ISOLATION AND CHARACTERIZATION OF SMALL ANTIMICROBIAL PEPTIDES FROM *Punica granatum* AGAINST MULTI-DRUG RESISTANT BACTERIA

SALHA KHAMES MOHAMED ALI KHAMES

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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SALHA KHAMES MOHAMED ALI KHAMES

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ISOLATION AND CHARACTERIZATION OF SMALL ANTIMICROBIAL PEPTIDES FROM *Punica granatum* AGAINST MULTI-DRUG RESISTANT BACTERIA

ABSTRACT

Pomegranate *(Punica granatum)* has long been used as a traditional medicinal plant, and its extracts are widely used for the treatment of diarrhoea and dysentery. Although *P. granatum* is known to possess potent antimicrobial compounds, its antimicrobial peptides (AMPs) have been poorly isolated and well characterized. In this study, peptides were isolated from pomegranate peel, leaf and stem, and their antibacterial activities against 11 different gram-negative enteropathogenic strains were screened using an agar well diffusion assay. The peptides were partially purified by 80% ammonium sulphate precipitation, and their antimicrobial activities were analysed. Gel filtration chromatography and semi-preparative high-performance liquid chromatography (HPLC) were performed to purify the peptides. The molecular weights of active peptides were identified by liquid chromatography-quadrupole time-of-flight mass spectrometry (Q-TOF LC/MS) analysis. Three potential antimicrobial peptides, two from the leaf fraction (LVF-AMP1 and LVF-AMP2) and one from the stem fraction (STF-AMP), with molecular weights of 386.17, 414.52 and 386.17 Da, respectively, were obtained. These active peptides inhibited the multidrug-resistant pathogenic strain *Shigella flexneri* serotype Y, with minimum inhibition concentration (MIC) values of 6.45, 7.55 and 6.05 μg ml^{-1}, respectively. The results suggest that *P. granatum* is a source of plant antibacterial peptides, which may serve as potent and specific antimicrobial agents for the treatment of gastrointestinal diseases.

Keywords: antimicrobial peptides, *Punica granatum*, enteropathogenic strains, antimicrobial activity, resistant bacteria

PENGASINGAN DAN PENCIRIAN PEPTIDA ANTIMIKROBIAL KECIL DARI *Punica granatum* TERHADAP BAKTERIA TAHAN MULT-IDRUG

ABSTRAK

Delima (*Punica granatum*) telah lama digunakan sebagai tumbuhan perubatan tradisional, dan ekstraknya digunakan secara meluas untuk rawatan cirit-birit dan disentri. Walaupun *P. granatum* diketahui memiliki sebatian antimikroba yang kuat, peptida antimikrobial (AMP) telah kurang terisolasi dan dicirikan dengan baik. Dalam kajian ini, peptida telah diasingkan dari kulit buah, daun dan batang buah delima, dan aktiviti antibakteria mereka terhadap 11 jenis strain enteropatogenik gram-negatif telah disaring menggunakan ujian penyebaran agar. Peptida telah dibersihkan sebahagiannya oleh pemendakan ammonium sulphate 80%, dan aktiviti antimikrobanya dianalisis. Kromatografi penapisan gel dan kromatografi cecair prestasi tinggi separa persediaan (HPLC) telah dilakukan untuk membersihkan peptida. Berat molekul peptida aktif telah dikenalpasti oleh analisis spektrometri jisim kromatografi-quadrupole (Q-TOF LC / MS) cecair. Tiga AMPs, dua daripada pecahan daun (LVF-AMP1 dan LVF-AMP2) dan satu daripada fraksi batang (STF-AMP), dengan berat molekul 386.17, 414.52 dan 386.17 Da, masing-masing diperolehi. Peptida aktif ini menghalang tegasan patogenik tahan multidrug *Shigella flexneri* serotype Y, dengan nilai kepekatan pencerobohan minimum (MIC) masing-masing sebanyak 6.45, 7.55 dan 6.05 μg ml-1. Hasilnya menunjukkan bahawa *P. granatum* adalah sumber peptida antibakteria tumbuhan, yang boleh berfungsi sebagai agen antimikrobial yang kuat dan spesifik untuk rawatan penyakit gastrousus.

Kata kunci: peptida antimikrobial, *Punica granatum*, strain enteropatogenik, aktiviti antimikroba, bakteria tahan

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LIST OF SYMBOLS AND ABBREVIATIONS

^{o}C	**:**	Degree Celsius
µg/ ml	**:**	Microgram per milliliter
µl	:	Microliter
µm	:	Micrometer
ACN	:	Acetonitrile
AML	:	Amoxicillin
AMP	:	Ampicillin
AMPs	:	Antimicrobial peptides
ANOVA	:	Analysis of variance
BSA	:	Bovine serum albumin
C	:	Chloramphenicol
CEF	:	Cefipime
CFU	:	Colony-forming unit
CIP	:	Ciprofloxacin
CLSI	:	Clinical and Laboratory Standards Institute
cm	:	Centimeter
CRO	:	Ceftriaxone
Da	:	Dalton
EC	:	*E. coli*
g	:	Grams
h	:	Hour
HCl	:	Hydrochloric Acid
HPLC	:	High performance liquid chromatography
I	:	Intermediate

K	:	Kanamycin
kDa	:	Kilodalton
LB	:	Luria Bertani
LC/MS Q-TOF	:	Liquid chromatography-quadrupole time-of-flight mass spectrometer analysis system
LV	:	Leave extract
LV8	:	Active chromatography fraction from leaf extract
LVF7	:	Purified fraction of leaf
M	:	Molarity
MDR	:	Multidrug resistant bacteria
mg	:	Milligram
mg/ml	:	Milligram per milliliter
MIC	:	Minimum inhibition concentration
min	:	Minute
ml	:	Milliliter
mm	:	Millimeter
NA	:	Nalidixic Acid
nm	:	Nanometer
MW	:	Molecular weight
OD	:	Optical density
PBS	:	Phosphate-buffer saline
PC	:	Positive control
PE	:	Peel extract
PE20	:	Active chromatography fraction from peel extract
PEF8	:	Purified fraction of peel
R	:	Resistant

RP-HPLC	:	Reversed-phase high performance liquid chromatography
Rpm	:	Revolutions per minute
S	:	Sensitive
S	:	Streptomycin
SB	:	*S. boydii*
SD	:	*S. dysentery*
Sd	:	Standard division
SE	:	Seed extract
Sf	:	*S. flexneri*
SMsp	:	*S. typhimurium*
SMt	:	*S. typhi*
sp.	:	Species
spp.	:	Subspecies
SS	:	*S. sonnei*
ST	:	Stem extract
ST15	:	Active chromatography fraction from stem extract
STF6	:	Purified fraction of stem
V	:	Volts
v/v	:	Volume per volume
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Diseases caused by enteric pathogens are a fundamental reason for morbidity and mortality worldwide and majorly give rise for a public health concern (Pai et al., 2011). Clinically, enteric pathogens have a critical role in causing millions of cases of food poisoning and gastroenteritis all over the world every year (Pai et al., 2011). These infections primarily affect the gastrointestinal tract, and eventually, have the ability to spread to the different parts of the body (Pradeep et al., 2008).

Diarrhea and dysentery are the most observed gastrointestinal symptoms. Among the enteric bacteria; *Salmonella*, *Shigella*, *E. coli*, *Pseudomonas*, *Vibrio cholerae* and *Staphylococcus aureus* are the main causative agents of occasional and epizootic diarrhea in young and adults as well (Pai et al., 2011). The infection with diarrhea is an important public health issue in underdeveloped countries (Geetha et al., 2011), diarrhea infections are the second reason of mortality; approximately 25 million of individuals are infected annually (Pradeep et al., 2008).

Enteropathogenic *Enterobacteriaceae* species have shown multidrug resistance in different geographical areas that results in a critical hurdle in controlling of diarrhea and create real challenges with the determination of applicable treatment (Pai et al., 2011; Tribble, 2017). The misuse such as; underdose, overuse, or incomplete course of antibiotic in medical practice is known to result in an increase of the number of microorganisms species that can develop resistance against these antibiotics (Subramani et al., 2017).

Recently, the antimicrobial agents that derived from natural sources have been assessed in order to establish their efficacies towards different of microorganisms (Dahham et al., 2010). These natural products may present attractive alternative medicines that could be safer than chemicals and cost-effective as well. They do not

show side effects in most of the cases, especially when they utilized in limit doses (Aparecida Pro et al., 2016).

Medicines that extracted from plants have been practiced frequently in the treatment of human diseases for centuries. Besides they were used as a food source, plant outputs were practiced historically as a folk remedy (Sajjad et al., 2015). As stated by the World Health Organization (WHO), medicinal plants are those plants comprise some ingredients that can be utilized for treatment applications or manufacturing of synthetic medicines (Aparecida Pro et al., 2016). The utilization of plant sources in the diseases therapy and in pharmaceutical applications has gradually grown (Nascimento et al., 2000).

For several centuries, plants have been known as a considerable source of natural products for human health-sustaining. Therefore a significant number of studies have been conducted for well establishment of natural therapies (Nascimento et al., 2000). Furthermore, natural products that were derived from medicinal plants are proven to have antimicrobial effects against various types of microorganisms (Sajjad et al., 2015). Plants are rich in various secondary metabolites such as alkaloids, flavonoids, tannins, and terpenoids. It has been reported that these metabolites have antimicrobials effect (Sajjad et al., 2015). The multi-drug resistance toward the clinical antibiotics with the promising results rising from researchers in plant resources, lead the world to conduct extensive studies in order to utilize these promising natural antibacterial agents (Aliahmadi et al., 2011).

Punica granatum (pomegranate) is one of the oldest eatable fruits. It has been referred to in the verses of Qur'an and Bible (Duman et al., 2009; Pai et al., 2011). Pomegranate has been studied broadly (Proc et al., 2016). In U.S., Asia, Africa and Europe, *P. granatum* has been commonly utilized as a conventional pharmaceutical to treat various types of illnesses (Duman et al., 2009). It has been practiced in the

treatment of acidosis, microbial infections, haemorrhage, dysentery, diarrhea, helminthiasis, and respiratory infections (Duman et al., 2009). Pomegranate extracts are known to possess antimicrobial properties. They have shown to have significant effect when utilized in therapeutic treatments. Over the most recent couple of years, various investigations have been directed in many countries in order to provide evidence to demonstrate such efficiency (Aparecida Pro et al., 2016; Dahham et al., 2010; Duman et al., 2009; Nascimento et al., 2000; Naz et al., 2007). Similar to many plants, *P. granatum* has phenolic compounds that have high molecular weight which have a considerable antimicrobial effect (Al-Zoreky, 2009). Furthermore, not only the secondary metabolites, phenols, and alkaloids showed antimicrobial properties, plant antimicrobial peptides have antimicrobial efficacy and they could be used against the pathogenic bacteria as well (Aliahmadi et al., 2011).

Antimicrobial peptides are small-molecular-weight compounds that can be created by different multicellular life from both the plant and animal kingdoms (Agarwal et al., 2016). The majority of AMPs have broad-spectrum activity and a net positive charge, and they form clusters of hydrophobic and hydrophilic residues. Therefore, they are amphiphilic in nature (Agarwal et al., 2016).

Although pharmacological industries have produced many new antibiotics in the last three decades, the problem of microbial resistance is growing and becoming a global issue that affects public healthcare around the world (Salazar et al., 2017). Actions must be taken to eliminate this problem, including offering effective natural antibacterial drugs to patients (Pradeep et al., 2008). Antimicrobial peptides (AMPs) have shown capability to fight alongside with antibiotics against pathogens that have developed drug resistance (Golla et al., 2016). Thus, the aim of the present study is to isolate and characterize antimicrobial peptides from different parts of *P. granatum*.

Research Objectives:

- To screen the crude extracts from different parts of *Punica granatum* parts (seed, leaves, stem and peel) which may have antimicrobial activities against resistant gastrointestinal pathogens.
- To isolate potential antimicrobial peptides (AMPs) from the different organs of *P. granatum*.
- To purify the isolated peptides from the active extracts.
- To characterize the antimicrobial peptides and determine their molecular weight.

CHAPTER 2: LITERATURE REVIEW

2.1 *Punica granatum* as a medicinal plant

A wide range of plants have an enormous fortune of medical value. A large part of these medicinal plants has been utilized to medicate different systemic conditions effectively (Lansky & Newman, 2007).

P. granatum (PG) is one of the very old eatable fruits; it has been broadly consumed in different nations for centuries, and it has prolonged time through history as a medical plant (Moorthy et al., 2013; Prasad & Kunnaiah, 2014; Viuda-Martos et al., 2010). It is consumed as fruit and juice (Hajifattahi et al., 2016). Not only the fruit, other parts of the plant including; flowers, trunk skin, roots, and seeds are used for medicinal practices (Hajifattahi et al., 2016).

P. granatum is popularly named pomegranate. Its genus name is *Punica,* which originates from the Roman name of Carthage (Moorthy et al., 2013). Pomegranate is a bush or small tree; it branches with numerous spines and forms many upright, spiky shanks, the leaf appears oval in shape, it is about 2×1 inches, the flower is white or red in colour, and it is double-flowered traits (Moorthy et al., 2013). The fruit when ripen is approximately five inches in width and acquires dark red colour; it has rough skin and grenade in shape with a pointed crown. The fruit is abundant in seeds. Almost it is filled with seeds that form multiple aggregates detached with white, membranous pericarp. The seed is surrounded by sour red juice (Prasad & Kunnaiah, 2014).

The fruit can be differentiated into many anatomic compartments: (1) seed, (2) juice, (3) peel, (4) leaf, (5) flower, (6) bark, and (7) roots. Each of these compartments has its fascinating medication advantage (Lansky & Newman, 2007). PG is originated in Iran and Northern India and has been long planted in the Middle East, spreading among the Mediterranean nations, eastbound to China. Then it continued extending to the

American Southwest, California and Mexico in the New World continents (Hajifattahi et al., 2016; Ismail et al., 2012; Lansky & Newman, 2007).

The pomegranate tree, *P. granatum* owns a broad traditional therapeutic history. It provides storage of phytochemicals that are heuristic in their medical importance (Lansky & Newman, 2007). It was praised in the past era; in the Old Testament of the Bible, the Jewish Torah, and the Babylonian Talmud as a sacramental fruit (Jurenka, 2008).

Several investigations have reported many activities of pomegranate active ingredients of its extract that including the antimicrobial, antifungal, antihelminthic, and antioxidant effect. These activities are proposing their preventive and therapeutic role in several cases such as; gastro-mucosal trauma, ulceration caused by ethanol and acetone, diabetic, oxidative damage and cancer chemoprevention (Dahham et al., 2010; Hajifattahi et al., 2016; Nascimento et al., 2000; Sasidharan et al., 2011).

2.1.1 Components of pomegranate

Over the nineties of the past century, considerable effort has been performed to elucidate the mechanisms by which the pomegranate extract and active ingredients accomplish their effects and what is the role of a single component. Therapeutic effects could be seen from any parts of PG extracts, and some investigations have demonstrated that the roots, bark, and leaves of pomegranate have medicinal value as well (Jurenka, 2008). The fruit itself which includes three components; 3% of the weight of the fruit is the seeds that in turn containing about 20% oil, and the juice which represents 30% of the fruit weight. The peel also includes the inner network of membranes that are useful as well. Other valuable parts of the plant incorporate the roots, bark, leaves, and flowers (Lansky & Newman, 2007).

Over the last decade, some reviews demonstrated the chemical components of different parts of pomegranate that have a significant role in prevention and therapy of inflammation and cancer (Ismail et al., 2012; Jurenka, 2008; Miguel et al., 2010). In those reviews, it has been stated that the most therapeutic advantageous *P. granatum* components including; flavonoids, punicic acid, anthocyanidins, ellagic acid, ellagitannins, and phenols present in flower, leaf and peel. In addition, polysaccharides have been identified in the peels of *P. granatum* (Jurenka, 2008; Miguel et al., 2010). Additionally, pomegranate peels are featuring by notable measures of phenolic compounds comprising; flavonoids such as anthocyanins, catechins and other flavonoids.

Besides their free radical-scavenging characteristics, punicalagin, ellagic acid and Gallic acid also have an antibacterial effect against enteric microflora, mostly pathogenic organism such as; *Salmonella* spp., *Shigella* spp., *V. cholerae*, as well as *E. coli* (Ismail et al., 2012; Lansky & Newman, 2007).

2.2 Plants derived antimicrobial

Wheat a-purothionin is the first antimicrobial peptide that was found out from a eukaryotic organism in 1942 by Balls and co-workers. The following peptide in this class was revealed only after about 30 years (Hammami et al., 2008). The conducted studies that illustrating the finding out of new antimicrobial peptides derived from plant tissues have increased considerably in the last decade onward (Hammami et al., 2009). Generally, AMPs are detected in many tissues; however, they are greater expressed when tissues are in contact with the surrounding environment as they are persistently subjected to the microbial biota. These peptides are commonly formed as pre-proteins. However, some are accompanying with a C-terminal pro-domain and producing bigger precursors (Benko-Iseppon et al., 2010). Antimicrobial peptides can be isolated from a

broad variety of plants or their parts (roots, seeds, leaves, flowers, and fruits); they are known as essential parts of the innate immune system and functioning as a complex signaling process (Srivastava et al., 2014). These compounds could be identified as indiscriminate proteins because of their broad-spectrum effect as they interact with many Gram-positive and Gram-negative bacteria species, viruses, fungi, yeast, protozoa and yeast. Furthermore, they can be utilized as a part of the therapeutic strategy in infectious diseases, inflammatory response, tumoral diseases and immunosuppressive (Franco, 2011).

2.2.1 Fruit antimicrobial peptides

Antimicrobial peptides (AMPs) are essential constituents of natural plant defences (Franco, 2011). These peptides are tiny small (<10kDa), and they possess cationic and amphipathic molecules that cause one surface acquires elevated positive charge and the other being hydrophobic. These features encourage the peptide binding to the target membrane (Srivastava et al., 2014). AMPs are short chain amino acids and mostly rich in cysteine (Broekaert et al., 1997). Despite the fact that they have usually been isolated from various species in many kingdoms, few numbers of distinct structural scaffolds have been analyzed in plants up to now. They are mostly correlated to one indiscriminate class with different role functions (Franco, 2011).

2.2.2 Classification of plant AMP

Plant AMPs have been widely investigated in plant parts, seeds, bark, leaves and roots (Benko-Iseppon et al., 2010). They are classified into many families, and they possess some features in common. These features are including; general positive charge, the existence of disulfide bonds which serve in stabilization of the structure. The

mechanism of action is directing external membrane structures, for example, ion channels (Hammami et al., 2008).

2.2.3 Main families of plant AMPs

Thionins

Thionins are a group of AMPs that characterized with small molecular weight (approximately 5 kDa), which enrich in lysine, arginine, and cysteine amino acids (Nawrot et al., 2014). Among more than 15 diverse plant species, there are about 100 singular thionin chains have been recognized (de Souza Cândido et al., 2014). These chains comprise two antiparallel α- helices and an antiparallel double-stranded β-sheet accompanying three or four preserved disulfide bonds**.** At neutral pH, they carry positive charges and the membrane interactions of which may be associated with cell leakage which appears to be a common mechanism of cell lysis of thionins (Nawrot et al., 2014).

Defensins

Defensins are the best understandable AMPs and seem to be the most plentiful with membranolytic cabacity based on information of selected plant genomes (Tam et al., 2015). The initial plant defensins was obtained from wheat T. aestivum and barley Hordeum vulgare. Because of their molecular weight of 5 kDa and the existence of four disulfide bonds, they are primarily identified as γ-thionins (de Souza Cândido et al., 2014). These are known to be cationic peptides. They comprise 45–54 amino acids and have four to five disulfide bonds (de Souza Cândido et al., 2014; Tam et al., 2015). Defensins showed many activities such as; antibacterial, antifungal, proteinase, and inhibition of amylase in insects (Nawrot et al., 2014). Defensins group are considerable

class of antimicrobial peptides with a well stable structure. They have been obtained from multiple organisms including; mammals, plants and insects (de Souza Cândido et al., 2014).

Lipid transfer proteins

Lipid transfer proteins (LTPs) are tiny peptides characterized by cationic charge and a preserved pattern of Cys-Cys bonds. Their molecular weight is determined between 7 and 10 kDa (de Souza Cândido et al., 2014). LTPs have been sub-grouped into two families (LTP1 and LTP2), that comprise 70–95 amino acids long. They are proven to have the capacity to reversibly bind and transfer hydrophobic molecules within in vitro models (Zoccatelli et al., 2007). As LTPs appear to have antimicrobial effects against bacteria and fungi, they are presently thought to be plant pathogenesis associated proteins (PR-14) (Zoccatelli et al., 2007).

Puroindolines

These peptides are basically small proteins and comprise a distinctive tryptophan-rich domain. They were isolated from wheat endosperm. Their molecular weight is approximately 13 kDa and they comprise five disulfide bonds (Tam *et* al., 2015). Not less than two main isoforms of these peptides are available. These isoforms are called puroindoline (PIN)-a and PIN-b. Each of the two proteins comprise a basic structure of ten preserved Cys residues accompanying a tertiary framework like that of LTPs containing four α- helices that separated by loops of different lengths, together with the tertiary structure are held by five disulphide bonds. Four of these bonds are similar to

those in ns-LTPs and the fifth bond is available in PINs because of the two extra Cys (Nawrot et al., 2014).

Snakins

These peptides have been isolated from potato tubers. They contain the cell wall-incorporated peptide snakin-1 (StSN1) and snakin-2 (StSN2), that are identified as antimicrobial peptides consists of 63 amino acid residues (6.9 kDa) (Nawrot et al., 2014). Snakins are the proteins that contain the greatest number of S S bonds comparing to AMPs plant protein families (de Souza Cândido et al., 2014). Snakins demonstrate just 38 % sequence identity and have similar antimicrobial effect against bacterial and fungal microorganism of various plant species. The mechanism of action by which snakins perform its effect have not been elucidated yet (Nawrot et al., 2014).

Cyclotides

Cyclotides seem to show high sequence identity and a structural symmetry. Plant cyclotides consist of 28–37 amino acids; comprise a head-to-tail cyclised backbone. There are three disulfide bonds among molecular level. These bonds organized in a cysteine backbone loop structure (cyclic cysteine knot, CCK) (Nawrot et al., 2014). cyclotides are a group of tiny cyclic peptides, that have been obtained from various plant families, for example, Rubiaceae, Violaceae, Poaceae and Fabacea (de Souza Cândido et al., 2014).

Hevein-like proteins

The first peptide of Hevein-like proteins was firstly isolated in 1960 and it was given the name, hevein by Archer. It was isolated from rubber tree (Hevea brasiliensis) latex

(de Souza Cândido et al., 2014). Hevein-like peptides are fundamental peptides consists of about 29–45 amino acids that have three to five disulfide bonds. They are demonstrated to be enriching in Gly and comprise preserved aromatic residues present in the hevein domain of lectins (Tam et al., 2015). These peptides are tiny with 4.7 kDa molecular wieght. They are plentyful with cysteine (*Hevea brasiliensislatex*). The antimicrobial Hevein-like proteins have been recognized in various plants, and they vary in the number of disulfide bonds (Nawrot et al., 2014).

Many investigators and reviewer, for example, Tam et al. (2015), Meneguetti et al. (2016), and Subramani et al. (2017) reported extensive details on the fundamental secondary metabolites and antimicrobial peptides that derived from plants. In 2011, antimicrobial peptides (AMPs) were isolated from seed/pod of *Pisum sativum* (garden pea) by Rehman and Khanum. These peptide are approximately 19 kDa, 22 kDa, 10 kDa and 11 kDa in their molecular weight. They have strong effect against various microorganism such as; *Staphylococcus epidermidis*, *Micrococcus luteus*, *E. coli*, *Klebsiella pneumonia*, *S. typhi*, *S.aureus*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* (Rehman & Khanum, 2011).

In addition, the antifungal effect of small molecular weight (7–9 kDa) polypeptides that obtained from pomegranate juice were demonstrated in a study conducted by Zoccatelli et al. (2007), the isolated polypeptides were characterized and described. The two proteins, called LTP1a and LTP1b, which they are demonstrated alike molecular weight.

2.3 AMP- mechanism of action and their therapeutic potential

AMPs also can play important role as an effector molecule in the time of inflammation, immune activation, and wound healing (Benko-Iseppon et al., 2010).

Because of the previously mentioned reasons, plant AMPs are being one of the most promising substances for new antimicrobial and anti-inflammatory drugs development in the future (Benko-Iseppon et al., 2010).

The main mechanism by which AMPs work is to disrupt the cell membrane and/or penetrate the lipid membranes of the microbes followed by attack the intracellular targets. This mechanism can cause cell lysis and rapid killing of microbial pathogens (Tam et al., 2015). Many mechanistic models have been suggested, such as the carpet model, barrel-stave model, and toroidal pore model, and all of them cause cell death (Tam et al., 2015).

Commonly, plant AMPs are effectively capable to kill microorganisms by decreasing pH, expanding membrane permeability, changing efflux pumping (Srivastava et al., 2014). These substances can target the cytoplasmic membrane by cleavage, fragmentation and creating of pores (Nguyen et al., 2011).

Beside the mechanisms of action that are targeting the plasma membrane, positive charge peptides may have the ability to inhibit proteins or enzymes transfer, interfering with DNA and RNA, inhibition of ion channels, targeting in the steroid hormone regulation, improvement of prospective redox, and inhibition of peptidoglycan formation (Srivastava et al., 2014). Therefore, investigation and isolation of plant peptides incorporated in the fight against pathogenic microbes may provide possibly beneficial novel antimicrobial molecules (Benko-Iseppon et al., 2010). Additionally, the broad spectrum of antimicrobial effects in some substances suggests they may have the ability to treat various types of cancer as well as viral and parasitic infections. Progressively, AMPs are being integrated as a modern group of antibiotics, because of their broad mechanism of action, comprise overcoming of multi-resistant

microorganisms and their medical usage seems to be expanding as anti-infective and immune regulator therapeutics (Srivastava et al., 2014).

The discovery of new groups of AMPs from fruit could provide a novel source of drug generation. That can be used for the treatment of human infectious diseases.

2.4 The history and epidemiology of Multi-Drug Resistant bacteria

In 2011, WHO proclaimed "combat drug resistance: no action today, no cure tomorrow." (Basak et al., 2016). The issue of rising antimicrobial resistance brings a critical concern as there is a very limited number of new antimicrobial agents that are under development, and this problem would be a serious threatening (Magiorakos et al., 2012). In the last few years, species of multidrug resistant microorganisms have increased four folds all over the world. Antimicrobial resistance (AMR) is a major threat to patient's undergoing treatment as it causes an increase in the probability of morbidity and mortality, increase in hospital stay, and big economic loss to the patient and nation (Basak et al., 2016).

Gram-positive and Gram-negative bacteria are affected by the emergence and the development of antimicrobial resistance. The challenge is being complicated by the fact that while the geographical spread of infection may be the same, grade of antibiotic resistance may vary significantly from one nation to another (Das et al., 2013).

As this issue progressively growing, tight coordination to investigate, describe and classify the multidrug-resistant bacteria is highly needed. Data that received from epidemiological screening can be dependably gathered and compared across healthcare framework among countries (Magiorakos et al., 2012).

2.5 The concept of Multi-Drug-Resistant bacteria (MRD)

It has been stated by the health care planners that; "Health for All by the year 2000." over the recent two decades, considerable rising in infectious diseases prevalence has been detected, and the quality of public health in various parts of the world appears to restored to pre-antibiotic era (Basak et al., 2016).

Many various definitions for multidrug-resistant (MDR), pan drug-resistant (PDR) and extensively drug-resistant (XDR) bacteria are always utilised in the literature to describe the various models of resistance found in the healthcare system where antimicrobial resistant bacteria has occurred (Magiorakos et al., 2012; Marinelli & Genilloud, 2013). The term, MDR refer to 'resistant to more than one antimicrobial agent'. However, there are no uniform definitions for this term yet, to be agreed among the medical society. Several definitions are being applied in order to describe models of multidrug resistance in all pathogenic bacteria groups (Magiorakos et al., 2012).

Many organizations aim to standardize international terminology. These organizations include; European Centre for Disease Control (ECDC) and Centre for Disease Control & Prevention (CDC). A group of international experts in the diagnosis, therapy and surveillance of antimicrobial-resistant bacteria integrated to create a standardized international terminology in order to identify of acquired resistance profiles in *Enterococcus* spp, *S. aureus*, *Enterobacteriaceae* including *Salmonella* and *Shigella*), *Acinetobacter* spp, and *P. aeruginosa* (Basak et al., 2016). All of these bacteria are usually responsible for infections and capable to develop multidrug resistance. All sort of drug resistance such as; multidrug-resistant (MDR), extensively pandrug-resistant (PDR), and drug-resistant (XDR) bacteria have been well elucidated (Basak et al., 2016; Magiorakos et al., 2012).

MDR is recognized as non-susceptibility of bacteria to at least one agent in three or more antimicrobial classes. XDR is recognized as non-susceptibility of bacteria to at least one antimicrobial agent in all but two or less antimicrobial classes. PDR is recognised as non-susceptibility to all agents in all antimicrobial classes (Basak et al., 2016; Marinelli & Genilloud, 2013). It must be considered that just in 2012 internationally acknowledged definitions of the terms were stated (Marinelli & Genilloud, 2013). To confirm the proper application of these definitions, bacterial isolates must be examined encounter all or most of the antimicrobial agents among the antimicrobial classes (Magiorakos et al., 2012).

A review by Subramani et al. (2017) demonstrated that from the year 2005 to 2015, a sum of 110 refind compounds, and 60 plant extracts were derived from 112 various plants which possess secondary metabolites which can overcome multidrug-resistant bacteria.

2.6 The Resistant bacteria

Antimicrobial resistance is progressively increasing and acquired importance in the therapeutic of intestinal infections, especially those caused by *Shigella*, enterotoxigenic *E. coli*, and *S. typhi*. In the developing countries, the rate of antimicrobial resistance is the highest, where the usage of antimicrobial medication is unsystematic (Sack et al., 1997).

Resistance against antimicrobial medications occurs among the decrease in bacteria affinity to bind to the medications. This reduction in the affinity can take place by the modification in drug target site. Bacteria destroys or modifies the drug target site through enzymes production or drug outflow from the cell (Tribble, 2017).

2.6.1 *Escherichia coli*

2.6.1.1 Pathogen

Multidrug-resistant *E. coli* has become a major public health concern in many countries, causing failure in treatment. Presently, the emergence and wide dissemination of *E. coli* strains developing resistance to wide spectrum of antibiotics has been reported (Ibrahim et al., 2012).

E. coli is among the three most common pathogens found in specimens that come from human clinical (Marinelli & Genilloud, 2013). It is one of the major widespread human pathogenic bacteria that is responsible for a wide range of diseases (Kayser et al., 2005). *E. coli* is gram-negative bacilli bacteria; it is classified under Enterobacteriaceae family. In human, the normal habitat of *E. coli* is the gastrointestinal tract (Sack et al., 1997). In spite that the *E. coli* is a commensal organism and it can exist among enteric microflora in human, major of species are pathogenic and have improved their capacity to cause gastrointestinal disorders in human (Jafari et al., 2012).

E. coli can be cultured easily from collected specimens in general or special media at 37°C in aerobic conditions. The bacteria in stool usually is cultured on MacConkey or eosin media that selectively allow growing Enterobacteriaceae bacterial family. Therefore, this selective media facilitates differentiation of enteric bacteria based on morphology (Nataro & Kaper, 1998). Three common clinical syndromes can be caused by the infection with pathogenic *E. coli* strains. These syndromes including; urinary tract infection, meningitis, and diarrheal disease (Jafari et al., 2012).

2.6.2 *Salmonella* spp

2.6.2.1 Pathogen

The occurrence of multi-drug-resistant (MDR) in *Salmonella* serotypes results in a substantial impact on the effectiveness of antibacterial medication, and a rising in prevalence of MDR strains may can cause an elevation of mortality rates due to *Salmonella* infections (Eng et al., 2015).

Salmonella is a Gram-negative facultative pathogenic bacteria that belong to the Enterobacteriaceae family. Approximately 2600 serotypes under the *Salmonella* genus have been recognized (Pui et al., 2011).

Salmonella strain is among the most common foodborne pathogens. It is widespread all over the world, and it increases the public health concern. *Salmonella* accounts for 93.8 million foodborne sickness and 155,000 death per year (Eng et al., 2015). In human pathology, the pathogenicity pattern of salmonellosis can be distinguished into four patterns including gastroenteritis, bacteremia, enteric fever and other complications excluding typhoidal salmonellosis and chronic carrier case too (Pui et al., 2011).

2.6.2.2 Enteric fever

Salmonella typhi is responsible for typhoid fever. However *Salmonella paratyphi* A, B and C are responsible for paratyphoid fever that characterized with symptoms that are milder and the mortality rate is lower relatively to typhoid fever (Kayser et al., 2005). These two serotypes are exclusively human pathogens. Normally, the infection happens when the host uptakes food or water that infected with contaminants from human waste. People with low immunity such as children below the age of 5 years and elderly and people who acquire immunosuppression are most susceptible to *Salmonella* infection comparing to healthy individuals (Eng et al., 2015).

Salmonella spp. with resistance to antimicrobial drugs are now widespread in both developed and developing countries. Multiple resistance in *salmonella* stains to four or more antibiotics is being found in various *salmonella* serotypes in several developed countries, and has been associated with treatment failures. Such multi-resistant strains have been responsible for several epidemics and some of these have been associated with contaminated water supplies. Furthermore, an increasing number of multi drugs resistant strains of *S. typhi* are currently showed decreased susceptibility to ciprofloxacin (Threlfall, 2002).

2.6.3 *Shigella* spp

2.6.3.1 Pathogen

Shigella is known to be as a major reason of dysentery and acute watery diarrhoea. It has a considerable role in causing epidemics and usually needs treatment with antibiotics to reduce the severity of disease (Das et al., 2013). *Shigella* is Gram-negative bacilli that classified under Enterobacteriaceae family. It does not have flagella; thus it is nonmotile and subsequently have no flagellar (H) antigens (Bhattacharya & Sur, 2017). *Shigella* species are the causative pathogen of infectious disease "Shigellosis", which are treated by clinical antibiotics included tetracycline, chloramphenicol, ampicillin, co-trimoxazole, and nalidixic acid. Currently, *Shigella* spp have developed resistance against ciprofloxacin, azithromycin, and ceftriaxone (Puzari et al., 2018; Klontz et al., 2015). Emergence of multidrug-resistant (MDR) *Shigella* strains is increasing rapidly and have been responsible for several epidemics (Ud-Din et al., 2013).

2.6.3.2 Pathobiology of *Shigella*

When *Shigella* left a host through feces, they tolerate the acid pH. This gives *Shigella* the capacity to remain alive with gastric acid and accounts in great part for the little inoculum needed. Once leave the stomach, the organisms lock the genes that control acid resistance and unlock the genes that permit them to infest the host's colon (Bhattacharya & Sur, 2017). Humans are the only source of infection as *Shigella* is solely infected humans. The pathogens are transferred straightway, more commonly indirectly through food and drinking water. Infective dose is only a few hundred of microorganism that would be enough to cause infection (Kayser et al., 2005).

Shigella can cause Shigellosis which is acute enteritis. Shigellosis is characterized by fever and mucosal inflammation (Koh et al., 2012). Pathogenicity of this disease is varies from watery to bloody diarrhoea. Dysentery may occur with a trio of signs and symptoms that are including; frequent small-amount of bloody faeces per day, abdominal colic, and an aching straining with the urgency to evacuation (Bhattacharya & Sur, 2017). Most of shigellosis infections in developing countries are because of *S. flexneri* strains, with a significant number caused by *S. dysentery* (Bhattacharya & Sur, 2017).

CHAPTER 3: MATERIALS AND METHODS

3.1 Selection and collection of plant material

Different plant parts (peel, leaf, stem and seed) of *P. granatum* were selected for this study. Plant samples were collected from the housing area in Negeri Sembilan, Malaysia in January 2017. A specimen was deposited in the Rimba Ilmu Herbarium at the University of Malaya, Kuala Lumpur, Malaysia for identification of the plant. Plant sample has been registered under (voucher no. KLU 49255).

3.2 Bacterial pathogens used in the study

The crude extracts of parts of the selected medicinal plant were tested against 11 drug-resistant enteropathogens. The following clinical strains were obtained from the microbiology laboratory of the Institute of Biological Sciences, Faculty of Science, University of Malaya. Bacteria strains were Gram-negative which used as indicators namely *S. flexneri* 3a (SF1025), *S. flexneri* 4a (SF1032), *S. flexneri* 1b (SF1014), *S. flexneri* Y (SF1013), *S. sonnei* (SS1001), *S. boydii* (SB1003), *S. dysentery* (SD1007), *E. coli* (EC3003), *E. coli* (EC3001), *S. typhi* (SM4002), *S. typhimurium* (SM4001) as shown in (Table 3.1). Antibacterial activity was screened using an agar well diffusion assay. Antimicrobial activity assays were performed in triplicate. Each well was filled with 100 µl of extracts and cultured on Luria Bertani (LB) agar.

Table 3.1: Test bacteria used in study**.** This displays the strains of the test bacteria used as indicators in this study.

Bacteria Name	**Type**	**Strain**
***S. flexneri* 3a**	Gram negative	SF1025
***S. flexneri* 4a**	Gram negative	SF1032
***S. flexneri* 1b**	Gram negative	SF1014
***S. flexneri* Y**	Gram negative	SF1013
S. sonnei	Gram negative	SS1001
S. boydii	Gram negative	SB1003
S. dysentery	Gram negative	SD1007
E. coli	Gram negative	EC3003
E. coli	Gram negative	EC3001
S. typhi	Gram negative	SM4002
S. typhimurium	Gram negative	SM4001

3.3 Preparation of bacterial suspension

At least two or three well-isolated colonies that have same morphology were picked from the agar plates with a sterilized wire loop and inoculated into a tube containing 5 ml of LB broth medium. The inoculated tubes were incubated at 37°C for 18- 24 hours. After the completion of the incubation period, the turbidity was observed and compared to a 0.5 McFarland standard (10^8 CFU /ml). The bacteria suspension was adjusted by adding sterile normal saline 0.85% sodium chloride (Merck, Germany) or more organism in case the suspension was too heavy or too diluted. This suspension was used for inoculation within 15 minutes of preparation.

3.4 Antibiotic susceptibility test for isolated bacterial strains

The antimicrobial susceptibility of pathogenic isolates was determined using a Kirby–Bauer disk diffusion assay according to the standards and criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2015). The isolates were inoculated into LB broth and incubated at 37ºC for 24 hours. After incubation, the turbidity of the culture was adjusted to 0.5 McFarland standards. The standard inoculum was then swabbed over LB agar plates using sterile cotton swabs.

The inoculated plates were then dried in an incubator at 37ºC for 30 minutes. Antibiotic discs mentioned in (Table 3.2) were aseptically placed on the agar surface using forceps. The antibiotics disks were purchased from Oxoid (Basingstoke, UK). The plates were incubated at 37ºC for 18-24 hours. After incubation, the diameter of inhibition zones was measured and interpreted as per CLSI recommendations (CLSI, 2015) and the isolated pathogenic bacteria were classified as resistant, intermediate and sensitive. The resistance rate was calculated as the ratio of pathogens resistant to specific antibiotic over pathogenic bacteria tested for susceptibility (Bauer et al., 1966).

Table 3.2: Antibiotics used for antibiotic susceptibility test. This table display desk antibiotics used to determine the sensitivity of isolated pathogens.

ANTIBIOTIC		CONCENTRATION	CLASS
Amoxicillin	AML	(5µg)	PENICILLINS
Tetracycline	TE	(30µg)	TETRACYCLINES
Ampicillin	AMP	(10µg)	PENICILLINS
Streptomycin	S	(25µg)	AMINOGLYCOSIDES
Kanamycin	K	(30µg)	AMINOGLYCOSIDES
Ciprofloxacin	CIP	(5µg)	FLUOROQUINOLONES
Cefipime	CEF	(20µg)	CEPHEMS
Nalidixic Acid	NA	(30µg)	QUINOLONES
Ceftriaxone	CRO	(30µg)	CEPHEMS
Chloramphenicol	C	(30µg)	PHENICOLS

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3.5 Protein crude extraction

Fresh parts of *Punica granatum* (peel, leaf, stem, and seed) were washed with distilled water, and dried using tissue paper, then weighed and cut into small pieces. The extraction was carried out based on the method described by Rehman and Khanum (2011), with some modifications. The plant materials of *P. granatum*; 10 g of each sample was crushed into a paste and blended with 100 ml of phosphate-buffered saline (PBS) (10 mmol-1 phosphate buffer, 2.7 mmol-1 potassium chloride and 0.137 mol-1 sodium chloride, pH 7.4) (Sigma, St. Louis, Missouri, USA), in an electric blender (National, Philips) at 4°C in a ratio of 1:10 w/v. The homogenate was frozen, thawed three times, and then frozen overnight. The extracts were filtered through Whatman no.1 filter paper, and the filtrates were then centrifuged using (Beckman J2-MI Centrifuge) at 10,000 rpm for 25 min at 4 0C. The residues were discarded, and the supernatant (crude extract) of peel, stem, leaf and seed were collected and stored at -20 ^{0}C for further examinations. The antimicrobial activity of the crude extracts was then tested (Rehman & Khanum, 2011).

3.6 Purification of peptides

3.6.1 Partial purification of peptides (Ammonium sulfate precipitation)

The precipitation process of the peptides was carried out using ammonium sulphate. The crude solution of peel, leaf and stem that showed antibacterial activity were treated with solid ammonium sulphate (R&M marketing Essex U.K).

Ammonium sulphate salt was weighed (6.60g) depending on the volume of the extract solution which was 10 ml and the percent saturation of the salt needed which was 80% relative ammonium sulphate saturation at 4 ºC. The resulting supernatant was filtered using 0.2µm membrane filter (Sartorius, Germany). The crude extracts then

were added into an 80 ml beaker. Ammonium sulphate salt was added slowly and subsequently with a spatula into the equase extract in order to allow dissolving the salt before further addition. The extract was stirred with a magnetic stirrer gentle stirring in order to allow dissolving the salt before further addition to prevent foaming (Basseri et al., 2016). Once the weighed amount of ammonium sulphate salt was dissolved, the solution was incubated overnight at 4 ºC. Lastly, the mixture was centrifuged at 10,000 g for 25 min at 4 ºC (Beckman J2-MI Centrifuge), and the supernatant was carefully removed. The pellet containing the precipitated peptides was collected and dissolved in PBS pH 7.4, then precipitate solution was stored at - 20 ºC for further analysis. The antimicrobial activity of the precipitate solution was then tested, and the protein concentration was estimated before and after ammonium salt precipitation, using the Bradford assay (Kamala et al., 2016).

3.6.2 Gel filtration chromatography (SEC)

A Sephadex G-25 Fine (Pharmacia, Sweden) was used for the separation of the active peptides. The dissolved ammonium sulphate precipitates obtained from stem, leaves, and peel that displayed inhibitory activity were further purified by gel filtration chromatography using a column (30 cm × 1 cm) of a Sephadex G-25 equilibrated with 0.02 M sodium acetate buffer (pH 5).

The column was eluted with the same buffer (3 folds bed volume) at the constant flow rate of 1ml/min. The eluates were collected, and their protein concentration monitored using Bradford assay with spectrophotometer at 595 nm (Bradford, 1976). Antibacterial activity of the fractions were tested against *S. flexneri* 1b (SF1014), *S. flexneri* 3a (SF1025), *S. flexneri* Y (SF1013), *S. dysentery* (SD1007), *S. boydii* (SB1003), *S. typhimurium* (SM4001) which were used as test microorganisms throughout purification procedures (Al Hazzani et al., 2013).

3.6.3 Separation of peptides by RP- HPLC

The active fraction collected by gel filtration chromatography with the higher protein content was introduced into a semi-preparative HPLC system (GX-271, Gilson, USA) using a Luna 5 micron C18 column (150*21.20 mm). Freeze-dried samples (PE20, LV8, and ST15) from peel, leaf and stem respectively, were dissolved in one half of their original volume with deionized water and then filtered through a 0.22 µm membrane filter. The injection volume was 500 µl. The protein fractions were eluted from the column by linear gradient separation, using two solvents: A (95% Mili-Q water and 5% acetonitrile) and B (100% acetonitrile). The fractions were collected differentially based on the increase and decrease of the UV absorbance of the most significant peaks during the respective HPLC run for each sample. Next, the collected fractions were lyophilized and redissolved in distilled water. The antimicrobial activity of the dissolved fractions against *S. flexneri* Y (SF1013) was then tested (Goh & Philip, 2015).

3.7 Antibacterial assay

3.7.1 Well diffusion assay

The antimicrobial activities of crude extract, dissolved ammonium sulphate precipitates, fractions of gel filtration chromatography, and purified peptides obtained after RP-HPLC were determined using an agar well diffusion assay and tested against standard cultures of pathogenic bacteria, as described earlier (Kamala et al., 2016). Wells of 8 mm diameter were bored on LB agar plates, onto which 100 µl of culture were spread. Then, 100µl of sample containing protein were added to the wells. The plates were incubated overnight at 37°C, and the zone of inhibition was measured. Antimicrobial activity assays were performed in triplicate, and the antibiotics

ciprofloxacin tetracycline, amoxicillin and ampicillin were used as positive controls. Distilled water was used as a negative control.

The results expressed as the mean ± SD, indicate the standard deviation of the triplicate incubations in millimeter (mm) (Kamala Golla et al., 2016).

3.7.2 Minimum inhibition concentration (MIC) test

The broth micro-dilution assay was performed to determine the MIC values of the three purified HPLC fractions against the multidrug-resistant isolate *S. flexneri* Y, which was inoculated into LB broth containing different prepared concentrations of protein (12.9- 0.12 µg/ml), (15.1- 0.18 µg/ml), and (24.2- 0.10 µg/ml) derived from peel, stem, and leaf of pomegranate respectively, and a standard antibiotic Ampicillin (1000–7.8 µg/ml), as described in (Balouiri et al., 2016) following CLSI protocol. The procedure of preparing two-fold dilutions involves: 200 μl of each of sample stock solutions were pipetted into the first row of a 96-well plate. Then, 100 µl of LB broth were pipetted into each of the remaining wells. A serial dilutions of the stocks were performed such that each of the following wells contained. One hundred microliters of standardized microbial suspension (*S. flexneri* Y) adjusted to 0.5 McFarland scale culture (108 CFU/ml) were added to each well. The 96-well plates were incubated at 37ºC for 18-20 hours.

The MIC was established by determining the lowest concentration of extract showing a lack of turbidity (no visible bacteria growth) in the wells.

Statistical analysis

The results are presented as the mean ± standard deviation of three replicates. Statistically significant differences of the experiments achieved in the various assays were evaluated by analysis of variance (ANOVA) and Tukey test. Statistical analyses were performed using SPSS software (version 22.0) and a significant difference was considered at a value of $P < 0.05$.

3.8 Determination of protein content

The protein concentration of the samples; crude extract, dissolved ammonium sulfate precipitates, gel filtration chromatography fractions and purified peptide antibacterial were determined using Bradford assay (1976).

Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G (No. B-1131) 100mg in 50 ml of 95% ethanol then filtered through a filter paper No.1 (Whatman, England). The solution was added to 100 ml of 85% phosphoric acid and diluted to 1000 ml distilled water. The reagent was filtered to remove the precipitates and then stored in a dark bottle at room temperature.

The 3ml aliquot of the Bradford reagent added to 0.1ml aliquot of the sample and gently mixed thoroughly. Then, the samples were incubated at water bath for 10 min, and then the absorbance was measured using spectrophotometer at 595 nm (JASCO V 730, JAPAN). Bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) was used as a standard. The protein concentration of the samples was determined by comparing the net absorbance values obtained at 595 nm against the blotted standard curve (Bradford, 1976).

3.9 Identification of purified peptides by Q-TOF LC/MS

The fractions of peptides were isolated by RP-HPLC (as described at 3.6.3). Those that showed antimicrobial activity were subjected to Q-TOF LC/MS analysis for identification purposes.

The analysis of samples (10 ppm) was conducted on an Agilent 6500 series Accurate-Mass Q-TOF LC-MS system (Agilent Technologies, Santa Clara, USA) at Centre for Research Services (PPP), INFRA Laboratory, University of Malaya. The injection volume was 10.00 µl, with a flow rate of 0.500 ml/min using solvent composition as described in Table 3.3. Mass spectra were acquired with a TOF/Q-TOF mass spectrometer with gas temperature 200°C; gas flow 14 l/min; and nebulizer 35 psig. The mass spectrometer was operated in the positive ion mode, with a scanning range of 100–1000 m/z. Liquid chromatography separation was performed with an Agilent Technologies Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 µm). The gradient profile for LC-MS showed in the (Table 3.4). The spectra of the peptides were identified using the METLIN Metabolomics Database (Sornwatana et al., 2013).

Table 3.3: Solvent composition. Table shows the solvent Composition of solvent A and B for LC-MS Q-TOF mobile phase.

Channel	**Ch.1 solvent**	**Name**	**Ch.1 solvent**	**Used**	**Percent**
A	100% Water V.03	5% ACN	100.0% Acetonitrile V.03	Yes	100.0%
B	100% Acetonitrile V.03		100.0% Methanol in Water V.02	Yes	0.00 %

Table 3.4: Solvent gradient profile in Q-TOF LC-MS. Table shows the solvent gradient that was performed by varying the proportion of Solvent A and Solvent B at respective time in minutes.

	Time	A	B	Flow	Pressure
1	3.00 min	100.00 %	0.00 %	0.5 mL/min	400 bar
2	60.00 min	60.00 %	40.00 %	0.5 mL/min	400 bar
3	53.00 min	0.00 %	100.00 %	0.5 mL/min	400 bar

CHAPTER 4: RESULTS

4.1 Antimicrobial susceptibility testing

Total 11 Gram negative pathogenic isolated bacteria selected as indicators in this study. They were tested for their susceptibility and resistance to verity of antibiotics which used in clinical laboratories. All the 11 strains were sensitive to Ceftriaxone and Chloramphenicol and resistant to the rest of antibiotics. The resistance rates of other antimicrobials agents are as follows: Streptomycin (45.5%), Tetracycline (27.2%), Ampicillin (54.5%), Amoxicillin (36.5%), Kanamycin (27.2%), Ciprofloxacin (18.1%), and Nalidixic acid (72.7%). Moreover, nine isolated strains (82%) were resistant to more than one antimicrobial agent as described in (Table 4.1). Out of 11 stains, three isolates namely: *S. flexneri* Y (SF1029), *S. boydii* (SB1003), and *E. coli* (EC1039; were multidrug resistant isolates (MDR) which defined as resistance to three or more classes of antimicrobial agents.

Table 4.1: Antimicrobial susceptibility testing (AST)**.** This table displays the antimicrobial susceptibility test of the pathogenic bacteria indicators. (S) presents sensitive. (R) refers to resistant. (I) presents intermediate. (MRD) = multidrug resistant bacteria (resistant to three or more classes of antibiotics). (R) = resistant to more than one agent. AML refers to Amoxicillin, TE to Tetracycline, AMP to Ampicillin, S to Streptomycin, K to Kanamycin, and CIP to Ciprofloxacin. CEF represents to Cefipime, NA to Nalidixic Acid, CRO to Ceftriaxone, and C to Chloramphenicol.

		Antibiotics									
Bacterial strains	**code**	**AML (10Mg)**	**TE (30Mg)**	**AMP (10Mg)**	**S (25Mg)**	**K (30M)**	**CIP (5Mg)**	**CEF (20Mg)**	**NA (30Mg)**	**CRO (30Mg)**	**C (30Mg)**
S. flexneri 3a^{R}	SF1025	S	S	S	R	S	I	S	R	S	S
S. flexneri 4a^{R}	SF1032	R	S	R	R	R	S	S	I	S	S
S. flexneri Y^{MRD}	SF1029	S	R	S	R	R	R	S	R	S	S
S. flexneri 1b^{R}	SF1014	S	S	S	R	S	S	R	S	S	S
S. boydii 1^{MRD}	SB1003	S	R	R	R	I	S	S	R	S	S
S. dysentery^{R}	SD1007	S	S	S	S	S	I	S	R	S	S
S. sonnei^{R}	SS1001	S	S	S	S	S	R	S	R	S	S
S. typhimurium^{R}	SM1037	R	S	R	S	S	S	S	R	S	S
S. typhi^{R}	SM402	S	S	R	S	S	S	S	R	S	S
E. coli^{R}	EC1033	R	S	R	S	R	S	S	I	S	S
E. coli^{R}	EC1039	R	R	R	I	R	S	S	S	S	S

4.2 Screening of medicinal plant for antimicrobial activity by well diffusion assay

In traditional medicine, whole plant or parts (leaves, bark, roots, stem, bark, and bulbs) were used for the preparation of remedies for the treatment of diarrhoea. In this study, parts of the medicinal plant (*P. granatum*) peel, leaves, stem, and seeds were used to extract and characterize potential peptide(s) having antimicrobial activity. Firstly, all the four tested pomegranate parts were screened for antimicrobial activity against different strains of enteropathogens: two *E. coli* species, two *Salmonella* species and one *S. boydii,* one *S. sonnei*, one *S. dysentery* and four serotypes of *S. flexner*i. Antimicrobial activity assays were performed in triplicate and each well was filled with 100 µl of various crude extracts. The positive control was 100 µl of antibiotic Ciprofloxacin Tetracycline, Amoxicillin, and Ampicillin.

The plant extracts exhibited varying degrees of antimicrobial activity towards resistant enteric pathogens, as shown in (Appendix D, Table 3). Peel, leaf and stem crude extracts with protein content (258, 351 and 351 µg/ml respectively) showed a clear and noticeable inhibition zone against all the tested diarrhoea causative bacteria, with the diameters of the zone of inhibition ranging between 16.3 and 30.0 mm. However, the seed extract did not show obvious antimicrobial activity against the test strains.

Among the active crude extracts, *P. granatum* peel extract showed the strongest antimicrobial activity against tested bacteria strains except *S. flexneri* Y (SF1013) which mostly affected by leave extract (23.0±4.36) mm, whereas crude extract obtained from stem was less active and showed the lowest inhibitory activity against all the challenge pathogens (Figure 4.1).

Furthermore, there were significant differences ($P < 0.05$) in the antibacterial activity of peel crude extracts and that of the two other extracts (leaf and stem) against most of the bacterial strains. However, there were no significant differences ($P<0.05$) the efficacy of leaf and stem extracts.

S. flexneri 3a (SF1025) exhibited the highest sensitivity toward the peel extract followed by *S. flexneri* 4a (SF1032) and *S. typhi* (SM4002) with inhibition zone 30.00, 28.00, and 27.30 mm respectively; whereas *S. flexneri* Y (SF1013) was the least sensitive to this extract (21.7 mm). In the case of leaves crude extract, *S. flexneri* 4a (SF1032) and *E. coli* (EC3003) showed the highest sensitivity with (24.3 and 23.0 mm) inhibition zone, respectively; whereas *S. flexneri* 4a (SF1032) was most sensitive to the stem extract and *E. coli* (EC3001) was less sensitive with inhibition zone (16.3mm). There were no significant differences ($P<0.05$) between the efficiency of leaves and stem extracts against the majority of tested pathogens.

Regarding the efficiency of the crude extracts, as compared with the antibiotics, which were used as a positive control, the majority of crude extracts showed higher efficacy than antibiotics against the tested resistant pathogens as shown in (Appendix D, Table 3). However, the efficiency of leaf and stem extracts against *E. coli* (EC3001) was lower than that of ciprofloxacin.

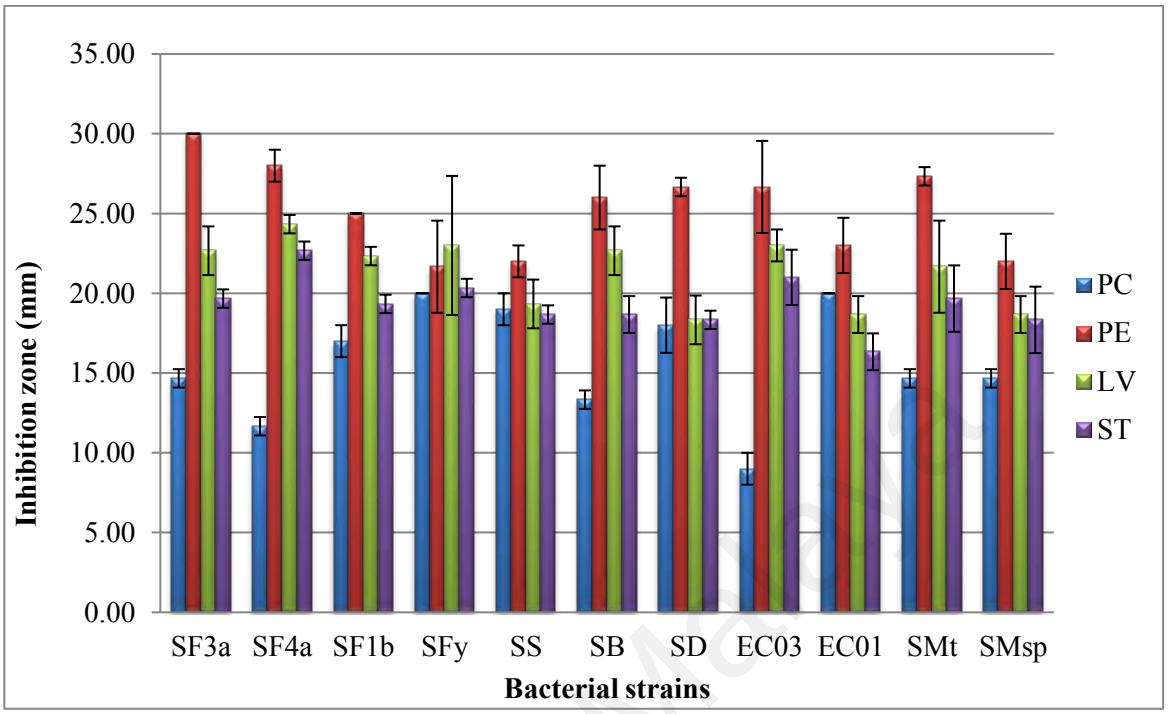


Figure 4.1: Antimicrobial activity of crude extracts. This figure shows the antimicrobial activity of crude extracts by well diffusion assay of *P. granatum* extracts (peel, leaves, and stem). PE presents peel extract. LV means leave extract. ST presents stem extract. PC presents positive control. SD refers to *S. boydii*, Sfy to *S.flexneri y*, Sf 1b to *S. flexneri1b*, Sf 3a to *S.flexneri 3a*, SD to *S.dysentery*, and SMsp to *S. typhimurium,* Sf4a refers to *S.flexneri* 4a, EC03 to *E.coli* (EC3003), EC01 to *E.coli* (EC3001), SMt to *S. typhi* (SM4002), SS to *S.sonnei* (SS1001), and SB to *S. boydii* (SB1003).

4.3 Partial purification of crude protein extracts with ammonium sulfate precipitation and antimicrobial activity

Crude protein of peel, leaves, and stem was partially purified by ammonium sulfate precipitation (80 % saturation) for each. After 80% ammonium sulphate precipitation, the pellet was tested for their antimicrobial activity against the test microorganisms. The solubilized ammonium sulphate precipitates of the peel, leaf and stem extract with protein concentrations of 298, 530 and 390 μg/ ml, respectively, displayed various levels of antimicrobial activity as shown in (Figure 4-2).

Solubilized ammonium sulphate precipitates of peel showed the highest activity against *S. boydii* (SB1003) followed by *S. typhimurium,* (SM4001), then *S. flexneri* 3a (SF1025) with inhibition zone (29.7mm, 25.3mm, and 20.3mm respectively), and the lowest activity against *S. sonnei* (SS1001), as described in (Appendix E, Table 2). Similarly, dissolved peptide precipitates of leaf exhibited highest activity against *S. boydii* (SB1003), followed by *S. flexneri* Y (SF1013) and *E. coli* (EC3001) with inhibition zone (24.3mm, 24.3mm, and 24.0 mm respectively), and lower antimicrobial activity against *S. typhi* (SM4002). In the case of solubilized precipitates of the stem, it showed the highest activity against *S. boydii* (SB1003) and lower activity against *S. flexneri* 4a (SF1032).

In general, the solubilized precipitates of peel extract exhibited less antimicrobial activity than the crude extracts. Moreover, *S. boydii* (SB1003) was most sensitive pathogen to all of the three solubilized protein precipitates. Furthermore, there were no significant differences ($P<0.05$) between the antibacterial activity of three solubilizes precipitates (peel, leaf, and stem) against *S. typhi* (SM4002) and *S. flexneri* 3a (SF1025).

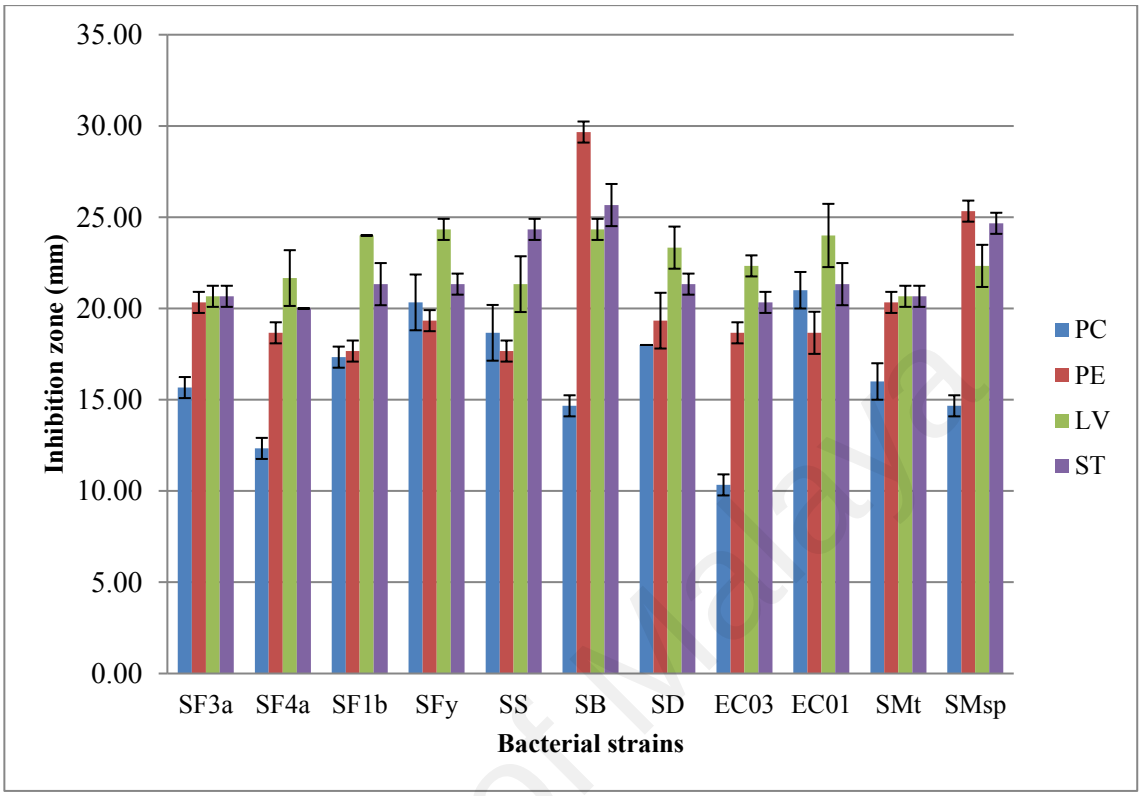


Figure 4.2: Antimicrobial activity of solubilized ammonium sulphate precipitates. This figure displays the zone of inhibition for antimicrobial activity of solubilized ammonium sulphate precipitates pellet by well diffusion assay of *P. granatum* extracts (peel, leaves, and stem). PE presents peel extract. LV means leave extract. ST presents stem extract. PC indicates positive control. SD refers to *S. boydii*, Sfy to *S.flexneri y*, Sf 1b to *S. flexneri1b*, Sf 3a to *S.flexneri 3a*, SD to *S.dysentery*, and SMsp to *S. thyphimurium*. Sf4a refers to *S.flexneri* 4a, EC03 to *E.coli* (EC3003), EC01 to *E.coli* (EC3001), and SMt to *S. typhi* (SM4002). SS refers to *S.sonnei* (SS1001), and SB to *S. boydii* (SB1003).

4.4 Gel filtration chromatography

After the determination of antimicrobial activity, the solubilized ammonium sulphate precipitates of peel, leaves, and stem were subjected to the gel filtration column chromatography using Sephadex G-25 to separate proteins based on their size.

In case of peel, total 20 peaks were obtained ranging from high molecular weight to low molecular weight protein(s) / peptide(s) which were designated as P1 to P20. All peak fractions were then checked separately for antimicrobial activity against *S. boydii* (SB1003), *S. flexneri* Y (SF1013), *S. flexneri* 1b (SF1014), *S. flexneri* 3a (SF1025), *S. dysentery* (SD1007), and *S. typhimurium* (SM4001) which were used as indicators to test all chromatography peaks activities. Fractions P4, P16, P17, P19, and P20 showed activity against various pathogens as shown in (Table 4.2). Among the active peel fractions 20 (PE20) displayed the highest activity against *S. flexneri* Y, with a 29.6 mm zone of inhibition (Appendix E, Figure 4). The protein content of the peel fraction (PE20) was 16.7 µg/ ml, and this fraction was chosen for further purification.

It was interesting to note that there was a significant antimicrobial activity ($p < 0.05$) of PE20 towards *S. flexneri* Y compared to the standard antibiotic Ampicillin (1 mg/ml). The peel fraction PE20 showed higher activity than that of Ampicillin with inhibition of zone (29.7mm).

In case of leaves, total 10 peaks were obtained ranging from high molecular weight to low molecular weight protein(s)/peptide(s) which were designated as L1 to L10. All peak fractions were checked separately for antimicrobial activity. Peak fractions (L6, L7, L8, L9, and L10) showed activity against all tested bacteria used. Fraction eight derived from leaf (LV8) showed significant antibacterial activity against *S. flexneri* 1b (SF1014), *S. dysentery* (SD1007) and *S. flexneri* 3a (SF1025), with a protein content of 21.6 µg/ml (Table 4.4). Similarly to the peel sample, total 20 peaks were obtained from

column chromatography of stem which were designated as S1 to S20. Peak fractions (S1, S3, S4, S5, S11, and S15) showed antimicrobial activity against all tested bacteria. Analysis of the antimicrobial activity of peak fractions of the stem revealed that fraction 15 (ST15) showed the highest antibacterial activity against S. *boydii* (SB1003), *S. flexneri* 1b (SF1014) and *S. flexneri* 3a (SF1025), with a protein content of 30 μg/ ml (Table 4.3).

Table 4.2: Antimicrobial activity of column chromatography of peels fractions. This table displays diameter of the zone of inhibition after 18-24 hours of incubation against the 6 test pathogenic bacteria in well diffusion assay. Each well was filled with 100 µl of semi purified extract. (PE) presents different chromatography peel fractions. Negative control was distilled water. PC presents positive control (tetracycline ampicillin, amoxicillin and ciprofloxacin). (*)= No further examination. Assay was performed in triplicate and results were presented as mean ± SD. (abcde) = Different letters in the same rows represent significant differences between the means.

Bacterial strains	**Negative control**	**Positive control**	**Diameter of the zone of inhibition (mm)**				
	NC	**PC**	**PE4***	**PE16***	**PE17***	**PE19***	**PE20**
S. flexneri 3a	0.00±0.0	10.6±0.58^{a}	14.7±0.58^{b}	17.3±0.58^{c}	18.7±1.15^{c}	19.3± 1.15^{cd}	21.3± 0.58^{d}
*S. flexneri*1b	0.00±0.0	17.3±0.58^{c}	9.67±1.15^{a}	12.3±0.58^{b}	13.7±0.58 ^{b}	10.3± 0.58^{a}	12.3± 0.58^{b}
S .flexneri Y	0.00±0.0	19.7±0.58^{a}	24.0±1.73^{b}	28.3±1.53^{cd}	25.7±1.15^{bc}	23.0± 1.73^{ab}	29.7± 0.58^{d}
S. boydii	0.00±0.0	10.0±1.00^{a}	24.7±0.58^{b}	24.0±0.00^{b}	25.7±0.58^{bc}	24.7± 1.15^{b}	27.7± 0.58^{c}
S. dysentery	0.00±0.0	20.0±0.00^{a}	21.7±0.58^{b}	23.3±0.58^{cd}	23.0±0.00^{bc}	24.7± 0.58^{d}	24.0± 1.00^{cd}
S. typhimurium	0.00±0.0	15.0±1.00^{b}	11.0±0.00^{a}	13.7±1.15^{b}	15.7±0.58^{b}	14.7± 1.15^{b}	14.7 ±0.58^{b}

Table 4.3: Antimicrobial activity of column chromatography of stem fractions. The table displays diameter of the zone of inhibition after 18-24 hours of incubation against the 6 test pathogenic bacteria in well diffusion assay. Each well was filled with 100 µl of stem fraction. ST presents different chromatography stem fractions. Negative control was distilled water. PC presents positive control (tetracycline ampicillin, amoxicillin and ciprofloxacin). (*)= No further examination. Assay was performed in triplicate and results were presented as mean ± SD. (abcde)= Different letters in the same rows represent significant differences between the means.

Bacterial strains	**Negative control**	**Positive control**	**Active peaks column chromatography of stems**					
	NC	**PC**	**ST1***	**ST3***	**ST4***	**ST5***	**ST11***	**ST15**
S. flexneri 3a	0.00±0.0	10.0±0.00^{a}	16.3±1.15^{bc}	18.3±0.58^{cd}	15.3±0.58^{b}	21.0± 1.73^{e}	19.0± 1.00^{de}	24.3 ±1.00^{f}
S. flexneri 1b	0.00±0.0	17.0±0.00^{ab}	15.0±0.00^{a}	16.3±2.31^{ab}	18.7±1.15^{bc}	21.3± 0.58^{c}	17.3± 0.58^{ab}	25.0 ± 0.58^{d}
S. flexneri Y	0.00±0.0	20.0±0.00^{abc}	16.3±1.15^{a}	19.3±0.58^{ab}	21.3±1.15^{bc}	26.3± 2.31^{d}	23.0± 3.46^{bcd}	24.3 ±3.46^{cd}
S. boydii	0.00±0.0	10.3±0.58^{a}	15.7±0.58^{b}	25.7±0.58^{c}	25.7±1.15^{c}	27.7± 1.15^{c}	25.7± 2.89^{c}	29.3 ±2.89^{c}
S. dysentery	0.00±0.0	20.0±0.00^{b}	11.0±0.00^{a}	15.0±0.00^{a}	12.0±0.00^{a}	11.0± 0.00^{a}	21.0± 0.00^{b}	15.0 ±0.00^{a}
S. typhimurium	0.00±0.0	15.00±0.00^{a}	19.0±0.00^{a}	19.3±0.58^{a}	21.0±0.00^{b}	23.7± 0.58^{e}	18.3 ±0.58^{b}	22.3 ±0.58^{d}

Table 4.4: Antimicrobial activity of column chromatography of leaf fractions**.** The table displays diameter of the zone of inhibition after 18-24 hours of incubation against 6 test pathogenic bacteria in well diffusion assay. Each well was filled with 100 µl of leaf fraction. LV presents different chromatography leaves fractions. Negative control was distilled water. PC presents positive control (tetracycline ampicillin, amoxicillin and ciprofloxacin). (*)= No further examination. Assay was performed in triplicate and results were presented as mean ± SD. (abcde) Different letters in the same rows represent significant differences between the means ($p< 0.05$).

Bacterial strains	**Negative control**	**Positive control**	**Active peaks column chromatography of leaves**				
	NC	**PC**	**LV6***	**LV7***	**LV8**	**LV9***	**LV10***
S. flexneri 3a	0.00±0.0	10.3±0.58^{a}	13.00±0.0^{b}	20.7±0.58^{e}	18.3±1.15^{d}	15.7± 0.58^{c}	14.7± 0.58^{bc}
S. flexneri 1b	0.00±0.0	17.0±1.00^{c}	12.7±0.58^{a}	12.7±0.58^{a}	19.0±0.00^{d}	15.0± 0.00^{b}	19.0± 0.00^{d}
S. flexneri Y	0.00±0.0	19.7±0.58^{c}	19.3±1.15^{c}	17.7±0.58^{bc}	16.3±0.58^{ab}	15.0± 0.00^{a}	16.7± 1.15^{ab}
S. boydii	0.00±0.0	9.67±0.58^{a}	19.3±0.58^{d}	18.7±0.58^{d}	18.0±0.00^{d}	14.3± 1.15^{c}	11.7± 0.58^{b}
S. dysentery	0.00±0.0	20.0±0.00^{b}	19.0±0.00^{b}	17.0±0.00^{a}	18.0±0.00^{a}	21.0± 0.00	20.0± 0.00^{b}
S.typhimurium	0.00±0.0	15.00±2.00^{bc}	15.7±0.58^{bc}	12.7±0.58^{ab}	17.0±1.73^{c}	11.3± 1.15^{a}	12.7 ±0.58^{ab}

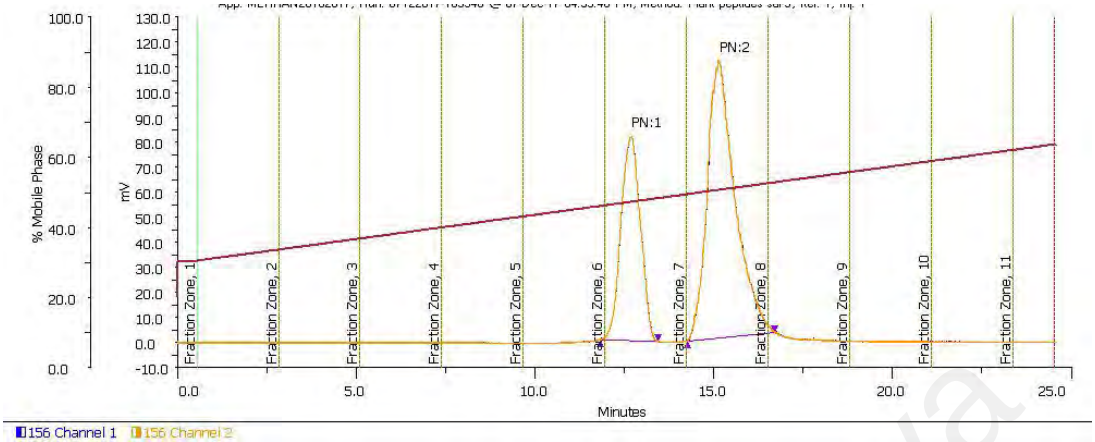
4.5 Separation of peptides by RP- HPLC and antimicrobial activity test

The active chromatography fractions (PE20, LV8, and ST15) were subjected to more purification by RP-HPLC. Two different peaks were obtained from each sample. Figure 4.3 shows the RP-HPLC chromatogram. Each fraction was separately collected and freeze-dried, dissolved in 3ml of distilled water and subjected to antibacterial activity test against *S. flexneri* Y (SF1013). The antimicrobial activity varies among the fractions.

In case of peel sample (PE20), two peak fractions (PEF7 and PEF8) were obtained. PEF8 fraction showed higher activity than PEF7. Similarly, LVF7 and LVF8 were derived from leaf sample (LV8), and higher antimicrobial activity observed from the HPLC fraction LVF7. In addition, two peak fractions (STF6 and STF9) were obtained from the stem sample (ST15). As a result, the fraction (STF6) showed higher activity than (STF9) as described in (Table 4.5).

The minimum inhibitory concentrations (MICs) for the three purified fractions (PEF8, LVF7, and STF6) against a representative multidrug-resistant isolate, *S. flexneri* Y were (6.45, 7.55, and 6.05 μg/ml), respectively. They are the lowest concentration that able to inhibited the growth of tested bacteria are shown in (Table 4.6).

The most active fractions (PEF8, LVF7 and STF6) derived from the peel, leaf and stem, respectively, showed antibacterial activity and then were selected for further studies.



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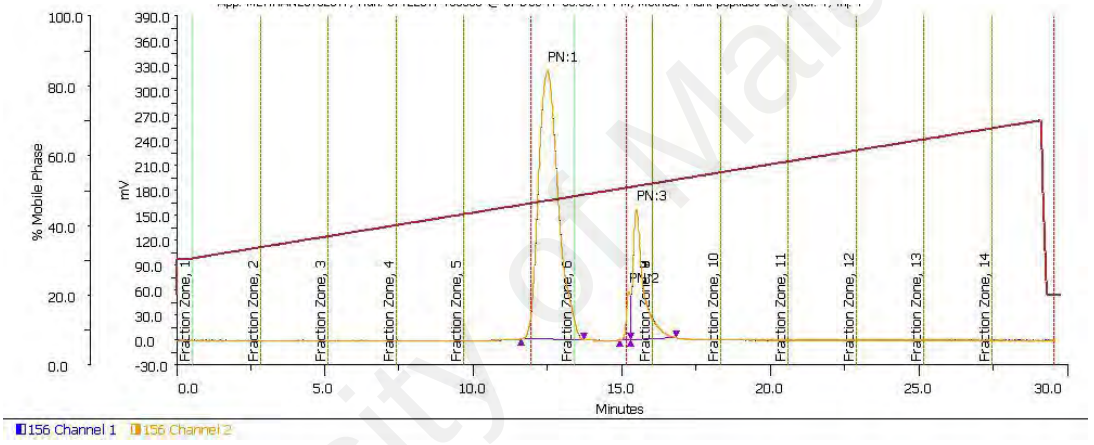

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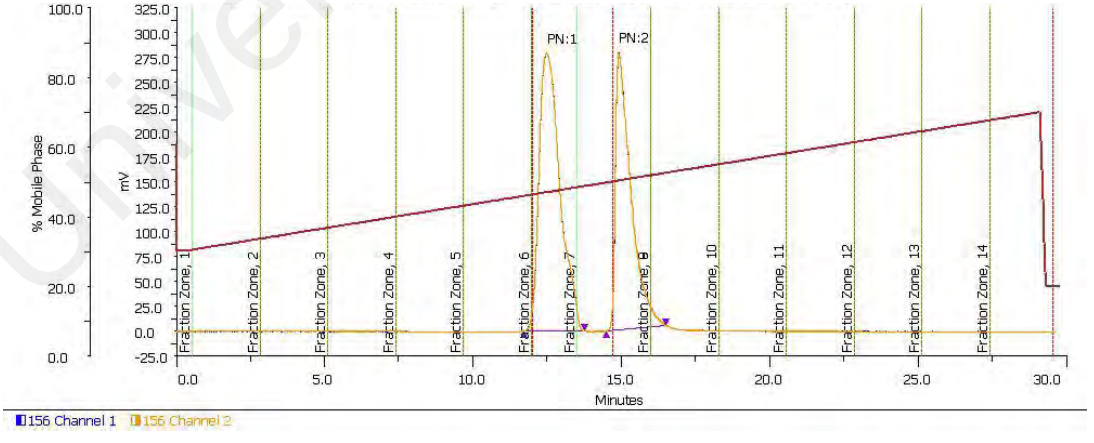

C

Figure 4.3: UV chromatography of RP-HPLC. Figure shows RP-HPLC chromatogram of samples derived from column chromatography, with A: PE20 (peel); B: LV8 (leaf); and C: ST15 (stem). The absorbance was measured at 230 nm.

Table 4.5: Antimicrobial activity of fractions obtained from HPLC separation**.** This table displays diameter of the zone of inhibition after 18-24 hours of incubation against *S.flexneri* Y isolate in well diffusion assay. Results are mean values of triplicate determinations ± SD. PE20a = active chromatography fraction from peel extract. LV8b = active chromatography fraction from leaf extract. ST15c = active chromatography fraction from stem extract. * Further analysis.

Fractions1	**Inhibition zone (mm)**
PE20[a]	
PEF7	11.6± 0.57
PEF8*	17.6± 0.57
LV8[b]	
LVF7*	16.3± 0.57
LVF8	14.6± 1.15
ST15[c]	
STF6*	19.3± 0.57
STF9	13.0± 0.00
Ampicillin (1 mg/ml) **Positive control**	19.7±0.58

Table 4.6: Minimum inhibition concentration of collected HPLC fractions. This table shows MIC the minimum inhibition concentration of the active purified fractions to inhibit the growth of *S. flexneri* Y isolate.

	MIC (µg/ml)			
Bacteria strain	**PEF8**	**LVF7**	**STF6**	**Positive control (Ampicillin)**
S.flexneri Y	6.45	7.55	6.05	31.2

4.6 Estimation of protein concentration using Bradford assay

The protein concentration of the samples; crude protein extract, solubilized ammonium sulfate precipitates, gel filtration chromatography fractions and purified HPLC fractions was determined using the of Bradford.

In general, all crudes extracts of (peel, leaf, and stem) have protein content (258, 351, and 351 µg/ml) respectively. However, the three crude extracts have lower protein content than their content after precipitation of the protein using ammonium sulphate. Whereas the concentration of protein significantly decreased through the purification procedure as shown in Table 4.7.

Table 4.7: Protein concentration of plant samples. This table display the protein content on plant sample extracts through purification process.

	Protein concentration (µg/ml)			
	Crude protein	**Dissolved ammonium sulfate precipices**	**Gel filtration chromatography fractions (active)**	**Active fractions collected from HPLC**
Peel	258 µg/ml	298 µg/ml	16.7 µg/ml	12.9 µg/ml
leaves	351 µg/ml	530 µg/ml	21.6 µg/ml	15.1 µg/ml
Stem	351 µg/ml	390 µg/ml	30 µg/ml	24.2 µg/ml

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4.7 Characterization of AMPs and estimation of their molecular weight

To identify the molecular weights of the peptides, the active fractions (PEF8, LVF7 and STF6) were subjected to liquid chromatography-quadrupole time-of-flight mass spectrometry (Q-TOF LC/MS) analysis. The acquired time, molecular weight, molecular formula and mass spectra of the peptides were identified.

Two potential antimicrobial peptides were detected in LVF7 (derived from leaf extract). The amino acid sequence of the first small peptide (LVF-AMP1) was Lys-Cys-His, and it had a molecular weight of 386.17 Da as shown in Figure 4.4. The second small peptide (LVF-AMP2) was composed of amino acids (Met-Lys-His), and its molecular weight was 414.52 Da (Figure 4.5). Another small peptide with a molecular weight of 386.17 Da and composed of amino acids (Lys-Cys-His) was detected in STF6 (derived from the stem extract) and named (STF-AMP). This peptide was similar to that identified in the leaf fraction shown in (Table 4.8).

Surprisingly, no peptides were detected in PEF8 derived from pomegranate peel extract. Although the peel purified fraction showed antimicrobial activity against the tested bacteria. However, other bioactive secondary metabolites (C16 Sphinganine, Phytosphingosine, and Xestoaminol C) have been identified in PEF8 peel fraction.

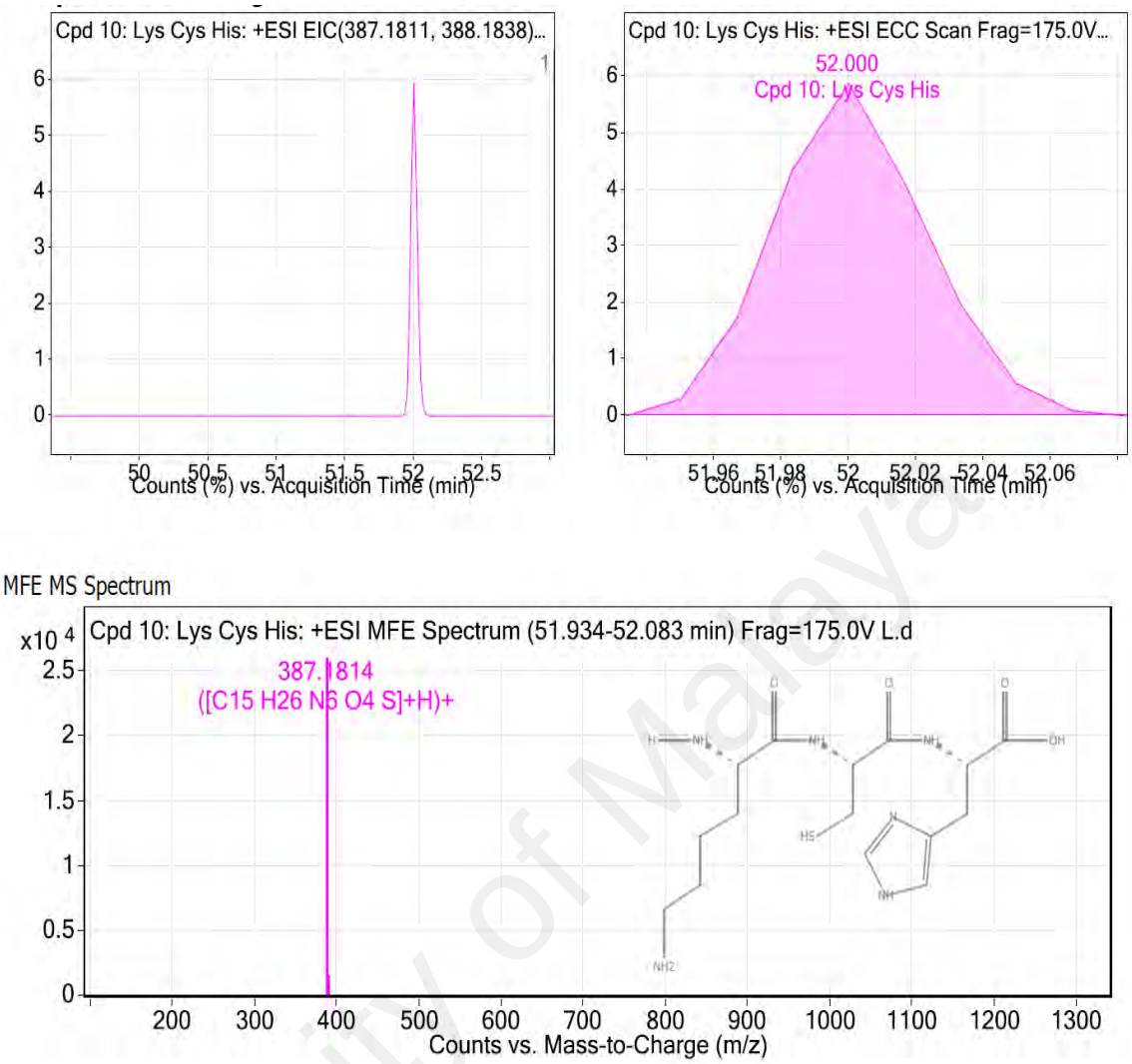

Figure 4.4: LC-MS Q-TOF profiling of antimicrobial peptide (LVF-AMP1) in leaf fraction. Figure shows LC-QTOF MS profiling, the panels show chromatogram of antimicrobial peptide (LVF-AMP1) identified in LVF7 fraction, the detected molecular weight, and molecular formula.

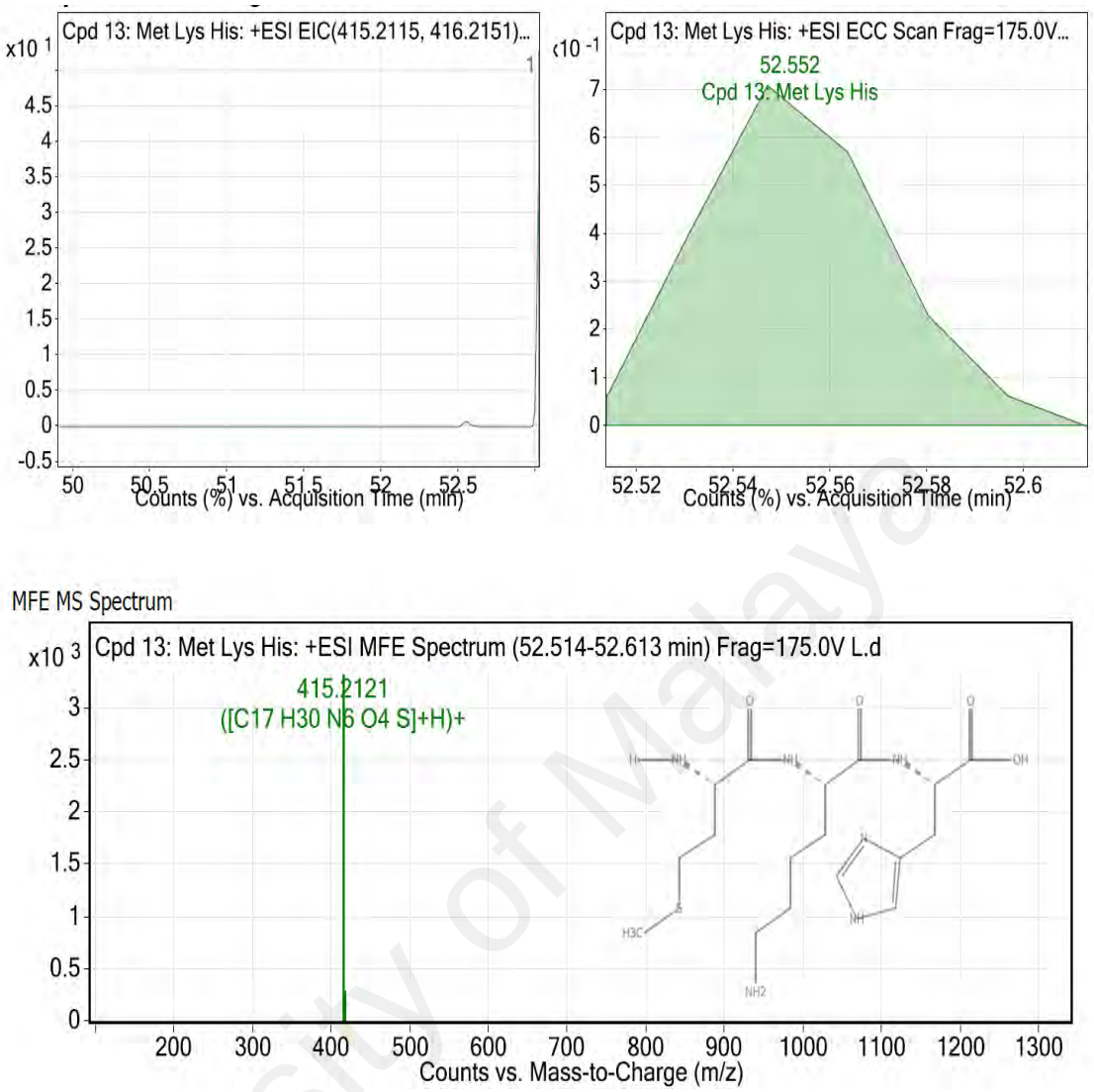

Figure 4.5: LC-MS Q-TOF profiling of the antimicrobial peptide (LVF-AMP2) in leaf fraction. Figure shows chromatogram of antimicrobial peptide (LVF-AMP2) identified in LVF7 fraction, as well as the detected molecular mass and molecular formula.

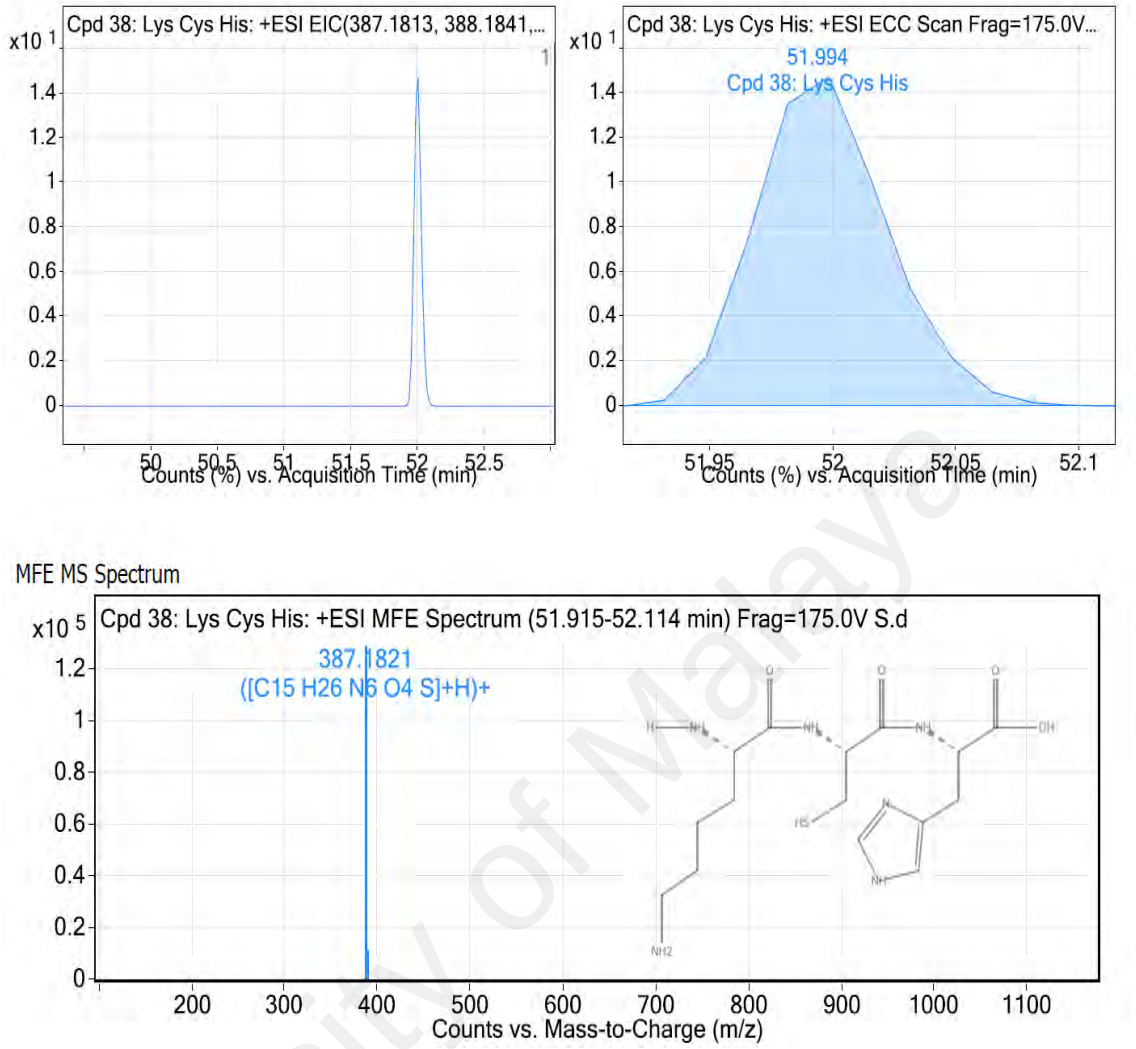

Figure 4.6: LC-MS Q-TOF profiling of the antimicrobial peptide in stem fraction. Figure show LC-QTOF MS profiling, the panels show chromatogram of antimicrobial peptide (STF-AMP) identified in STF6 fraction, as well as the detected molecular mass and the molecular formula.

Table 4.8: The identified antimicrobial peptides. The table displays the identified antimicrobial peptides in purified fractions (lVF7 and STF6), with their molecular weight, molecular formula and amino acid sequences.

Fractions	Peptide	Molecular weights of fragment (Da)	Molecular Formula	Amino acid content
LVF7	LVF-AMP1	386.17	C15 H26 N6 O4 S	Lys Cys His
	LVF-AMP2	414.52	C17 H30 N6 O4 S	Met-Lys-His
STF6	STF-AMP	386.17	C15 H26 N6 O4 S	Lys Cys His

CHAPTER 5: DISCUSSION

Different parts of medicinal plant *Punica granatum* (peel, leaves, stem, and seeds) were selected in this study. This plant and its parts were chosen specifically because *P.granatum* was widely used in folklore medicine and had a wide range of health benefits. In addition to its ancient historical uses, pomegranate was used in diverse systems of medicine as ailments.

P. granatum has been used extensively as a traditional medicine in many countries for the treatment of dysentery, diarrhoea (Moorthy et al., 2013). Various extracts derived from pomegranate were shown to possess antimicrobial activity (Dey et al., 2015). Furthermore, several compounds have been isolated from *P. granatum* such as tannins, punicalagin, ellagic acid, gallic acid, hydroquinone pyridinium, delphinidin, cyanidin, and pelargonidin which are well known for their therapeutic properties (Baindara et al., 2013; Qnais et al., 2007).

According to the review article made by Jurenka (2008) about the therapeutic applications of pomegranate, almost all parts of this plant are used in traditional medicine for the treatment of various diseases. Moreover, the bark and rind of pomegranate are used to treat dysentery, diarrhoea, to reduce the risk of cardiovascular disease, and as an anthelmintic.

Therefore, the main aim for selecting this plant in our study was due to its therapeutic history, antimicrobial activity, and the possibility of antimicrobial peptides presence which may be significant in therapeutic treatments.

In this study, the screening of the selected parts of *P.granatum* and their antimicrobial property was determined by well diffusion assay. Enteric pathogens (*Salmonella, Shigella*, and *E. Coli*) species were chosen for this study since they cause acute infectious diarrheal diseases which continue to be one of the most frequent causes of childhood deaths in the developing world (Basak et al., 2016; Sack et al., 1997). Furthermore, the chosen Gram-negative bacteria are resistant to a wide range of antimicrobials including Tetracycline, Ampicillin, Streptomycin, Ciprofloxacin, Cefixime, Nalidixic Acid, and Chloramphenicol. Gram-negative bacteria are generally more resistant to antibiotics. Although all bacteria have an inner cell membrane, gram-negative bacteria have a unique outer membrane. This outer membrane excludes certain drugs and antibiotics from penetrating the cell (Fair & Tor, 2014).

The bacteria selected as indicators in this study were tested for their susceptibility to antibiotics. It was seen that, all chosen bacteria are resistant to the antibiotics. Among these resistant bacteria, three pathogens were multi-drug resistant (MRD). The presence of an outer membrane in enteric bacteria (Gram negative bacteria) generally excludes antibiotics from penetrating the cell. Additionally, these organisms have the ability to exchange the genetic material (DNA) among strains of the same species and even among different species (Alam et al., 2013; Duedu et al., 2017). Enteric pathogens tend to be associated with mobile genetic elements such as plasmids and transposons, which usually carry multiple resistance genes to other members of the Enterobacteriaceae especially *E. coli*, *Salmonella*, and *Shigella* etc. This causes the problem greater issue to clinicians and hospitals, particularly in controlling infection diseases like typhoid, dysentery, diarrhoea and other gastrointestinal infections (Alam et al., 2013).

In this research, crude protein extracts from *P. granatum* were obtained by the extraction method (as described earlier). Peptides were extracted from all four tested

pomegranate parts and screened for antimicrobial activity against different strains of enteropathogens: two *E. coli* species, two *Salmonella* species, one *S. boydii,* one *S. sonnei*, one *S. dysentery* and four serotypes of *S. flexner*i.

Antimicrobial activity assay results revealed that peel, leaves, and stem with protein concentration (258, 351, and 351 μg/ml) respectively, inhibited all the bacterial strains. However, pomegranate seed did not show any inhibitory activity against the test pathogens. This result conflict with a previous study by Das and others (1999), which reported that methanol extract of *P. granatum* seed has antidiarrheal activity, and the extract also cause a significant reduction in gastro-intestinal motility in charcoal meal test in rats. The difference in results is probably because of the different extraction solvent used in our study than the Das's group. According to Sajjad et al. (2015), there were variations in phytochemistry of different *P. granatum* extracts. In addition, the composition depends on many factors for instance environmental factors, processing, cultivation and post harvesting.

P. granatum peel exhibited the strongest antimicrobial activity against tested bacteria strains. These results are corroborated by Alan´ıs et al. (2008) who evaluated the effect of *P. granatum* peel water extract on *E. coli, Shigella* spp *and Salmonella* spp which were isolated from cases with bloody diarrhea. This study showed that *P.granatum* were active against all tested pathogens with a percentage of inhibition up to 100%, and exhibited a strong activity against *S. flexneri* species.

Furthermore, the crude protein extracts of *P. granatum* leaf exhibited significant antimicrobial activity against tested pathogens strains. These findings are similar to another study by Madduluri et al. (2013) reported that pomegranate leaves exhibited

significant antibacterial activity against gram negative pathogens *E. coli, Klebsiella pneumonia,* and *S. typhi*.

Although there are many studies have reported the inhibitory activity of *P. granatum* parts (Mathabe et al., 2006; Moorthy et al., 2013; Nascimento et al., 2000), there is a lack of studies conducted on pomegranate stem or bark part which have not been widely studied. In our study we prove that the *P. granatum* stem extract has significant antimicrobial properties, similar to other pomegranate parts.

The results from our antimicrobial activity screening of *P. granatum* parts (peel, stem, and leaf) are in agreement with the previously mentioned studies. These findings confirmed the therapeutic properties of pomegranate. On this basis, *P. granatum* was selected as a candidate plant for the isolation of antimicrobial peptides.

In this study isolation and partial purification of proteins/ peptides from crude extracts was performed. Firstly, the total proteins were precipitated using ammonium sulphate (80% saturation). The total protein concentration of dissolved precipitates was determined. All three samples derived from (peel, leaf, and stem) contain higher protein contents than the crude extracts. This is probably due to the precipitation and concentration of protein.

The obtained peptides precipitation were tested for their antimicrobial activity to determine whether this activity due to the presence of antimicrobial peptides. Based on the results, the dissolved peptides precipitates of three parts (peel, leaf, and stem) extracts with protein concentration (298, 530, 390 μg/ml) respectively, displayed high antimicrobial activity against the majority of tested pathogens. In general, the solubilized precipitates of peel exhibited less antimicrobial activity than the crude extracts. It is probably because the presence of phenolic compounds in which proven that they play important role in antimicrobial activity of pomegranate peel crude extract as reported by Sajjad et al. (2015).

These findings, which are summarized in Table 4.3, are in accordance with those of Mahmood et al. (2012). In their study, the latter precipitated 683 μg/ml of peptides/proteins using 80% ammonium sulphate from *Momordica charantia* L. crude extract, and found that these peptides exhibited significant antimicrobial activity against *E. coli*, *Staphylococcus epidermidis*, *S. typhi* and *Lactobacillus bulgaricus*. Likewise, their results indicated that the active peptides isolated from the crude extract were partially purified which is similar to our study. In sum, this confirms that the antimicrobial peptides are present in *P. granatum*.

In order to remove salt and separate the peptides according to size, the obtained ammonium sulphate precipitates of peels, leaves and stems were subjected to gel filtration column chromatography (Sephadex G-25). Peak fractions were collected from the three samples, and their antimicrobial activity and protein content were then tested separately using the Bradford assay.

The fractions showed significant antimicrobial activity against all the tested pathogenic isolates. Among the active peel fractions, fraction 20 (PE20) displayed the highest activity against *S. flexneri* Y. The protein content of the peel fraction was 16.7 μg/ ml. In the case of leaves, fraction eight derived from leaf (LV8) showed significant antibacterial activity against *S. flexneri* 3a and *S. dysentery*, with a protein content of 21.6 μg/ ml. Meanwhile, an analysis of the antimicrobial activity of peak fractions of the stem revealed that fraction 15 (ST15) showed the highest antibacterial activity against the majority of tested bacteria strains *S. boydii*, *S. flexneri* 1b and *S. flexneri* 3a, with a protein content of 30 μg/ ml. As a result, PE20, LV8 and ST15 were chosen for further purification.

The above results are similar to those reported by (Dahot, 1998), who separated a *Moringa oleifera* sample on Sephadex G-25 column chromatography and found three

peaks containing small peptides, all of which showed antibacterial and antifungal activity. Recently, (Jabeen & Khanum, 2017) purified AMPs from *Momordica charantia* by using Sephadex G-100. They obtained four peaks, with peak 3 showing the highest level of activity against *E. coli*, *S. aureus*, *S. typhi* and *P. aeruginosa*. Similarly, Jamil and others (2013) purified antimicrobial peptides from *Psoralea corylifolia* and *Solanum nigrum* using Sephadex G-100 gel column. A significant activity was observed in many fractions of seed extracts of the plants. The results indicated that gel filtration chromatography is one of the effective techniques for separating proteins based on their size in order to obtain semi-purified antimicrobial peptides.

Semi-preparative RP-HPLC used for the separation of antimicrobial peptides. This method used previously by Wang et al. (2009) to purify defense peptides isolated from plants source and it has proven to be a reliable method.

The fractions with antibacterial activity (PE20, LV8 and ST15) were subjected to semi-preparative RP-HPLC for separation and purification of peptides.

Two different unknown peaks were detected in each sample. The antibacterial activity of each of the collected fractions was tested by the agar diffusion method against the multidrug-resistant isolate *S. flexneri* Y. This reduction in indicators numbers is due to the limitation of the recovery volume. The active fractions (PEF8, LVF7, and STF6) which were derived from peel, leaf, and stem respectively, showed antibacterial activity against the multi-drug resistant isolate *S. flexneri* Y and were selected for further studies.

Recently, a study by Sornwatana et al. (2013) purified antimicrobial peptides from B. javanica (L.) using reversed-phase HPLC, four separated fractions (F1, F2, F3 and F6)

were collected. Fraction 3 (F3) showed antibacterial activity against *S. typhi, S. aureus, and S. pyogenes* and then selected for further investigation which is similar to our study.

Microtiterplate turbidity assays were used to monitor the inhibitory effect of the purified fractions on the growth of tested bacteria where effective protein concentrations required for 80 % inhibition of bacterial growth were determined.

The differing minimum inhibition concentrations (MICs) for the three purified fractions derived from pomegranate (peel, leaf, and stem) against a representative multiple drug-resistant isolate, *S. flexneri* Y, showed that they all inhibited the growth of the tested bacterium at different concentrations. The purified stem fraction STF6 exhibited the highest level of activity at the lowest concentration, with an MIC value of 6.05 μg/ml, whereas the purified fractions PEF8 and LVF7 inhibited the growth of *S. flexneri* Y only at concentrations of 6.45 μg/ml and 7.55 µg/ml respectively. With wide spectrum antibacterial activity of the purified fractions described earlier, it is likely that these HPLC fractions contained more than one peptide and other active compounds. Therefore, these fractions were potent enough to exhibit bacterial growth inhibition.

The active fractions subjected to LC-MS Q-TOF for identification of peptides. Liquid chromatography coupled with mass spectrometry (LC/MS) is also a powerful technique for the analysis of complex botanical extracts. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable (Sasidharan et al., 2011).

In this study, LC-MS Q-TOF analysis revealed the presence of three small peptides. Two potential antimicrobial peptides were detected in LVF7 (derived from leaves extract) with molecular (386.17 Da) and (414.52 Da). However, another peptide has been detected in STF6 (derived from stem extract) with 386.17Da molecular weight

which exhibited antimicrobial activity toward the resistant pathogen. A blast search (http://web.expasy.org/blast/) and DRAMP database search (http://dramp.cpu-bioinfor.org/) were conducted to identify similar sequences, but found no record of matching sequences. These peptides can therefore be classified as novel peptides with antimicrobial properties.

Another finding was that the amino acid sequences of the detected antimicrobial peptides in this study displayed that their C-terminal residue is a basic amino acid, histidine. This can be a key factor in their antimicrobial activity. This result are consistent with those of Brown and Hancock (2006) and Sedaghati et al.(2014), who reported that cationic side chains of histidine, arginine, and lysine can mediate in the interaction between peptides and negatively charged bacterial membranes or cell walls, including lipopolysaccharide.

Similar to our study, Hou et al. (2007) isolated small antimicrobial peptide *Musca domestica* with molecular weight 8 kDa after purification using gel filtration chromatography (Sephadex G-15) and RP-HPLC. Their peptides inhibited the growth of *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. dysenteriae*, *S. aureus* and *Bacillus subtilis* with MIC ranging from 18 to 72 μg/ml. In another study carried out by Sedaghati and others (2016) they isolated antibacterial peptides (bC8, bC12, and bC14) in the plasmin digest of b-casein with molecular weight 872.08, 911.09, 1590.92 Da, which showed antimicrobial activity against Gram negative and Gram positive pathogens. furthermore, Jabeen and Khanum (2017) also isolated antibacterial peptide with molecular weight 10 kDa had inhibitor activity against *E. coli, S. aureus, S. typhi and P. aeruginosa*. However, this groups of researchers did not perform an amino acid analysis of the AMP, nor did they clarify the identity of the peptide through a protein database search.

In this study, the active peptides in leaf and stem fractions of *P. granatum* proved to be more effective than the antibiotic penicillin (MIC value 31.2 μg/ml). Recent results were reported by (Sornwatana et al., 2013) who isolated an antimicrobial peptide called Brucin, from dried fruit protein of *Brucea javanica*. This AMP exhibited inhibitory activity against *Streptococcus pyogenes* with 16-fold and 12.5-fold higher than penicillin G and chloramphenicol, respectively which is similar to our finding.

Surprisingly, no peptides were detected in PEF8 derived from pomegranate peel extract, although the peel purified fraction showed antimicrobial activity against the tested bacteria. However, secondary metabolites (C16 sphinganine and phytosphingosine) were identified in PEF8. The antimicrobial activity of these identified compounds was reported in a study by Farwick and Santonnat (2008). This latter study suggested that these metabolites have strong antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts, even at low concentrations, due to their antibacterial and anti-inflammatory properties.

Our study further identified, SW 163C, pelletierine and theobromine in pomegranate extracts as shown in (Appendix J, Table 3). Previous studies had shown these bioactive secondary metabolites to have significant antibacterial effects against resistant bacteria, and to exhibit antihelminthic, antiviral and anti-tumoral activity (Nidhi Singh, 2015; Sharmin, 2016; Takahashi et al., 2001). It is therefore reasonable to conclude that the presence of these bioactive metabolites in our study may have contributed to the inhibitory activity of pomegranate fractions which may need further purification to recover the pure peptides.

CHAPTER 6: CONCLUSION

Different parts of the medicinal plant *Punica granatum* (peel, leaf, stem, and seed) were screened for antimicrobial activity against resistant pathogens isolates. The plant was chosen based on historical therapeutics in treatment of diarrhea and dysentery. The protein crude extracts of peel, leaf, and stem inhibited the growth of all tested bacteria, therefore; these parts chosen for investigation the presence of antimicrobial peptides. The protein isolated by ammonium sulphate and the dissolved ammonium sulphate precipitates pellet showed significant antimicrobial activity with high protein content. Thus, the three pomegranate extracts contain peptides having antimicrobial activity

The purification of the active peptides was carried out using gel filtration chromatography and the semi-preparative HPLC. LC-MS Q-TOF analysis identified three antimicrobial peptides which showed inhibitory activity against multi-drug resistant isolate bacteria.

To sum up, this study identified the presence of small peptides with short amino acid sequences in *P. granatum* extracts, which showed inhibitory activity against a multidrug resistant gastrointestinal pathogen. Specifically, by screening through extracts from a range of pomegranate plant parts, we identified three short peptides with significant antimicrobial activities. In future, the extracted peptides could be chemically synthesized and their inhibitory activities determined to prove their responsibility activity. Furthermore, further structural studies using Nuclear Magnetic Resonance (NMR) analysis could be done to provide a more complete structural analysis of these peptides. Meanwhile, the AMPs identified in this study could help in the fight against gastrointestinal bacteria resistance as well as being a potential resource for the synthesis of antibiotics.

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