UNIVERSITI PUTRA MALAYSIA

ANTIMICROBIAL ACTIVITY OF Rhus coriaria L. FRUIT EXTRACTS AGAINST SELECTED BACTERIAL AND FUNGAL PATHOGENS ON TOMATO

TAVGA SULAIMAN RASHID

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By

TAVGA SULAIMAN RASHID

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

May 2016

DEDICATION

THIS THESIS IS DEDICATED TO

MY BELOVED HUSBAND HAYMAN

MY LOVELY TWINS AHMED AND TLOVA

MY MOTHER'S SOUL

MY GRETEST FATHER

MY LOVELY BROTHERS

Abstract of thesis submitted to the Senate of Universti Putra Malaysia in fulfillment of the requirements of the Degree of Doctor of Philosophy

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May 2016

Chairman : Associate Professor Kamaruzaman Sijam, PhD
Faculty : Agriculture

An effective and efficient nanoemulsion biopesticide derived from *Rhus coriaria* was developed for the control of selected tomato bacterial and fungal pathogens. Fruit extracts of *Rhus coriaria* were screened for antifungal and antibacterial activities in vitro and extract that showed strongest activity was screened in vivo. Aqueous, methanol, acetone and ethanol extracts of *R. coriaria* resulted in the production of antifungal and antibacterial substances that significantly inhibited the growth of *Rhizoctonia solani, Fusarium oxysporum, Fusarium solani, Fusarium acuminatum, Phoma destructiva, Colletotrichum acutatum* and *Colletotrichum boninense* and three selected bacterial pathogens *Pseudomonas syringae, Ralstonia solanacearum* and *Xanthomonas vesicatoria* ($P<0.05$). The best inhibitory effect was elicited by aqueous extract against *C. bonininse* and *C. acutatum* (82.3% radial growth inhibition), while crude ethanol extract showed the least significant effect 61.62%. Aqueous extract showed the best inhibitory effect against *X. vesicatoria* the inhibition zone was 30mm followed by *P. syringae* and *R. solanacearum* (26.5 and 23.5 mm respectively). The result showed that *R. coriaria* fruit extract has inhibitory effect on all types of fungi and bacteria. Light and scanning electron microscopic observations showed that active crude extract resulted in degraded, twisted, shriveled, burst and with distorted morphology of fungal cell wall and bacterial cells. The aqueous crude extract was further purified through High Performance Liquid Chromatography (HPLC) fractionation and resulted in the total of 55 fractions. Results showed that most of the fractions have antibacterial activity where the inhibition zones were between 31.6-17.3mm, while only nine fractions showed antifungal activity of 75-65 %. Antimicrobial effects of the most effective extract of *R. coriaria* crude extract was supported by the presence of chemical constituents identified by GC-MS. Furfural, 2,5-Furandione, 1-Cyclopetene, Phloroglucinol, Succinic acid, p-Tolylacetic acid, Malic acid and Coumalic acid were detected as major compounds in *R. coriaria* fruit extract that were possibly responsible for the antimicrobial activity. The non-volatile compounds present were identified by Liquid chromatography–mass spectrometry (LC-MS). Gallic acid, gallic acid conjugate, tannic acid, phenylvaleric acid, quercetin conjugate, malic acid, 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid,

and 3,30-di-O-methyl ellagic acid conjugate were detected as major compounds in aqueous fruit extract that were possibly responsible for antimicrobial activities. From the five surfactants used Emereen 1820 showed to be the best surfactant and was stable after six months of storage. A surface tension value of nanoemulsion solution was 27.174 mN/m, while particle size was 70.44 nm. The formulation was evaluated for its efficacy in controlling two of the most destructive fungi and two common bacteria under glasshouse condition. Overall, the nano-emulsion formulation showed strong activity in reducing disease incidence and the disease reduction was between (82.58-64.80%) at the concentration 10% w/w while reduction was between (66.43-54.07%) at the concentration 5%, in comparison with antibiotic and fungicide. Nano-emulsion formulation was able to increase significantly shoot height (45%), root length (33%), shoot height dry weight (65%) and root dry weight (59.3%) compared to the positive and negative control at the 8^{th} weeks of plant growth.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah.

AKTIVITI MIKROBIAL EKSTRAK BUAH *Rhus coriaria* KE ATAS BAKTERIA TERPILIH DAN KULAT PATOGEN TOMATO

Oleh

TAVGA SULAIMAN RASHID

Mei 2016

Pengerusi : Profesor Madya Kamaruzaman Sijam, PhD
Fakulti : Pertanian

Bio-pestisid nano-emulsi yang efektif yang diambil dari sumac telah dibangunkan untuk mengawal patogen balkteria dan kulat pada buah tomato. Ekstrak buah-buahan Rhuscoriaria telah disaring untuk aktiviti-aktiviti anti-kulat dan anti-bakteria in-vitro dan ekstrak menunjukkan bahawa aktiviti terkuat telah disaring in vivo. Ekstrak akua, metanol, aseton dan alkohol *R. coriaria* telah menghasilkan pengeluaran bahan-bahan anti-kulat dan anti-bakteria yang merencatkan pertumbuhan *Rhizoctonia solani, Fusarium oxysporum, Fusarium solani, Fusarium acuminatum, Phoma destructiva, Colletotrichum acutatum, Colletotrichum boninense* dan tiga patogen bakteria terpilih iaitu *Pseudomonas syringae, Ralstonia solanacearum* dan *Xanthomonas vesicatoria* ($P<0.05$). Kesan rencatan terbaik telah dipaparkan oleh ekstrak akua ke atas *C.boninense* dan *C. acutatum* (82.3% rencatan tumbesaran radial), sementara ekstrak alkohol mentah menunjukkan kesan yang paling kurang signifikan, iaitu 61.62%. Ekstrak akua menunjukkan kesan rencatan terbaik ke atas *X.vesicatoria* di mana zon rencatan ialah 30mm diikuti dengan *P.syringae* dan *R.solanacearum* (26.5 dan 23.5 mm, masing-masing). Keputusan menunjukkan bahawa ekstrak buah R. coriaria mempunyai kesan rencatan ke atas semua jenis kulat dan bakteria. Pemantauan mikroskopik elektron imbasan ringan menunjukkan bahawa ekstrak mentah yang aktif menghasilkan morfologi dinding sel kulat dan sel bakteria yang pecat, berselirat dan berpintal. Ekstrak mentah akua telah disuling dengan lebih lanjut melalui pemeringkatan Kromatografi Cecair Berpresatasi Tinggi (HPLC) dan menghasilkan sejumlah 55 pecahan. Keputusan menunjukkan bahawa kebanyakan pecahan mempunyai aktiviti anti-bakteria di mana zon rencatan adalah di antara 31.67.3 mm, sementara hanya lapan pecahan menunjukkan adanya aktiviti anti-kulat sebanyak 75-65 %. Kesan anti-mikrobial ekstrak yang paling efektif R. coriaria ekstrak mentah disokong oleh kehadiran konstituen kimia yang dikenalpasti oleh GC-MS. Furfural, 2,5-Furandione, 1-siklopeten, floroglusinol, asid sukinik, acid p-tolilasetik, asid malik dan asid koumalik dikesan sebagai sebatian utama dalam ekstrak buah *R. coriaria* yang mungkin bertanggungjawab ke atas aktiviti anti-mikrobial. Sebatian tidak berubah-ubah dikenalpasti oleh spektometri jisim cecair (LC-MS). Asid galik, konjugat asid galik, asid tanik, asid fenilvalerik, konjugat kuersetin, asid malik, asid 2(3,4-dihikdroksifenil)-7-hidroksi-5-benzin propanoik, dan konjugat asid elagik

3,30-di-O-metil telah dikesan sebagai sebatian utama dalam ekstrak buah akua yang berkemungkinan bertanggungjawab ke atas aktiviti-aktiviti anti-mikrobial. Daripada lima bahan permukaan yang digunakan, Emereen 1820 menunjukkan yang ia adalah bahan permukaan terbaik dan stabil selepas enam bulan disimpan. Nilai regangan permukaan larutan nano-emulsi ialah 27.174 mN/m, sementara saiz partikel ialah 70.44 nm. Keberkesanan pembentukan telah dinilai dalam mengawal dua jenis kulat yang paling merbahaya dan dua bakteria umum dalam rumah kaca. Secara keseluruhannya, formulasi atau pembentukan nano-emulsi menunjukkan aktiviti yang kuat dalam mengurangkan insiden pengurangan penyakit ini adalah di antara (82.58- 64.80%) pada kepekatan 10% w/w manakala pengurangan adalah di antara (66.43-54.07%) pada kepekatan 5%, berbanding dengan antibiotik dan fungisid. Formulasi nano-emulsi berjaya meningkatkan ketinggian pucuk dengan signifikan (45%), kepanjangan akar (33%), berat kering ketinggian pucuk (65%) dan berat kering akar (59.3%) berbanding dengan kawalan positif dan negatif pada minggu ke-8 tumbesaran pokok.

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I certify that a Thesis Examination Committee has met on 12 May 2016 to conduct the final examination of Tavga Sulaiman Rashid on her thesis entitled "Antimicrobial Activity of *Rhus coriaria* L. Fruit Extracts against Selected Bacterial and Fungal Pathogens on Tomato" 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 Doctor of Philosophy.

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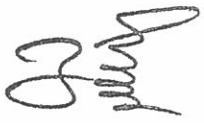

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy.
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This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated Rules 2003 (Revision 2012-2013) were adhered to.

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Signature:	
Name of Member of Supervisory Committee:	Associate Professor Dr. Jugah Kadir

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Name of Member of Supervisory Committee:	Associate Professor Dr. Halimi Mohd Saud

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APG	Alkyl Polyglucoside
cfu/ml	Colony forming unit per milliliter
$^{o}C\ min^{-1}$	Degree centigrade per minute
CRD	Completely Randomized Design
cm	Centimeter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTPs	Deoxynucleoside triphosphates
DPPH	2,2-diphenyl-1-picryl-hydrazyl
DW	Dry weight
EDTA	Ethylene Diamine Tetraacetic acid
EIC	Effective Inhibitory Concentration
FDW	Freeze- dried weight
Fwd_seq	Forward sequence
GC-MS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
Kb	Kilo base
KCl	potassium chloride
Kg	Kilogram

LCB	lactophenol-cotton blue
LSD	least significant difference
M	Molar
MeOH	Methanol
MBC	Minimum Bactericidal Concentration
MIC	Minimum inhibitory concentration
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
M	Molar
µl	Microlitre
Mm	Millmolar
Ml	Millilitre
mL min^{-1}	Millilitre per minute
µm	Micrometer
Mm	Milli molar
$MgCl_2$	Magnesium chloride
NA	Nutrient agar
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NB	Nutrient broth
Nm	Nanometer
NO	Nitric oxide
OD	Optical density
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
Ph	Logarithm of hydrogen ion activity
PIRG	Percentage inhibition radial growth
Rdna	Ribosomal deoxyribonucleic acid
Rev_name	Reverse name
Rev_seq	Reverse sequence
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscopy
± SEM	Standard error of means
Temp	Temperature
TBE	Tris- Borate- EDTA
Uv	Ultraviolet
V	Volt
v/v	Volume per volume
Ver	Version
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Lycopersicon esculentum, or the tomato plant, of the *Solanaceae* family is considered one of the most important vegetable in the world. It is an economically attractive crop among farmers due to its relatively quick maturity and high yield. The economic importance of tomato includes domestic consumptions, exports and food industries. Production of tomatoes tends to be more successful in highland areas in Malaysia, mainly due to the milder temperatures there. Hence, Cameron Highlands is a popular location for large-scale tomato cultivation. Only well-nourished plants that are free from diseases can results in satisfactory crops of top-grade tomatoes.

In many parts of the world, plant disease is the limiting factor for tomato production. There are around 200 known diseases (Agrios, 2005) that afflict tomato crops and around 70 % of all major crop diseases can be traced to infestation by fungi and bacteria (Janisiewicz et al., 2001).

In the area of disease control, chemicals have been proven to be expensive and at times ineffective. It is therefore important to come up with some sort of biological control for such diseases. This biological control should also encompass economic attributes to make it commercially viable. In crop protection, biological control methods are on the uptrend as an alternative to chemical fungicides to control diseases in vegetable crops caused by fungal and bacterial plant pathogens. This is lined to concerns related to environmental and human safety, plant pathogens progressively gaining resistance to chemicals and increasing regulations of chemical pesticide use (Elliott et al., 2009).

Natural plant products, also known as botanical pesticides, have long been utilized in stemming microorganisms from causing plant and human diseases. The use of synthetic and botanical pesticides in agriculture has significantly diminished in recent years. Though extremely effective, synthetic pesticides often have detrimental side effects such as animal toxicity and environmental pollution. Of the herbal medicine and botanical pesticides, much attention has been focused on the extracts of phenolic-rich plants. Tannins are important water-soluble plant phenolics that have been traditionally used as medicine to treat infectious human diseases (Schofield et al., 2001**;** Tegegne, 2008**)** and display antimicrobial activities against phytopathogenic fungi and bacteria **(**Chung, 1998**)**.

In order to create an improved agro-based product with new promising compounds, it is imperative to have a pesticide formulation, similar to commercial fungicides, that are formulated with active ingredients made up of inert materials that: are stable during production, processing and storage; assist application; protect the active compounds from unfavourable environmental conditions and promote activity on the target (Pindi

and Satyanarayana, 2013). Nanoemulsion is a useful technology for delivering chemical compounds across the cuticle. The efficacy of this approach is based on the small size of nanoemulsion droplets, the large surface area of the emulsion, the low surface tension of the entire system, and the low interfacial tension of the droplets (Bouchemal et al., 2004).

The stability of antimicrobial agents during distribution and storage, assistant to handling and application of the product, protection against adverse environmental factors and enhancement of microbial agents in the field are among the important issues during formulation development (Nisisako et al., 2005). There is very little information available on pesticide formulations and their efficacy even though studies on pesticides formulation have received much emphasis as with herbicides. It is imperative to create pesticide formulations that are efficient and environmental friendly.

Scientists have recently found that *R. coriaria* contains high levels of phenolic mixtures (Kossah et al., 2010); and also rich in oleic acids, vitamins, minerals and organic acids (Kossah et al., 2009). Prior research has been conducted on the use of *R. coriaria* for certain applications related to antibacterial properties against human pathogens (Nasar-Abbas and Halkman, 2004; Kosar et al., 2007), antifungal (Hashem and Alamri, 2010), antioxidant, anti-inflammatory/chondroprotective (Panico et al., 2009), anti-ischemic, vasorelaxant (Baretta et al., 2009), etc. To date, there have been several reports on the control of human pathogens using *R. coriaria*, but there is as yet no reports for the biological properties against plant pathogens and nanoemulsion prepared from *R. coriaria* extract; hence, the great interest in studying this indigenous plant.

The objectives of the study:

The general objective of the study was to investigate possible antimicrobial activities of *Rhus coriaria* crude extracts and its nano-emulsion formulation for the control of tomato diseases.

1. To identify common tomato bacterial and fungal pathogens in Cameron Highlands Malaysia and to test their pathogenicity.
2. To determine the antimicrobial properties of *R. coriaria* fruit extracts against selected bacterial and fungal tomato diseases.
3. To determine the phytochemical constituents in the active crude extracts of *R. coriaria.*
4. To formulate and characterise the physical properties of nano-emulsion using *R. coriaria* crude extract.
5. To evaluate the antimicrobial effect of nano-emulsion formulation on selected bacterial and fungal tomato diseases under glasshouse condition.

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