



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi



**NANOEMULSION FORMULATION OF *Vernonia amygdalina* Delile
AGAINST *Botrytis cinerea* CAUSING GRAY MOLD DISEASE IN TOMATO
AND THEIR EFFECTS ON POSTHARVEST QUALITY**



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi

By

SITI FAIRUZ BINTI YUSOFF

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

April 2022



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi



05-4506832



pustaka.upsi.edu.my



Perpustakaan Tuanku Bainun
Kampus Sultan Abdul Jalil Shah



PustakaTBainun



ptbupsi



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

**NANOEMULSION FORMULATION OF *Vernonia amygdalina* Delile
AGAINST *Botrytis cinerea* CAUSING GRAY MOLD DISEASE IN TOMATO
AND THEIR EFFECTS ON POSTHARVEST QUALITY**

By

SITI FAIRUZ BINTI YUSOFF

April 2022

Chair : Siti Idera Ismail, PhD
Faculty : Agriculture

Gray mold disease caused by *Botrytis cinerea* is one of the significant postharvest losses mostly observed during tomato storage and transportation. Synthetic fungicide is currently used to control this disease, but it poses adverse effects to human health, environment, and development of resistance to synthetic fungicides. *V. amygdalina* extract showed antifungal activity which could be a sustainable tool as biofungicide for plant disease management. The present study aimed to screen phytochemical compounds in *V. amygdalina* leaf extract, develop nanoemulsion formulations containing *V. amygdalina* crude extract, evaluate antifungal activities of nanoemulsions against *B. cinerea* and preserve postharvest quality. Ten fungal isolates were obtained from symptomatic tomato fruits sampled from Cameron Highlands, Pahang, Malaysia. Fungal colonies on PDA appeared cottony and white to gray color. Conidia were ovoid in shape, hyaline, and measured $10.03\text{-}16.08 \times 7.37\text{-}11.15 \mu\text{m}$. To confirm molecular identification, the primer pair ITS4/ITS5 of rDNA was used for amplification and sequencing of isolates. The sequences with GenBank accession MT012053-MT012062 were the closest match to *Botrytis cinerea* with query coverage was 98-99%. Based on pathogenicity assay, the isolates indicated highly pathogenic with the maximum disease severity was 90% (Isolate MT012058). *In vitro* test showed leaf extracts of aqueous, hexane, dichloromethane (DCM) and methanol at 100-500 mg/mL were significantly inhibited mycelial growth of *B. cinerea*. DCM was the most effective, which inhibited up to 75.74% of the mycelial growth of *B. cinerea*. The top major chemical compounds identified in DCM extract using GC-MS analysis were squalene, phytol, triacontane, heptacosane, and neophytadiene. For *in vivo* bioassay, the fruits treated with dichloromethane extract at 400 and 500 mg/mL showed the lowest disease incidence with mild severity of infection. The SEM observation proved that the treatment altered the fungal morphology, which leads to fungal growth inhibition. The nanoemulsion system containing oil, water and surfactant was obtained using spontaneous emulsification technique by





employing four surfactants. From eight selected formulations, two formulations, F5 and F7 showed stability in storage, remarkable thermodynamic stability, small-sized droplet (66.44 and 139.63 nm), highly stable in zeta potential (−32.70 and −31.70 mV), low in polydispersity index (0.41 and 0.40 Pdl), low in viscosity (4.20 and 4.37 cP) and low in surface tension (27.62 and 26.41 mN/m) compared to other formulations. *In vivo* efficacy on tomato fruits showed F5 formulation had a fungicidal activity against *B. cinerea* with zero disease incidence and severity, whereas F7 formulation reduced 62.5% disease incidence compared to a positive control with scale 1. F5 and F7 nanoemulsions exhibited higher enzyme activities of PAL, POD, and SOD compared to benomyl and control fruits. Meanwhile, F5 nanoemulsion triggered significantly higher PPO and CAT activities compared to F7 nanoemulsion. F5 nanoemulsion showed delays in fruit maturity, minimal weight loss, slower changes in firmness, TA, SSC and pH, retained the vitamin C content, fair in phenolic content and execute high antioxidant activities. In conclusion, F5 nanoemulsion has a fungicidal effect on *B. cinerea*, induces higher defense-enzymes activities, and gives optimum postharvest quality in tomato. Thus, F5 nanoemulsion containing *V. amygdalina* leaf extract could be useful for inhibiting gray mold disease on tomato fruit and has the potential as a natural antifungal agent.





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

**FORMULASI NANOEMULSI *Vernonia amygdalina* Delile TERHADAP
Botrytis cinerea YANG MENYEBABKAN PENYAKIT KULAT KELABU
DALAM TOMATO DAN KESANNYA KE ATAS KUALITI LEPAS TUAI**

Oleh

SITI FAIRUZ BINTI YUSOFF

April 2022

Pengerusi : Siti Izera Ismail, PhD
Fakulti : Pertanian

Penyakit kulat kelabu yang disebabkan oleh *Botrytis cinerea* adalah satu kehilangan lepas tuai ketara yang sering diperhatikan semasa penyimpanan dan pengangkutan tomato. Racun kulat sintetik sering digunakan untuk mengawal penyakit ini tetapi ia menimbulkan kesan buruk terhadap kesihatan manusia, persekitaran, dan meningkatkan kerintangan terhadap racun sintetik. Ekstrak *V. amygdalina* menunjukkan aktiviti antikulat yang boleh menjadi alat mampan sebagai biofungisida untuk pengurusan penyakit tumbuhan. Kajian ini bertujuan untuk menyaring sebatian fitokimia dalam ekstrak daun *V. amygdalina*, membangunkan formulasi nanoemulsi yang mengandungi ekstrak mentah *V. amygdalina*, menilai aktiviti antikulat nanoemulsi terhadap *B. cinerea* dan memelihara kualiti lepas tuai. Sepuluh pencilan kulat telah diperolehi daripada buah tomato bergejala yang diambil dari Cameron Highlands, Pahang, Malaysia. Koloni kulat pada PDA kelihatan seperti kapas dan berwarna putih ke kelabu. Konidia berbentuk ovoid, hialin, dan berukuran $10.03\text{-}16.08 \times 7.37\text{-}11.15 \mu\text{m}$. Untuk mengesahkan identifikasi molekul, pasangan primer ITS4/ITS5 dari rDNA digunakan untuk amplifikasi dan penjujukan pencilan. Jujukan dengan GenBank aksesori MT012053-MT012062 adalah berpadanan paling hampir dengan *Botrytis cinerea* dengan liputan kuir adalah 98-99%. Berdasarkan ujian patogenik, pencilan menunjukkan patogenik sangat tinggi dengan keparahan penyakit maksimum iaitu 90% (Pencilan MT012058). Ujian *in vitro* menunjukkan ekstrak daun akueus, heksana, diklorometana (DCM) dan metanol pada 100-500 mg/mL telah menghalang pertumbuhan miselia *B. cinerea* dengan ketara. DCM adalah yang paling berkesan menghalang pertumbuhan miselia *B. cinerea* sehingga 75.74%. Sebatian kimia utama teratas yang dikenal pasti dalam ekstrak DCM menggunakan analisis GC-MS ialah squalena, fitol, triakontana, heptakosana, dan neofitadina. Untuk ujian bio *in vivo*, buah yang dirawat dengan ekstrak diklorometana pada 400 dan 500 mg/mL menunjukkan kejadian penyakit yang paling rendah dengan keterukan jangkitan yang ringan. Pemerhatian SEM membuktikan bahawa rawatan telah mengubah morfologi kulat, yang membawa





kepada perencatan pertumbuhan kulat. Sistem nanoemulsi yang mengandungi minyak, air dan surfaktan diperoleh menggunakan teknik pengemulsi spontan dengan menggunakan empat surfaktan. Daripada lapan formulasi terpilih, dua formulasi, F5 dan F7 menunjukkan stabil dalam simpanan, kestabilan termodinamik yang luar biasa, titisan bersaiz kecil (66.44 dan 139.63 nm), sangat stabil dalam potensi zeta (-32.70 dan -31.70 mV), rendah dalam indeks polidispersiti (0.41 dan 0.40 Pdl), kelikatan rendah (4.20 dan 4.37 cP) dan tegangan permukaan rendah (27.62 dan 26.41 mN/m) berbanding formulasi lain. Keberkesanan *in vivo* pada buah tomato menunjukkan formulasi F5 mempunyai aktiviti racun kulat terhadap *B. cinerea* dengan sifar kejadian dan keterukan penyakit, manakala formulasi F7 mengurangkan 62.5% kejadian penyakit berbanding kawalan positif dengan skala 1. Nano-emulsi F5 dan F7 menunjukkan lebih tinggi aktiviti enzim PAL, POD, dan SOD berbanding dengan buah benomil dan kawalan. Sementara itu, nanoemulsi F5 mencetuskan aktiviti PPO dan CAT yang lebih tinggi berbanding nanoemulsi F7. Nanoemulsi F5 menunjukkan kelewatan dalam kematangan buah, penurunan berat minimum, perubahan keanjalan, TA, SSC dan pH yang lebih lambat, mengekalkan kandungan vitamin C, kandungan fenolik yang baik dan menunjukkan aktiviti antioksidan yang tinggi. Kesimpulannya, nanoemulsi F5 mempunyai kesan fungisida ke atas *B. cinerea*, mendorong aktiviti enzim pertahanan yang lebih tinggi dan memberikan kualiti lepas tuai yang optimum tomato. Oleh itu, nanoemulsi F5 yang mengandungi ekstrak daun *V. amygdalina* berguna untuk menghalang penyakit kulat kelabu pada buah tomato dan berpotensi sebagai agen antikulat semulajadi.





ACKNOWLEDGEMENTS

Alhamdulillah, I am very grateful for this wonderful journey. I am indebted and grateful especially to Dr. Siti Izera Ismail, Dr. Farah Farhanah Harun and Prof. Dr. Mahmud Tengku Muda Mohamed for their great supervision and support, invaluable guidance, tireless advice, constructive comments, and patience throughout my study. All of you are really inspired me to be a successful lecturer, proactive researcher and good academic writer. Then, I would like to acknowledge SLAB, Ministry of Higher Education Malaysia, for awarding scholarship sponsor to complete this study. My sincere gratitude goes to the Human Resource and Faculty of Technical and Vocational, UPSI to give me a full-time study leave with allowance. It is an utmost pleasure to thank my laboratory colleagues (Safari, Indah, Mazumder, Sirah, Rohasmizah, Ainnur, Shahara and Waziri) and PG roommates for their friendship, support and ideas that make my PhD journey very enjoyable. I am delighted and grateful to know all of you. To my lovely circles (Cik Mai, Pika Conco and Jimah), thank you for always being there for me, especially during my late pregnancy, giving me endless support, and those beautiful moments that we have spent together. Last but not least, my most profound appreciation to my husband (Zulkefli Abdullah) for his sacrifice, care, love and ongoing support. Also, I am blessed to have a little princess (Zara Aisyah) in May 2021 after 12 years of waiting. This success would not have been realized without my Happy Family Members (Father: Hj. Yusoff; Siblings: Dr. Zabri, Zanariah, Zainura, Zakiah, Shahrum Nizam, and Siti Noor Adila; In-laws and also my lovely nephews and nieces). Million thanks for your trust, sincere love, continuous encouragement and financial support. Al-fatihah to my late mother, Hajah Remlah Derahman.





I certify that a Thesis Examination Committee has met on 12 April 2022 to conduct the final examination of Siti Fairuz Binti Yusoff on her thesis entitled Nanoemulsion formulations of *Vernonia amygdalina* against *Botrytis cinerea* causing gray mold disease in tomato and their effects on postharvest quality 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 Doctoral of Philosophy.

Members of the Thesis Examination Committee were as follows:

Nur Azura Adam, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Noor Azmi Shaharuddin, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ganesan Vadamalai, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Elhadi Yahia, PhD

Professor
Facultad De Ciencias Naturales,
Mexico Universidad Aut Noma De Quer Taro
Avenida De Las Ciencias S/N, Juriquilla Quer Taro
Mexico
(External Examiner)

Zuriati Ahmad Zulkarnain, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:





This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Siti Izera binti Ismail, PhD

Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Norhayu binti Asib, PhD

Senior lecturer, Ts.
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Siti Zaharah binti Sakimin, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Farah Farhanah binti Haron, PhD

Deputy Director
Biological Control Programme
Agrobiodiversity & Environment Research Centre
Malaysian Agricultural Research and Development Institute (MARDI)
(Member)

ZALILAH MOHD SHARIFF, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 August 2022





Declaration by Graduate Student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any institutions;
- intellectual property from the thesis and the copyright of the thesis are fully-owned by Universiti Putra Malaysia, as stipulated in the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from the supervisor and the office of the Deputy Vice-Chancellor (Research and innovation) before the thesis is published in any written, printed or electronic form (including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials) as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld in accordance with the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software



Signature: _____ Date: _____

Name and Matric No.: Siti Fairuz Binti Yusoff, GS48580





Declaration by Members of Supervisory Committee

This is to confirm that:

- the research and the writing of this thesis were done under our supervision;
- supervisory responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

Signature: _____

Name of Chairman
of Supervisory
Committee:

Siti Izera binti Ismail,

Signature: _____

Name of Member of
Supervisory
Committee:

Norhayu binti Asib

Signature: _____

Name of Member of
Supervisory
Committee:

Siti Zaharah binti Sakimin

Signature: _____

Name of Member of
Supervisory
Committee:

Farah Farhanah binti Haron



TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xv
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xx
 CHAPTER	
 1 INTRODUCTION	 1
 2 LITERATURE REVIEW	 4
2.1 <i>Lycopersicon esculentum</i> Mill	4
2.1.1 Botanical Description and Taxonomy of the Tomato	4
2.1.2 Tomato Production and Health Benefits	5
2.1.3 Tomato Losses Due to Pathological Damage	6
2.2 Postharvest Fungal Diseases of Tomato	6
2.3 Gray Mold Disease Caused by <i>Botrytis cinerea</i>	8
2.3.1 Disease Impact, Symptoms, Signs and Etiology	8
2.3.2 Host Range	9
2.3.3 Epidemiology	9
2.3.4 Pathogen Biology	10
2.3.5 Pathogenesis	13
2.3.6 Genetic Diversity and Virulence	15
2.4 Strategies to Control <i>B. cinerea</i> Infection	16
2.4.1 Cultural Control	17
2.4.2 Physical Control	17
2.4.3 Fungicides	18
2.4.4 Induced Systemic Resistance	19
2.4.5 Biological Control Agents	19
2.4.6 Plant-Based Compound	20
2.4.7 Integrated Control	20
2.5 <i>Vernonia amygdalina</i> (Bismillah)	21
2.5.1 Botanical Description and Uses	21
2.5.2 Phytochemical Constituents	23
2.5.3 Potential of <i>V. amygdalina</i> as Antifungal Agent and Their Toxicity	25
2.6 Biofungicide	25



2.6.1	Plants as Source of Biochemical Fungicides	26
2.6.2	Biofungicide Formulation	26
2.6.3	Emulsion Formulations	29
2.6.4	Nanoemulsions and Their Materials	30
2.6.5	Preparation and Characterization of Nanoemulsion	31
2.7	Biofungicides Application on Postharvest Fruits	33
2.7.1	Effect of Biofungicide on Postharvest Quality of Fruit	34
2.7.2	Effect of Biofungicide on Antioxidant Properties	34
2.7.3	Effect of Biofungicide on Defense-related Enzymes	35
3	MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF <i>B. cinerea</i> ISOLATED FROM TOMATO FRUITS	37
3.1	Introduction	37
3.2	Materials and Methods	38
3.2.1	Sample Collection	38
3.2.2	Isolation of <i>B. cinerea</i>	38
3.2.3	Morphological Characterization of <i>B. cinerea</i>	38
3.2.4	Molecular Identification of <i>B. cinerea</i> Based on Ribosomal DNA Internal Transcribed Spacer (rDNA-ITS)	39
3.2.5	Pathogenicity Test	41
3.2.6	Experimental Design and Statistical Analysis	42
3.3	Results	43
3.3.1	Gray Mold Disease Symptoms	43
3.3.2	Identified <i>Botrytis</i> Species Based on Morphological Characteristics	43
3.3.3	Molecular Identification of <i>B. cinerea</i>	46
3.3.4	Pathogenicity Assay	47
3.4	Discussion	51
3.5	Conclusion	53
4	ANTIFUNGAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF <i>V. amygdalina</i> LEAF EXTRACT AGAINST <i>B. cinerea</i>	54
4.1	Introduction	54
4.2	Materials and Methods	55
4.2.1	Plant Material Preparation	55
4.2.2	Preparation of <i>V. amygdalina</i> Crude Extracts	56
4.2.3	Preparation of <i>B. cinerea</i>	57
4.2.4	<i>In vitro</i> Evaluation for <i>V. amygdalina</i> Antifungal Activity	58





4.2.5	Microscopic Observation Using a Scanning Electron Microscope (SEM)	58
4.2.6	Antifungal Activities of <i>V. amygdalina</i> by <i>In vivo</i> Bioassay	59
4.2.7	Screening for Chemical Constituents of <i>V. amygdalina</i> Hexane, Dichloromethane and Methanol Crude Extracts Using Gas Chromatography-Mass Spectrometry (GCMS) Analysis	61
4.2.8	Screening for Chemical Constituents of <i>V. amygdalina</i> Aqueous Extract Using Liquid Chromatography-Mass Spectrometry (LCMS) Analysis	61
4.2.9	Experimental Design and Statistical Analysis	62
4.3	Results	62
4.3.1	Yield of <i>V. amygdalina</i> Crude Extract	62
4.3.2	<i>In vitro</i> Antifungal Activities of <i>V. amygdalina</i> Crude Extract Against <i>B. cinerea</i>	62
4.3.3	Effect of <i>V. amygdalina</i> Crude Extract on the Morphology of <i>B. cinerea</i>	64
4.3.4	<i>In vivo</i> Antifungal Activities of <i>V. amygdalina</i> Crude Extract Against <i>B. cinerea</i>	65
4.3.5	Phytochemical Screening of <i>V. amygdalina</i> Hexane, Dichloromethane and Methanol Crude Extracts Using GCMS	67
4.3.6	Phytochemical Screening of <i>V. amygdalina</i> Aqueous Extract Using LCMS-QTOF	71
4.4	Discussion	75
4.5	Conclusion	78

5 EMULSION FORMULATION DEVELOPMENT AND CHARACTERIZATION OF DICHLOROMETHANE *V. amygdalina* LEAF EXTRACT

5.1	Introduction	80
5.2	Materials and Methods	81
5.2.1	Materials for Formulation Components	81
5.2.2	Preparation of Emulsion by Ternary Phase Diagram	82
5.2.3	Selection of Emulsion Formulation Composition from Ternary Phase Diagrams	82
5.2.4	Characterization of Emulsion Formulation	82





5.2.5	Experimental Design and Statistical Analysis	84
5.3	Results	84
5.3.1	Ternary Phase Diagrams of Emulsion Formulations	84
5.3.2	Point Selection of Emulsion	88
5.3.3	Characterization of Emulsion Formulations	88
5.4	Discussion	91
5.5	Conclusion	94
6	IN VIVO EFFICACY OF <i>V. amygdalina</i> NANOEMULSION FORMULATION IN CONTROLLING GRAY MOLD DISEASE AND INDUCIBLE OF DEFENSE-RELATED ENZYMES IN TOMATO FRUITS	95
6.1	Introduction	95
6.2	Materials and Methods	96
6.2.1	<i>In vivo</i> Efficacy of Selected Nanoemulsion Formulations on Artificially Inoculated Tomato Fruits	96
6.2.2	Extraction of Tomato Tissues Enzyme	97
6.2.3	Determination of Protein Content	97
6.2.4	Determination of Peroxidase Activity	98
6.2.5	Determination of Superoxide Dismutase Activity	98
6.2.6	Determination of Catalase Activity	99
6.2.7	Determination of Polyphenol Oxidase Activity	100
6.2.8	Determination of Phenylalanine Ammonia Lyase Activity	100
6.2.9	Experimental Design and Statistical Analysis	100
6.3	Results	101
6.3.1	<i>In vivo</i> Efficacy of <i>V. amygdalina</i> Nanoemulsion Formulations Against <i>B. cinerea</i> in Tomato	101
6.3.2	Effect of <i>V. amygdalina</i> Nanoemulsion Formulations on Defense Enzymes Activities in Tomato Fruit as Indicator of Resistance Mechanism	103
6.4	Discussion	107
6.5	Conclusion	110
7	EFFECTS OF <i>V. amygdalina</i> NANOEMULSION FORMULATION ON POSTHARVEST QUALITY AND ANTIOXIDANT ACTIVITIES OF TOMATO DURING STORAGE	111
7.1	Introduction	111
7.2	Materials and Methods	112





7.2.1	Application of Selected Nanoemulsion Formulations on Artificial Inoculated Tomato Fruits and Storage Condition	112
7.2.2	Determination of Postharvest Physical Quality Characteristics	113
7.2.3	Determination of Postharvest Chemical Quality Characteristics	115
7.2.4	Determination of Antioxidants	116
7.2.5	Experimental Design and Statistical Analysis	118
7.3	Results	119
7.3.1	Effect of <i>V. amygdalina</i> Nanoemulsion Formulations on Physical Quality Characteristics	119
7.3.2	Effect of <i>V. amygdalina</i> Nanoemulsion Formulations on Chemical Quality Characteristics	122
7.3.3	Effect of <i>V. amygdalina</i> Nanoemulsion Formulations on Antioxidants	123
7.4	Discussion	129
7.5	Conclusion	133
8	SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	134

**REFERENCES****APPENDICES****BIODATA OF STUDENT****LIST OF PUBLICATIONS**

137

186

188

189





LIST OF TABLES

Table		Page
2.1	<i>Botrytis cinerea</i> classification	11
2.2	Virulence factors of <i>B. cinerea</i>	16
2.3	Chemical fungicides against gray mold disease: mode of action, intrinsic toxicity and application rate	18
2.4	Ethnomedicine uses of <i>V. amygdalina</i> based on plant parts and extraction method	22
2.5	Isolated compounds and bioactivities of <i>V. amygdalina</i>	24
2.6	Commercial biopesticides of plant extract	28
3.1	The size of conidiophore, conidia and sclerotia of <i>Botrytis</i> isolates	46
3.2	Identity of isolates based on ITS region comparison with nucleotide sequences from GenBank	47
3.3	Aggressiveness description based on lesion diameter and disease severity for <i>B. cinerea</i> isolates on tomato after 5 days of inoculation	48
4.1	Main and interaction effects of <i>V. amygdalina</i> crude extracts and concentration level on PIRG of <i>B. cinerea</i> . PIRG: percentage of radial growth	63
4.2	Percentage of <i>B. cinerea</i> incidence on tomato treated fruits	66
4.3	Percentage of disease severity index on tomato treated fruits. DSI: Disease severity index.	66
4.4	Chemical composition in hexane, DCM and methanolic extract of <i>V. amygdalina</i> .	69
4.5	Identified compounds in <i>V. amygdalina</i> aqueous extract by LCMS-QTOF analysis	73
5.1	Components used in the formulation development	81
5.2	Selected points component composition without the active ingredient	88
5.3	Emulsion formulations composition with DVLE as the active ingredient	89
5.4	Homogeneity and stability of emulsion formulations after centrifugation, storage, and thermostability test	90
5.5	Physical characteristics of emulsion formulations	91





6.1	Amount of reagent used in the test tube (T), control tube (C), blank tube (B1), and blank tube (B2)	98
6.2	Main and interaction effects of formulation types and storage duration on POD, SOD, CAT, PPO, and PAL activities in tomato	104
7.1	Main and interaction effects of formulation types and storage duration on weight loss and firmness of tomato	121
7.2	Main and interaction effects of formulation types and storage duration on TA, SSC, and pH of tomato	123
7.3	Main and interaction effects formulation types and storage duration on vitamin C and TPC of tomato	124
7.4	Main and interaction effects formulation types and storage duration on DPPH, ABTS, and FRAP of tomato	126
7.5	Correlation between antioxidants and antioxidant activities of the treated tomato	128



LIST OF FIGURES

Figure		Page
2.1	Top 10 countries for tomato production in 2019	5
2.2	Gray mold lesion on tomato fruit. (A) After 3 days infected (B) After 8 days infected	8
2.3	Infection of <i>B. cinerea</i> on plant host	10
2.4	Life cycle of asexual and sexual stage of <i>B. cinerea</i>	12
2.5	Stages of conidial germination of <i>Botrytis cinerea</i> on <i>Eucalyptus urograndis</i> leaves using SEM. (a and b) conidia with 2-4 short germ tubes at 6 hours after inoculation (hai); (c) formation of an infection cushion-like structure (*) at 6 hai; (d) direct fungal penetration, as evidenced by the thickened hyphae curved at their tips, at 12 hai	14
2.6	Droplet structure of an amphiphile stabilizing oil-in-water and water-in-oil emulsions	29
2.7	Preparation of high energy emulsification method and low energy emulsification method	32
3.1	Fruit storage condition after inoculation	42
3.2	Gray mold symptom on tomato fruits sampled from Cameron Highlands	43
3.3	Influence of temperature on mycelial growth of <i>B. cinerea</i> isolated from disease tomato fruits. Growth after 5 days on PDA	44
3.4	Front view (left) and reverse view (right) of <i>B. cinerea</i> on PDA after 7 days incubation	44
3.5	Formation of sclerotia of <i>Botrytis cinerea</i> isolates on PDA. (A) three-week-old culture with grayish mycelium containing a few sclerotia formation; (B) four-week-old culture showing small whitish to grayish sclerotia scattered on the PDA surface; (C) five-week-old culture, the sclerotia turned to larger harden and black in color	45
3.6	Morphological characteristics of <i>B. cinerea</i> . (A) Branched mycelium under light microscope at 4x magnification; (B) Conidiophore producing blastoconidia at 10x magnification; (C) Conidia at 40x magnification	45
3.7	Amplified PCR products with ITS4/ITS5 Primers and electrophoresed in 1 % Agarose Gel. The bands of PCR products were approximately 550 bp. Lane P1, P2, P3, P4, P5, P6, P7, P8, P9, and P10 indicate DNA form	47



Botrytis spp. Isolated from Cameron Highlands. M is referred to 1 kb DNA ladder (Promega, USA)

3.8	Gray mold disease symptoms on tomato fruits from day 2-5 after inoculation with spores of <i>B. cinerea</i>	49
3.9	Disease severity levels of <i>B. cinerea</i> isolates on artificial inoculated tomato. (A to C) Highly pathogenic; (D to I) Moderately pathogenic; (J and K) Mild Pathogenic; and (L) Symptomless on control fruits after 5 days of inoculation	50
4.1	Appearance of plant material prepared for crude extract. (a) Fresh leaves of <i>V. amygdalina</i> harvested from plant; (B) Dried leaves; and (C) Powdered leaves	56
4.2	Sequential extraction procedure of <i>V. amygdalina</i>	57
4.3	Diagrammatic scale for evaluation of gray mold severity on tomato fruits	60
4.4	Effect of crude extraction of <i>V. amygdalina</i> at the various concentration on PIRG of <i>B. cinerea</i> after eight days of incubation	64
4.5	Effects of dichloromethane (DCM) crude extract on <i>B. cinerea</i> at 400 and 500 mg/mL on mycelium morphology viewed under SEM. (A) Healthy mycelium are slender and uniform, with a smooth surface and an intact structure in the control plate; (B) Healthy conidiophore from the control plate; (C) Mycelia were ruptured, folded with edge burrs, and sheet-like structure at 400 mg/mL; (D) The hyphae tip was wrinkled and deformed at 400 mg/mL; (E) Agglutinated mycelia at 500 mg/mL; (F) The conidia were shrunken at 400 mg/mL	65
4.6	Ion chromatograms of hexane (A), DCM (B) and methanolic (C) crude extract using GCMS analysis	67
4.7	Ion Chromatogram of aqueous crude extract using LCMS-QTOF	72
5.1	Steps of surface tension measuring by du Nouy Ring method	83
5.2	Phase diagram of Glucopon 225: AMD810: water system	85
5.3	Phase diagram of Glucopon 215: Agnique AMD810: water system	86
5.4	Phase diagram of 50% Agnique MBL510 and Agnique 50% MBL530: Agnique AMD810: water system	87
5.5	Phase diagram of Agnique MBL530: Agnique AMD810: water system	87





5.6	Phase diagram of Agnique MBL510: Agnique AMD810: water system	87
6.1	Effect of formulation types on gray mold (A) Disease incidence and (B) Disease severity index in tomato fruits after 12 days of storage	102
6.2	Effect of formulation types on gray mold of tomato fruits	103
6.3	Effect of formulation types on POD specific activity in tomato fruits after 12 days of storage	105
6.4	Effect of formulation types on SOD specific activity in tomato fruits after 12 days of storage	105
6.5	Effect of formulation types on CAT specific activity in tomato fruits after 12 days of storage	106
6.6	Effect of formulation types on PPO specific activity in tomato fruits after 12 days of storage	107
7.1	Maturity indices of tomatoes as described by Malaysian Standard (MS 893:2010)	114
7.2	Effect of formulation types on maturity indices of tomato fruits in 12 days of storage	120
7.3	Relationships between weight loss and storage duration of formulation treated tomato. F5 nanoemulsion (\blacktriangle)= $0.01+0.28x+0.0007x^2$ ($R^2=0.97$), F7 nanoemulsion (x)= $0.11+0.17x+0.014x^2$ ($R^2=0.99$), benomyl (\blacksquare)= $-0.02+0.42x+0.006x^2$ ($R^2=0.99$), and control (\blacklozenge)= $0.26-0.19x+0.084x^2$ ($R^2=0.99$)	122
7.4	Effect of formulation types on TPC of tomato fruits during 12 days of storage	125
7.5	Effect of formulation types on DPPH radical scavenging of tomato fruits during 12 days of storage	127
7.6	Effect of formulation types on FRAP of tomato fruits during 12 days of storage	128





LIST OF ABBREVIATIONS

%	percent
<	less than
>	greater than
≤	less than equal
°C	degree celcius
μM TE/g FW	micromolar Trolox Equivalent per gram fresh weight
a.i	active ingredient
ABTS	2, 2-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid
ACQ	acquisition
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCA	biological control agent
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C ₂ H ₃ NaO ₂ . 3H ₂ O	sodium acetate anhydrous
C ₂ H ₄ O ₂	acetic acid
CA	control atmosphere
CAT	catalase
CE	crude extract
CFB	corrugated fiber board
CL	concentration levels
cm	centimeter
CO ₂	carbon dioxide
cP	centipoise
CRD	completely randomized design
CS	capsule suspension
DCM	dichloromethane
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DOA	Department of Agriculture





DP	dust powder
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	dressing seed
DSI	disease severity index
DVLE	DCM <i>V. amygdalina</i> leaf extract
EDTA	ethylenediaminetetraacetic acid
EIP	emulsion inversion point
ESI	electrospray ionization
FAO	Food Agriculture Organization
FDA	Food and Drug Administration
FeCl ₃ .6H ₂ O	iron trichloride hexahydrate
FRAP	ferric reducing antioxidant power
FT	formulation types
g	gram
g/kg	gram per kilogram
g/L	gram per liter
GCMS	Gas Chromatography-Mass Spectrometry
GR	granule
GRAS	generally recognized as safe
h	hour
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
hai	hour after inoculation
HCl	hydrochloric acid
HLB	hydrophilic-lipophilic balance
HPLC	High Performance Liquid Chromatography
HR	hypersensitive response
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemistry
JA	jasmonic acid
K ⁺	potassium ion
kb	kilobyte
kg	kilogram





KJ m ⁻²	kilojoule per square meter
kPa	kilopascal
kV	kilovolt
L	Liter
LC	liquid Chromatography
LCMS	Liquid Chromatography-Mass Spectrometry
LSD	Least significant difference
M	Molar
m/s	meter per second
m/z	mass-to-charge ratio
MA	modified atmosphere
MAE	microwave-assisted extraction
MeJA	methyl jasmonic acid
mg	milligram
MG	micro granules
mg/kg	milligram per kilogram
mg/kg/day	milligram per kilogram per day
mg/mL	milligram per milliliter
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MI	maturity index
min	minute
mL	milliliter
mL/min	milliliter per minute
mm	millimeter
mM	millimolar
mN/m	millinewton per meter
mPa.S	millipascal per second
MPWL	maximum permissible weight loss
MS	mass spectrometer
mV	millivolt
MΩ.cm	megohm centimeter
N	newton





NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	nanometer
O/W	oil-in-water
O ₂	oxygen
OD	oil dispersion
<i>p</i>	probability
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDA	potato dextrose agar
pDI	polydispersity index
PIC	phase inversion composition
PIRG	percentage inhibition of radial growth
PIT	phase inversion temperature
POD	peroxidase
PPO	polyphenol oxidase
psi	pound per square inch
PVPP	polyvinylpolypyrrolidone
QTOF	Quadrupole time-of-flight
R ²	R square
rDNA	ribosomal DNA
RH	relative humidity
ROS	reactive oxygen species
rpm	revolutions per minute
s	second
SA	salicylic acid
SAS	statistical analysis system
SC	suspension concentrate
SD	storage duration
SE	suspo-emulsion
SEM	scanning electron microscopy
SOD	superoxide dismutase





sp.	species
SSC	soluble solids concentration
TA	titratable acidity
TBE	tris-borate-EDTA
TPC	total phenolic content
TPTZ	2, 4, 6- tripyridyl-s-triazine
u/mg	unit per milligram
U/ μ L	unit per microliter
UV	ultraviolet
UV-C	ultraviolet-C
UV-VIS	UV–visible spectrophotometry
v/v	volume per volume
v/v/v	volume per volume per volume
w/kg	watt per kilogram
W/O	water-in-oil
w/v	weight per volume
w/w	weight per weight
WG	water-dispersible granules
WP	wettable powder
μ g/mL	microgram per milliliter
μ L	microliter
μ m	micrometer
μ M	micromolar





CHAPTER 1

INTRODUCTION

Tomato is a Solanaceae family member and is extensively cultivated as an annual vegetable crop worldwide, either in the greenhouse or in the open-field system. The global tomato industry for both fresh and processed has sharply increased in the past five decades (Heuvelink, 2018). FAO (2021) revealed that in 2019, Asia dominates world tomato production up to 62.02%, followed by the Americas (13.16%), Europe (12.62%), Africa (11.98%), and Oceania (0.23%). Tomatoes in Malaysia are the second highest vegetable production value after brassica and the largest planted area is in Cameron Highland (DOA, 2019). Among the tomato type, the classic round is the most popular variety that is consumed in salad, soup, grilling, baking and sauces (Heuvelink, 2018). Besides that, the tomato provides significant antioxidants such as lycopene, carotenoid, vitamins and phenolic compounds that are beneficial to human health (Salehi et al., 2019).

Botrytis cinerea is an air-borne plant pathogenic fungus with a necrotrophic lifestyle that can infect dicotyledonous plant species, including tomato. Subjected to scientific and economic importance, *B. cinerea* was ranked as the second top plant pathogen in the world (Dean et al., 2012). *B. cinerea* infection is considered the primary pathogen of harvested tomatoes. *B. cinerea* causes gray mold in fruit, affects vegetative tissues, postharvest decay, or remains latent until storage.

Spore germination of this pathogen grows vigorously in higher relative humidity and low temperature (Leyronas et al., 2015). Thus, in cold storage, it leads to the development of gray mold symptoms, and this disease spreads rapidly among fruits in the same packaging. Multiple applications of chemical fungicides are made per season to control gray mold on tomato fruits, but the repeated use of synthetic fungicide can develop fungal resistance and be harmful to consumer health. *Botrytis* species can develop resistance to multiple modes of action. A few modes of action from a new fungicide provide adequate protection for fresh tomatoes (Siviero et al., 2003). However, the residue and toxicity concerns may limit their use.

Among the postharvest strategies in controlling plant diseases, natural products offer a promising treatment to reduce the disease incidence of postharvest diseases. Natural products from plant-based contain advanced chemical novelty compared to chemically synthesized products, and for this reason, researchers try to discover new bioactive compounds in plants (Ma et al., 2015). Plant extracts also contain beneficial secondary metabolites such as phenolics, tannins, coumarins, quinones, flavonoids, saponins, terpenoids, and alkaloids. These compounds have been proven to be potentially significant in plant protection as antimicrobial agents (Compean and Ynalvez, 2014). Many studies





have been conducted using plant extracts to control *B. cinerea* pathogen causing gray mold disease. Soylu et al. (2010) reported that essential oils extracted from rosemary and lavender could cause hyphae shriveled, protoplast leakage, conidia loss, and cytoplasmic coagulated on the morphology of *B. cinerea*. The extracted essential oil of fennel, cinnamon, and anis also showed fungicidal effects on *B. cinerea* during *in vitro* and *in vivo* tests (Mohammadi et al., 2012). Moreover, the extraction of oregano and lemon effectively lowered the disease severity of gray mold disease in tomatoes, strawberries, and cucumbers (Vitoratos et al., 2013). In recent findings, stilbene extracted from grapevine leaves possessed antifungal activity of *B. cinerea* by inhibiting the mycelium growth and simultaneously reducing the necrotic lesion (De Bona et al., 2019).

Bitter leaf is scientifically known as *Vernonia amygdalina*. In Africa and Asia, it is commonly used as a medicinal plant (Alara et al., 2017). Various parts of *V. amygdalina*, including the leaf, root, and stem, have been used for their antidiabetic, antioxidant, antimicrobial, anticancer, anti-inflammatory, and antiparasmodial effects (Kadiri and Olawoye, 2016). Among the plant parts, researchers identified that the leaf part contains the highest chemical constituents and nutritional compositions (Toyang and Verpoorte, 2013). Detailed investigations in the compound purification of *V. amygdalina* extract discovered many promising active compounds; for example, flavonoids, triterpenoids, saponins, tannins, sesquiterpene lactones, alkaloids, terpenes, phenolics, and steroidal glycosides (Alara et al., 2017). According to Akowuah et al. (2015), the extract from *V. amygdalina* was non-toxic in mice when exposed up to 2000 mg/kg/day for 28 days.

To date, most researchers have focused on *V. amygdalina* crude extract in order to uncover its potential as an antifungal agent for the management of plant disease. Recent findings found that *V. amygdalina* ethanol extracts showed a good ability to inhibit postharvest fungal pathogens *Rhizopus stolonifer* and *Fusarium moniliforme* (John et al., 2016; Okey et al., 2016). In another study, an *in vitro* test using an ethanol crude extract of *V. amygdalina* at 300 mg/mL were completely inhibited the growth of *Cercospora persica* and *Curvularia lunata* obtained from leafspot disease of ground nut (Ilondu, 2013).

The active ingredient in this precious medicinal plant should be innovated and upgraded into an efficient plant-based biopesticide to compete with the synthetic pesticides in the current market. Thus, the formulation should be reliable in terms of handling, storage life, and competency (Asib et al., 2015). Nowadays, nanotechnology in biopesticides is getting attention due to outstanding characteristics, including smaller droplet size that is efficient in delivery target, good in stability in varied storage temperature, low surface tension to allow them to stick better and widely spread on the target (Fakari and Nezamzadeh-Ejhieh, 2017; Mukhopadhyay, 2014). Besides this, technology also could decrease their quantity use and be non-toxic to the ecosystem as well as human health (Khandelwal et al., 2016).





Up to now, there is no comprehensive study on the effects of fungicide formulation from *V. amygdalina* extract on gray mold disease control in tomato at postharvest and their application effects on postharvest quality, antioxidants and changes of resistance mechanism of the fruits. Therefore, the general research aimed was to develop nanoemulsion formulation of *V. amygdalina* crude extract on gray mold disease control in tomato and their effects on the changes of postharvest physicochemical quality of fruits during storage. The specific objectives were:

1. to isolate and identify *Botrytis cinerea* isolates causing gray mold disease on tomato based on morphological and molecular characteristics;
2. to evaluate antifungal activities of *V. amygdalina* leaf extracts against *B. cinerea* and to screen the phytochemical compounds in the crude extracts;
3. to formulate and characterize nanoemulsion formulation of *V. amygdalina* leaf extract;
4. to evaluate the *in vivo* efficacy of *V. amygdalina* nanoemulsion formulation in controlling gray mold disease on tomato fruits and their effects on host defense-related enzymes activities; and
5. to determine the effect of *V. amygdalina* nanoemulsion formulation on the postharvest quality and antioxidants of tomato during storage.

