Extraction and quantitation of total peptides and proteins from the whole plant *Crotalaria pallida* and determination of antimicrobial activity of the isolated peptides and proteins

A project submitted

by

Shishir Ahmed Sikta

ID: 13146053

Session: Spring 2013

to

The department of pharmacy

In partial fulfillment of the requirements for the degree of

Bachelor of Pharmacy (Hons.)

Dhaka, Bangladesh

June, 2017

This Research Paper is lovingly dedicated to my respective parents who have been my constant source of inspiration. They have given me the drive and discipline to tackle any task with enthusiasm and determination. Without their love and support this project would not have been made possible.

Certification Statement

This is to certify that this project titled 'Extraction and quantification of total peptides and proteins from whole plant *Crotalaria pallida* and determination of antimicrobial activity of the isolated peptides and proteins' submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University, constitutes my own work under the supervision of Dr. Hasina Yasmin, Associate Professor, Department of Pharmacy, BRAC University and this project is the result of the author's original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the project paper itself.

Signed,

Countersigned by the supervisor,

Acknowledgement

First of all, I express my greatest gratitude to Almighty Allah for endowing me with health, patience, protection and faith in all aspect of my life.

I would like to express my sincere gratitude to my supervisor Dr. Hasina Yasmin, Associate Professor, Department of Pharmacy, BRAC University, for her continuous support, patience, motivation, enthusiasm, immense knowledge and guidance in this project work. Her guidance, positive attitude and scholastic knowledge helped me during the research and writing of this project paper. Without her guidance, my study and research would not be complete.

I would also like to thank Dr. Eva Rahman Kabir, Chairperson of Department of Pharmacy, BRAC University, for providing me with an opportunity and necessary support to carry out the project at an individual level.

I am thankful to Arifur Rahman, Lecturer of Department of Pharmacy, BRAC University for his advice and immense patience in guiding me to investigate the antimicrobial effect of *Crotalaria pallida*.

Further thanks to Dr. Zakir Sultan, Senior Scientist, Center for Advance Research and Science (CARS) for his complete guidance, knowledge and opening his laboratory and allowing me to work with specialized equipment's without which my work would remain incomplete.

Additionally, I would like to thank all the Lab Officers and Lab Assistants for their support and concerns.

Finally, I would like to thank the faculties of Department of Pharmacy at BRAC University, my friends and my family who constantly encouraged me and pushed me to get through and complete my project successfully.

Abstract

Crotalaria pallida belongs to the family 'liguminosae' and has been used for treating various diseases. In traditional medicine, the plant is used to treat urinary problems and fever. In addition, a poultice of the roots is also applied to swelling of joints and an extract of the leaves is used to expel intestinal worms. In general, plants of liguminosae family contain peptides and proteins to protect themselves against microbial pathogens. The objective of this study was to extract and quantify peptides and proteins from *Crotalaria pallida* and investigate antimicrobial activity of this extracted peptides and proteins. A cold extraction buffer was used for the extraction of peptides and proteins and for quantitative determination different analytical methods were applied such as Lowry method, fluorescence spectroscopy, UV-Vis spectroscopy and enzymatic method. BSA was used as the standard protein. The concentrations calculated by different methods were in close proximity (Lowry method: 217 ± 5 µg/mL; fluorescence spectroscopy: 219 ± 3 µg/mL; UV-Vis spectroscopy: 226 ± 4 µg/mL; enzymatic method: 204 ± 3 µg\mL). The antimicrobial activity of the peptides and proteins extract was observed against two gram positive bacterial strains, *Staphylococcus aureus & Bacillus subtilis* and two gram negative bacterial strains, *Escherichia coli & Vibrio cholera* by disc diffusion method. Kanamycin 30, Amoxicillin 10 and Streptomycin 10 were used as standard antibacterial agents. Kanamycin 30 and Streptomycin 10 showed inhibition against *Escherichia coli* only whereas Amoxicillin 10 showed inhibition against *Staphylococcus aureus.* No inhibition was observed in other strains of bacteria by the standard antibacterial agents which might be due to the resistance of the strains to the agents. However no zone of inhibition was found against the four experimental species by the extract of peptides and proteins also.

Table of contents

CHAPTER 02: METHODOLOGY

List of tables

List of figures

List of abbreviations

AMP: Antimicrobial Peptide

BSA: Bovine Serum Albumin

WHO: World Health Organization

IC_{50}: Half maximal Inhibitory Concentration

EDTA: Ethylenediaminetetraacetic acid

Ile: Isoleucine

Arg: Arginine

Lys: Lysine

Phe: Phenylalanine

Glu: Glutamic acid

Ala: Alanine

Val: Valine

Pro: Proline

PR: Pathogenesis-related

mg: Milligram

mL: Milliliter

mm: Millimiter

nm: Nanometer

µg: Microgram

Chapter 01: Introduction

1.1 General Information

From the beginning of human existence on the planet, diseases have played a critical role in the events of every era and it would be difficult to overstate the impact of disease on history. To get alleviated from the effects of some of the worst diseases, different medicines, vaccines have been invented for the diagnosis, treatment and prevention. But still now, there are some diseases for the treatment of which, no medicines have been discovered and scientists are doing research on it to discover the appropriate medicines for the treatment of those diseases. For the treatment of bacterial infections, people are now taking antibiotics; for gastric disorders like peptic ulcer, proton pump inhibitors, beta blockers etc. have been used for the treatment and others types of drugs are being used in the treatment of other diseases. But every medicine has some side effects and it may cause serious harmful effects for the long term use. For example, long term use of proton pump inhibitor increases the risk of fractures in hip, wrist and spine; decreases the levels of magnesium in the blood, which may lead to arrhythmias, seizures and muscle spasms (Neill *et al*., 2013). People who take NSAID (Non-steroidal anti-inflammatory drugs) regularly, have a greater risk for heart attacks, strokes and heart related diseases. So, now people are searching for the alternative way of treatments which have no side effects. Medicinal plants will be a better choice in that case because plants contain substances which have therapeutic effects with no or little side effects.

Plants have been used for therapeutic purposes much sooner than ancient period. Various chemical compounds isolated from plants, have biological functions including defense action against different kinds of fungi, insects and herbivorous mammals. Some plants like green tea, ginger, aloe, pepper, walnuts and turmeric etc are supposed to be nutritious and also have

therapeutic values. Besides this, some plants and their derivatives are also used as active ingredients in the formulation of different drugs like aspirin. Formulas for the treatment of basic illnesses, for example, diarrhea, constipation, low sperm count, hypertension, dysentery and weak penile erection, coated tongue, piles, bronchial asthma, menstrual disorders, leucorrhoea and fevers are given by the traditional medicine practitioners very effectively (Singh, 2015). As of late, World Health Organization (WHO) assessed that 80% of individuals are now using herbal medicines as their primary health care needs. According to WHO, there are more than 21,000 plant species which have the efficiency to cure diseases and is being used as therapeutic plants (Rafieian-Kopaei, 2012). Treatment with therapeutic plants is viewed as extremely protected as there is no or negligible side effects. Though in the course of recent decades, the usage of herbal medicines is increasing, there is still some lacking in site of research activity.

1.2 Medicinal plants

According to World Health Organization (WHO), medicinal plants are such kind of plants which contain different properties or components that can be utilized for therapeutic purposes and also their synthesized metabolites are used to produce useful drugs (WHO, 2008). Actually, medicinal plants have a recognized therapeutic use and the usage of medicinal plants ranges from the preparation of herbal medicines to pharmaceutical products. In human history, herbal medicine is the oldest form of medical treatment. People used medicinal plants for different therapeutic purposes from the ancient time and now, it is the precursor of the modern pharmaceutical field.

Seeds, berries, roots, leaves, bark or flowers of the medicinal plants are being utilized for medicinal purposes. For example, Neem inhibits allergic reactions when applied externally or eaten. The green leaves of Aloe-vera are used in the treatment of Burn healing, wound healing and sunburn. Turmeric stimulates digestion, boost liver function and Curcumin is a means of reducing breast cancer risk among women (Grundmann, 2012). Nowadays, research on plants to

find out the therapeutic effect is growing phenomenally at the international level and traditional medical practice has become the integral part of their culture in most of the developing countries.

Figure 1.1: Examples of some medicinal plants

1.2.1 History of medicinal plant

According to the earliest record of 4000 years old, Sumerian clay slab was founded as the first written evidence of medicinal plants utilization for preparation of drugs which contained information about more than 250 medicinal plants and twelve formulas for drug preparation. Emperor Shen Nung wrote the Chinese book 'Pen T'Sao' (2500 BC) and this book treats 365 drugs which is prepared from medicinal plants (Petrovska, 2012). Pliny (23 AD-79), wrote about

more than 1000 medicinal plants in his book 'Historia naturalis'. The first list of drugs which have similar or identical action and also interchangeable, was compiled by Galen (131 AD–200). 'De re medica', a book written by Celsus (25 BC–50 AD), quoted about more than 250 medicinal plants which include aloe, flax, henbane, poppy, cinnamon, pepper, cardamom, the star gentian, false hellebore, etc. Hippocrates (459–370 BC) classified 300 medicinal plants according to their physiologic action, for example Wormwood is active against fever; garlic is active against intestine parasites, etc. Theophrast (371-287 BC), in his book "De Historia Plantarium" described about plant history. A new Law on Drugs and Medical Devices was established on September, 2007 and according to this law fresh or dry parts of therapeutic plants might be utilized for formulation of herbal medicines. In the middle of 19th Century, first synthetic drug was developed based on the medicinal plants. Nowadays, in the rural area of the developing nations, 75-90% people are relay on herbal medicines as their primary health care (Petrosvska, 2012).

1.2.2 Contribution of medicinal plants to modern drug

At the present time, substances from plants are used in the following main ways in modern medical treatment:

- Directly as source of active pharmaceutical agents – either as single purified drugs, for example, morphine (extracted from the opium poppy *Papaver somniferum*) or in advanced extract form often in admixtures with other ingredients, for example, senna extract from *Cassia senna.*
- As blue-prints for the manufacture of synthetic drugs of a similar structure, for example the plant alkaloid cocaine extracted from *Erythroxylum coca* which has provided the chemical structure for the synthesis of procaine and other related anaesthetics.
- Medicinal plants are used in a variety of dosage forms as- powders, pastes, juices, infusions, decoctions medicinal preparations.

- Isolation of other important plant-derived drugs of modern medicine rapidly followed and many useful drugs have since been discovered and introduced into modern medicine. Drugs like caffeine from *Thea sinensis,* quinine from *Cinchona* spp. and colchicines from *Colchicum autumnale*, constitute some examples of such early drugs.

- More than 100 drugs of defined structures are in common use nowadays throughout the world and about half of them are accepted as useful drugs in the industrialized countries. These include drugs like atropine, colchicine, digitoxin, L-dopa, emetine, ephedrine, ergotamine, hyoscine, hyoscyamine, morphine, quinidine, quinine, rescinnamine, reserpine, sennosides, vinblastine, vincristine, etc.

- In addition to these, there are other plant-derived chemical substances of known structures that are used as drugs or necessary components of many modern medicinal preparations. These include camphor capsaicin, eucalyptol, menthol, minor cardiac glycosides, various volatile oils etc. These are only a few examples of vast number of drugs that are derived from plants.

1.3 Research of traditional drugs in Bangladesh

In Bangladesh, ayurvedic, homeopathic, unanie/kabiraji are so common and famous in rural areas and different parts of plants with medicinal properties, are being used as traditional medicines. People of rural areas are mostly depended on medicinal plants for their primary health care and remedy of diseases. Because of unavailability of drugs and commodities, lack of trained providers, imposition of unofficial fees, a rural-urban imbalance in health provider's distribution, unfavorable opening hours and weak referral mechanisms; poor people have a little access to services. Thus, these are contributing to low utilization of public facilities in Bangladesh (Nawaz *et al*., 2009). Medicinal plants are low in cost so that it is affordable and accessible to the poor and easy to get. They comprise a precious asset of a country and donate to its health care system. Well-judged and scientific investigation of these plants can significantly

contribute to the public health. There are more than 500 medicinal plants in Bangladesh which are extensively used in the preparation of herbal medicines (Ghani, 2003).

Figure 1.2: Traditional uses of medicinal plants

Table 1.1: Some crude drugs used as medicine in Bangladesh (Ghani, 2003)

Bangali Name	Scientific Name	Part Use	Used
Nayantara	*Catharanthus roseus*	Flowers	Cancer, insomnia, blood pressure and diabetes.
Sarpagandha	*Rauvolfia serpentian*	Root	pressure and dysentery
Ghritokumari	*Aloe indica*	Leaves	Constipation, antihelmintic, fistula,

			piles, leucorrhoea, burns and jaundice
Lajjabati	*Mimosa pudica*	Whole plant	Blood purification, toothache, convulsion fistula and piles
Assamlata	*Makania cordata*	Leaves	Dysentery.
Ulatkambal	*Abroma augsta*	Bark, leaves	Gonorrhea, diarrhea, urethritis and irregular menstruation
Jogyadumur	*Ficus hispida*	Bark, root	Insects bites, boils, asthma, piles, cough, bronchitis, and diarrhea
Shatamuli	*Asparagus racemosus*	Roots	Cancer, bacterial and fungal disease, tonic, appetizer, jaundice and diabetes.
Anatamul	*Tylophora indica*	Leaves	Asthma, cough, bronchitis, diarrhea, dysentery and stimulant
Mahedi	*Lawsonia inermis*	Leaves, flower	Skin disease, pox, burns, dandruff and insomnia.
Bohera	*Terminalia Billerica*	Fruit, bark	Constipation, diarrhea, dysentery, leprosy, rheumatisms

			and piles.
Bherenda	*Ricinus communis*	Roots, seeds	Constipation and rheumatisms.
Ghandabadal	*Paederiafoetida*	Leaves	Diarrhea, uriticaria, paralysis, piles and toothache
Haritaki	*Terminalia chebula*	Fruit, Bark	Indigestions, jaundice, piles, skin disease and ulceration of gum.
Thankuni	*Cliotoria ternatea*	Whole plant	Weakness, dermatitis, jaundice and stomach disorder
Neem	*Azadirachta indica*	Leaves	Anathematic, fever dermatitis, stomach disorder, jaundice, nausea, and ruminates
Tulshi	*Ocimum sanctum*	Leaves, flower, seeds	Stomach disorder, malaria, common cold, and hypertension.
Nishinda	*Vitex negunda*	Leaves, barks	Weakness, cough, headache, malaria, and kalazar
Basak	*Adhatoda vasica*	Root, leaves, flowers	Cough, asthma, arthritis, dysentery, and malaria

Arahar	*Cajanus cajan*	Leaves, seeds	Jaundice, mouth sore and leprosy.
Arjun	*Terminalia arfuna*	Bark	Heart disease
Kalojira	*Nigella sativa*	Seeds	Common cold, rheumatisms, galactagogue and carminative
Hatishur	*Heliotropoum indicum*	Root, leaves	Fever, rheumatisms, wound
Amloki	*Phyllanthus emblica*	Bark flower, fruit	Hair tonic, cough, diuretic, stomach ache, dysentery, jaundice, dermatitis.
Halud	*Curcuma longa*	Rhizomes	Blood purification, skin disease, eye disease, tonic, and stomachache
Methi	*Trigonella foenumgraceum*	Seeds	Hypertension and diabetes

1.4 General approaches to drug discovery from natural sources

Now-a-days, different compounds that are derived from plants, are being used as drugs in the treatment of various diseases either in their original form or semi-synthetic form and also the secondary metabolites of plants can be used as drug prototypes, drug precursors, and different pharmacological tests. Moreover, there are some plant extracts or "phytomedicines" which are in clinical trials for the treatment of different kind of diseases (Salim *et al*., 2008). In general, there

are different approaches for the selection of plant materials for drug discovery from natural product such as chemosystematic approach, random approach, ethnopharmacological approach, computational approach and ecological approach.

For selecting plant material for natural product drug discovery, the approaches have been mentioned in table 1.2.

Table 1.2: Approaches to select plant material for natural product drug discovery (Katiyar *et al.*, 2012)

Approach	Characteristics
1. Random approach	According to this approach, extracts from various plant species are selected randomly based on their availability.
2. Chemosystematic approach	According to this approach, the test samples are selected on basis of their chemotaxonomy and phylogeny and considering that plant species from different families or genera will produce different types of compounds which have a certain bioactivity or therapeutic potential.

3. Ethnopharmacological approach	According to this approach, test samples are selected on basis of the medicinal usage of the plant species.
4. Computational approach	According to this approach, test samples are selected on basis of counting on *in silico* bioactivity predictions for constituents of various plant species.
5. Ecological approach	According to this approach, on basis of the interactions between organisms and their environment, test samples are selected and it also has been considered that a potential therapeutic use for humans can be derived from the plants secondary metabolites which occupied some ecological functions.

Development of new drug is a complex, time-consuming, and costly process. It takes around 12 years from discovery of a new drug to its achieving the center, involving more than 1 billion US$ of investments in today's context (Katiyar *et al*., 2012).

Schematic diagram of different steps involving in the development of drugs from a medicinal plant is given below:-

Figure 1.3: Schematic diagram of development of drugs from a medicinal plant.

1.5 Bioactive peptides and proteins from plants

According to Sharma *et al.* (2012), bioactive peptides are specific protein fragments that have a positive impact on body functions and may influence health condition. Plants are good source of bioactive peptides and proteins and these are found in seeds, leaves, stems, flowers, and roots of the plants. Those bioactive peptides produced in plants show therapeutic activity and most of them possess antimicrobial properties and also play important role in cellular signaling (Salas *et*

al., 2014). Besides these, many researches have been conducted about the therapeutic activity of peptides in vitro and from the results of these researches, it has been invented that bioactive peptides produced in plants, have a wide range of therapeutic activities including cholesterol-lowering ability, blood pressure-lowering(ACE inhibitory) effects, antimicrobial properties, antioxidant activities, cyto- or immune modulatory effects, increase of the mineral absorption and opioid-like activities (Malaguti *et al.,* 2014). For example, potato tuber is a good source of proteins and these proteins are classified into three major groups such as protease inhibitors, patatins, and other proteins. It is proved that patatin possess antioxidant or antiradical activity and also an allergen for some people (Pihlanto & Mäkinen, 2013). The low molecular weight protease inhibitor possesses enzymatic and inhibitory effect and protease is considered as the second major potato tuber storage protein. They can inhibit a variety of enzymes such as protease, invertase etc and also show defense mechanisms against pathogens (Pihlanto & Mäkinen, 2013). Different types of plants of leguminosae family have been identified as a source of bioactive peptides which can show their activity as ACE-inhibitor, anti-oxidative agents and so on. For example, peptides from soybean, chickpea and pea are ACE-inhibitor. Some plants can show a systemic defense response because of the concomitant accumulation of the respective defense proteins, for example tomato plants can respond to local injury by herbivorous insects (Schaller, 2001).

1.5.1 Antimicrobial activity of plant peptides

According to Salas *et al*. (2014), antimicrobial peptides works as the first line defense system against invading pathogens and also play a significant role in the immunity. Nowadays, alternative microbicides is needed to control infectious diseases because the rapid increase in drug-resistant infections poses a challenge to conventional antimicrobial therapies and bioactive peptides can fulfill this role because they display antibacterial, antifungal, antiviral and/or antiparasitic activities (Salas *et al*., 2014). To protect against microbial attacks from bacteria, viruses and fungi and to counter physical and chemical stress like drought, cold, pollutants and heavy metals; higher plants possess a broad range of defense mechanisms. Plants release

different types of secondary metabolites like tannins, polyphenolic and phytoalexins compounds and they also generate pathogenesis-related (PR) proteins and by this way, they response to the infections caused by a variety of pathogens. In the early 1970s, PR proteins were first discovered in tobacco leaves, in response to tobacco mosaic virus infections which was defined as the induced proteins and they are released during pathogenic attacks. In a recent review, 17 families are estimated which can be detected and isolated and they also possess a wide range of defense-related properties, including antifungal, antibacterial, antiviral, anti-oxidative activity and proteinase inhibitory activities and the families are defensin (PR-12 family), thionin (PR-13 family), knottin, α-hairpinin, hevein-like peptide, lipid transfer protein (PR-14 family) and snaking (Tam *et al.*, 2015).

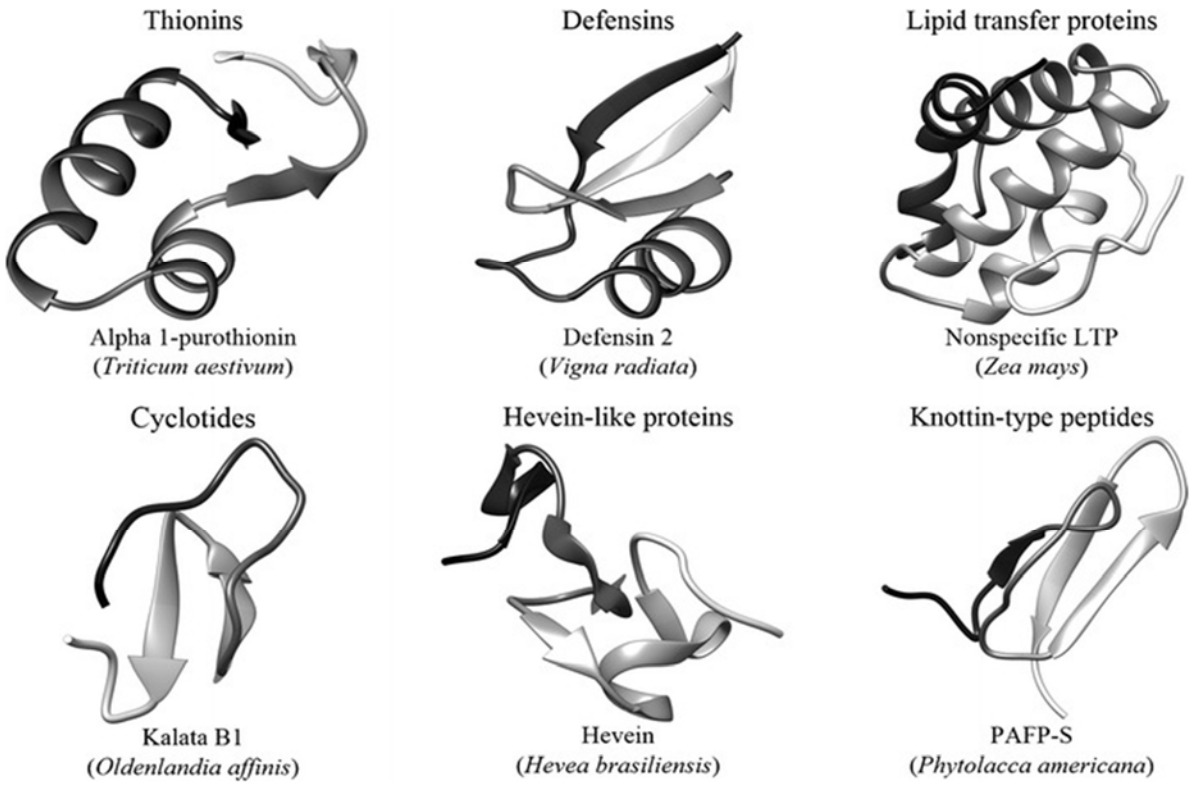

Figure 1.4: Examples of antimicrobial peptides of plants

1.5.2 Plant sources of peptides and proteins

Different types of vegetables, fruits and legumes are good sources of protein and legumes have a higher content of protein than vegetables and fruits. Examples of different plant peptides and proteins which have antimicrobial effect are mentioned in table 1.3.

Table 1.3: Sources of different plant peptides and proteins having antimicrobial effects Nawrot *et al.* (2014)

Source of Peptides	Activity
Thionins (types I–V)	Antibacterial
Thionein: alpha-1-purothionin (*Triticum aestivum)*	Antibacterial
Cyclotides: kalata B1 and B2 (*Oldenlandia affinis*)	Antibacterial, antifungal,
Lipid transfer proteins (LTPs) (*Zea mays*)	Antibacterial
Knottin-peptides: PAFP-S (*Phytolacca americana*)	Antibacterial
Puroindolines: PIN*A* and PIN*B* (*Triticum aestivum*)	Antibacterial
Snakins (*Solanum tuberosum*)	Antibacterial
Heveins (*Hevea brasiliensis*)	Antibacterial and antifungal
Peptides (*Phaseolus vulgaris*)	Antibacterial and antifungal

Peptide PvD1 (*Phaseolus vulgaris*)	Antibacterial and antifungal
Defensins (*Triticum aestivum* and *Hurdeum vulgare*)	Antifungal and antiviral
Lunatusin (*Phaseolus lunatus*)	Antiviral and antibacterial
Vulgarinin (*Phaseolus vulgaris*)	Antifungal, antibacterial and antiviral
Hispidulin (*Benincasa hispida)*	Antifungal and antibacterial
Lc-def (*Lens culinaris*)	Antifungal
Cicerin (*Cicer arietinum*)	Antiviral and antifungal
Arietin (*Cicer arietinum*)	Antiviral and antifungal
Peptide So-D1 (*Spinacia oleracea*)	Antibacterial and antifungal
Shepherins (*Capsella bursa-pastoris*)	Antifungal and antibacterial
Peptides (*Brassica napus*)	Antibacterial

1.5.3 Peptide and protein extraction techniques from plants

Different types of extraction techniques for proteins and peptides have been established according to the physicochemical and structural characteristics of the peptides and proteins such as molecular weight, solubility, hydrophobicity, isoelectric point. Some extraction techniques are given below:

1.5.3.1 Centrifugation

Among the methods which are used for the isolation and fractionation of peptides and proteins, centrifugation method is one of the simplest and easiest methods. In order to isolate proteins, the first step is to separate different cell substructures because the proteins that we want to isolate are locally concentrated on membrane, mitochondria, or nucleus of the cell. Multiple centrifugation steps are involved in this process because it will cause separation of each component into different layers based on the molecular weight, size, and shape. After that, in order to isolate the protein fraction from the selected layer, solubilization steps should be carried out (Jiang *et al.,* 2011).

1.5.3.2 Precipitation

Ammonium sulphate is used to make aggregation and precipitation of protein and this salt is the most widespread precipitants. Firstly, a large amount of this salt is added into a protein solution and other salts such as sodium chloride can also be used instead of ammonium sulphate. By increasing protein interaction, it causes protein aggregation and precipitation which is known as salting out process. The salt concentration which is needed for protein precipitation varies from one protein to another and thus this process allows selective protein separation because (Bodzon-Kulakowska *et al*., 2007).

1.5.3.3 Electrophoretic method

Electrophoretic method is mainly used for analytical and preparative purposes. Proteins are separated from the mixtures based on their charge/mass ratio, size, charge, or shape in electrophoresis method (Guttman *et al*., 2004). Sometimes, one-dimensional gel electrophoresis (1DE) has been used for protein purification. Bioactivity of the protein has been maintained in the solution.

1.5.3.4 Chromatographic method

In liquid chromatography (LC) method, different proteins are separated according to their hydrophobicity, size, charge or specificity. In most of the cases, to remove some interruptive substances (e.g., salts), chromatographic methods can also be used (Schmidt *et al.*, 2007).

1.5.3.5 Aqueous solutions

Water is more advantageous than alcohols because water is non-inflammable, neither toxic nor explosive and that is why, water is more preferable. Maximum proteins are solubilized in water at acidic, neutral, or alkaline pH and for this reason water can be used for the extraction of proteins. Type of extraction process influences the extraction yield and properties of protein and it is also influenced by different factors like salts pH, concentration, the ionic strength of the medium, electrostatic repulsions and net charge (Tan *et al*., 2011).

1.5.3.6 Aqueous enzymatic extraction

Aqueous enzymatic extraction technique is a safe, cheap and environmentally friendly alternative method for the extraction of peptides and proteins from plants (Latif & Anwar, 2009). This process protects proteins from serious damages which can be caused in the purification steps (Moure *et al.,* 2000). Moreover, in case of protein extraction, enzymes can aid in several ways, for example, carbohydrase is an enzyme which attacks the cell wall components to liberate more protein from the matrix source and also increase the protein yield (Tang *et al*., 2002).

1.5.3.7 Low-pressure liquid chromatography

It is a process that can fractionate peptides and proteins based on their molecular size and a bed of porous beads are used in this technique to separate the analytes (Ly & Wasinger, 2011). Different types of resins with various pore sizes are most commonly used and as stationary phase, polyacrylamide or other types of polymers are also used. Peptides and proteins are eluted with water, ammonia, ammonium salts or organic acids, depending on the resin composition.

1.5.4 Quantitative determination of peptides and proteins

Many methods have been established for determining the concentration of protein and among these methods, some are depended on the reactions of reagents with peptide bonds or amino acid side chains of the protein and others are depended on the binding of a reagent (dye) to the protein. Some of the methods are described below:-

1.5.4.1 The biuret method

A dilute copper sulfate solution is used in this method. Copper sulfate reacts with peptides and proteins at alkaline pH and it creates a transformation of color from blue to violet. This process of color change requires five minutes for completion. Then, absorbance of protein is measured at 540 nm and for detection; relatively large quantities of proteins or peptides (1 - 20 mg protein / mL) are required in this method.

1.5.4.2 The Lowry test

Actually, the Lowry test is the modification of the biuret method and it is the most sensitive quantitative colorimetric assay for protein detection. It can detect only only 0.005 to 0.3 mg protein per mL and an intense blue-green color is formed which comes from the reaction of the phosphomolybdotungstate in the Lowry reagent with the **W** and **Y** residues in the protein.

1.5.4.3 The Bradford method

In this method, Coomasie Brilliant Blue G-250, a negatively charged dye has been used and it binds to the positive chains of the peptide or protein and a blue complex has been formed. It can detect from 0.2 to 1.4 mg of protein per mL. The color intensity depends on the concentration of the protein; if the concentration is high, the more intense the blue color will be produced. The time needed for the color formation is only 2-5 minutes and the color is stable up to 24 hours and that is why, it is the most popular method of protein quantitation.

1.5.4.4 Fluorometric method

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which can analyze fluorescence from a sample. A beam of light has been used to excite the electrons in molecules of certain compound and usually ultraviolet light has been used. It causes the emission of light from that compound. A molecule moves from its ground states to excited state by absorbing energy in the form of UV and visible radiation. Then the excited molecules emit radiation for transition from the excited state to the ground state. The intensity of fluorescence increases as the concentration of fluorescing species increases. Peptide and protein concentration can also be determined by measuring the intrinsic fluorescence property of protein which is due to the aromatic amino acids, tryptophan, tyrosine and phenylalanine.

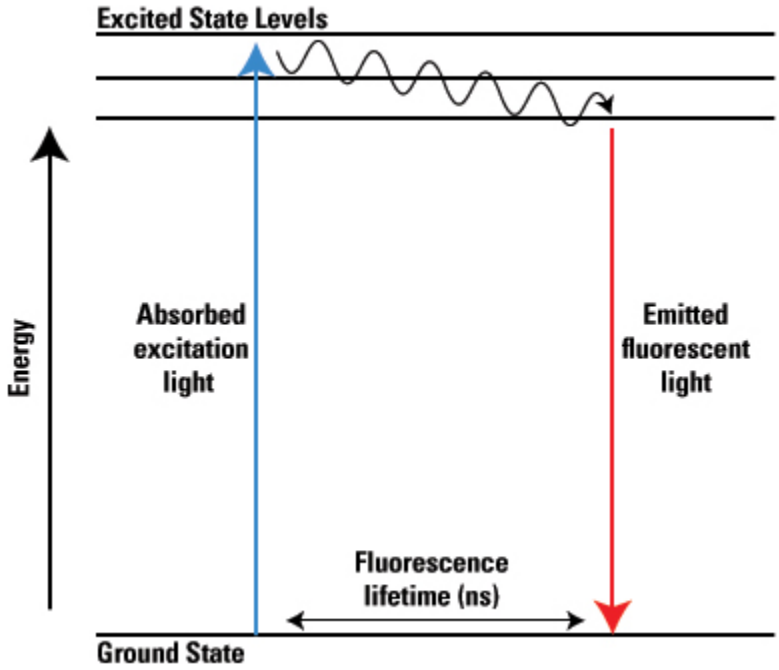

Figure 1.5: Absorption and emission of light in fluorescence spectrophotometer

1.5.4.5 UV-Vis spectrometry

UV-Vis Spectroscopy is a type of absorption spectroscopy that uses the UV and visible part of the electromagnetic radiation. All atoms and molecules absorb light according to their own structure variation and that is why, the kind and amount of radiation absorbed by a molecule depend upon the structure of the molecule and the number of molecules interacting with the radiation. Absorption of the UV radiations causes the excitation of the electrons from the ground energy state to higher energy state. The energy difference between the ground state and higher energy states is equal to the energy of the ultra-violet radiation that is absorbed. UV spectroscopy obeys the Beer-Lambert law, which states that, "when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution." The concentration of peptides and proteins can also be determined by using UV-Vis spectroscopy.

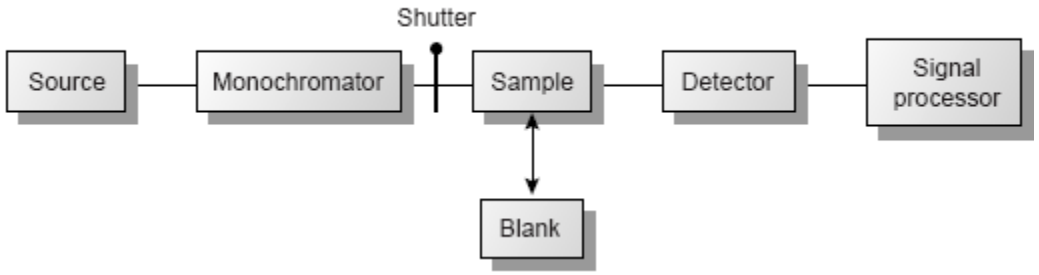

Figure 1.6: Schematic diagram of UV spectrophotometer

1.6 General information about *Crotalaria Pallida*

Crotalaria pallida is a plant belonging to the liguminosae family, prominently known as "rattle or rattlesnake" and utilized as a part of traditional medicine. This is an annual or short-lived perennial herb. The stout stem is hairy and the height of the plant is 1.5m or more. Flowers are yellow in color and leaves are trifoliate with a 2-8.5 cm long petiole. Fruits are 3-5 x 0.6-0.8 cm in length and 30-40 seeded that are heart-shaped (Chong, 2009).

Crotalaria pallida is most likely a local plant of tropical Africa. In Asia it is common in Sri Lanka and India and throughout South-Earth Asia (Hamem, 1997). The plant is grown particularly in the between columns of rubber trees and coconut palms. Flowers are eaten as a vegetable in Cambodia, where the seeds are roasted and grounded for use as a sort of coffee beverage. The roots are sometimes chewed with betel nuts in Vietnam. In traditional medicine, the plant is used to treat urinary problems and fever, a poultice of the roots is applied to swelling of joints and an extract of the leaves is taken to expel intestinal worms (Chong, 2009).

Common Names of *Crotalaria Pallida*:

Bangla: Jhunjhuni

English: Rattlepod, smooth crotalaria, smooth rattlepod, smooth rattlebox

1.6.1 Botanical Classification of *Crotalaria Pallida*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae/Liguminosae

Genus: *Crotalaria* L.

Species: *Crotalaria pallida*

Figure 1.7: Leaves of *Crotalaria pallida*

Figure 1.8: Flowers of *Crotalaria pallida*

Figure 1.9: Fruits of *Crotalaria Pallida*

Figure 1.10: Whole plant of *Crotalaria pallida*

1.6.2 Description

Height: It is an annual, terrestrial herb which is up to 150 cm in height.

Stem: Stems are 10-20 dm long, ascending or erect and short-pubescent.

Leaves: Leaves are 1.2-4.5 cm wide, trifoliolate, oblanceolate or ovate, 2.8-9.5 cm long and veins are 6-12 on each side of midvein.

Flowers: Flowers are 9-15 mm long, bracts are linear-triangular, 3-4 mm long and ascending. The lobes are 3.5-4.5 mm long. Petals are yellow in color.

Fruit: Pods are brown in color at maturity, 3.5-3.8 cm in length, stipe are less than 3 mm long.

Seeds: Each pod contains 36 seeds and they are brown in color and up to 2.8 mm in length (Wagner *et al.,* 1999).

Habitat/ecology: Commonly found in waste land and roadsides. In Hawai‘i, "naturalized in disturbed sites such as pastures, waste ground and roadsides, 5-1,070 m" (Wagner *et al.,* 1999). In Fiji, "abundantly naturalized from near sea level to about 800 m in open places, clearings, thickets, waste places, and cultivated areas, on sand dunes and open hillsides, and along river banks and roadsides" (Smith, 1985). In French Polynesia, "the plants grow on strands, motus, and upward into the mountains" (Welsh, 1998). "In Guam it is found in cultivated ground and other disturbed areas" (Stone, 1970).

Native range: "Pantropical, in part adventive and hence the original distribution obscure, but the species appears to be indigenous in parts of tropical Africa" (Smith, 1985). Tropical Africa and Asia; also cultivated and widely naturalized elsewhere in tropics (GRIN).

1.7 Literature review

1.7.1 Therapeutic activity of peptides and proteins isolated from *Crotalaria pallida*

1.7.1.1 Antimicrobial activity

Crotalaria pallida, a typical plant which belongs to the Liguminosae family, contains peptides in their seeds named Cp-AMP which is able to inhibit the growth of the gram-negative bacteria of *Proteus* species and filamentous fungi, *Fusarium oxysporum* (Patricia *et al.,* 2009). Moreover, peptide antibiotics are very potent, having higher specificity, showing higher activity, having less toxicology problems, few drug-drug interaction challenges, do not accumulate in organs and are biological and chemical diverse (Rehman & Khanum, 2011). Feng(2003) states that “plants protect themselves against different microbial pathogens by various defense responses including production of antimicrobial peptides (AMPs), secondary metabolites, lytic enzymes and membrane-interacting proteins. Thus, the peptide therapeutics market is providing new commercial opportunity to biotechnology and pharmaceutical industries.”

1.7.1.2 Anti-proliferative activity

Bioactive peptides and proteins show anti-proliferative activity on different types of cancer cell models. Lunasin has been the most commonly studied for its anticancer activities among these bioactive peptides and proteins. In 1999, "Galvez and Lumen researched about the anti-proliferative effect of lunasin and transfected *E. coli* with a lunasin encoding cDNA which show an arrest of mitosis. Besides this, when the lunasin gene was transfected into murine hepatoma and human breast cancer cells, it caused cell division arrest, chromosomal fragmentation, abnormal spindle fiber elongation and cell lysis and thus, lunasin shows its anti-proliferative effect." (Galvez & Lumen, 1999)

1.7.1.3 Antihypertensive activity

Nowadays, cardiovascular diseases have become one of the leading causes of death worldwide with many associated risk factors such as high cholesterol level, diabetes, obesity and high blood pressure (Calhoun *et al.,* 2008). From some researches, it has been demonstrated that peptides and proteins from legumes exert hypotensive activity. For example, "Ile–Arg, Lys–Phe and Glu–Phe, dipeptides from pea protein digestion, have been reported to show ACE-inhibitory effect. A peptide isolated from pepsin-hydrolysed soybean globulin which sequence was Ile–Ala–Val– Pro–Gly–Glu–Val–Ala, exerted a hypocholesterolemic effect by binding bile salts and reducing cholesterol absorption and bile salts reabsorption" (Pak *et al*. 2005). Moreover, in a recent study, it was showed that 28 days supplementation of soybean β-conglycinin (200 mg/day) lowered plasma triglycerides and cholesterol and decreased the low density lipoproteins/high density lipoproteins (LDL/HDL) ratio (Ferreira *et al.,* 2011).

1.7.1.4 Antioxidant activity

Oxidative stress can cause the onset and the progression of several chronic diseases like cancer, neurodegenerative and cardiovascular diseases. Macromolecule like lipids, protein and DNA can be damaged by reactive oxygen species. Many foods which are obtained from plants may contain

bioactive compounds which can counteract oxidative stress, but recently it has been invented that some peptides and proteins can exert an antioxidant effect. From the hydrolysis and digestion of proteins, the antioxidant peptides can be obtained. According to Wang *et al.* (2005), some amino acids such as, Histidine, Tryptophan, Tyrosine and Lysine, have antioxidant properties. It has recently demonstrated that Valine and Leucine exert antioxidant properties when found at the N-terminus of a peptide. On the contrary, Tyrosine and Tryptophan exert antioxidant properties when found at the C-terminus (Medina *et al*., 2012). Antioxidant activity of other peptides and proteins was also confirmed from other sources such as pea, wheat gliadin and soy proteins (Wang *et al.,* 2007). According to Jeong et al. (2010), lunasin able to chelate Fe2+ ions, can prevent hydroxyl radical formation through a Fenton reaction and thus it shows antioxidant properties.

1.7.2 Therapeutic activity of non-peptide constituents of *Crotalaria pallida*

Crotalaria pallida contains tannin, flavonoids, stigmasterol, pterocarpanoids, reducing suger, glycosides which give different therapeutic activity like anthelmintic activity, antimicrobial activity, anti-oxidant activity, anti-inflammatory activity, estrogenic and mutagenic activity (Alam *et al*., 2014)

1.7.2.1 Anti-inflammatory activity

Inflammation is the reaction of living tissues to injury, infection or irritation. It involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane and Botting, 1995). According to Govindappa *et al.* (2011), "the *in vitro* anti-inflammatory activitiy of *Crotalaria pallida* was evaluated by using albumin denaturation, membrane stabilization and proteinase inhibitory activities using the solvent extracts. Aspirin was used as standard drug for the study of anti-inflammatory activity. The ethanol, ethyl acetate and petroleum ether showed activity by inhibiting the heat induced albumin denaturation and red blood cells membrane stabilization with 83.17, 71.33 and 58.14 and 68.21, 61.44 and 60.72 g/mL, respectively. The proteinase activity was significantly inhibited by the

ethanol (82.53), ethyl acetate (74.31) and petroleum ether (62.92) g/mL. In addition, the ethanol, ethyl acetate and petroleum ether extracts showed anti-lipoxygenase activity and they also exhibited a moderate xanthine oxidase and acetylcholinesterase inhibitory activity."

1.7.2.2 Anthelmintic activity

Helminthiasis is one of the most significant animal diseases. Panda *et al.* (2015) evaluated the anthelmintic activity of ethanol, n-butanol, petroleum ether and ethyl acetate extract of leaves of *Crotalaria pallida* and in their study they used indian earthworms *Pheretimaposthuma* as the test worm. The in-vitro anthelmintic activity was determined by released into 10 mL of desired formulation containing three different concentration, each of crude extract i.e. ether extract, ethyl acetate, n-butanol and ethanol extract (20,40 and 60 mg/mL) were prepared. In this study, as reference standard albendazole (10 mg/mL) was used and distilled water was also used as control. Then, six worms were placed in it. The result showed that ethanol and petroleum ether showed anthelminitic activity. Between the two, the activity of petroleum ether was better than ethanol as compared to standard drug albendazole. The ethanol extract of *Crotalaria pallida* also shows dose dependent decrease in paralysis time and also cause death of worm *Paramphistomum cervi* (trematoda) (Alam *et al.,* 2014).

1.7.2.3 Antioxidant activity

According to Alam *et al. (*2014), the antioxidant activity of *Crotalaria pallida* leaves extract was evaluated by assessing its discoloration of 2, 2-diphenyl-1picryl-hydrazyl radical (DPPH) in ethanol. The value of IC_{50} of the extract was compared to the IC_{50} value of standard ascorbic acid. This study has shown that presence of polyphenols contribute significantly to the antioxidant activity.

1.7.2.4 Estrogenic and Mutagenic activity

According to Boldrin *et al.* (2013), they used the Recombinant Yeast Assay for the investigation of the estrogenic potential of *Crotalaria pallida* leaves. Stigmasterol and dichloromethane fraction was used by Ames test for assessing the mutagenic effect of the extract of *Crotalaria pallida*. The result of the estrogenic activity assessment was equivalent to estradiol and dichloromethane fraction and stigmasterol isolated from *Crotalaria pallida* leaves was proved to have the estrogenic activity and there was no mutagenic effect in the Ames assay.

1.7.2.5 Antimicrobial activity

According to Alam *et al*. (2014), four species of both gram positive (*Streptococcus pyrogens*) and gram negative bacteria (*Vibrio cholera, Shigella dysenteriae, Shigella flexneri)* were used for antibacterial test the study was performed by disc diffusion method. Kanamycin (30µg/disc) was used as reference standard. From the result of this study, it can be estimated that ethanol extract of *Crotalaria pallida* showed mild antibacterial activity against the bacterial strains *Shigella flexneri, Vibrio cholera, Shigella dysenteriae.*

1.8 Rationale of the study

At present, the continuous use of analogous antibiotics has resulted in multi resistant bacterial strains all over the world. Now, it can be predict that in the very near future antibiotic resistance will make healthcare professionals helpless toward effective therapies for bacterial infections. That is why, there is necessary to search for unconventional alternative antibiotics and therefore, interest in peptide antibiotics has been increased greatly during the past decade, as these are believed to be very potent, higher specificity, showing higher activity, having few toxicology problems, few drug-drug interaction challenges, do not accumulate in organs and are biologically and chemically diverse. From certain researches, it has been shown that plants protect themselves against microbial pathogens by various defense responses including production of antimicrobial peptides (AMPs), secondary metabolites, lytic enzymes and membrane-interacting

proteins (Feng *et al*., 2003) and their amphipathic nature allows them to persist at water-lipid interfaces and then to disturb microbial membrane components (Ruissen *et al*., 2001). For this reason, the peptide therapeutics market is providing new commercial opportunity to biotechnology and pharmaceutical industries.

1.9 Aim of the study

The aim of this study is to extract peptides and proteins from *Crotalaria pallida* and evaluate the antimicrobial activity of the extract of peptides and proteins.

1.10 Objectives of the study

The objectives of this study are:-

- To establish an extraction procedure for isolation of peptides and proteins from *Crotalaria pallida*.
- To quantify peptides and proteins.
- To evaluate the antimicrobial activity of the extract of peptides and proteins.

Chapter 02: Methodology

2.1 Extraction of peptides and proteins from *Crotalaria pallida*

2.1.1 Preparation of extraction buffer

Used chemicals

1. Distilled Water
2. NaCl
3. Ethylene diamine tetraacetic acid (EDTA)
4. glycerol
5. phosphate buffer

Used apparatus

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

Procedure

Extraction buffer was prepared by dissolving 0.074 g EDTA, 0.292 g NaCl, 0.68 g monopotassium phosphate (KH_2PO4) and 0.87 g dipotassium phosphate (K_2HPO4) into distilled water and then 10 mL glycerol was added with the solution. Volume was adjusted to 100 mL and pH 7 was maintained. Then it was kept in the refrigerator for several minutes (Aliahmadi *et al*., 2011).

2.1.2 Peptides and proteins extraction technique

- Whole plant was selected, rinsed with water and distilled water and dried under a chemical hood.
- After milling, it was subjected to total protein extraction using extraction buffer containing 50 mM phosphate buffer (pH 7), 2 mM EDTA, 5% glycerol and 50 mM NaCl.
- 50 mL of the cold extraction buffer was added to 2.5 g of the plant powder and the mixture was put on a shaker for up to 4h at 4ºC.
- Then it was filtered using Whatman filter paper and temperature was maintained at 4ºC

2.2 Methods for Quantification of peptides and proteins

2.2.1 Lowry method

Lowry method is the most widely used method to estimate the amount of peptides and proteins in samples. First the peptides and proteins are pre-treated with copper ion in alkali solution, and then the aromatic amino acids in the treated sample reduce the phosphomolybdatephosphotungstic acid present in Folin Reagent. A blue color is formed in the end product by this reaction. The concentration of peptides and proteins in the sample can be determined by measuring the absorbance at 750 nm (Gerhardt *et al*., 1994)

Used chemicals

1. Sodium hydroxide (NaOH)
2. Sodium carbonate (Na_2CO_3)
3. Copper sulfate ($CuSO_4$)
4. Na_2 Tartrate.2(H_2O)
5. Folin Reagent

6. Bovine Serum Albumin (BSA)

7. Distilled water

Used apparatus

1. Glass tubes

2. Volumetric flasks

3. Vortex machine

4. Spectrophotometer

Preparation of Lowry solution

- Firstly, 0.57 g of NaOH and 2.86 g of Na_2CO_3 was dissolved in 100 mL distilled water to prepare solution A.
- 0.71 g of $CuSO_4.5(H_2O)$ was dissolved in 50 mL distilled water for the preparation of solution B.
- 1.74 g of Na_2 Tartrate was dissolved in 50 mL distilled water to prepare solution C.
- Then, 1 mL of solution B and 1mL of solution C was added with 100 mL of solution A to prepare Lowry solution.

Preparation of BSA standard protein solution

10 mg of BSA was dissolved in 10 mL distilled water to prepare the stock solution. The concentration of the stock solution is 1 mg/mL. Then it was diluted in several times to make different concentrations for the standard curve.

Procedure

- Firstly, 2 mL of sample and 2 mL of standard (dilutions) were transferred to a 10 mL glass tube.
- Then, 2.8 mL of Lowry solution was added to the each tube and mixed properly.
- After that, the glass tubes were kept into dark place for 20 minutes and at the last 5 minutes, diluted Folin Reagent was prepared.
- After 20 minutes of incubation, glass tubes were taken out and 0.4 mL of diluted Folin Reagent was added to each tube and mixed properly.
- Then the glass tubes were incubated for 30 minutes in dark at room temperature.
- After 30 minutes of incubation, the absorbance of sample and standards was recorded at 750 nm using UV-Visible spectroscopy.

2.2.2 Fluorescence spectroscopy

Peptide and protein concentration can also be determined by measuring the intrinsic fluorescence property of protein which is due to the aromatic amino acids, tryptophan, tyrosine and phenylalanine.

Used chemicals

- BSA
- Distilled water
- Cold extraction buffer

Used apparatus

- Fluorescence spectrophotometer
- Beakers
- Volumetric flasks

Procedure

- 10 mg BSA was dissolved in 10 mL distilled water to make the concentration 1 mg/mL.
- BSA solution was used as standard and temperature was maintained at 4ºC for both the sample solution and standard.
- The excitation and emission spectra of the standard BSA solution and sample solution were measured at 290 nm and 340 nm by using Hitachi-850 spectrofluorimeter.

2.2.3 Enzymatic method

In enzymatic method, a chelate is formed in the biuret reaction between the Cu^{2+} ion and the peptide bonds of the proteins in alkaline solutions to form a violet colored complex whose absorbance is measured by UV spectroscopy.

Reagent composition

In this method, a reagent is used which is composed of:

- Cupric sulfate 6 mmol/L
- Sodium potassium tartrate 21 mmol/L,
- Potassium iodide 6 mmol/L
- Sodium hydroxide 0.75 mol/L

Procedure

- Firstly, three clean test tubes were taken; one for blank, one for sample and another for standard BSA solution (7g/L).
- Then 3 mL of reagent was taken into the each tube.
- After that 60 µL of sample and 60 µL of standard was added and mixed properly.
- The tubes were incubated for 10 min at 37º C.
- Then the absorbance of the sample and the standard was taken at 540 nm against the reagent blank.

2.2.4 UV-Vis Spectroscopy

Used chemicals

- BSA
- Distilled water
- Extraction buffer

Used apparatus

- UV spectrophotometer
- Volumetric flasks
- Beakers
- Conical flasks

Procedure

- Firstly, 10 mg BSA was dissolved in 10 mL distilled water and it was used as standard protein solution.
- Then the standard BSA solution was diluted to several times to make different concentrations (0.20, 0.40, 0.60, 0.80, 1 mg/mL) for the preparation of standard curve.
- Then absorbance of the different concentrations of BSA solution and sample solution was measured at 280 nm by using UV-1800 spectrophotometer.

2.3 Antibacterial activity study by Disc Diffusion method

2.3.1 Apparatus and reagents

- Filter paper discs
- Autoclave
- Nutrient Agar Medium
- Laminar air flow hood

- Petri-dishes
- Sterile cotton
- Micropipette
- Inoculating loop
- Sterile forceps
- Screw cap test tubes
- Spirit burner
- Refrigerator
- Incubator
- Dichloromethane
- Ethanol
- Nose mask and Hand gloves

2.3.2 Test organisms

Both gram positive and gram-negative organisms were taken for the test and they are listed in the table 3.1.

Table 2.1: List of bacteria used in the study

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus	*Escherichia coli*
Bacillus subtilis	*Vibrio cholera*

2.3.4 Preparation of medium

Firstly, 3.8 mg Mueller Hinton Agar was dissolved in 100 mL distilled water to prepare the medium. Then it was mixed thoroughly until the agar was completely dissolved. After autoclaving for 20 min at 121^0C, the mixture was cooled up to (45-50) ^{0}C and poured into 4 sterile petri dishes equally to form uniform depth in each plate. Each 120 mm petri dish got approximately 25 mL of agar solution. Then it was allowed to cool for solidifying at room temperature.

2.3.5 Culturing of bacterial strains

Firstly, Nutrient Broth medium was prepared by dissolving 0.25 g Nutrient Broth in 10 mL distilled water. Strains were collected from long term preserved medium containing skim milk, trypsine, glucose and glycerin which were preserved in Ultra Low Temperature. The bacterial strains were taken from there by a loop and then transferred into Nutrient Broth medium. Then it was incubated for 24 hr at 37ºC for reviving of those bacteria. New bacterial colony was formed after 24 hr of incubation and that was ready to use.

2.3.6 Preparation of disc

The disc was made of Whitman paper with 6mm diameter and it was autoclaved in a test tube. Then solutions of plant extract were added to the disc. Then it was allowed to soak all the plant extracts for 10-15 min.

2.3.7 Procedure

Firstly, a cotton swab was dipped into the suspension of bacteria. The swab was gently squeezed against the tube to get rid of excess fluid. Then, the swab was streaked the bacterial suspension to the nutrient agar plate in one direction to another and after that in was streaked diagonally. After that, the agar plates were dried for 5 min. Then, the discs containing extract of peptides and proteins were placed individually by using forceps, on the surface of the place. Kanamycin 30, Amoxicillin 10, Streptomycin 10 were used as reference standard and also placed on the surface of the plate. After using every time the forceps were sterilized by flame. After that, the petri dishes were incubated 24 hr at 37^0C to get the lawn growth of bacteria.

Chapter 03: Results

3.1 Extraction of peptides and proteins

The extraction of peptides and proteins was done by using a cold extraction buffer which was prepared by distilled water, NaCl, EDTA, glycerol and phosphate buffer (Aliahmadi *et al*., 2011). Then, 50 mL of the cold extraction buffer was added to 2.5 g of the powder of dried plant and the mixture was put on a shaker for up to 4 h at 4ºC. After that, it was filtered using whatman filter paper. Conditions were optimized for our analysis.

3.2 Quantification of peptides and proteins using different methods

Quantity of peptides and proteins was determined by using different methods such as Lowry method, fluorescence spectroscopy, UV-Vis spectroscopy and enzymatic method.

3.2.1 Lowry method

The principle behind the Lowry method of determining peptide and protein concentrations lies in the reaction of the peptide nitrogen with the copper [II] ions under alkaline conditions. Firstly, the peptides and proteins are pre-treated with copper ion in alkali solution. Then the phosphomolybdatephosphotungstic acid present in Folin Reagent is reduced by the aromatic amino acids presented in the treated sample. A blue color has been formed at the end product of this reaction. The concentration of peptides and proteins in the sample can be determined by measuring the absorbance at 750 nm (Gerhardt *et al*., 1994)

In this method, Bovine Serum Albumin (BSA) was used as standard solution. 1 mg/mL BSA solution was prepared in H_2O as stock solution and it was diluted with H_2O to obtain 0.031,

0.062, 0.125 & 0.25 mg/mL solutions. The absorbance of the different concentrations of BSA solutions was measured in UV-Vis Spectroscopy and the results are given in Table 4.1. Standard curve was constructed by plotting the concentrations against absorbance. A linear equation was obtained from concentration range 0.031 mg/mL to 0.25 mg/mL ($R^2 = 0.994$).

Table 3.1: Absorbance of the different concentrations of BSA solution in Lowry method

Concentration (mg/mL)	Absorbance	Standard Deviation n=3
0.031	0.326	± 0.139
0.062	0.528	± 0.101
0.125	0.812	± 0.122
0.25	1.327	± 0.093

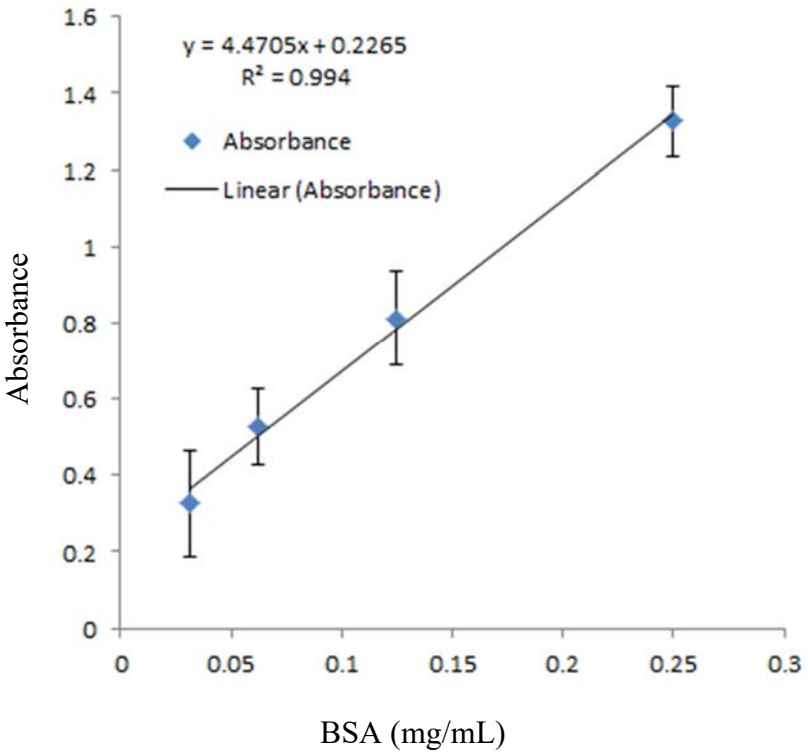

Figure 3.1: Standard curve of BSA solution for Lowry method

The regression equation from the standard curve is:

$$y = 4.4705x + 0.2265$$

$$=> \ x = \ (y - 0.2265)/4.470$$

Where, x = Concentration of peptides and proteins

y = Absorbance

The absorbance of sample, $y_1 = 1.197$

Concentration of peptides and proteins, $x_1 = (1.197 - 0.2265)/ 4.470$

= 0.217 mg/mL

= 217 µg/mL

Accordingly, x_2 =212 µg/mL

x_3 = 222 µg/mL

Avg. x = 217 ± 5 µg/mL

So, Concentration of peptides and proteins in the extract of *Crotalaria pallida* is 217 ± 5 µg/mL by using Lowry method.

3.2.2 Fluorescence Spectroscopy

Peptide and protein concentration can also be determined by measuring the intrinsic fluorescence property of protein which is due to the aromatic amino acids, tryptophan, tyrosine and phenylalanine. A beam of light usually ultraviolet light has been used which excites the electrons in molecules that causes the emission of light; typically the visible light. A molecule moves from its ground states to excited state by absorbing energy in the form of UV and visible radiation. Then the excited molecules emit radiation for transition from the excited state to the ground state. The intensity of fluorescence increases as the concentration of fluorescing species increases.

The absorption and emission of light for the sample solution and BSA solution were measured at 290 nm and 340 nm. The fluorescence spectra of BSA solution and sample solution are shown in figure 4.2 and 4.3.

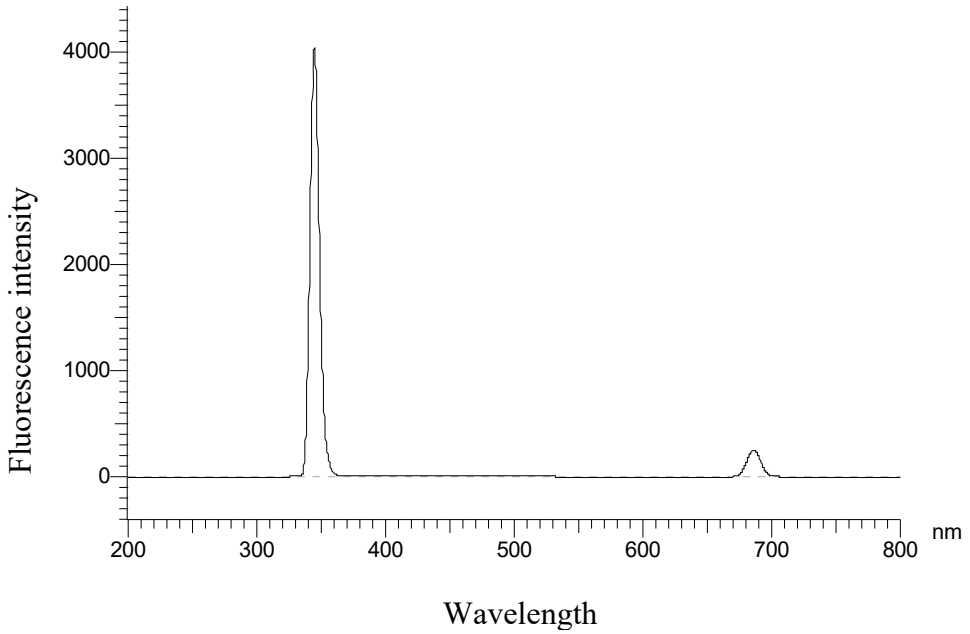

Figure 3.2: Fluorescence spectrum of BSA solution

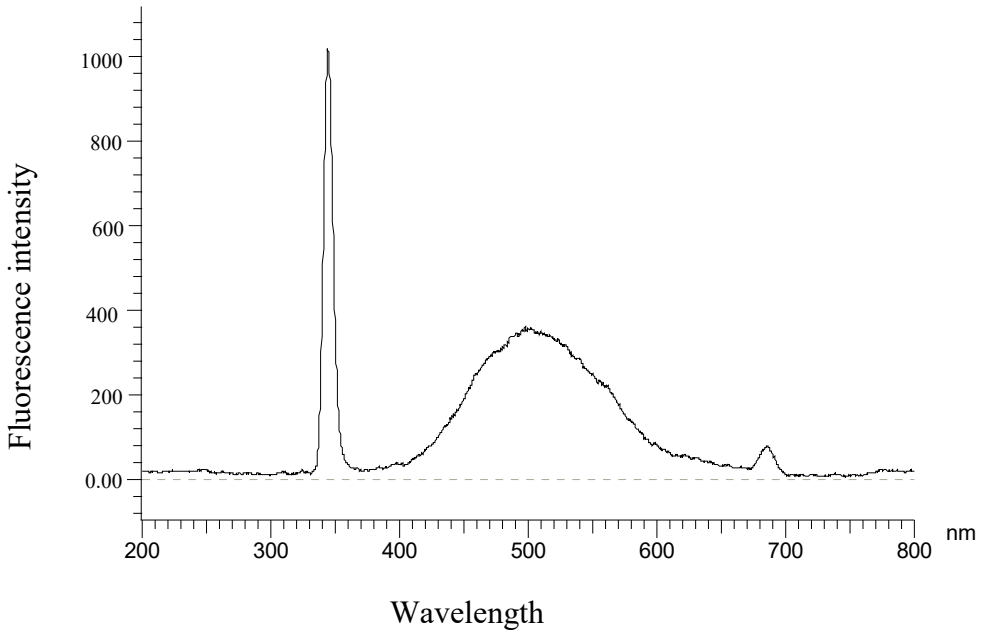

Figure 3.3: Fluorescence spectrum of peptide and protein extract from *Crotalaria pallida*

From the calculation using Beer-Lambert's Law,
Concentration of peptides and proteins, x_1 = 222 µg/mL

x_2 = 219 µg/mL

x_3 = 216 µg/mL

Avg. x = 219 ± 3 µg/mL

So, Concentration of peptides and proteins in the extract of *Crotalaria pallida* was found 219 ± 3 µg/mL by using fluorescence spectroscopy.

3.2.3 Ultraviolet-visible spectroscopy

UV-Vis Spectroscopy uses the UV and visible part of the electromagnetic radiation. All atoms and molecules can absorb energy according to their own structure variation. The amount of radiation absorbed by a molecule generally depends upon the structure of the molecule and the number of molecules interacting with the radiation.

After absorbing the ultra-violet radiations, the electrons get excited and move from the ground state to higher energy state. The energy difference between the ground state and higher energy states is equal to the energy that is absorbed by the atom. UV spectroscopy obeys the Beer-Lambert law, which states that "when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution."

In this study, BSA solution was used as standard and was diluted to several times to make different concentrations for the preparation of standard curve. The absorbance of the different concentrations of BSA solution was measured at 280 nm and the result is given in Table 4.2. A linear range was obtained from 0.2- 01 mg/mL (R^2= 0.991)

Table 3.2: Absorbance of the different concentrations of BSA solution for UV spectrophotometric method

Concentration (mg/mL)	Absorbance	Standard Deviation n= 3
0.20	1.083	± 0.112
0.40	1.238	± 0.155
0.60	1.398	± 0.091
0.80	1.615	± 0.131
1.0	1.911	± 0.098

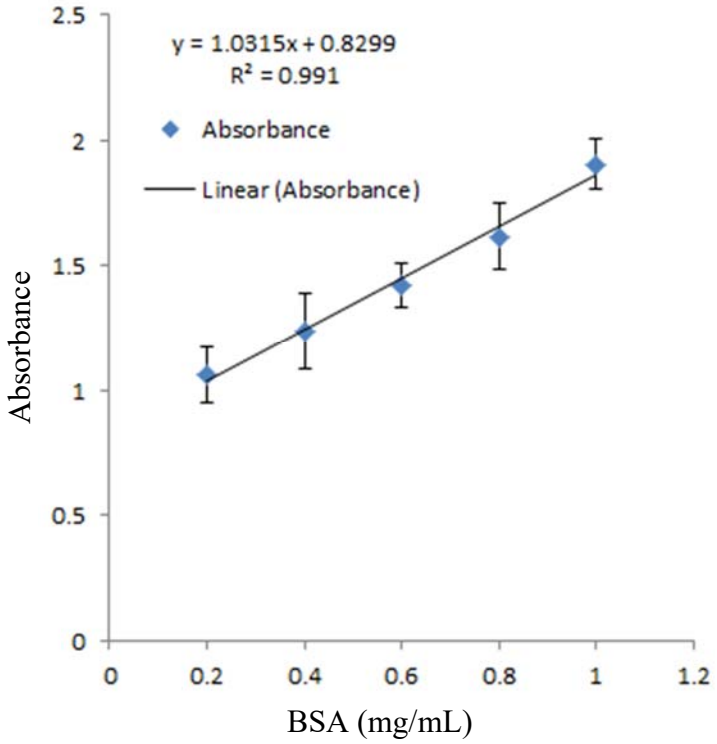

Figure 3.4: Standard Curve of BSA Solution for UV spectrophotometric method

The linear equation from the standard curve is:

$$y = 1.0315x + 0.8299$$
$$\therefore x = (y - 0.8299)/1.0315$$

Where, x = Concentration of peptides and proteins

y = Absorbance

The absorbance of sample, $y_1 = 1.063$

So, Concentration of peptides and proteins, $x_1 = (1.063 - 0.8299)/ 1.0315$

= 0.226 mg/mL

= 226 µg/mL

Accordingly, $x_2 = 222$ µg/mL

$x_3 = 230$ µg/mL

Avg. x = 226 ± 4 µg/mL

So, Concentration of peptides and proteins in the extract of *Crotalaria pallida* was found 226 ± 4 µg/mL by using UV-Vis spectroscopy.

3.2.4 Enzymatic method

In enzymatic method, a chelate is formed in the biuret reaction between the Cu^{2+} ion and the peptide bonds of the proteins in alkaline solutions to form a violet colored complex whose absorbance is measured by UV spectroscopy.

In this method, a reagent was used which is composed of cupric sulfate 6 mmol/L, sodium potassium tartrate 21 mmol/L, potassium iodide 6 mmol/L and sodium hydroxide 0.75 mol/L. BSA solution was used as standard which concentration was 7 g/L. The absorbance of the sample and standard was measured at 540 nm using UV-Vis spectroscopy.

Absorbance of the sample, $A_{sample} = 0.015$

Absorbance of the standard, $A_{standard} = 0.514$

Concentration of the standard, $C_{standard}$ = 7 g\L

Concentration of the sample, C_{sample} (x_1) = $(A_{sample} \times C_{standard}) / A_{standard}$

$$= (0.015 \times 7)/0.514$$

= 0.204 g\L

= 204 µg\mL

Accordingly, x_2 = 201 µg\mL

x_3 = 207 µg\mL

Avg. x = 204 ± 3 µg\mL

So, Concentration of peptides and proteins in the extract of *Crotalaria pallida* was found 204 ± 3 µg/mL by using enzymatic method.

The concentrations of peptides and proteins in the extract of Crotalaria pallida obtained by four different methods such as Lowry method, fluorescence spectroscopy, UV-Vis spectroscopy and enzymatic method, has been mentioned in table 4.3.

Table 3.3: The concentrations of peptides and proteins using different methods

Name of methods	Lowry method	Fluorescence spectroscopy	UV-vis spectroscopy	Enzymatic method
Concentration of peptides and proteins	217 ± 5 µg/mL	219 ± 3 µg/mL	226 ± 4 µg/mL	204 ± 3 µg/mL

3.2 Antimicrobial activity

This study focused on the determination of the antimicrobial activity of the extract of peptides and proteins from *Crotalaria pallida* using two gram positive bacterial strains, *Staphylococcus aureus & Bacillus subtilis* and two gram negative bacterial strains, *Escherichia coli & Vibrio*

cholera by disc diffusion method. Kanamycin 30, Amoxicillin 10 and Streptomycin 10 was used as standard. Kanamycin 30 showed zone of inhibition against *Escherichia coli* and the inhibiting zone of kanamycin was 17 mm and the inhibiting zone of Streptomycin was 12 mm against *Escherichia coli*. Amoxicillin 10 showed zone of inhibition against *Staphylococcus aureus* and the inhibiting zone was 22 nm. No zone of inhibition was found against *Bacillus subtilis* and *Vibrio cholera* and the peptides and proteins extract of *Crotalaria pallida*, do not show any zone of inhibition against *Staphylococcus aureus, Bacillus subtilis, Vibrio cholera and Escherichia coli.*

Table 3.4: The antimicrobial activity of peptides and proteins extracted from *Crotalaria pallida*

Antimicrobial agent	**Diameter of zone of inhibition in mm**			
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Vibrio cholerae*
Peptides and proteins	-	-	-	-
Kanamycin 30	-	-	17 mm	-
Amoxicillin 10	22 mm	-	-	-
Streptomycin 10	-	-	12 mm	-

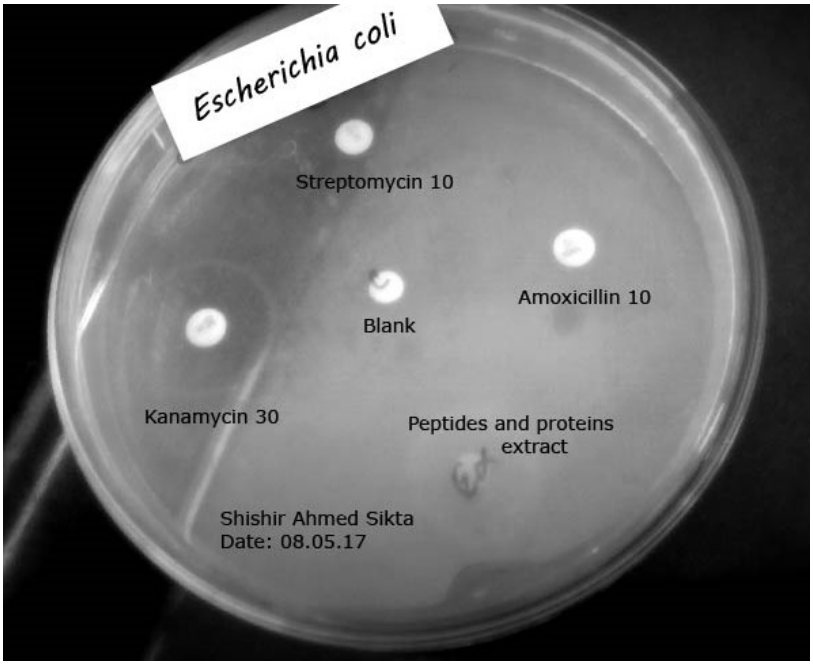

Figure 3.5: Antimicrobial study of peptides and proteins extracted from *Crotalaria pallida* against *Escherichia coli*

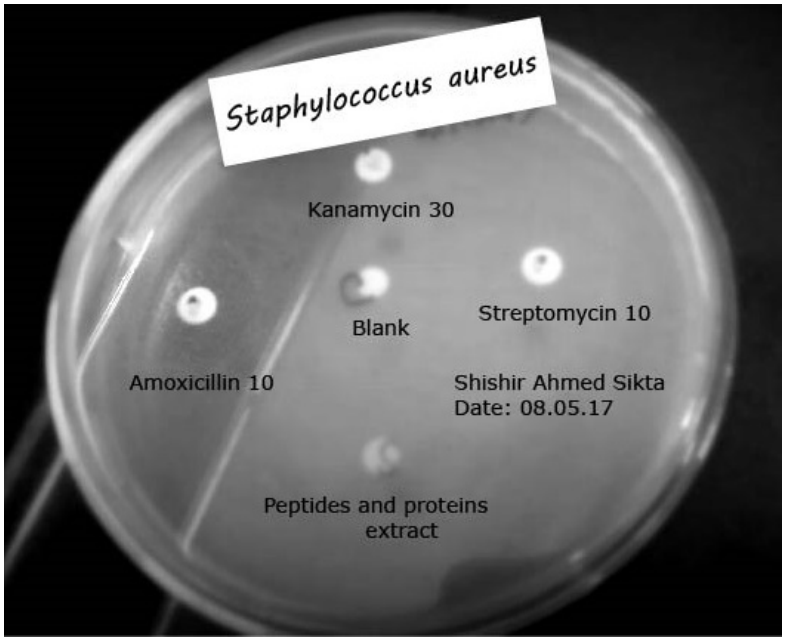

Figure 3.6: Antimicrobial study of peptides and proteins extracted from *Crotalaria pallida* against *Staphylococcus aureus*

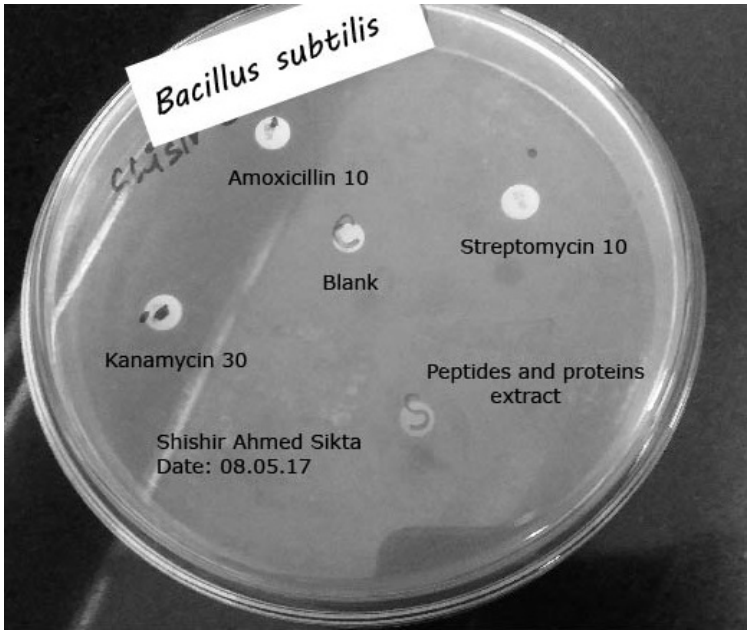

Figure 3.7: Antimicrobial study of peptides and proteins extracted from *Crotalaria pallida* against *Bacillus subtilis*

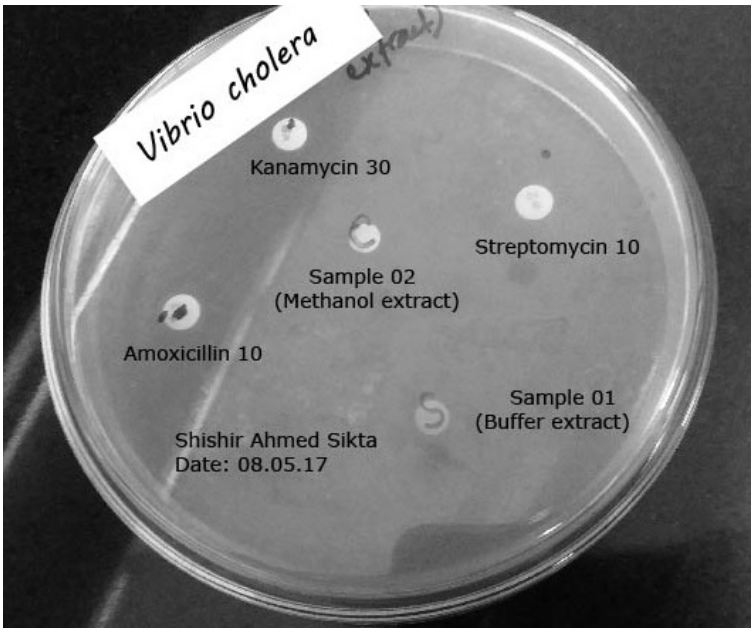

Figure 3.8: Antimicrobial study of peptides and proteins extracted from *Crotalaria pallida* against *Vibrio cholerae*

Chapter: 04
Discussion

Bioactive peptides and proteins produced in plants show therapeutic activity and most of them possess antimicrobial properties and also play important role in cellular signaling (Salas *et al*., 2014). Nowadays, alternative microbicides are needed to control infectious diseases in human because of the rapid increase in drug-resistant infections poses a challenge to conventional antimicrobial therapies. Bioactive peptides may fulfill this role as they display antibacterial, antifungal, antiviral and/or antiparasitic activities (Salas *et al*., 2014).

Crotalaria pallida belongs to the family, 'liguminosae' and utilized as traditional medicine. It is used to treat urinary problems and fever, the root of this plant is applied to swelling of joints and an extract of the leaves is taken to expel intestinal worms (Chong, 2009). Generally, plants of liguminosae family contains peptides and proteins which show different biological activities like antimicrobial effect, antihypertensive effect, anti-proliferative effect and so on (Patricia *et al.,* 2009; Galvez & Lumen*,* 1999; Ferreira *et al.,* 2011). In this study, a peptide and protein extraction method from *Crotalaria pallida* has been described, quantity of the extracted peptides and proteins were determined by different analytical methods and the antimicrobial activity of the extracted peptides and proteins has also been investigated.

The extraction of peptides and proteins was done by using a cold extraction buffer which was prepared by distilled water, NaCl, EDTA, glycerol and phosphate buffer (Aliahmadi *et al*., 2011). 50 mL of the cold extraction buffer was added to 2.5 g of the plant powder and the mixture was put on a shaker for up to 4h at 4ºC. After that, it was filtered using whatman filter paper and then the solution was collected. During the entire process 4ºC temperature was maintained (Aliahmadi *et al*., 2011). Without further processing, the solution was used for quantitative analysis and antimicrobial study. For quantitative determination we applied different analytical methods such as Lowry method, fluorescence spectroscopy, UV-Vis spectroscopy and

enzymatic method. In every analytical method, BSA was used as the standard protein. The concentrations calculated by different methods were in close proximity (Lowry method: 217 ± 5 µg/mL; fluorescence spectroscopy: 219 ± 3 µg/mL; UV-Vis spectroscopy: 226 ± 4 µg/mL; enzymatic method: 204 ± 3 µg\mL) which shows the presence of peptides and proteins in the sample and success of the extraction method.

This study also focused on the antimicrobial activity of the peptides and proteins extracted from *Crotalaria pallida*. Previously, the antimicrobial study of various parts of *Crotalaria pallida* has been performed in filamentous fungi *Fusarium oxysporum* and gram-negative bacteria of *Proteus* species (Patricia *et al.,* 2009). So in this study, antimicrobial study has been done using other strains. Two gram positive bacterial strains, *Staphylococcus aureus & Bacillus subtilis* and two gram negative bacterial strains, *Escherichia coli & Vibrio cholera* were used for the study by disc diffusion method. Kanamycin 30, Amoxicillin 10 and Streptomycin 10 was used as standard antimicrobial agents. Kanamycin 30 and Streptomycin 10 showed inhibition against *Escherichia coli* only whereas Amoxicillin 10 showed inhibition against *Staphylococcus aureus.* No inhibition was observed in other strains of bacteria by the standard antibacterial agents which might be due to the resistance of the strains to the agents. However no zone of inhibition was found against the four experimental species by the extract of peptides and proteins also. It might be due to the low concentration of peptides and proteins in the extract, resistance or personal errors.

In conclusion, peptides and proteins are successfully extracted from the plant Crotalaria pallida and the concentration of peptides and proteins were estimated by different analytical methods showed results in close proximity. No antimicrobial activity was found for the extracted peptides and proteins due to low concentration, resistance or other experimental errors.

References

References:

Alam M. H., Murshid G.M.M., Bokshi, B., Suma, S.A., Sathi S.I., & Rahman, A. (2014). Assessment of antioxidant, antibacterial and anthelmintic activities of ethanol extract of leaves of crotalaria pallida (aiton). *International Journal of Pharmacognosy,* 1(7), 438-444.

Aliahmadi, A., Roghanian, R., Emtiazi, G., Ghassempour, A. (2011). A simple method for primary screening of antibacterial peptides in plant seeds. *Iranian Journal of Microbiology,* 3(2), 104-108.

Agyeia, D. & Danquah, M. K. (2011). Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnology Advances,* 29, 272-277.

Bayer, A.W., Kirby, W.M.M., Sherris, J.C. & Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol*, 45, 493-496.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K. & Tomita, M. (1992). Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *Journal of Applied Bacteriology,* 73(6), 472-479.

Belovic, M.M., Mastilović, J.S., Torbica, A.L., Tomić, J.M., Stanić, D.R. & Džinić, N.R. (2011). Potential of bioactive proteins and peptides for prevention and treatment of mass non-communicable dieseases. *Food Feed Res,* 38, 51–61.

Bodzon-Kulakowska, A., Bierczynska-Krzysik, A., Dylag, T., Drabik. A., Suder, P., Noga, M., Jarzebinska, J. & Silberring, J. (2007) Methods for samples preparation in proteomic research. *J Chromatogr B*, 849, 1–31.

Boldrin, P. K., Resende, F. A., Höhne, A. P., Camargo, A. D., Espanha, L. G. & Nogueira, K. H., (2013). Estrogenic and mutagenic activities of Crotalaria pallida measured by recombinant yeast assay and Ames test. *BMC Complementary and Alternative Medicine* , 13,216

Calhoun, D.A., Jones, D., Textor, S., Goff, D.C., Murphy, T.P., Toto, R.D., White, A. Cushman, W.C., White, W. & Sica, D. (2008). Resistant hypertension: Diagnosis, evaluation, and treatment. A scientific statement from the American Heart Association Professional Education Committee of the Council for High Blood Pressure Research. Hypertension.51, 1403–1419.

Chong, K. Y., Tan, H. T. W. & Corlett, R. T. (2009). *A checklist of the total vascular plant flora of Singapore: native naturalised and cultivated species*. *Raffles Museum of Biodiversity Research,* 273.

Farrokhi, N., Whitelegge, J.P., Brusslan, J.A. (2008). Plant peptides and peptidomics. Plant Biotechnol, 6, 105–134.

Feng, J., F. Yuani, Y. Gao, C. Liang, J. Xu, C. Zhang & L. He. (2003). A novel antimicrobial protein isolated from potato (Solanum tuberosum) shares homology with an acid phosphatase. *Journal of Biochem*, 376(2): 481-487.

Ferreira Ede, S., Silva, M.A., Demonte, A. & Neves, V.A. (2011) Soy β-conglycinin (7S Globulin) reduces plasma and liver cholesterol in rats fed hypercholesterolemic diet. *J. Med. Food,* 14, 94–100.

Galvez, A.F., de Lumen & B.O. (1999). A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. Nat. *Biotechnol.* 17, 495–500.

Gani, A., 2005, Practical phytochemistry (including methods of phytochemical studies), 22-23.

Gerhardt, P., Murray, R. G., Wood, W. A. & Krieg, N. R. (1994). Methods for general and molecular bacteriology. *American Society for Microbiology*, 518.

Gesler, W.M. (1992). Therapeutic landscape: medicinal issue in light of the new cultural geography. *Soc Sci Med,* 34, 735-46.

Ghani A (2005). Text Book of Pharmacognosy. Rajbari Printing Press, 197-204.

Govindappa M., Bharath, N., Shruthi, H. B., Sadananda, T. S. & Sharanappa P. (2011). Antimicrobial, antioxidant and in vitro anti-inflammatory activity and phytochemical screening of Crotalaria pallida Aiton. *African Journal of Pharmacy and Pharmacology*, 5(21), 2359-2371.

Grundmann, O. (2012). Aloe Vera Gel Research Review: An overview of its clinical uses and proposed mechanisms of action. *Natural medicine journal,* 4(9), 117-132.

Guttman, A., Varoglu, M. & Khandurina, J. (2004). Multidimensional separations in the pharmaceutical arena. *Drug Discov Today*, 9,136–144

Hamem, F., L.J.G & Vander Macsen. (1997). Plant resources of south-east Asia, 11 auxiliary plants.

Hélène L. Citerne, R. Toby Pennington & Quentin C. B. C. (2006). An apparent reversal in floral symmetry in the legume Cadia is a homeotic transformation" *PNAS.* 103 (32): 12017–12020

Jahan, F.I., Hasan, M.R.U., Jahan, R., Seraj, S., Chowdhury, A.R., Islam,M.T., Khatun, Z. & Rahmatullah M. (2011). A comparison of medicinal plant usage by folk medicinal practitioners of two adjoining villages in Lalmonirhat district, Bangladesh. *Am Eur J Sust Agri.* 5(1), 46–66.

Jiang, J., Xiong, Y.L. & Chen, J. (2011). Role of b -conglycinin and glycinin subunits in the pH-shifting-induced structural and physicochemical changes of soy protein isolate. *J Food Sci,*76,293–302

Judd, W. S., Campbell, C. S. Kellogg, E. A. Stevens, P.F. Donoghue, M. J. (2002), Plant systematics: a phylogenetic approach, *Sinauer Axxoc,* 287-292.

Katiyar C., Kanjilal S., Katiyar S. (2012). Drug discovery from plant sources: *An integrated approach. Journal of Research in Ayurveda,* 33(1), 10-19.

Ko, H., Weng, J., Tsao, L., Yen, M., Wang, J., Lin, C. (2003). Anti-inflammatory flavonoids and pterocarpanoid from Crotalaria pallida. *Bioorganic & Medicinal Chemistry Letters,* 14, 1011-1014.

Ly, L., & Wasinger, V. C. (2011). Protein and peptide fractionation, enrichment and depletion: tools for the complex proteome. *Proteomics,* 11,513–534

Malaguti, M., Dinelli, G., Leoncini, E., Bregola, V., Bosi S., Arrigo, F., Cicero, G., & Hrelia, S. (2014). Bioactive Peptides in Cereals and Legumes: Agronomical, Biochemical and Clinical Aspects. *International Journal of Molecular Sciences,* 15, 21120-21135.

Marco Malaguti , G. D., Emanuela, L., Bregola, V., Bosi, S. &. Hrelia, S. (2014). Bioactive Peptides in Cereals and Legumes: Agronomical, Biochemical and Clinical Aspects. *International Journal of Molecular Sciences,* 15, 21120-21135.

Medina-Godoy, S., Ambriz-Pérez, D.L., Fuentes-Gutiérrez, C.I., Germán-Báez, L.J., Gutiérrez-Dorado, R., Reyes-Moreno, C. & Valdez-Ortiz, A. Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *J. Sci. Food Agric.* 2012, 92, 1974–1981.

Nawaz, A.H.M.M., Hossain, M., Karim, M., Khan, M., Jahan, R.& Rahmatullah, M. (2009). An ethnobotanical survey of Rajshahi district in Rajshahi division, Bangladesh. *Am Eur J Sust Agri,* 3,143–150.

Nawrot, J., Barylski,G.Nowicki, J., Broniarczyk,W., Buchwald, A. & Go´zdzicka-J´ozefiak. (2014). "Plant antimicrobial peptides," *Folia Microbiologica,* 59(3), 181–196.

Neill L. W., Benjamin L. C., John A. Galdo. (2013). Long-term Consequences of Chronic Proton Pump Inhibitor Use. *Us Pharmacist,* 38(12), 38-42.

Pak, V.V., Koo, M.S., Kasymova, T.D. & Kwon, D.Y. (2005). Isolation and identification of peptides fromSoy 11S-globulin with hypocholesterolemic activity. *Chem. Nat. Compd,* 41, 710–714.

Panda, S. K., Debajyot, D. & Niraj, K. T. (2014). Phytochemical investigation and anthelmintic activity of various leaf extracts of Crotalaria pallida aiton. *World Journal of Pharmacy and Pharmaceutical Sciences,* 4(2), 336-342.

Pelegrini B. P., Luciana R. Farias, Amanda C. M. Saude, Fabio T. Costa, Carlos Bloch Jr, Luciano P. Silva, Adeliana S. Oliveira Carlos, E. M. Gomes, Maurı´cio P. Sales, Octa´vio L. Franco. (2009). A Novel Antimicrobial Peptide from Crotalaria pallida Seeds with Activity Against Human and Phytopathogens. *Curr Microbiol* ,59:400–404

Petrovska, B. B. (2012). Historical review of medicinal plant's usage. *Pharmacogn Rev.* 6(11), 1–5.

Pihlanto, A., & Mäkinen, M. (2013). Antihypertensive Properties of Plant Protein Derived Peptides. 145-182.

Rafieian-Kopaei, M. (2012). Medicinal plants and the human needs. *Journal of HerbMed Pharmacology,* 1(1), 1-2.

Rehman S. & Khanum A., (2011). Isolation and characterization of peptide(s) from pisum sativum having antimicrobial activity against various bacteria. *Pak. J. Bot*., 43(6): 2971-2978

Robert Nawrot, J. B., Grzegorz Nowicki, and W. B. Justyna Broniarczyk, Anna Goździcka-Józefiak (2014). Plant antimicrobial peptides. *Folia Microbiol*, 59, 181-196.

Ruissen, A.L.A., J. Groeninck, E.J. Helmerhorst, E. Walgreen-Weterings, W. Van't Hof, E.C.I. Veerman and A.V. Nieuw-Amerongen. (2001). Effects of Histatin 5 and derived peptides on Candida albicans. *J. Biochem.,* 356(2): 361-368.

Salas C. E., Guadalupe R., Oliver-Salvador C. (2014). Biologically Active and Antimicrobial Peptides from Plants. *BioMed Research International 2015*,1-11.

Salim, A. A., Chin, Y.-W., Kinghorn, A. D. (2008). Drug Discovery from Plants. *Bioactive Molecules and Medicinal Plants*, 4(1), 1-24.

Schaller A. (2001). Bioactive peptides as signal molecules in plant defense, growth and development. Bioactive Natural Products, 25, 367-411.

Schmidt, S.R., Schweikart, F. & Andersson, M. E. (2007). Current methods for phosphoprotein isolation and enrichment. *J Chromatogr B*, 849:154–162

Smith, A. C. (1985). Flora Vitiensis nova: a new flora of Fiji. National Tropical Botanical Garden, Lawai, Kauai, Hawaii, 3, 758.

Sharma, S., Singh, R., Rana, S. (2011). Bioactive Peptides: A Review. *International Journal of Bioautomation*, 15(4), 223-250.

Singh, R. (2015). Medicinal Plants. A Review. *Journal of Plant Sciences*, 3(1), 50-55.

Sushobhan Ukila, S. L. & Roy, R. N. (2015). Physicochemical characterization and antibacterial activity of the leaf oil of Crotalaria pallida Aiton. *Journal of Taibah University for Science,* 10(2), 490-496.

Stone, B. C. 1970. The flora of Guam. Micronesica, 6,1-659.

Tang, S., Hettiarachchy, N.S., Shellhammer, T.H. (2002). Protein extraction from heat-stabilized defatted rice bran, Physical processing and enzyme treatments. *J Agric Food Chem*, 50:7444–7448.

Tam, J. P., Wang, S., Wong, K. H., & Tan, W. L. (2015). Antimicrobial Peptides from Plants. *Pharmaceuticals*, *8(4)*, 711-757.

Vane, J. R. & Botting, R.M. (1995). Ne insight into the mode of action of anti-inflammatory drugs. *Inflammation Res*., 44: 1-10.

Wagner, Warren L., Herbst, Derral R., Sohmer, S. H. (1999). Manual of the flowering plants of Hawaii. Bernice P. *Bishop Museum Press.*

Wang, W.; de Mejia, E.G. (2005). A new frontier in soy bioactive that may prevent age-related chronic diseases. *Compr. Rev. Food Sci. Food Saf*, 4, 63–78.

Watson, L. & Dallwitz, M. J.(2007). "The families of flowering plants: Leguminosae"

Welsh, S. L. (1998). Flora Societensis: A summary revision of the flowering plants of the Society Islands. 420.