# Extraction of Peptides and Proteins from *Senna tora* Seeds and Determination of its Antimicrobial Activity

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Deparment of Pharmacy
Brac University
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## Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

**Student's Full Name & Signature:**

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# Approval

The project titled “Extraction of Peptides and Proteins from *Senna tora* Seeds and Determination of its Antimicrobial Activity” submitted by Sudipta Saha (15146080) of Spring 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 20th August, 2019.

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## Ethics Statement

This study does not involve any kind of animal or human trial.

## Abstract

*Senna tora (L.) Roxb* is a plant of Liguminosae family and this family is known to be rich in protein content. The objective of this study is to extract and quantify the amount of peptides and proteins present in *Senna tora* seeds as well as to analyze its antimicrobial efficacy and these experiments has been done for the first time on *Senna tora* seeds. To extract the peptides and proteins a cold extraction buffer was used and their presence was determined by SDS-page and its concentration was quantified by Lowry method. Antimicrobial efficacy of the extracted peptides & proteins and methanolic crude extract of the seeds were examined against both gram positive and gram negative bacteria. No antimicrobial activity was found for peptides and proteins but the methanolic crude extract formed clear zone against *E.coli* and *Pseudomonas aeruginosa*.

**Keywords:** Plant peptide; Extraction of peptide and protein; Antimicrobial activity; SDS-page.

# Dedication

*Dedicated to my parents for their immense support and for having faith in me.*

# Acknowledgement

First of all, I would like to thank almighty God for giving me an opportunity to complete my project and overcome all the obstacles faced doing this project. I would never finish this project without His mercy.

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## List of Acronyms

| | |
|---|---|
| AMPs | Antimicrobial Peptides |
| WHO | World Health Organization |
| LPS | Lipopolysaccharides |
| LTP | Lipid Transfer Protein |
| TNF | Tumor Necrosis Factor |

# Chapter 1

# Introduction

## 1.1 A brief introduction of medicinal plants

The quest for medicinal plants is an age old process as the diseases were inevitable for human from the beginning. At that time because of the deficit of the knowledge about the diseases and its cure the selection of medicinal plants were solely based on perception. However, as the time passes people identified a certain type of plants work for a particular disease and started to treat diseases with their grounded knowledge (Petrovska, 2012). It is evident that the medicinal plants are very popular amongst developing countries as more than 3.3 billion people of these region uses medicinal plants on a regular basis (Singh R, 2015).

According to the definition of  World Health Organization (WHO), medicinal plants are those kind of plants which poses different kind of compounds and properties that can be used for their therapeutic purposes or synthesis of various metabolites is possible from them which can be used for effective drug production (WHO, 2008).  In an report of World Health Organization (WHO), for primary healthcare needs almost 80% of world's population is dependent on traditional medicines (Muthu, Ayyanar, Raja, & Ignacimuthu, 2006). In Asia the uses of medicinal plants are widely popular in China, India, Thailand and Japan. In China almost 40% medicines being consumed are traditional medicines and in Japan the popularity of conventional traditional medicines is higher than pharmaceutical products. Another popular source of traditional medicines is Africa. *Catharanthus roseus* is very popular in Africa because it poses vincristine and vinblastine which are effective against tumor cells. *Nothadoytes foetida* is a medicinal plant which is mainly found in Southern part of India and in Sri Lanka is best known for its anticancer property are even being smuggled in UK, Japan. USA, Switzerland, France and Germany (Singh R, 2015).

## 1.2 Antimicrobial Resistance and Importance of New Drug or Antibiotic

In the history of medicines one of the most successful forms of chemotherapy is antibiotic (Aminov, 2009). The first antibiotic Penicillin was discovered in 1928 and the very first antibiotic was prescribed in 1940s. During that time it was thought by many that all the diseases associated the intervention of pathogenic microbes will be wiped out from the world (Aminov, 2009). But in the late 1950s to 1960s for the first time *E.coli*, Salmonella and Shigella were found to be become resistant against antibiotic. Later in 1970s it was found that *Neisseria gonorrhoeae* and *Haemophilus influenza* were showing resistance against Ampicillin. After 70 years later in the year of 2015 bacteria has become a life threat to mankind once again (Aslam et al., 2018).

There are three main mechanisms by which bacteria can grow resistance against an antibiotic and they are antibiotic inactivation, modification of drug binding site and prevention of accumulation of antibiotics.

**Prevention of accumulation of antibiotics:** In this mechanism bacteria increase the efflux or decrease the influx of antibiotics by several processes.

- Drug molecules entered inside the bacterial cell by diffusion through porins, diffusion through the bilayer and by self-uptake. Porin channels are located on the outer membrane of gram negative bacteria and β-lactams and quinolones can pass through the outer membrane only by porins. So, bacteria can grow resistance against these bacteria by decreasing the number of porin channels.
- Another way that bacteria grow resistance against antibiotic is by the help of efflux pump. These are the pumps by which bacteria export antibiotics out of their cells before they can reach their target and keep a lower concentration of antibiotic inside

their body. Except polymixin all antibiotics are susceptible to the activation of efflux system

**Modification of drug binding site:** In this process, as the drug binding site is get modified antibiotics cannot bind to their target and cannot exert their activity.

- If the alteration takes place in 30s subunit and 50s subunit , then the designated antibiotics like tetracycline , macrolides cannot bind with them and the protein synthesis of bacterial cell cannot be disrupted, thus bacteria become resistant against them.
- Bacterial cell wall synthesis can be hampered by glycopeptides binding with D-alanyl-D-alanine residues of peptidoglycan precursors. But in some cases D-alanyl-D-alanine changed to D-alanyl-lactate and then glycopeptides cannot form link with them and cells synthesis cannot be inhibited.
- Mutation of DNA gyrase and topoisomerase IV inhibited the activity of fluroquinolone while activity of tetracyclines gets imparted by ribosomal protection mechanisms.

**Inactivation of antibiotics:** Several enzymes such as aminoglycoside-modifying enzymes, β-lactamases, and chloramphenicol acetyltransferases are responsible for inactivation of antibiotics. These enzymes are responsible for changes in the molecular structure of antibiotics like β-lactamases hydrolyses the β-lactam ring of β-lactam antibiotics. The modified version of chloramphenicol cannot bind to a ribosomal 50s subunit as chloramphenicol transacetylase that acetylates hydroxyl groups of chloramphenicol (Kapoor, Saigal, & Elongavan, 2017).

### 1.2.2 Necessity of New Drug or Antibiotic

Though the antibiotics were termed as "Magic bullets" that can cure any sorts of bacterial infection but with times bacteria are becoming resistant and many well-known organizations like Centers for Disease Control and Prevention (CDC), World Economic Forum, Infectious Diseases Society of America and the World Health Organization (WHO) have marked antibiotic resistance to be a "global public health concern". Only in USA 99,000 people died every year due to Hospital Acquired Infections (HAIs) which are associated with antibiotic resistant bacteria. Because of unawareness amongst people and easy availability, antibiotics have spread everywhere. In the year 2010 the total antibiotic consumption throughout the world was 63,151 tons which is estimated to rise around 67% by the year of 2030. Because of improper uses of antibiotic microbes grow resistance against antibiotic through mutation. Nowadays, "Superbug" is a term which represents microbes that have grown resistance against multiple antibiotics. If the number of superbugs started increasing by the year 2050 around 444 million people will be affected by infections and birthrate will decrease rapidly.

Though the time frame from 1930s to 1960s were termed as "golden era" of antibiotics but scientist could not keep the pace of innovating new antibiotics as the microbes were becoming resistant (Aslam et al., 2018). As the rapidity of microbes being resistant is so high that if new antibiotics or antimicrobial agent is not being invented it is very much possible that the world will sink into a "post antibiotic era''. The present antibiotics are so limited and conventional in their mode of actions that it is high time to find new antibiotics (Mahlapuu, Håkansson, Ringstad, & Björn, 2016). That is why a lot of money is being invested in search for new antibiotics and various plans are being submitted in search for new antibiotics as soon as possible to combat the treat against resistant microbes. (Aslam et al., 2018).

## 1.3 Antimicrobial Peptides

Protein is a versatile molecule because of its different kind of functions like it helps in metabolism providing different kind of enzymes, works as body's defense mechanism, as a nutrient, as storage and so on (Salas, Badillo-Corona, Ramírez-Sotelo, & Oliver-Salvador, 2015a). In every form of life whether it is a microorganism, animal or plant, innate immune system acts a defense system and anti-microbial peptides (AMPS) is an integral part of it and works as a first line of defense (Salas, Badillo-Corona, Ramírez-Sotelo, & Oliver-Salvador, 2015). The number of amino acids present in a antimicrobial peptide can vary from five to over a 100.These AMPs work against all sorts of pathogens e.g. Viruses, gram positive and gram negative bacteria, fungi and other kind of parasites to protect against any type of infections (Park, Okhovat, & Kim, 2017).

### 1.3.1 Structure of AMPs

In general, the AMPs being reported till now have four basic secondary structures α-helix, β-sheet, extended, and loop. Amongst them α-helix and β-sheet are most common. The demonstration of the structures are shown in figure 1 where LL-37 and human lactoferricin represent α-helical peptides, human β-defensin 1 represents β-sheet peptides, and indolocidin represents extended/random-coil structures (Mahlapuu et al., 2016).

### 1.3.2: Mechanism of Antimicrobial peptides

- **Interaction with bacterial membrane**

  The basic mechanism of antimicrobial peptides are that they are cationic peptides means they are positively charged and the bacterial surface are negatively charged which helps them to interact with each other by surface binding. Surface binding is the determinant factor for the AMPs to exert their antimicrobial activity. The most interesting part is the phospholipids present in bacterial cell surface have negatively charged heads and the other hands the mammalian cell, the outer surface has

zwitterionic phospholipids and if any negatively charged phospholipids are present there it remains in the inner surface facing the cytoplasm. That is why using AMPs inside human body will only disrupt bacterial cell surface but no disruption will occur in mammalian cell surface (Mahlapuu et al., 2016).

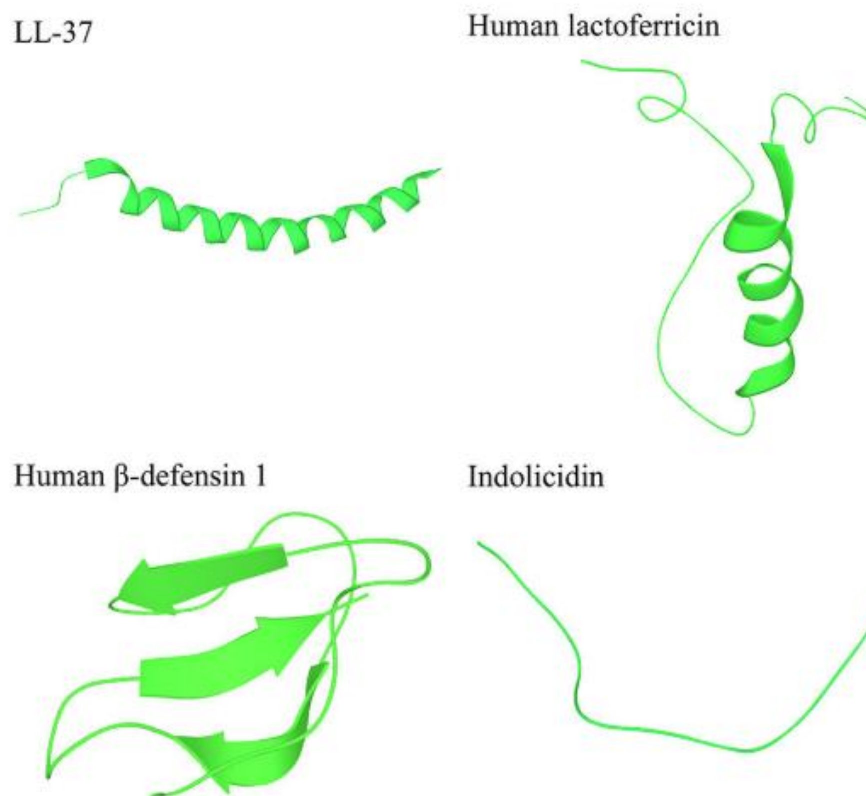


*Figure 1: Structure of AMPs (Mahlapuu et al., 2016)*

- **Membrane Disruption and Intracellular Targets in Bacterial Cells:**

The outer membrane of the bacteria gets stabilized as the **Ca2+ and Mg2+** that bind to the phosphate groups of the inner core of LPS and form an entrance barrier for many macromolecules inside the cytoplasm. But due to the higher affinity towards lipopolysaccharides AMPs dislocate the cations and bind with lipopolysaccharides.

As the AMPs are bulky it forms transient cracks on the outer membrane and pass through it. The AMP derived deeper into the membrane as the peptide allow for interaction with the hydrophilic head groups of the phospholipids due to its charged domains and the hydrophobic domains of the peptide interact with the hydrophobic core of the lipid bilayer. Thus, it disrupts the cell surface and destroys bacteria (Mahlapuu et al., 2016).

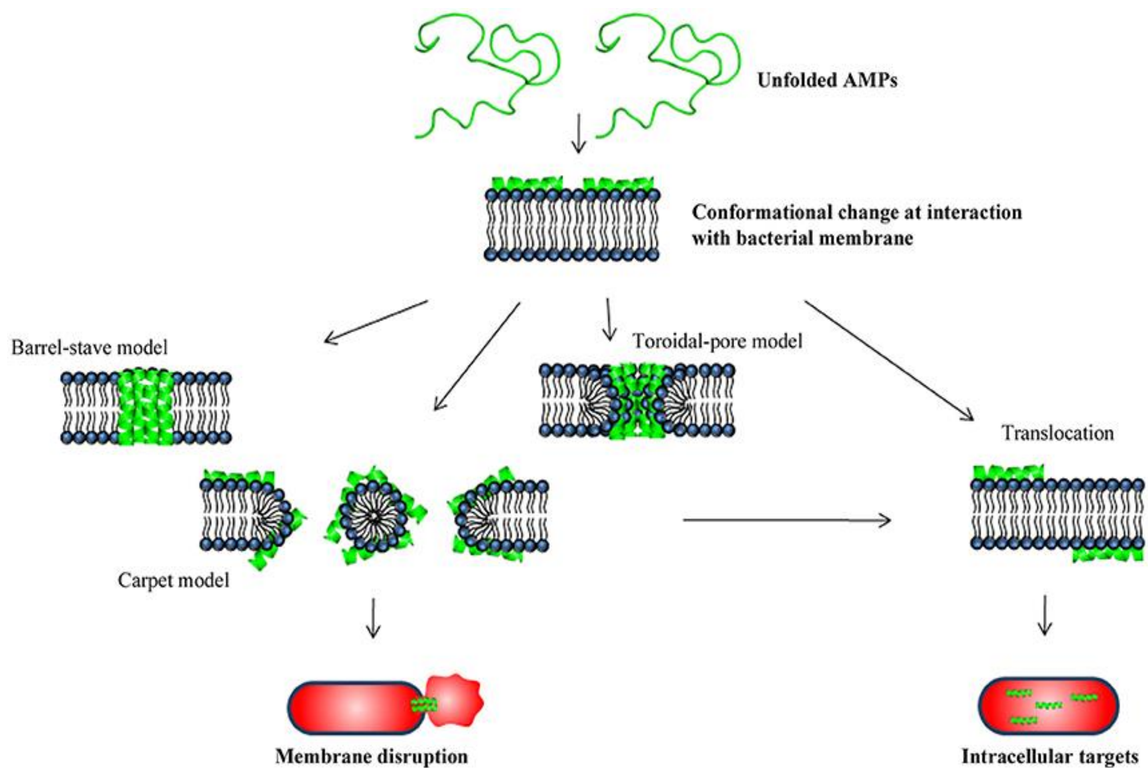


*Figure 2: Cell disruption mechanism of AMPs(Mahlapuu et al., 2016)*

- **Immunomodulatory activities:**

In recent studies it being evident that the AMPs are not only responsible for bacterial cell disruption but it also enhances the immune response inside the human body by stimulating chemotaxis, modulating immune cell differentiation and initiating adaptive immunity . All these activities together contribute to the bacterial clearance of the host (Mahlapuu et al., 2016).

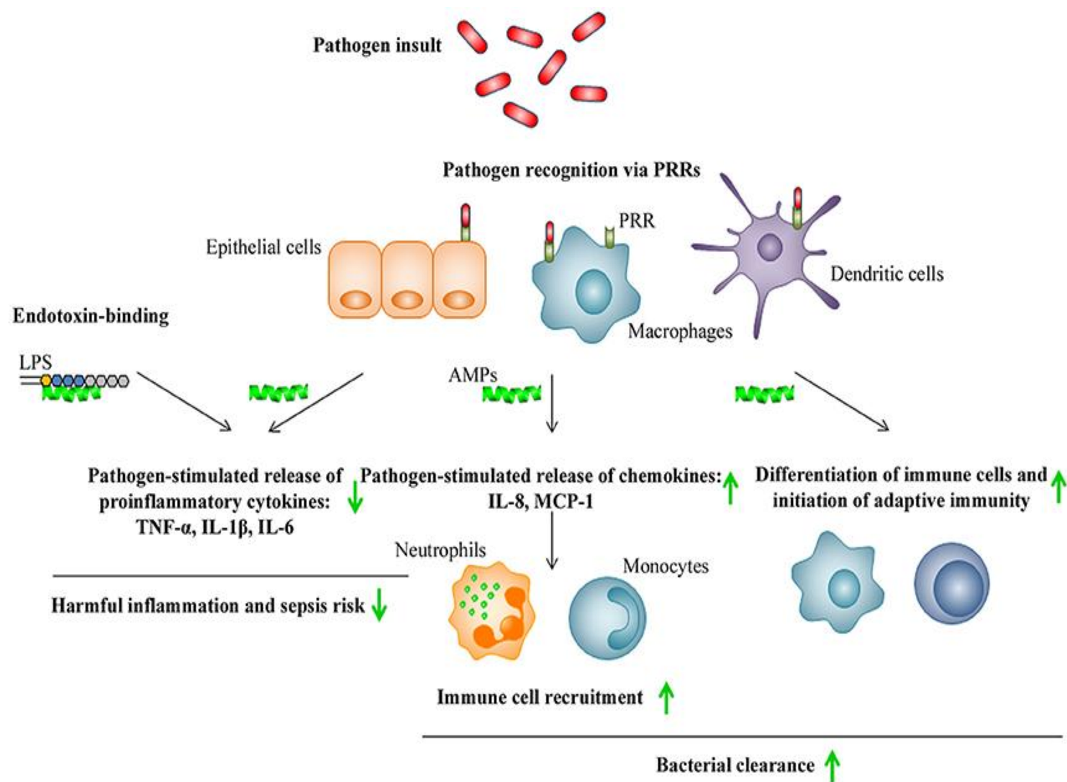


*Figure 3: Immunomodulatory activities of Amps (Mahlapuu et al., 2016)*

## 1.4 Plant Peptides and Proteins as a Possible Source of Antimicrobial Agents

Although a number of antibiotics have been produced by pharmaceutical industries but microbes are becoming resistant against them. In search of new antibiotics many resources have already been explored like soil, microbes, plants and animals. Through systematic screening of medicinal plants used in our folk medicines have showed positive antimicrobial activity (Ertürk, 2010). The therapeutic potentials of medicinal plants are humongous because at the same time they can eliminate the side effects of synthetic antibiotics and work against infectious diseases (Saranraj & Sivasakthi, 2014). Pant AMPs are pretentious compound produced in plant cells and the first plant based AMP was found in wheat flower called purothinin. From then on many AMPs have been identified and many of them have potent

antimicrobial effect. In plant AMPs can be found in different parts of it e.g. root, leaf, steam, seed. As the present antibiotics are becoming resistant day by day scientist are looking for new antimicrobial agents to treat infections. Only 343 AMPs are from plants amongst more than 5000 identified AMPs till date. For most of the AMPS clinical trials have not been performed and those were sent for clinical trials shows toxicity or low potency. As a result, the search for potent and safe AMPs is much needed now that can be used for therapeutic purposes to combat with microbes (Tang, Prodhan, Biswas, Le, & Sekaran, 2018).

### 1.4.1 Classification of plant based antimicrobial peptides

#### a. Thionins

Thionins represents a family of antimicrobial peptides and their molecular weight is low which about 5 kDa. They are rich in lysine, arginine and cysteine residues. They are positively charged at neutral pH and in their structure two antiparallel α-helices and an antiparallel double-stranded β-sheet with three or four conserved disulfide linkages are present. In 1942 the first thionin was isolated from wheat endosperm known as purothionin and till now in more than 15 different plant species around 100 individual thionin sequences have been identified (Nawrot et al., 2014).

#### b. Defensins

Plant defensins are basic in nature and are positively charged. They are also small with a molecular weight of around 5 kDa and they are composed of cysteine-rich peptides ranging from 45 to 54 amino acids. According to various reports about plant defensins state that it has antifungal, antibacterial, proteinase, and insect amylase inhibitor activities. The first plant defensins were initially classified as γ-thionins and were isolated from wheat *T. aestivum* and barley *Hordeum vulgare*. Plant defisins have three dimentional structure comprises of a

triple-stranded β-sheet with an α-helix in parallel stabilized by four disulfide bridges (Nawrot et al., 2014).

**c. Lipid transfer proteins**

Lipid transfer protein founds in the nature are of two different sizes 9kDa (LTP1s) and 7kDa (LTP2s) having a hydrophobic cavity enclosed by four α-helices which are held in a compact fold by four disulfide bonds. The nonspecific small lipid transfer proteins (ns-LTPs) are found to be present in various monocotyledonous and dicotyledonous plant species which are capable of in vitro lipids exchange between membranes. Membrane biogenesis, involvement in defense reactions against phytopathogens, regulation of the intracellular fatty acid pools, cutin formation, embryogenesis and the adaptation of plants to various environmental conditions are their main functions. Though it's being estimated that ns-LTP may insert themselves in fungal membranes which will form a pore resulting in an efflux of intracellular ions resulting in cell death but it's not well established yet (Nawrot et al., 2014).

**d. Puroindolines**

The puroindolines are basic in nature containing a unique tryptophan-rich domain. They were isolated from wheat endosperm. They have five disulfide bridges with a molecular mass of around 13 kDa. Puroidolines are found to be have antimicrobial and antifugal properties. They may have membranotoxins which might play an important role in plants to fight against microbial pathogens (Nawrot et al., 2014).

**e. Cyclotides**

The cyclotides are circular proteins comprise 28–37 amino acids which occur naturally in bacteria, plants, and animals. Their structural identity and sequence are highly similar (Nawrot et al., 2014).

**f. Snakins**

Snakins having 63 amino acid residues were isolated from potato tubers. The two different types of them are snakin-1 and snakin-2 which are cell wall-associated peptide and have antimicrobial activity (Nawrot et al., 2014).

**g. Hevein-like proteins**

Heveins have 43 amino acid residues with a molecular mass of 4.7 kDa and they are cysteine-rich chitin-binding peptide are found to be present in the lutoid bodies of rubber tree *Hevea brasiliensis* latex. They bind with chitin and inhibit the hyphal growth of fungi. In different plants hevein-like proteins with antimicrobial activity have also been identified (Nawrot et al., 2014).

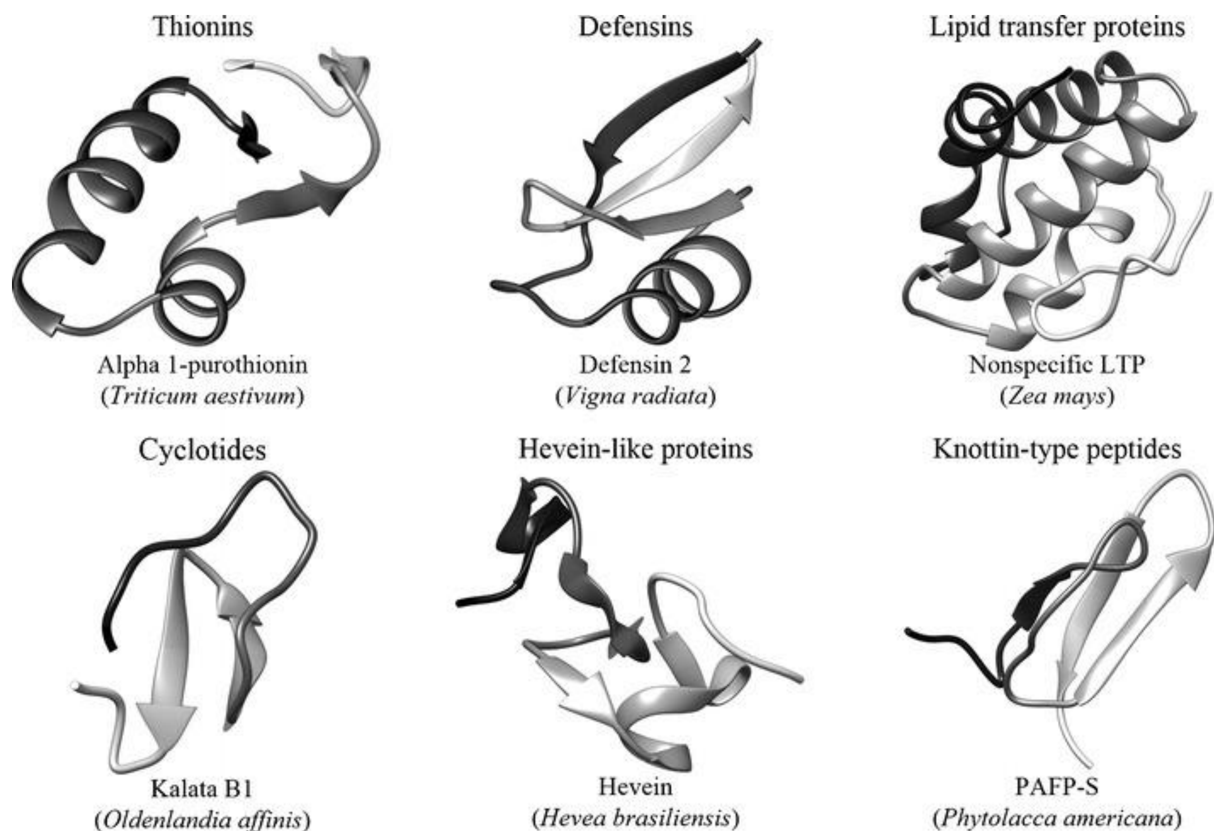


*Figure 4: Plant based antimicrobial peptides (Nawrot et al., 2014)*

*Table 1:List of some antimicrobial peptides with their origin (Tang et al., 2018)*

| Family: Representative peptide | Plant | Plant organs |
|---|---|---|
| Cyclotides: Kalata B1 and B2 | *Oldenlandia affinis* | Leave & flowers |
| Puroindolines: PINA and PINB | *Triticum aestivum* | Endosperm |
| Thionein: α-1-purothionin | *Triticum aestivum* | Endosperm |
| Defensins: Peptide PvD1 | *Phaseolus vulgaris*<br>*Hordeum vulgare* | Seeds |
| Lipid transfer proteins | *Zea mays* | Seeds |
| Vicilin-like AMPs: PMAPI | *Macadamia integrifolia* | Seeds |
| Hevein-like AMPs: PMAPI | *Broussonetia papyrifera syn.*<br>*Morus papyrifera L.* | Leaves |

## 1.5 General information about *Senna tora (L.) Roxb*

*Senna tora (L.) Roxb.* is a plant of Leguminosae family mainly grows in tropical countries like Bangladesh, India, Pakistan and West China. It is a weed of rainy season and grows well on wasteland, riverbanks and uncultivated lands. In different culture of medicines it named differently, e.g. 'Chakramard' in Ayurveda, and 'Jue Ming Zi' in Chinese system of medicine and 'Panwar' in Unani.

5 (a)

5 (b)

5 (c)

5 (d)

*Figure 5: Different parts of Senna tora*

*Senna tora* plant uses in Folk and Traditional Medicines. Almost all parts of the plant uses in traditional medicine, e.g. leaves, roots and seeds. In folklore practice it is believed to have antirheumatic and laxative activity. According to the Chinese traditional medicine practice believed that *Senna tora* has aperients, antiasthmatic and diuretic activity. It helps in eye diseases and in liver disorders. In Korea, for the protection of liver hot seed extract are taken orally. In skin disorders like ringworm and itch seeds and leaves are effective (Harshal A. Pawar and Priscilla M. D'mello, 2011).

*Table 2: Photochemistry of Senna tora .(Harshal A. Pawar and Priscilla M. D'mello, 2011)*

| Plant parts | Chemical compounds |
|---|---|
| Leaves | Anthraquinone glycosides ,Flavonoids and |

| | sennosides |
|---|---|
| Seeds | Anthraquinone and naphthopyrone like componds |
| Flowers | Kaemferol and leucopelargonidine |
| Roots | 1, 3, 5 trihydroxy 6, 7 dimethoxy-2-methyle anthraquinone, leucopelargonidine and β-Sitosterol |
| Stem bark | Arachidic acid, isostearic acid, linolineic acid, palmitic acid, marginic acid, behenic acid, phenolics like rhein, emodine, Hexahydroxy flavones and a Hydroxycoumarin |
| Pods | Sennosides |

### 1.5.1 Pharmacological activity of *Senna tora (L.) Roxb*

In traditional form of medicines *Senna tora* is being used as liver tonic, in eye diseases, skin problems and so on (Harshal A. Pawar and Priscilla M. D'mello, 2011). But studies shows that it has several other pharmacological activities e.g. antibacterial, antihelmentic, antihypotensive, antimutagenic, antioxidant, antifungal , anti-inflammatory , hypolipidemic and so on (Jain & Patil, 2010)

- **Antioxidant Activity:** The methanolic extract of seeds of *Senna tora* (MECT) is found to be exerting stronger antioxidant activity than Alpha-tocopherol. The component responsible for this activity is Emodin (Jain & Patil, 2010).
- **Antibacterial activity:** Torachrysone, aloe-emodin, toralactone, emodin and rhein isolated from the seeds showed potent antimicrobial effect against methicillin resistant *Staphylococcus aureus* but the phenolic glycosides extracted from the seeds did not show potential antimicrobial effect against *E. coli* and *P. aeruginosa* (Jain & Patil, 2010).

- **Antifungal activity:** Due to the presence of chrysophenol and crysophanic acid- 9-anthrone and other anthraquinones such as emodine, physcion and rhein in the leaf extract, it shows antifungal activity against *Aspergillus niger, Candida albicans, Sachharomyces cerevisiae* and *Trichophyton mentagrophyte* (Maity & Dinda, 2003) .
- **Antitumor Activity:** Emodine which is an anthraquinone, present in root and bark of *Senna tora* shows inhibitory effect on angiogenic and metasis regulatory process. Emodine may interfere with electron transport process and in altering cellular redox status because of its quinine like structure, which may account for its cytotoxic property (Choi, Lee, Park, Ha, & Kang, 1997).
- **Antimutagenic Activity:** Methanolic extract of roasted *Senna tora* seeds showed antimutagenic activity against Aflatoxin-B1 (AFB1) was demonstrated with Salmonella typhimurium assay. On that assay it was found that the number of relevant per plate decreased significantly when the extract was added to assay system using Salmonella typhimurium TA100 and or TA98 35. Alaternin and isorubrafusarin gentiobioside found to be responsible antimutagenic activity (Jain & Patil, 2010).
- **Antihelmintic Activity:** Antihelmintic activity was found in the alcohol and aqueous extracts of *Senna tora* seeds showed against *Pheretima posthuma* and *Ascardia galli* due to the presence of flavonoids using a concentration of 100 mg/mL (Jain & Patil, 2010).
- **Antifertility Activity:** Antifertility activity in Female rats was observed after application of *Senna tora* leaves extract (200 mg/100 mg/Kg body weight) (Jain & Patil, 2010).
- **Hepatoprotective Activity:** A significant hepatoprotective effect against toxicity of galactosamine in primary cultured rat hepatocytes was found by methanolic extract of

seeds at a dose of 400 mg/mL orally in rats (Dhanasekaran, Ignacimuthu, & Agastian, 2009).

- **Purgative Effect:** Probably due to the presence of emodine, aloe-emodine and anthraquinone glycosides the methanolic extract of C. tora leaves was found to possess purgative action (Wu, Hsieh, Song, & Yen, 2001).

## 1.6 Purpose of this study

The first antibiotic was invented in 1928 and at that time it could cure any types of infections but with time it found to become resistant against several bacteria. Scientists have invented several antibiotics but due to over use and unawareness most of the antibiotics have become resistant against several microbes. Bacterial infections have become threat to the mankind once again. It has been estimated that in 2050 around 444 million people will be infected with bacterial infection and by that time if enough new antibiotics will not be invented it will be a disaster for mankind (Aslam et al., 2018). Moreover the synthetic antimicrobials have several side effects , so scientists are trying to search bioactive antimicrobials from natural sources (Saranraj & Sivasakthi, 2014). The purpose of the study is to find antimicrobial peptides and proteins from *Senna tora (L.) Roxb* and determine antimicrobial effect of its methanolic extract. In this study, *Senna tora (L.) Roxb* has been selected because not enough experiment has been done on this plant to find whether any antimicrobial peptide is present in this plant or not. Moreover, in this study only seeds have been used because seeds are an excellent source of protein.

# Chapter2

# Methodology

## 2.1 Collection of plant materials

In the month of November-December plant materials were collected from Shailkupa, Jhenaidah, Bangladesh. Locally the plant is known as “Jhunti Plant”. From the plants seeds were collected carefully, rinsed with water, shade dried and stored in an air tight container. The authentication of the plant as *Senna tora (L.) Roxb*. was done by Bangladesh National Herbarium situated at Mirpur, Dhaka, Bangladesh with an accession number 47200.

## 2.2 Extraction of peptides and proteins from *Senna tora* seeds

### 2.2.1 Preparation of extraction buffer (Aliahmadi, Roghanian, Emtiazi, & Ghassempour, 2011)

Apparatus used:

1. Volumetric flask
2. Beaker
3. Glass Rod
4. Balance

Used chemicals:

1. Ethylene diamine tetraacetic Acid (EDTA)
2. Glycerol
3. NaCl
4. Distilled Water
5. Phosphate Buffer

**Procedure**

- Firstly, to prepare 1M solution of $KH_2PO_4$, 6.8 g of $KH_2PO_4$ was dissolved in 50 mL of water (Solution A).
- 1M solution of $K_2HPO_4$, 8.71 g of $K_2HPO_4$ was dissolved in 50 mL of water (Solution B).
- In a beaker 2.11 mL of solution A added with 2.89 mL of solution B and added 20 mL of distilled water and mixed properly.
- Then 5 mL of Glycerol, 0.075 g EDTA and 0.29 g NaCl were added in the same beaker and mixed properly by adding more distilled water.
- After that pH was checked and pH 7 was maintained.
- The final volume of the solution was 100 mL.

**2.2.2 Peptides and proteins extraction technique**

- The collected seeds were subjected to milling machine to convert the seeds into fine powder.
- After milling, a cold extraction buffer was used for total protein extraction from the seed powder.
- The extraction buffer contained 50 mM phosphate buffer (pH 7), 2 mM EDTA, 5% glycerol and 50 mM NaCl.
- In a conical flask 100 mL cold extraction buffer and 10 mg powder were added and with the help of a magnetic stirrer it was stirred for 2 hours.
- After the stirring was done the seed particles in the mixture were allowed to settle down and then the solution from the top was filtered with the help of a sterile syringe filter whose pore size is 0.22 μm.

- Throughout the extraction process the temperature was maintained below 4ºC.
- After filtration through syringe filter the protein extract was taken in sterilize eppendrof tube and kept at -20ºC for preservation.

### 2.2.3 Precipitation of protein by Ammonium sulphate

The precipitation was performed according to the method described by Tripathi (2011). Protein extract were taken in several test tubes and ammonium sulphate solution (80%) was added into the test tubes at a slow rate. After protein precipitation was done it was centrifuged at 12000 rpm at 4ºC for 20 minutes. Then the protein pellets were collected and stored at -20ºC for preservation (Tripathi, Kumar, & Garg, 2011).

### 2.2.4 Protein purification by dialysis (Harcum, n.d.)

To purify the protein from undesired salt particles dialysis was done by a 9kDa semipermeable membrane. At first, 500 mL of ice cold distilled water was taken in beaker. Then, the semipermeable membrane was cut in desired size, soaked in ice cold distilled water and one side was sealed with the help of a cotton thread so that no liquid can pass through that side. Around 15 mL of protein extract was poured inside that dialysis membrane and other side was also sealed with the same manner. Then the dialysis bag was put inside that beaker and with the help of a magnetic stirred the water inside that beaker was stirred slowly. The water was changed with fresh ice cold distilled water for several times and the dialysis was done for 28 hours. The salt particles passes from higher concentration to lower concentration by passive diffusion and then solution was collected from the dialysis bag and filtered again through a syringe micro filter (Pore size, 22 µm) and stored in eppendrof tube at -20ºC.

## 2.3 Gel electrophoresis/ SDS Page

Gel electrophoresis or SDS page is a protein purification technique by which protein gets separated according to their size and charge particles. To purify protein polyacrylamide gel was used so that the protein does not get denatured. In lane 1, only filtered protein sample; in lane 2, blank; in lane 3, filtered and dialyzed protein sample and in lane 4, protein marker was applied. Then, an electric current was applied on the top of the gels and the protein started to move through the matrix. Depending on some factors protein gets separated and those are size, shape and charge density of proteins. Protein with higher charge density moves faster through the gels. The gel used here worked as a sieve because of its pore and the proteins which have higher molecular size moves slowly and gets entangled inside the gels. Protein which is fibrous and elongated moves slowly compared with globular shaped protein (Kumar & Sharma, 2015). In this process, four samples were subjected to run through the gel which were marker (Standard whose band size are known), blank, filtered protein extract, filtered and dialyzed protein extract.

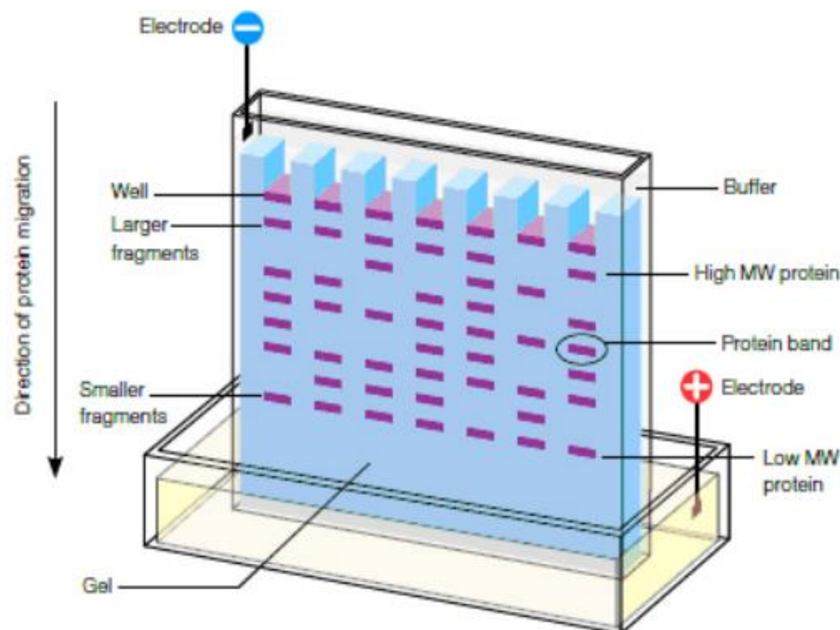


*Figure 6: Instrument for SDS Page (Kumar & Sharma, 2015)*

## 2.4 Quantification of peptides and proteins

To determine the protein concentration Lowry method is one of the most frequently used methods. The only disadvantage in this this method is that it is relatively more time consuming than other methods. It has been observed that after incubating at 37ºC, the mixture of sample and reagent changes its color (Shakir, Audilet, Drake, & Shakir, 1994).

**Reagent composition**

- At first, 0.57 g of sodium hydroxide and 2.86 g of sodium carbonate were dissolved in 100 mL of distilled water (Solution A).
- To prepare solution B, 0.71 g of copper sulphate pentahydrate was dissolved in 50 mL of distilled water.
- To prepare solution C, 1.74 g of sodium potassium tartrate was dissolved in 50 mL of distilled water.
- The final composition was 1 mL of solution B, 1 mL of solution C and 98 mL of solution A.

**Preparation of BSA (Bovine serum albumin)**

- 10 mg of BSA dissolved in 50 mL of phosphate buffer in a volumetric flask to make the concentration of the solution 0.2 mg BSA/mL.
- Then the prepared BSA solution was diluted in different concentration with distilled water in several test tubes.

*Table 3: Dilution of BSA with distilled water*

| BSA solution (mL) | Distilled water (mL) | Final concentration (µg/mL) |
|---|---|---|
| 10 | 0 | 100 |
| 8 | 2 | 80 |
| 6 | 4 | 60 |
| 4 | 6 | 40 |
| 2 | 8 | 20 |

**Preparation of BSA solution and protein extract for UV spectrophotometer**

- At first, 2 mL of solution from each dilution and plant protein sample were taken in separate test tubes.
- 2.8 mL of Lowry solution were added in each test tube and kept in dark for 20 minutes.
- Dituted Folin-Ciocalteu reagent (2 mL reagent diluted with 2 mL distilled water) was prepared in the 5 minutes of 20 minutes of incubation.
- 0.4 mL of Folin-Ciocalteu reagent was added in each test tube and kept in dark for 30 minutes to allow the reaction.
- Finally, the reading was taken at 750 nm.

### 2.4.1 Scanning of protein samples with UV-Vis Spectroscopy

First of all, BSA (Bovine Serum Albumin) solution of different concentrations were prepared dissolving standard BSA with phosphate buffer and scanned through UV-Vis spectrophotometer in the range of 200-400 nm. The seed protein extract was also scanned at the same wavelength. This was done to find out the $\lambda_{max}$ of each sample to detect the presence of protein in the extract by comparing with standard BSA solution.

## 2.5 Crude extract preparation

Around 200 g of seed powder was mixed with 250 mL of methanol in an amber glass container in a way so that all the powder remains submerged into the methanol. The mixture was kept in a dark place for 72 hours. After 72 hours the mixture was filtered by using a whatman filter paper by using a vacuum pump. After filtration the liquid portion was collected and the methanol was separated by using a rotary evaporator. Then the crude extract was collected in petri dish and kept under laminar air flow hood for complete evaporation of the remaining methanol in the extract. After complete evaporation of the methanol the crude extract was taken in a small glass bottle and kept in the refrigerator at 4ºC.

## 2.6 Antibacterial activity of protein extract

### 2.6.1 Apparatus and reagents

| | |
|---|---|
| ➢ Laminar air flow hood | ➢ Autoclave |
| ➢ Spirit burner | ➢ UV-Vis spectrophotometer |
| ➢ Screw cap test tube | ➢ Petri dish |
| ➢ Nose mask & hand gloves | ➢ Filter paper disk |
| ➢ Sterile cotton | ➢ Sterile forceps |
| ➢ Refrigerator | ➢ Micropipette |
| ➢ Sterile cotton | ➢ Inoculating loop |
| ➢ Ethanol | ➢ Cotton swab |
| ➢ Muller Hinton Agar | ➢ Muller Hinton Broth |

### 2.6.2 Culturing of bacterial strains

To culture each bacterial strain 5 mL of Muller Hinton broth was used which was prepared by dissolving 0.105 g of agar medium in 5 mL of distilled water in separate test tubes. All the bacterial strains were collected from a previously preserved bacterial medium. To prepare an inoculum, with the help of a loop bacterial colonies were taken and mixed in the broth medium. All the medium, test tubes and loop were autoclaved beforehand. Then the inocula were kept in the digital shaking incubator at 37ºC and 150 rpm to facilitate the production of bacteria. In the meantime the number of bacterial colonies had increased and became turbid. After 24 hours the bacteria containing broth medium was subjected to UV reading at 600 nm. The target was to keep the bacterial concentration in 0.5 McFarland standard solution and for that the desired dilutions of the bacterial suspension were done to keep the absorbance in between 0.08-0.13 at 600 nm (FO, ZS, & CV, 2018).

### 2.6.3 Preparation of culture medium

First of all, 7.6 g of Muller Hinton agar was dissolved in 200 mL of distilled water in a conical flask. For sterilization, all the culturing media, petri dishes necessary for preparing culturing plate were autoclaved at 121ºC for 20 minutes. After sterilization, the temperature of the agar medium was cooled down to around 50ºC and all the petri dishes were dried under a laminar air flow hood. Then in each plate approximately 25 mL of agar medium was poured in a way so that the uniformity remained maintained in each plate and kept them at room temperature to solidify the agar medium.

### 2.6.4 Preparation of disc

The discs were prepared from whatman filter paper and each disc was 6 mm in diameter. Before using the discs were autoclaved at 121ºC for 20 minutes for sterilization. After

sterilization the sample solution were applied onto the discs and allow them to soak the materials.

### 2.6.5 Antimicrobial activity test of protein extract by disk diffusion & well diffusion method

At first, to streak bacteria onto the Muller Hinton agar plate a cotton swab was dipped inside the bacterial suspension and then the cotton swab was squeezed gently against the test tube to get rid of excess fluid. After that the cotton swab was streaked throughout the agar plate starting from one direction to another and then streak again diagonally. Then the plate was kept aside for five minutes and the previously prepared disc which had already been soaked with protein extract was applied on the agar plate. Three whatman filter paper discs and one standard antibiotic disc were applied in each petri dish. The standard antibiotic disc was used as a reference to compare the antimicrobial activity produced by protein extract and standard antibiotic. Finally, it was kept in an incubator for 24 hours at 37ºC to allow the bacteria to grow.

In well diffusion method with the help of a sterilized micropipette tip four wells had been created on the agar plate. Eppendrof tube containing the protein extract of seed was taken out from -20ºC and de thawed to collect liquid from it.  In every well 150 µL of liquid protein extract was applied with the help of a micropipette. Then it was allowed for some time to soak the liquid protein extract by the agar medium and the kept in an incubator for 24 hours at 37ºC to allow the bacteria to grow.

*Table 4: Test organisms used for antimicrobial susceptibility testing for protein extract*

| Gram positive | Gram negative |
|---|---|
| *Staphylococcus aureus* | *E.coli* |
| *Bacillus subtillis* | *Pseudomonas aeruginosa* |

### 2.6.6 Antimicrobial activity test of crude extract by disk diffusion & well diffusion method

At first, crude extract were taken in different quantities and dissolved in sterilized distilled water to prepare 20 mg/mL, 40mg/mL and 60 mg/mL solution. Then with the help of a micropipette, solutions of different concentrations were applied on previously prepared sterilized discs and allowed them to soak all the materials. In the meantime, a cotton swab was dipped inside the bacterial suspension and then the cotton swab was squeezed gently against the test tube to get rid of excess fluid. After that the cotton swab was streaked throughout the agar plate starting from one direction to another and then streak again diagonally. Then the plate was kept aside for five minutes and the previously prepared discs which had already been soaked with crude extract solution of different concentrations were applied on the agar plate in a way so that each plate contains one disc of each concentration. One standard antibiotic disc was applied in each petri dish as a reference to compare the antimicrobial activity produced by crude extract of seeds and standard antibiotic. Finally, it was kept in an incubator for 24 hours at 37ºC to allow the bacteria to grow.

In well diffusion method, two wells had been made in each agar medium containing plate with the help of a sterilized micropipette tip. In this method only two different concentrations of crude extract solution were used. The previously prepared solutions having concentration of 40 mg/mL and 60 mg/mL were applied in two different wells of each plate and then it was allowed to soak the solution in agar medium. Finally, it was kept in an incubator for 24 hours at 37ºC to allow the bacteria to grow.

*Table 5: Test organisms used for antimicrobial susceptibility testing of crude extract of seeds*

| **Gram positive bacteria** | **Gram negative bacteria** |
|---|---|
| *Staphylococcus aureus* | *E.coli* |
| *Bacillus subtillis* | *Shigella Dysenteriae* |
| *Enterococcus  feacalis* | *Shigella Flexneri* |
| | *Salmonella typhi* |
| | *Pseudomonas aeruginosa* |

# Chapter 3

# Result

## 3.1 Detection of peptide & protein by SDS Page

The Gel electrophoresis or SDS page was performed to separate proteins according to their size and shape. After, completion of this process different band of protein was found which were separated according to their size and shape. By comparing with standard marker the band of different proteins were identified.

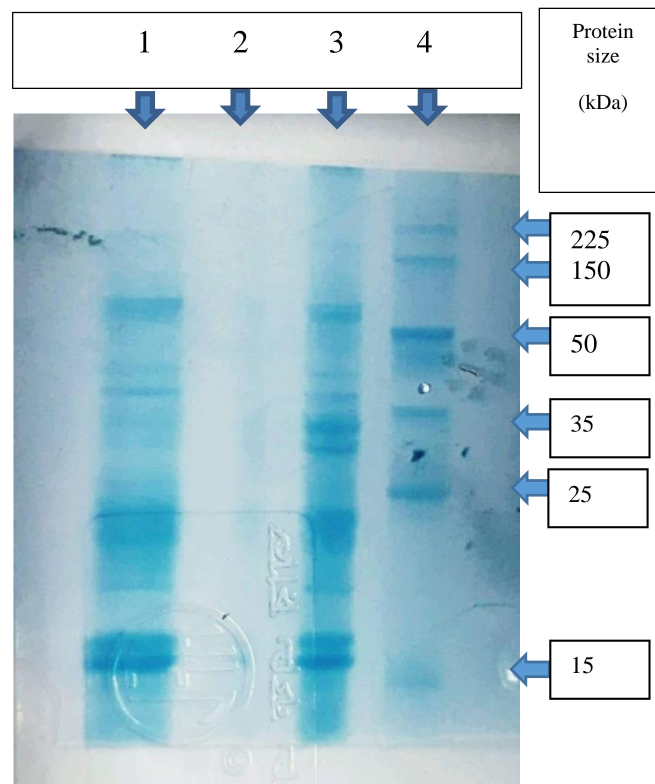


*Figure 7: Purification and detection of protein size by Gel Electrophoresis, 1= Filtered protein sample, 2=Blank, 3= Filtered and dialyzed protein sample, 4= Protein marker*

## 3.2 Quantitative determination of extracted peptides and proteins

The Lowry method was performed for the identification and quantification of protein. The main mechanism behind this experiment was it changes color after adding the reagents. After color formation UV reading was taken at 750 nm to identify the absorbance and using those data amount of protein present in the unknown concentration was identified (Oliver H. Lowry, Nira J.Roseebrough, n.d.).

The mechanism behind this experiment is that the Folin & Ciocalteu reagent being used here is a composition of phosphomolybdic-tungstic mixed acid. This mixed acid acts as a chromagen. When protein and this reagent come in contact proteins effect a reduction on the acid mixture and oxygen gets released from tunstate and/or molybdate. By this process a lot of reduced species get released which are responsible for color formation and copper form chelates with protein and transfer electron in the chromagen acid mixture (Peterson, 1979).

In this experiment, Bovine Serum Albumin (BSA) was used as standard solution .At first 100 µg/mL solutions were prepared which was further diluted into 80, 60, 40 and 20 µg/mL solutions. Then, by using UV-Vis spectroscopy absorbance of different concentrations were taken and the result is given in table 4. By plotting concentration against absorbance a standard curve was prepared and from the standard curve a linear equation was obtained.

*Table 6: Absorbance of the different concentrations of BSA solution in Lowry method*

| Concentration (µg/mL) | Absorbance |
|---|---|
| 100 | 0.460 |
| 80 | 0.413 |
| 60 | 0.350 |
| 40 | 0.227 |
| 20 | 0.145 |

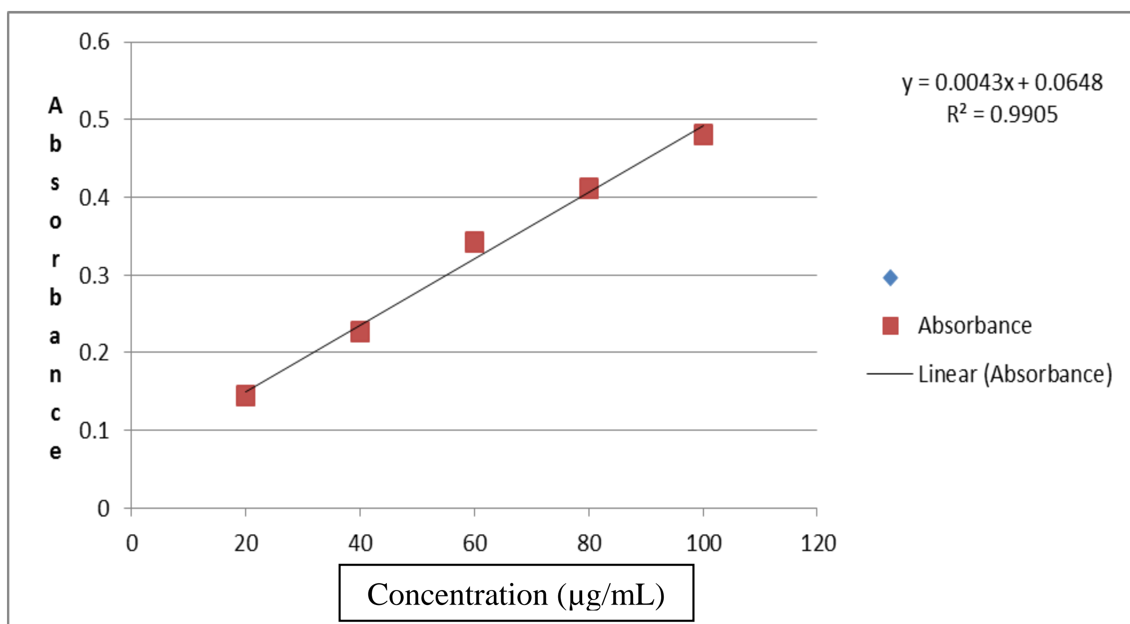


*Figure 8: Calibration curve of BSA solution for Lowry method*

The regression equation from the standard curve is:

$Y=0.0043x + 0.0643$

$=>X= (Y-0.0643)/0.0043$

Where, x = Concentration of peptides and proteins

Y = Absorbance

The absorbance of sample is $y_1= 4$

Concentration of peptides and proteins, $x_1= (4-0.0643)/0.0043$

= 915.163 µg/mL

= 0.915 mg/mL

So, Concentration of peptides and proteins in the extract of *Senna tora* is 915.163 µg/mL by using Lowry method.

## 3.3 Antimicrobial study

### 3.3.1 Peptide & protein extract

The antimicrobial activity of peptide and protein extract seeds of *Senna tora* plant was done by using two gram positive and two gram negative bacteria were used as mentioned in table 4. Penicilin and Streptomycin were used as standard. The data found after this study are given below in table no 8.

*Table 7: The antimicrobial activity of peptides and proteins extracted from seeds of Senna tora*

| Antimicrobial agent | Diameter of zone of inhibition in mm | | | |
|---|---|---|---|---|
| | *Bacillus subtilis* | *Staphylococcus Aureas* | *E.coli* | *Pseudomonas aeuroginosa* |
| Peptides and proteins | - | - | - | - |
| Penicillin (0.01 mg/mL) | - | - | - | - |
| Streptomycin (0.01 mg/mL) | 27 | - | 22 | 14.5 |

### 3.3.2 Methanolic extract of the seed

For studying the antimicrobial activity of methanolic extract of Senna tora seeds three gram positive and five gram negative bacteria were used as mentioned in table 2.4. Penicilin and Streptomycin were used as standard. The data found after this study are given below in table no 5.

*Table 8: The antimicrobial activity of methanolic extract of seeds of Senna tora by disk diffusion method*

| Bacterial Strain | Concentration of methanolic extract of seeds (mg/mL) | Standard |
|---|---|---|

| | 20 | 40 | 60 | Streptomycin (0.01 mg/mL) | Penicillin (0.01 mg/mL) |
|---|---|---|---|---|---|
| | Diameter of zone of inhibition in mm | | | | |
| *Staphylococcus aureas* | - | - | - | - | - |
| *Bacillus subtilis* | Lessen the growth around the disk | Lessen the growth around the disk | Lessen the growth around the disk | 27 | Lessen the growth around the disk |
| *Enterococcus fucalis* | - | - | - | Lessen the growth around the disk | Lessen the growth around the disk |
| *E.coli* | - | 8 | 13 | 22 | - |
| *Shigella dysenteriae* | - | - | - | 18 | - |
| *Shigella flexneri* | - | - | - | 18 | - |
| *Salmonella typhi* | - | - | - | 16 | - |
| *Pseudomonas aeruginosa* | - | - | - | 14.5 | - |

*Table 9: The antimicrobial activity of methanolic extract of seeds of Senna tora by well diffusion method*

| Bacterial Strains | Concentration of methanolic extract of seeds (mg/mL) | | Standard Antibiotic disc | |
|---|---|---|---|---|
| | 40 mg/mL (150 µL/well) | 60 mg/mL (150 µL/well) | Streptomycin (0.01 mg/mL) | Penicillin (0.01 mg/mL) |

| | Diameter of zone of inhibition in mm | | | |
|---|---|---|---|---|
| *Staphylococcus aureas* | - | - | - | - |
| *Pseudomonas aeruginosa* | 14 | 15 | 14.5 | - |

# Chapter 4

## Discussion

One of the greatest concerns of modern era is antibiotic resistance and this problem is being mounting day by day. Scientists are struggling to keep pace with discovering necessary antibiotics to combat with resistant pathogens. The number of multidrug resistant pathogens is increasing at an alarming rate and if necessary steps will not be taken, its being estimated that in 2050 around 444 million people will be affected by microbial infection throughout the world (Aslam et al., 2018).The necessity of new antibiotic has become an worldwide need now and bioactive compounds can play a vital role to fight against this global problem. Many peptides and protein has antimicrobial activity and against various pathogens antimicrobial peptide works as a first line of defense. Scientists and researchers have been continuously looking for antimicrobial peptides and the number of listed antimicrobial peptides are increasing for the last twenty years (Salas et al., 2015). *Senna tora* is an herb mainly growing in Asian countries is being used as a traditional medicine for ages. The medicinal values of *Senna tora* is mentioned in Ayurveda and the seeds of this plant is very famous in China as a herbal medicine and even it is mentioned in Chinese pharmacopeia. This study contains a peptide and protein extraction technique and an evaluation of presence of antimicrobial peptide and overall antimicrobial activity of *Senna tora* seeds.

The protein extraction has been done by using a buffer which contains phosphate buffer, EDTA, NaCl , Glycerol ,distilled water and the overall pH was 7 (Aliahmadi et al., 2011). At first, 10 g of powder was mixed with 100 mL of distilled water which was stirred by a magnetic stirrer for 2 hours and then filtered by a sterilized micro syringe filter. Throughout the process 4ºC was maintained. After that, the presence of protein was identified and the amount and size of protein was quantified by Lowry method, UV-Vis Spectrophotometry, ammonium sulphate precipitation and Gel electrophoresis. The concentration found by

Lowry method was 915 µg/mL. By gel electrophoresis proteins of different sizes were found which 15,17,23,32,34, and 75 kDa according to the marker.

Another focus of this study was to evaluate the antimicrobial activity of the protein founded here and the antimicrobial activity of methanolic extract of the seeds. To evaluate antimicrobial activity of peptides and proteins two gram positive and two gram negative bacteria were used mentioned in table 4 and the experiment was done by both disk diffusion and well diffusion method. Penicillin (0.01 mg/disk) and streptomycin (0.01 mg/disk) were used as standard and bacterial strains were found to become resistant against penicillin but streptomycin strongly inhibited all four pathogens. On the other hand, the peptides and protein could not inhibit the growth of microbes and this could happen for several reasons e.g. the lower concentration of protein, the resistance of pathogens or personal errors.

Antimicrobial activity of methanolic extract were also evaluated by using 3 gram positive and 5 gram negative bacteria mentioned in table 5. Disk diffusion method was used against all the pathogens but well diffusion method was used only against *Staphylococcus aureus* and *Pseudomonas aeruginosa* .In this case penicillin and streptomycin were used as standards. *Staphylococcus aureus* was found to be resistant against both the standards. The growth of *Enterococcus faecalis* were lessened but no clear zone of inhibition was found against any of the antibiotics. All the bacterial strains were found to resistant against penicillin but against *Shigella dysenteriae, Shigella flexneri, Salmonella typhi and Pseudomonas aeruginosa* only streptomycin was effective. By disk diffusion method only against *E.coli* clear zone was found for the methanolic extract (Conc. 40 mg/mL & 60 mg/mL) but by well diffusion method applying the solution of same concentrations strong inhibition zone was found for methanolic extract. May be this happened because of the amount being soaked the filter paper disk was not good enough to work against microbes.

# Chapter 5

## Conclusion

In conclusion, the extraction of proteins from the seeds of *Senna tora* was successful and purification and quantification have also been done by several methods. The peptides and proteins did not show any antimicrobial activity but the methanolic extract of the seeds showed antimicrobial activity against two of the microbes. May be it's not the proteins but there is some antimicrobial agents present in the seeds of *Senna tora.*

## Future Work

To identify extracted proteins and to search for anti-microbial activities of the proteins extracted from the other parts of *Senna tora*.

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