

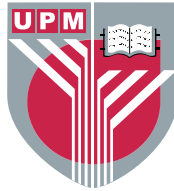


UNIVERSITI PUTRA MALAYSIA

***DEVELOPMENT OF GINGER ESSENTIAL OILS-NANOBACTERICIDES
FOR CONTROLLING BACTERIAL LEAF BLIGHT DISEASE OF RICE
CAUSED BY *Xanthomonas oryzae* pv. *oryzae****

ADAMU ABDULLAHI

FP 2022 31



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ADAMU ABDULLAHI

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

June 2022

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DEDICATION

This thesis is dedicated to my parents, Alh. (Mal) Abdullahi Muhammad Gada and Mlm. Fatima Abubakar Gada, as well as my entire family.



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

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ADAMU ABDULLAHI

June 2022

Chairman : Associate Professor Khairulmazmi bin Ahmad, PhD
Faculty : Agriculture

The bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) have been found to be the major rice disease that causes significant loss of yield upon infection. The susceptible plants can result in the production losses of up to 50%. Scientists and farmers are increasingly paying more attention to the development of plant-based nanobactericides for disease management in order to lessen overdependence on chemical pesticides. A series of laboratory experiments were conducted in order to promote this development. These experiments concentrated on the extraction and characterization of the phytochemical compounds found in ginger essential oils (EOs), the suppression of the development of Xoo biofilm, and microscopic examinations. It was followed by nanobactericide evaluation of the antibacterial activity of Xoo. Three blocks were made for each treatment and three plants per replicate in a completely randomized block design. The essential oils (EOs) were extracted from fresh ginger rhizomes using a modified Clevenger-type apparatus by hydro-distillation, which were purchased from local producers in Bentong, Pahang. The chemical compositions of the EOs were then discovered and profiled using the analytical techniques of gas chromatography-mass spectroscopy (GCMS) and headspace procedures. Chemical compounds present in the EOs were monoterpenes such as trans-caryophyllene, camphene, geranial, eucalyptol, and neral, as well as sesquiterpene hydrocarbons mainly α -zingiberene, ar-curcumen, β -bisabolene, β -sesquiphellandrene which differ in their composition and concentrations. Food poisoned and disc diffusion techniques were applied on the tested pathogens to determine the percentage inhibition of fungal mycelial and bacterial growth inhibition, respectively. The EOs produced mycelial growth inhibition in all the test fungal pathogens which include (*Fusarium oxysporum*, *Pyricularia oryzae*, *Colletotrichum falcatum*, *Ganoderma boninense*, and *Rigidoporus microporus* *Fusarium oxysporum*, *Pyricularia oryzae*, *Colletotrichum falcatum*, *Ganoderma boninense*, and *Rigidoporus microporus*) after five days of incubation. The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of the EOs on the tested fungi were in the range of 1- 3 μ L/mL and 5-6 μ L/mL, respectively. The bacterial growth of all the tested isolates (*Xanthomonas oryzae* pv.

oryzae- strain A, *X. oryzae* pv. *oryzae*- strain B, *Ralstonia solanacearum*, *Bacillus* sp. and *Klebsiella* sp.) was also affected by EOs at 100-500 $\mu\text{L/mL}$, from weak to strong antibacterial activity. The inhibition zone of positive control (streptomycin) at 15 $\mu\text{g/disc}$ was 25.00 mm and appeared to be efficient. The MIC values of the EOs were in the range of 100 $\mu\text{L/mL}$ to 200 $\mu\text{L/mL}$. The LC₅₀ value resulting from exposure to the EOs varied among fungal and bacterial pathogens. Metabolomics analysis to concurrently quantify variability among multiple compounds in the data sets and identify such compounds responsible for the *Xoo* inhibition were determined. The cross validated PLS model has shown a strong correlation between ginger EOs and bioactivity. The action of ginger EOs on the cell structure of *Xoo* was fully identified using scanning electron microscope (SEM), transmission electron microscope (TEM) and confocal laser scanning microscope (CLSM) as well as biofilm formation. It was done by observing the changes in morphology and integrity of *Xoo* cells. The Dimethyl sulfoxide (DMSO) treatment (control) showed a normal rod shape cell, while treatment with the ginger EOs showed irregular shape with sunken surfaces, and treatment with antibiotics display abnormal growth of the cells. The ginger EOs display a wide range of activity against the pathogen. The biofilm of the *Xoo* strain has been formed. The EOs showed different optical density values measured due to the formation of *Xoo* biofilms and the inhibition percentage of *Xoo* biofilm. The results showed that the control (broth containing untreated *Xoo* cells) had the highest mean optical density value with a strong biofilm formation (2.459 O.D), followed by 31.25 $\mu\text{L/mL}$ treatment. The inhibition percentage showed that the maximum inhibition was 76.33% at a concentration of 500 $\mu\text{L/mL}$. When the concentration of EOs was decreased, the *Xoo* biofilm inhibition declined. Problems of volatility, solubility, and stability of EOs lead to the use nanotechnology via nanoemulsions approach. The preparation of nano-emulsion of ginger EOs have prepared. A ternary phase diagram (TPD) was developed using a low-energy method based on nonionic surfactant Tween 20, water, and EOs as the active ingredient. Four formulations of nanobactericides coded as A1, A2, A3 and A4 were chosen from the single-phase ternary phase diagram for preliminary screening after constructing TPD. They were tested for their stability and thermostability over time at 54°C and 28°C. Finally, A4- is the best formulation that displays a single, low viscous and watery phase that is stable following physiochemical property measures. Usage of the developed nanobactericides have been applied both *in-vitro* and *in-vivo* to evaluate its efficiency to the pathogens. The *in-vitro* antibacterial activity of the nanobactericides was measured by the presence or absence of inhibition zones. The growth of the tested pathogens was affected by the different concentration of the formulation ranging from 50-125 $\mu\text{L/mL}$. The findings showed significant antibacterial activity against *Xoo*, *Burkholderia glumae* *Ralstonia solanacearum*, and least effective against *Erwinia chrysanthemi* at higher concentrations. Glasshouse application showed that the disease symptoms decrease more gradually to nanobactericides due to its phytoconstituents. Three concentrations of formulation were tested containing 75, 100, and 125 $\mu\text{L/mL}$. Findings of research revealed that treatment 125 $\mu\text{L/mL}$ was the best treatment based on suppression of disease severity index, area under the disease progress curve (AUDPC) value as well as plant height, physiological and yield parameters when compared to the positive control. In conclusion, developed nanobactericides could be used as a new antimicrobial agent in suppressing the growth of *Xoo in-vitro* and suppress disease severity index of bacterial leaf blight disease *in-vivo* trials and as a promising new alternative to synthetic bactericides.

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

**PEMBANGUNAN MINYAK PATI HALIA-NANOBAKTERISID UNTUK
MENGAWAL PENYAKIT HAWAR DAUN BAKTERIA PADI YANG
DISEBABKAN OLEH *Xanthomonas oryzae* pv. *oryzae***

Oleh

ADAMU ABDULLAHI

Jun 2022

Pengerusi : Profesor Madya Khairulmazmi bin Ahmad, PhD
Fakulti : Pertanian

Penyakit hawar daun bakteria (BLB) yang disebabkan oleh *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) merupakan penyakit padi utama yang mengakibatkan kehilangan hasil yang ketara. Pada masa ini, pembangunan nanobakterisid-berasaskan tumbuhan telah mendapat perhatian daripada para saintis dan petani untuk mengawal penyakit ini dan seterusnya mengurangkan kebergantungan kepada racun kimia. Untuk merangsang perkembangan ini, satu siri eksperimen makmal telah dijalankan. Penekanan diberikan kepada pengekstrakan dan pencirian sebatian fitokimia yang terdapat dalam minyak pati halia, diikuti dengan penindasan pembentukan biofilm *Xoo* dan pemeriksaan mikroskopik, pembangunan nanobakterisid dan penilaian aktiviti antibakteria patogen tumbuhan. Minyak pati halia adalah cecair volatil berminyak dengan banyak juzuk dan mempunyai aktiviti antimikrob yang ketara. Minyak pati diekstrak menggunakan peralatan jenis *Clevenger* yang diubahsuai oleh *hydro-distillation* kemudian diikuti dengan kaedah analisis *gas chromatography-mass spectroscopy* (GCMS) dan prosedur *headspace* untuk mengesan dan menentukan profil dan komposisi fitokimia. Fitokimia yang terdapat dalam minyak pati adalah *monoterpenes* seperti *trans-caryophyllene*, *camphene*, *geranial*, *eucalyptol*, dan *neral*, serta hidrokarbon *sesquiterpene* terutama *α -zingiberene*, *ar-curcumene*, *β -bisabolene* dan *β -sesquiphellandrene* yang berbeza komposisi dan kepekatan. Teknik media beracun dan difusi cakera digunakan terhadap fitopatogen yang diuji untuk menentukan peratusan perencatan miselium kulat dan perencatan pertumbuhan bakteria. Minyak pati menghasilkan perencatan pertumbuhan miselium bagi kesemua patogen kulat yang diuji selepas lima hari inkubasi. Nilai MIC dan MFC pada kulat yang diuji adalah dalam julat 1->3 $\mu\text{L/mL}$ dan 5-6 $\mu\text{L/mL}$. Pertumbuhan bakteria untuk semua isolate fitopatogen yang diuji juga berkesan pada 100-500 $\mu\text{L/mL}$, daripada lemah kepada aktiviti antibakteria yang kuat. Minyak pati berupaya mengawal *Xoo*-strain-A pada kepekatan yang rendah kepada lebih tinggi masing-masing pada 400-500 $\mu\text{L/mL}$ dengan purata perencatan masing-masing 20.66 mm dan 22.66 mm. Zon perencatan kawalan positif (streptomycin) pada 15 $\mu\text{g/cakera}$ adalah 25.00 mm dan kelihatan sangat berkesan. Nilai-nilai MIC adalah dalam julat 100-> 200 $\mu\text{L/mL}$. Nilai LC_{50} yang terhasil daripada pendedahan kepada minyak pati berbeza

di kalangan patogen kulat dan bakteria. Analisis metabolomik untuk mengukur kepelbagaian di kalangan sebatian dalam set data dan mengenalpasti sebatian yang bertanggungjawab untuk perencatan *Xoo* telah ditentukan. Model PLS yang telah divalidasi silang telah menunjukkan hubungkait yang kuat antara minyak pati halia dan bioaktiviti. Tindakan minyak pati halia pada struktur sel *Xoo* telah dikenalpasti sepenuhnya menggunakan *scanning electron microscope* (SEM), *transmission electron microscope* (TEM) dan *confocal laser scanning microscope* (CLSM) serta pembentukan biofilm. Ia dilakukan dengan memerhatikan perubahan morfologi dan integriti sel *Xoo*. Rawatan DMSO (kawalan) menunjukkan sel berbentuk rod normal, manakala rawatan dengan minyak pati halia menunjukkan bentuk yang tidak teratur dengan permukaan tenggelam, dan rawatan dengan antibiotik pula memaparkan pertumbuhan sel-sel yang tidak normal. Minyak pati halia memaparkan pelbagai aktiviti terhadap patogen. Biofilm *Xoo* telah terbentuk. Minyak pati memberi nilai ketumpatan optik yang berbeza disebabkan pembentukan biofilm *Xoo* dan peratusan perencatan biofilm *Xoo*. Keputusan menunjukkan kawalan (larutan yang mengandungi sel-sel *Xoo* yang tidak dirawat) mempunyai nilai kepadatan optik tertinggi dengan pembentukan biofilm yang kuat (2.459), diikuti oleh rawatan 31.25 $\mu\text{L/mL}$. Peratusan perencatan menunjukkan bahawa perencatan maksimum adalah 76.33% pada kepekatan 500 $\mu\text{L/mL}$. Apabila kepekatan minyak pati berkurangan, perencatan biofilm *Xoo* juga merosot. Masalah turun naik, kebolehlaksanaan, dan kestabilan minyak pati membawa kepada penggunaan nanoteknologi melalui pendekatan nano-emulsi. Penyediaan nano-emulsi minyak pati halia telah disediakan. Gambar rajah fasa ternary (TPD) dibangunkan menggunakan kaedah tenaga rendah berdasarkan gabungan Tween-20, air, dan minyak pati bukan ionik sebagai bahan aktif. Empat rumusan nanobakterisid yang dikodkan sebagai A1, A2, A3 dan A4 dipilih daripada gambar rajah fasa *single-phase ternary* untuk saringan awal selepas membina TPD. Ia diuji dari segi kestabilan dan ketegakan dari masa ke semasa pada 54°C dan 28°C. Rumusan A2 dan A3 bertukar menjadi *milky solution* manakala baki dua formulasi, A1 dan A4 mengekalkan kestabilan sebagai cecair yang jernih telah dipilih untuk meneruskan ciri-ciri fisiokimia. Akhirnya, A4- adalah formulasi terbaik yang memaparkan fasa tunggal, likat rendah dan berpasir yang stabil berikutan langkah-langkah harta fisiokimia, termasuk kelikatan (19.0 ± 0.2), pH (4.2 ± 0.4), saiz zarah (73 ± 0.8), potensi zeta ($-43.43.42 \pm 0.38$), pdi (0.21 ± 0.30), ketegangan permukaan (30.90 ± 0.5) dan struktur/morfologi zarah. Penggunaan racun bakteria-nano yang dibangunkan telah digunakan dalam *in-vitro* dan *in-vivo* untuk menilai kecekapannya kepada patogen. Aktiviti antibakteria *in-vitro* bakteria-nano diukur dengan kehadiran atau ketiadaan zon perencatan. Pertumbuhan patogen yang diuji terjejas oleh kepekatan formulasi yang berbeza antara 50-125 $\mu\text{L/mL}$. Penemuan ini menunjukkan aktiviti antibakteria yang ketara terhadap *Xoo*, *Bulkholderia glumae* *Ralstonia solanacearum*, dan paling kurang berkesan terhadap *Erwinia chrysanthemi* pada kepekatan yang lebih tinggi. Aplikasi rumah kaca menunjukkan gejala penyakit berkurangan dengan lebih beransur-ansur kepada bakteria-nano kerana campuran kompleks tersebut. Tiga kepekatan rumusan telah diuji mengandungi 75, 100, dan 125 $\mu\text{L/mL}$. Hasil penyelidikan mendedahkan rawatan 125 $\mu\text{L/mL}$ adalah rawatan terbaik berdasarkan penindasan indeks keterukan penyakit, nilai AUDPC serta pertumbuhan, parameter fisiologi dan hasil jika dibandingkan dengan kawalan positif. Kesimpulannya, pembangunan bakteria-nano boleh digunakan sebagai agen antimikrob baru dalam menindas pertumbuhan *Xoo in-vitro* dan menyekat indeks keterukan penyakit hawar daun bakteria dalam percubaan *in-vivo* dan sebagai alternatif baru kepada bakteria sintetik.

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

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Declaration by Members of Supervisory Committee

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LIST OF ABBREVIATIONS

%	Percent
&	And
±SD	Standard deviation of means
°	Degree
°C	Celsius
μL	microliter
ABI	Agro Biotechnology Institute
AI	active ingredient
ANOVA	Analysis of variance
AUDPC	Area under Disease Progress Curve BLB Bacterial leaf Blight
Cm	Centimeter
CRD	Complete Randomize Design
Conc.	Concentrations
CLSM	Confocal Laser Scanning Microscopy
CW	Control with water
DAI	Days After Inoculation
DAS	Days After Sowing
DMT	Domestic
DMSO	Dimethyl sulfoxide
DSI	Disease Severity Index
EOS	Essential oils
<i>et al.</i>	<i>et alli</i> , and others
FAO	Food and Agricultural

GC-MS	Gas Chromatography-Mass Spectrophotometry
i.e	<i>example gratia</i> , for example
IBS	Institute of Bioscience
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
mg	milligram
Mins	Minutes
ML	Milliliters
Mm	millimeters
mN/m	millinewtons per meter
mPas	Millipascal-second
NA	Nutrient Agar
NB	Nutrient Broth
NFO-AI	Nanoformulation- Active ingredients nm Nanometer
NIBM	National Institutes of Biotechnology Malaysia No. Number
O/W	Oil-in-water
PBD	Potato Dextrose Broth
PDA	Potato dextrose agar
PDI	Polydispersity Index
pH	Logarithm of hydrogen ion activity
PIRG	Percentage Inhibition Radial Growth
PSGI	Percentage Spore Germination Inhibition
pv	Pathovar
PEO	Pure essential oils
Rpm	Revolution per minute

RCBD	Randomized Complete Block Design RT Retention Times
SEM	Scanning Electron Microscopy
SI	Similarity Index
SIMCA	Sugud Islands Marine Conservation Area spp species
TC	Treatment control 1
TD	Treatment control 2
TE	Treatment control 3
TF	Treatment control with antibiotics
TA	Treatment control with pathogen
TEM	Transmission Electron Microscopy
Tw	Tween 20
UPM	Universiti Putra Malaysia
v/v	volume per volume
Ver.	Version
Viz	<i>videlicet</i> , that is, namely
Vol.	Volume
w/v	weight/volume
w/w	weight/weight
WLD	Wild
<i>Xoo</i>	<i>Xanthomonas oryzae</i>

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Rice (*Oryza sativa*) is the staple food crop globally and major important foodstuff of Asia (Kagale *et al.*, 2004) including Malaysia (Vaghefi *et al.*, 2011). Pathogenic microbes in plants are the major cause of plant diseases which lead to huge crop losses, especially in tropical regions. Fungal diseases of rice like sheath blight and bacterial blight caused by *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae*, respectively poses a serious problem which significantly affects rice productions and food security. The safety in food production play a very vital role, being food production not only to feed the growing world population but also one of the primary drivers of economy (Ali *et al.*, 2016; Son *et al.*, 2020).

Bacterial blight disease is the most important disease that affects the cultivation/production of rice in Malaysia. The BLB infections of susceptible plants can result in severe production losses of up to 50%. However, at the greatest tillering stage, yield losses are reduced by 20–40%, whereas early infection stage losses are seen at about 50%, thus, it led to crop losses (Chompa *et al.*, 2022). It is challenging to control this plant disease despite recent agricultural advancements. This is because readily available synthetic chemicals have a very limited ability to treat diseases without causing significant side effects. (Shaheen and Issa, 2020). These synthetic chemicals are attributed to the negative effects on the environment such as significant soil and water pollutions, long periods of degradation, residual accumulation in the food chain and less control efficacy against pathogenic microbes with long-term usage (Ghormade *et al.*, 2011; Nega, 2014; Bhavaniramya *et al.*, 2019). The existing antibiotics and copper-based fungicides are actually prohibited for agricultural practices in many countries of the world due to their adverse effects including severe or extreme pathogens resistance, higher cost of production, prolonged cycles of chemicals degradation, and environmental pollutions (Hajano *et al.*, 2012; Sundin *et al.*, 2016; Buttmer *et al.*, 2017). Moreover, Streptomycin was the primary antibiotic used in the management of plant diseases. It is sprayed on plants at a concentration of 0.5 g/L. Although it is only advised as an emergency measure for blight under the integrated disease and pest management schedule (IDIPM), farmers frequently use this antibiotic carelessly to prevent the occurrence of bacterial blight (Sharma *et al.*, 2022). But, later, the phytopathogens resistant to Streptomycin emerged and developed (Xu *et al.*, 2010). Therefore, these limitations heavily affected the control efficiency of plant diseases in the agriculture production system (Juroszek and Von Tiedemann, 2011; Dara, 2019). Nevertheless, the disease management systems are of the major concern with a strong need to look for the novel and potential natural antimicrobial agents or biopesticides that can be effective, non-toxic, and ecologically safe to control plant diseases. Research into alternative pesticides and antimicrobials, including natural plant products such as ginger essential oils, has tripled recognition. The introduction of new generation/innovation of biopesticides could provide solutions for the pathogenic microbes that have developed resistance to the available synthetic chemicals (Saha *et al.*, 2016).

It became evident from nowadays researches that these natural products have the ability to influence the modern agrochemical solutions for their biological and antimicrobial activities (Abdel-Kader *et al.*, 2015) as well as economic viability and low toxicity (Brusotti *et al.*, 2014). They are also well known for their antibacterial, antiviral, antifungal activities and as an insect repellent (Venkateshwarlu, 2014; Sendanayake *et al.*, 2017; Azhari *et al.*, 2017), biodegradable and generally embraced by many societies. Thus, it is a new solution in the plant protection sector (Lim *et al.*, 2012; Lanzotti *et al.*, 2013). Ginger essential oils (EOs) have demonstrated excellent antimicrobial activity against pathogenic microorganisms as it was well documented (Debbarma *et al.*, 2013; Nikolic *et al.*, 2014; Wonni *et al.*, 2016; Mostafa, 2018). However, because of their low water solubility and high sensitivity to oxygen, moisture, heat, and light make their uses very limited. Advancement in science and technology have emerged as solutions to these emerging challenges to improve their stability, water-solubility, and protect it from UV light degradation. The ginger EOs nano-based delivery systems such as nanoemulsions is one of the models for enclosure of these natural bioactive compounds to enhance antimicrobial activity. Thus, it can bring active compounds to the desired site, shield them from adverse environmental factors and increase their effectiveness (Donsi and Ferrari, 2016). Recently, nanoemulsions have become increasingly important as potential vehicles for the controlled delivery and optimized dispersion of active ingredients, particularly the ginger EOs. The penetration of the active ingredient is increased due to the small droplet with its high surface area, which allows the active to be efficiently transported to the desired parts of the plant (Ostrosky *et al.*, 2015). It is one of the promising delivery systems used in many field of researches such as plant protection in agricultural sector (Ram *et al.*, 2014) with a great advantages over typical emulsions in which droplets are smaller in sizes (Lee *et al.*, 2016). The system resulted in displaying greater spreadability, superior mechanical stability, and wettability (Nair *et al.*, 2010., Diaz-Blancas *et al.*, 2016).

Studies have been performed and solutions have been found on plant disease control, yet various diseases have been persisted, invariably incurring crop loss annually. In Malaysia, bacterial and fungal species had been isolated from various crops, but there is need to subject them for antimicrobial testing using ginger EOs as an alternative way that is eco-friendly for the management of rice diseases. Therefore, the recent effort is directed towards extraction and characterization of ginger EOs as well as control efficacy of developed nanobactericides in controlling *X. oryzae* pv. *oryzae* and this may serve as one of the new tools for controlling the BLB disease of rice. This is considered an interesting alternative to synthetic chemical control and to explore hidden potentiality of the herbs for its antimicrobial activity against the phytopathogenic bacteria.

1.2 Objectives of Studies

The main objective of this study is to develop nanobactericides through nanoemulsion approach to control *X. oryzae* pv. *oryzae* towards sustainable disease management.

The specific objectives are:

1. To extract essential oils, profile its chemical components, and determine the antibacterial efficacy against important phytopathogens from domestic and wild gingers.
2. To determine the mechanism of action of ginger EOs against *X. oryzae* pv. *oryzae*.
3. To develop and characterize nanobactericides formulated from ginger EOs
4. To evaluate the disease control efficacy of the developed nanobactericides both *in vitro* and *in-vivo* trials.

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