UNIVERSITI PUTRA MALAYSIA

IN- VITRO ANTIBACTERIAL ACTIVITY AN D PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS FROM POMEGRANATE (Punica granatum L.) CRUDE PEEL EXTRACTS

AYAD ISMAEL KHALEEL

FP 2016 40

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By

AYAD ISMAEL KHALEEL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

May 2016

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DEDICATION

This Thesis is dedicated to

The most precious people in my life;

My Father and mother

My brother and sisters

My wife and kids

For their unconditional everlasting love

They began my education

They motivated me to continue it

They will always contribute to it

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

IN-VITRO ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS FROM POMEGRANATE (*Punica granatum* L.) CRUDE PEEL EXTRACTS

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May 2016

Chairman : Associate Professor Kamaruzaman Bin Sijam, PhD
Faculty : Agriculture

Plant pathogenic bacteria are recognized to be harmful microbes able to decrease the quantity and quality of crop production in agriculture. *Punica granatum L.* peel was screened for its potential use as a biological control agent for plant pathogenic bacteria. *P. granatum* peel was successfully extracted using n-hexane, methanol and ethyl acetate. The highest percentage of crude extracts was obtained from ethyl acetate 1.37% followed by methanol crude extracts 1.17% and n-Hexane 0.89%. The highest yield obtained by ethyl acetate showed that ethyl acetate extracted more compounds that are readily soluble to methanol and n-hexane. For *in-vitro* antibacterial activity, three different species of plant pathogenic bacteria were used namely *Erwinia carotovorum* subsp. *carotovorum, Ralstonia solanacearum,* and *Xanthomonas gardneri*. For all crude extracts, four different concentrations of 25, 50, 100 and 200 mg/mL were used in cup-plate agar diffusion method. Streptomycin sulfate at concentration 30 µg/mL was used as positive control while each respective solvent used for peel extraction was used as a negative control. The results obtained from *in vitro* studies showed only ethyl acetate extract possessed antibacterial activity tested on the plant pathogenic bacteria. Methanol and n-hexane did not show any antibacterial activity against plant pathogenic bacteria selected where no inhibition zones were recorded. *R. solanacearum* recorded the highest diameter of inhibition zone for all ranges of concentrations introduced followed by *P. carotovorum* subsp. *carotovorum* and *X. gardneri.* For the minimum inhbitory concentration (MIC) and minimum bactericidal concentration (MBC), only the ethyl acetate extract was subjected to the assay as only ethyl acetate extract exhibited antibacterial activity. The minimum concentration of ethyl acetate extract that was able to inhibit plant pathogenic bacteria was recorded at a concentration of 3.12 mg/mL, which inhibited *R. solancearum* and *P. carotovorum* subsp. *carotovorum*, followed by *X. gardneri* at concentration of 6.25 mg/mL. For the minimum bactericidal concentration (MBC), the results showed that at the concentration of 12.5 mg/mL, the extract was still capable of killing the pathogenic bacteria, *R. solanacearum,* and *P. caratovora* sub.sp. *caratovora* while for the bacteria *X. gardneri*, the concentration that was able to kill the bacteria was 25 mg/mL. The

qualitative estimation of phytochemical constituents within *P. granatum* ethyl acetate peel extracts revealed the presence of tannins, flavonoids, phenols alkaloid, Saponins, and terpenoids. The thin layer chromatography (TLC) profiling of ethyl acetate extract using hexane, ethyl acetate, and chloroform with ratio 5:3:2 (v/v) gave eleven maximum colorful bands when visualized under short UV wavelength (254nm), six bands under long UV wavelength (365nm) and eight bands in daylight (normal light) with different retention factors, and R_f values that provined the presence of various secondary metabolites within ethyl acetate extract. The antibacterial activity of ethyl acetate peel extract was also screened through direct bioautography technique in order to detect the location of the progressive band on chromatograms developed in the same substance for TLC profiling. The recorded active R_f values that inhibited all selected plant pathogenic bacteria at the same R_f values location were 0.45, 0.83 and 0.92. The GC-MS spectrum range affirmed the vicinity of 292 different components with diverse retention times and chemical structure eleven elements in the high peak chosen were (DMSO; n-Hexadecanoic acid; 9,12-Octadecadienoic acid (Z,Z); cis-Vaccenic acid; Octadecanoic acid; Pentanoic acid; cis-9-Hexadecenoic acid; Tetradecanoic acid; 2-Heptenoic acid; Octanal diethyl acetal; Glycerin). The results obtained from this study suggest that *P. granatum* L ethyl acetate peel extracts have the potential to be industrialized as a novel bactericide.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI ANTIBAKTERIA *IN-VITRO* DAN PENYARINGAN KOMPOUN BIOAKTIF FITOKIMIA DARI EKSTRAK KULIT MENTAH DELIMA (*Punica granatum* L.)

Oleh,

AYAD ISMAEL KHALEEL

Mei 2016

Pengerusi : Profesor Madya Kamaruzaman Bin Sijam, PhD
Fakulti : Pertanian

Bakteria patogenik tumbuhan diketahui umum sebagai mikrob berbahaya yang dapat mengurangkan kuantiti dan kualiti hasil pertanian. Kulit *Punica granatum* L. telah disaring potensinya sebagai agen kawalan biologi bakteria patogenik tumbuhan. Kulit *P. granatum* L. ini telah berjaya diekstrak menggunakan n-heksana, metanol dan ethyl acetat. Hasil peratusan tertinggi adalah 1.37% melalui ekstrak mentah ethyl acetat, diikuti oleh ekstrak mentah methanol, 1.17% dan n-heksana, 0.89%. Hasil tertinggi diperolehi daripada ethyl acetate menunjukkan ethyl acetat mengekstrak lebih sebatian bahan mudah larut berbanding metanol dan n-heksana. Bagi aktiviti antibakteria *in-vitro*, tiga jenis spesis bakteria patogenik tumbuhan telah digunakan iaitu *Pectobacterium carotovorum* subsp. *carotovorum*, *Ralstonia solanacearum*, dan *Xanthomonas gardneri*. Bagi kesemua ekstrak mentah, empat kepekatan berbeza iaitu 25, 50, 100 dan 200 mg/ml telah diuji menggunakan kaedah resapan plat-cawan. Streptomisin sulfat pada kepekatan 30 µg/ml telah digunakan sebagai kawalan positif, manakala setiap bahan pelarut pengekstrakan kulit digunakan sebagai kawalan negatif. Keputusan yang diperolehi daripada kajian *in-vitro* ini menunjukkan hanya ekstrak ethyl acetat mempunyai aktiviti anti-bakteria terhadap bakteria patogenik tumbuhan yang diuji. .Metanol dan n-heksana tidak menunjukkan sebarang aktiviti antibakteria terhadap bakteria patogenik tumbuhan terpilih di mana tiada zon perencatan telah direkodkan. *Ralstonia solanacearum* merekodkan diameter perencatan tertinggi untuk semua julat kepekatan yang diuji dan diikuti oleh *P. carotovorum* subsp. *carotovorum* dan *X. gardneri*. Untuk kepekatan perencatan minima (MIC) dan kepekatan minima bakterisidal (MBC), hanya ekstrak ethyl acetat yang tertakluk kepada ujian kerana hanya ekstrak etil asetat menunjukkan aktiviti anti-bakteria. Kepekatan minima ekstrak ethyl acetat yang mampu untuk merencat bakteria patogenik tumbuhan dicatatkan pada kepekatan 3.12 mg/ml, untuk merencat *R. solancearum* dan *P. carotovorum* subsp. c*arotovorum* dan *X. gardneri* pula pada kepekatan 6.25 mg/ml. Untuk kepekatan minima bakterisidal (MBC), keputusan menunjukkan bahawa pada kepekatan 12.5 mg/ml, ekstrak itu masih mampu membunuh bakteria patogenik, *R. solanacearum* dan *P. caratovorum* subsp. *caratovorum* manakala bagi bakteria *X. gardneri*,

kepekatan yang mampu membunuh bakteria adalah 25 mg/ml. Anggaran kualitatif juzuk fitokimia dalam *P. granatum* L. ekstrak kulit ethyl acetat mendedahkan kehadiran tanin, flavonoid, fenol alkaloid, saponin dan terpenoid. Profil kromatografi lapisan nipis (TLC) bagi ekstrak ethyl acetat menggunakan heksana, ethil acetat, dan kloroform dengan nisbah 5:3:2 (v/v) memberi 11 jalur yang berwarna-warni maksim apabila digambarkan di bawah gelombang UV ringkas (254 nm), enam jalur di bawah gelombang UV panjang (365 nm) dan lapan jalur di bawah cahaya putih (cahaya biasa) dengan faktor pengekalan berbeza dan nilai Rf yang menunjukkan kehadiran pelbagai metabolit sekunder di dalam ekstrak ethyl acetat. Aktiviti antibakteria ekstrak kulit ethyl acetat juga telah disaring melalui teknik bioautography terus untuk mengesan lokasi jalur progresif pada kromatogram terbentuk dengan menggunakan bahan yang sama melalui pemprofil TLC. Direkodkan nilai Rf aktif yang merencatkan semua bakteria patogenik tumbuhan berada di nilai Rf yang sama iaitu 0.45, 0.83 dan 0.92. Julat spektrum GC-MS mengesahkan sebanyak 292 komponen berbeza dalam masa tahanan yang pelbagai dan 11 struktur kimia mempunyai puncak tinggi telah dipilih adalah (DMSO; n-Hexadecanoic asid; asid 9,12-Octadecadienoic (Z, Z); cis- asid Vaccenic; asid Octadecanoic; asid Pentanoic; cis-9-Hexadecenoic asid; asid Tetradecanoic; asid 2-Heptenoic; Octanal diethyl asetal; Glycerin). Keputusan yang diperolehi daripada kajian ini mencadangkan bahawa ekstrak kulit *P. granatum* L ethyl acetat mempunyai potensi untuk dikilangkan sebagai baktersidal yang baharu.

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I certify that a Thesis Examination Committee has met on 10 Mei 2016 to conduct the final examination of Ayad Ismael Khaleel on his thesis entitled "*In-Vitro* Antibacterial Activity and Phytochemical Screening of Bioactive Compounds from Pomegranate (*Punica granatum* L.) Crude Peel Extracts" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science.

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LIST OF ABBREVIATIONS

PG_E	*Punica granatum* L. ethyl acetate peel extracts
PG_N	*Punica granatum* L. n-hexane peel extracts
PG_M	*Punica granatum* L. methanol peel extracts
%	Percent
ANOVA	Analysis Of Variance
DCSO	dimethyl sulfoxide
$FeCl_3$	Ferric chloride
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
cm	Centimeter
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MTC	3-(4,5-dimethylthiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
FAO	Food and Agriculture Organization of the United Nations
g	Gram
n	Number of replicates
NA	Nutrient Agar
kb	Kilo base
L	Liter
LSD	Least Significant Difference
M	Molarity
min	Minute
mm	millimeter
NaOH	Sodium hydroxide
ºC	Degree
pH	potential Hydrogen
SAS	Statistical Analysis System
sec	Second
UPM	Universiti Putra Malaysia
UV	Ultra violet

V	Volts
NH_3	Ammonia
O.D.	Optical density
Pv.	Pathovar
R_f	Retention factor
rpm	Revolutions per minute
S.D	Standard deviation
Subsp.	Subspecies
TLC	Thin layer chromatography
TTC	2,3,5-triphenyltetrazolium
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfopheny)-2H-tetrazolium-5-carboxanilide
GCMS	Gas chromatography–mass spectrometry
IC_{50}	Inhibitory Concentration 50% of bacteria
IC_{90}	Inhibitory Concentration 90% of bacteria

CHAPTER 1

INTRODUCTION

1.1 Background

The history of human civilization offers an abundance of evidence that an amazing range of plants and plant materials have been used as medicinal agents and numerous natural products acquired from medicinal plants either as a crude extract or as purified products have been applied in disease control. Owing to the presence of various medicinal properties in these medicinal plants and plant parts, they have been extensively used for the extraction of natural drugs. They constitute credible sources for a huge number of modern drugs, several of which are based on their traditional folk homoeopathic efficacy. The World Health Organization (WHO) has stated that medicinal plants are the best source for obtaining a variety of therapeutic agents, and several medicinal plants have been employed as sources of medicine in daily life for treatment of various types of ailments globally (Alo et al., 2012). Clinical microbiologists have widely used medicinal plants for the screening of new therapeutic agents (Ashokkumar & Rajkumar, 2010). A great range of biotic molecules referred to as secondary metabolites are produced by plants (Dash et al., 2011), thereby making them a rich source for diverse forms of medicine. Additionally, the primary advantages of using these naturally derived products include safety for human consumption, environmentally friendly, and economical in treating microbial infections when they are used (Al-Zubaydi & Al-Hmdany, 2009).

Recently, much attention has been paid to the use and search for natural products. Extracts of plant origin are usually rich in a broad range of active compounds (Al-Zubaydi & Al-Hmdany, 2009) with the potential to be developed as natural anti-bacterial agents (Bhardwaj & Laura, 2009); (Dash et al., 2011) Plant extracts have been demonstrated to have antibacterial properties in *in-vitro* studies (Ghosh et al., 2008; Rios & Recio, 2005) Plant-based products have been shown to be safer than synthetic products, and they have gained more acceptance as they are non-toxic to humans, biodegradable and non-polluting. Additionally, they are less phytotoxic and possess greater systemic effected. These properties have led to plants being further investigated for their effectiveness in combatting diverse kinds of microbes in many fields of study (Abdollahzadeh et al., 2011).

The use of hazardous chemicals to control the spread of disease is of great concern to those who are aware of the dangers of the toxic effects on food crops. The consequences of short-term or long-term toxicity caused by toxic chemicals can affect human health negatively. In addition, it also may environmental pollution, particularly of water, soil and the air. This may could lead to serious ecological damage that would impact both humans and a whole range of living things.

Several studies have been conducted to address this issue in attempts for finding solutions of the problems associated with compere to harmful chemicals in agriculture. In recent years, biological control methods have been studied as a viable and safer alternative to compare to harmful chemicals as they have been proven to be effective, inexpensive and easily available. Biological control methods are also easily manipulated from natural resources and at the same time are friendly to the environment and ensure that the agricultural product safe for human consumption. Pathogens such as fungi, viruses and bacteria have been identified being capable of causing plant diseases that may lead to deterioration of yield in terms of a decline in quality and quantity of crops. Add to all that, it causes economic losses to countries that largely depend on agricultural produce both for food production and for export.

Biological control measures were therefore introduced to the agriculture industry owing to increased demand for safer crop products as more people are becoming aware of the harmful and toxic effects of chemical pesticides on crops. Biological control of plant pests and pathogens is continually inspiring research and development in diverse fields where plant pathogens act as a class of targets of biological control that are designed to limit other pests including insects, parasitic nematodes and weed (Gardener & Fravel, 2002). Among the diverse, the use of plant extract as a biological control measure has gained considerable acceptance as a way to develop safe pesticides or antibiotics taken from nature in the form of phytochemicals to control pathogenic microbes desired by scientists in diverse fields.

P. granatum (pomegranate) is one of the oldest known edible fruits. It has been commonly used in traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases. In addition to its older historical purposes, pomegranate is used in several systems of medicine for a variety of ailments (Olapour & Najafzadeh, 2010). In Ayurvedic medicine, pomegranate is viewed as "a pharmacy unto itself " and is used as an antiparasitic agent, a "blood tonic," and to heal aphthae, and ulcers (Jurenka, 2008). Pomegranate (*P. granatum* L.) is native to the Mediterranean region and has been used extensively in the folk medicine of many countries. In India, it is used in the form of juice, concentrate, canned beverage, wine, jam, and jelly (Tripathi et al., 2014). Fresh juice contains a small amount of pectin, ascorbic acid, and flavonoids. The soluble polyphenolic content of pomegranate juice (0.2-1.0%) includes anthocyanins, catechins, ellagic tannins, and gallic and ellagic acids (Aviram et al., 2000). Previous work carried out in the laboratory showed high antioxidant activities of the methanolic extracts of pomegranate peel in various in vitro models (Singh et al., 2002).

Although several studies have attempted using natural plant extract to protect crop plants from plant pathogens, the use of *P. granatum* L. peel extracts against plant pathogenic bacteria, and its MIC and MBC values have not been investigated and there are no previous studies done for the selected bacteria. In view of the increasing quest for safer biological control measures against plant pathogens, investigating the effect of *P. granatum* L. peel extracts as a plant pathogenic bacteria are imperative, and is therefore the primary objective of this study.

1.2 Research Objectives

In order to meet the requirements for safety and non-hazardous pesticides that can be used to control plant pathogenic bacteria, *P. granatum* L. peel was used and tested to fulfill the research objectives, which were:

1. To evaluate the efficacy of *P. granatum* L. peel extracts against plant pathogenic bacteria *in vitro* and determine the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of the extracts.
2. To determine the chemical constituents and identify the bioactive compounds of *P. granatum* L. peel extracts that exhibited the strongest antibacterial effect against plant pathogenic bacteria by using phytochemical screening test, Direct TLC bioautography assay and Gas Chromatography- Mass Spectrometry (GC-MS) Analysis.

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