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

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

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Chair : Siti Izera Ismail, PhD
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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 10.03-16.08 × 7.37-11.15 µm. 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 PdI), 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

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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 10.03-16.08 × 7.37-11.15 µm. Untuk mengesahkan identifikasi molekular, pasangan primer ITS4/ITS5 dari rDNA digunakan untuk amplifikasi dan penjujukan pencilan. Jujukan dengan GenBank aksesi MT012053-MT012062 adalah berpadanan paling hampir dengan *Botrytis cinerea* dengan liputan kuiri 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 PdI), 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 antioksida 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.

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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.

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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
$C_2H_3NaO_2.\ 3H_2O$	sodium acetate anhydrous
$C_2H_4O_2$	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 *V. amygdalina* leaf extract
EDTA	ethylenediaminetetraacetic acid
EIP	emulsion inversion point
ESI	electrospray ionization
FAO	Food Agriculture Organization
FDA	Food and Drug Administration
$FeCl_3.6H_2O$	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_2O	water
H_2O_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 mililiter
$MgCl_2$	magnesium chloride
MgSO4	magnesium sulphate
MI	maturity index
min	minute
mL	mililiter
mL/min	mililiter per minute
mm	milimeter
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
p	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/µL	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
µg/mL	microgram per milliliter
µL	microliter
µm	micrometer
µM	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 used 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 antiplasmodial 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 to 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 *Cercosporella persica* and *Curvularia lunatus* 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.