±0.0011	0.0068±0.0002 0.074±0.0008 0.0054±0.0004 0.003±0.0005
Bacillus subtilis (Gram-positive)	7.5±0.2	0	500 100 50 10	0.07±0.037 0.15±0.04 0.08±0.03 0.09±0.01	0.005±0.0008 0.14±0.023 0.004±0.0005 0.0025±0.0002	0.08±0.07 0.24±0..08 0.19±0.03 0.16±0.07

4. Discussion

A wide array of activities during metabolism in which proteins are seen to be engaged approves the fact that these major primary metabolites were patterned to be as flexible molecules. A collection of well-entrenched, undeniable roles played by proteins includes their function as protective entities in case of immune system-related organizations, as regulative as well as signalling substances, as a source of nourishment, as a pivotal component of the enzymatic machinery necessary during the metabolic processes and as contractile and storage units as well[13]. Another vital variety of functions that proteins possess is the property to inhibit the growth and development of microbial fauna and the most excellent class of protein falling in this category is referred to as Plant-derived Antimicrobial peptides. AMPs demonstrate their antimicrobial potential not only on microbes infecting the concerned plant species but also function against gram-positive, and gram-negative bacteria, fungi, viruses as well as pathogens responsible for causing disease in humans. They also play essential roles in case of wound restoration, inflammation and defence system stimulation. The major methodology linked with AMPs for the speedy destruction of microbial population is connected with the disruption of the cell membrane of the pathogen[14,15]. The absence of necessity for definite receptors if could be applicable in the case of maximum AMPs can prove to be very beneficial for the activity of AMP as well as can probably contribute as an advantage in cases that involve expansion of defiance in bacterial strains through mutations[16]. Though it is quite evident that peptides/proteins obtained from higher eukaryotic plant cells have antimicrobial capabilities that can entirely inhibit the growth of microbial fauna or opportunistic pathogens that cause infectious diseases in human beings however, still a considerable amount of data regarding plant species possessing peptides with antimicrobial capabilities is not studied assiduously Hence, the current study focuses on investigating the antibacterial activity of ethanol, methanol and chloroform peptides/ proteins isolate obtained from the pods of *A. nilotica*. To our knowledge, the present study is the first organized conclusive report on the antibacterial effectiveness of the ethanol, methanol and chloroform peptide isolate from pods of *A.nilotica*. The ethanol, methanol as well as chloroform peptide isolates were effective against *Bacillus* species and the effective concentration of the protein isolate for all the 3 solvents was reported to be at 100µg/ml in the case of Disc-Diffusion assay which was further supported by results obtained by MIC. However, none of the peptide isolates was found to be effective in inhibiting the growth of gram-negative *E.coli* and this kind of observation could be because of the destruction of AMPs by intracellular peptidases. One such instance which supports such kind of observation is the production of Oligopeptidase B by *E.Coli*that are effective in degrading AMPs which wereProline-rich and the level of defiance was interlinked with the amount of peptidase present[16,17]. Hence, the antibacterial action of the peptide isolates procured from the pods of *A.nilotica* against gram-positive *B. subtilis*proves the anti-infective efficacy of the plant species relevant towards inhibiting bacterial infections.

5. Conclusion

The current study provides information on the antibacterial action of the proteins/peptide isolate obtained from the pods of *A.nilotica*. The antibacterial activity displayed slightly by the Ethanol, Methanol and majorly by the Chloroform peptide/protein isolate against Gram-positive *B. Subtilis* on one hand confirms the antimicrobial potential of the major primary metabolite obtained from *A. nilotica* and on the other hand encourages the researchers belonging to the concerned field to further elucidate and investigate the possible mechanism of action through which the proteins are inhibiting the growth of bacterial strains since any sort of reports considering the protein isolates from the considered plant species is yet not seen to be studied so far. Further, the study also explains the antibacterial action of the peptide isolate by considering different concentration doses that suggest a therapeutic role of the peptide isolate against infectious bacterial species and hence in future can be utilized as an alternative plant-based antimicrobial drug therapy in place of antibiotics which will be effective at a minimal dose with no side-effects and will also help in controlling the increasing rate of antimicrobial resistance in contagious bacterial fauna. Hence, the study encourages the researchers to perform further pharmacological-based investigations related to the peptide isolate of *A. nilotica* and many other related medicinal plant varieties that possess therapeutic values for various microbe-associated conditions. Thus, the current study indicates a prospect for the development of a novel therapeutic antimicrobial agent.

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