# BIOFUNGICIDAL POTENTIAL OF SELECTED PLANT EXTRACTS AGAINST FRUIT ROT PATHOGENS OF BANANA, TOMATO AND MANGO

By

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

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**Chair : Nur Ain Izzati Mohd Zainudin, PhD**
**Faculty : Science**

Fruit rot caused by several fungal pathogens can be considered as a threat to economic loss due to quality defect and quantity loss, besides constitute health risk to the consumers due to mycotoxin contamination produced by the pathogens. Frequent and unselective use of fungicide to control the pathogens has ended up to resistant development of the pathogens and increase toxic accumulation in fruits. Previous studies reported that plant extracts might contain variety of bioactive constituents that able to control the pathogen's growth. Hence, the aims of this study were to screen antifungal activity of selected plant extracts against *Fusarium oxysporum, Fusarium proliferatum, Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* under *in vitro* condition, to examine morphological and cytological changes of the pathogens treated with selected plant extracts using scanning (SEM) and transmission (TEM) electron microscope, to evaluate the efficacy of the selected plant extracts against fruit rot disease and to identify phytochemical constituents of the selected plant extracts using Ultra High Performance Liquid Chromatography Mass Spectrometer (UHPLC MS/MS). The plant extracts of *Pilea microphylla, Peperomia pellucida, Persicaria odorata, Cymbopogon citratus, Tamarindus indica, Garcinia mangostana* and *Averrhoa bilimbi* were prepared using different types of solvents and *in vitro* screening was conducted using poisoned food bioassay. Eight out of 48 plant extracts showed high significant inhibition effect against mycelial growth of *F. oxysporum* and *F. proliferatum*, while no extracts showed high significant inhibition effect against *C. gloeosporioides* growth and no extracts showed significant inhibition effect against *L. theobromae* when compared to positive controls. The eight effective extracts were further used to examine their inhibition effect on conidial germination. The results showed *G. mangostana* pericarp and *A. bilimbi* fruit ethanolic extracts significantly lowered the conidial germination of *F. oxysporum* (14.33%) and (20.00%), *F. proliferatum* (28.33%) and (39.75%), *C. gloeosporioides* (14.67%) and (20.00%) and *L. theobromae* (18.89%) and (28.57%) when compared to the controls. Shrivelled mycelia were observed via SEM on pathogens treated with both plant extracts indicating morphological changes were occurred in the cell compared to the controls in which the mycelia were in normal form. Alterations in hyphae cellular structures of the treated pathogens were observed via TEM, indicating cytological changes occurred in the cell membrane when compared to the controls in which the

hyphae cells were in normal form. The selected plant extracts at different concentrations showed varied degrees in disease severity reduction percentages against all pathogens, especially to *F. oxysporum* and *F. proliferatum* that were inoculated on different type of fruits; banana, tomato and mango. *G. mangostana* pericarp ethanolic extract at concentration of 100 mg/mL exhibited the equivalent efficacy in supressing fruit rot disease in both banana and tomato fruits, while *A. bilimbi* fruit ethanolic extract at concentration of 100 mg/mL showed significant reduction in fruit rot development on mango when compared to fungicide carbendazim. Significant changes on fruit quality of banana and tomato were displayed by the treatment of *G. mangostana* pericarp ethanolic extract, while *A. bilimbi* fruit ethanolic extract treatment showed significant changes in fruit quality of mango, when compared to the control fruits. Identification of phytochemical constituents was exhibited the presence of some of vital component groups which contributed to the antifungal activity of the extracts. UHPLC MS/MS spectral analysis displayed 50 metabolites in the negative ion mode, while 68 metabolites were identified in the positive ion mode of *G. mangostana* pericarp ethanolic extract correspondingly. Meanwhile, 59 metabolites were tentatively identified in the negative ion mode, whereas 109 metabolites were identified in the positive ion mode of *A. bilimbi* fruit ethanolic extract respectively. The equivalent and greater effect of *G. mangostana* pericarp and *A. bilimbi* fruit ethanolic extracts when compared to fungicide carbendazim was due to the presence of phytochemical compounds that possessed antifungal properties. This innovation has potential to be applied as an eco-friendly and gentle approach to control fruit rot disease.

Keywords: fruit rot disease, fungal pathogens, plant extracts, TEM, SEM, UHPLC MS/MS

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**POTENSI BIOFUNGISIDA EKSTRAK TUMBUHAN TERPILIH TERHADAP PATOGEN PENYAKIT REPUT BUAH PISANG, TOMATO DAN MANGGA**

Oleh

**NUR BAITI BINTI ABD MURAD**

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Penyakit reput buah disebabkan oleh beberapa patogen kulat boleh dianggap sebagai ancaman terhadap kerugian ekonomi akibat kecacatan kualiti dan kehilangan kuantiti hasil, selain menyebabkan risiko kesihatan kepada pengguna akibat penghasilan mikotoksin oleh patogen. Penggunaan racun kulat yang kerap dan tidak selektif untuk mengawal patogen menyebabkan terjadinya kerintangan patogen dan pengumpulan sisa toksik dalam buah-buahan. Kajian terdahulu mendapati ekstrak tumbuhan mengandungi pelbagai sebatian bioaktif yang mampu mengawal pertumbuhan patogen. Oleh itu, tujuan kajian ini adalah untuk menyaring aktiviti antikulat ekstrak tumbuhan terpilih terhadap patogen reput buah iaitu *Fusarium oxysporum, Fusarium proliferatum, Colletotrichum gloeosporioides* dan *Lasiodiplodia theobromae* secara *in vitro*, untuk mengkaji perubahan morfologi dan sitologi pathogen yang dirawat dengan ekstrak tumbuhan terpilih menggunakan mikroskop elektron pengimbasan (SEM) dan pengaliran (TEM), untuk menilai keberkesanan ekstrak tumbuhan terpilih terhadap penyakit reput buah dan mengenal pasti sebatian fitokimia bagi ekstrak tumbuhan terpilih menggunakan Spektrometer Jisim Kromatografi Cecair Berprestasi Tinggi Ultra (UHPLC MS/MS). Ekstrak tumbuhan dari *Pilea microphylla, Peperomia pellucida, Persicaria odorata, Cymbopogon citratus, Tamarindus indica, Garcinia mangostana* dan *Averrhoa bilimbi* disediakan menggunakan pelbagai jenis pelarut dan saringan *in vitro* dijalankan menggunakan bioassai makanan beracun. Lapan daripada 48 ekstrak tumbuhan menunjukkan kesan perencatan yang sangat ketara terhadap pertumbuhan miselia *F. oxysporum* dan *F. proliferatum,* manakala tiada ekstrak menunjukkan kesan perencatan yang sangat ketara terhadap pertumbuhan *C. gloeosporioides* dan tiada ekstrak menunjukkan kesan perencatan ketara terhadap *L. theobromae* berbanding kawalan positif masing-masing. Lapan ekstrak yang efektif telah digunakan untuk mengkaji kesan perencatannya terhadap percambahan konidia. Hasil kajian menunjukkan bahawa ekstrak etanol kulit manggis dan buah belimbing buluh telah mengurangkan percambahan konidia *F. oxysporum* dengan ketara sebanyak (14.33%) dan (20.00%), *F. proliferatum* sebanyak (28.33%) dan (39.75%), *C. gloeosporioides* sebanyak (14.67%) dan (20.00%) dan *L. theobromae* sebanyak (18.89%) dan (28.57%) berbanding kultur kawalan. Pengecutan miselia diperhatikan melalui SEM pada patogen yang diberikan

kedua-dua ekstrak tumbuhan tersebut menunjukkan perubahan morfologi berlaku dalam sel berbanding kawalan di mana miselia berada dalam bentuk normal. Perubahan struktur selular hifa bagi patogen yang diberikan ekstrak tumbuhan yang sama diperhatikan melalui TEM dan menunjukkan perubahan sitologi berlaku dalam membran sel berbanding dengan kawalan di mana sel hifa berada dalam bentuk normal. Ekstrak tumbuhan yang terpilih dengan kepekatan yang berbeza menunjukkan peratusan pengurangan keparahan penyakit yang berbeza terhadap semua patogen terutamanya, *F. oxysporum* dan *F. proliferatum* yang diinokulasi pada buah yang berbeza iaitu pisang, tomato dan mangga. Ekstrak etanol kulit manggis pada kepekatan 100 mg/mL menunjukkan keberkesanan yang setara dalam menyekat penyakit reput buah pada buah pisang dan tomato, manakala ekstrak etanol buah belimbing buluh pada kepekatan 100 mg/mL menunjukkan pengurangan ketara dalam perkembangan reput buah mangga, jika dibandingkan dengan racun kulat carbendazim. Perubahan pada kualiti buah pisang dan tomato telah ditunjukkan oleh rawatan ekstrak etanol kulit manggis, manakala bagi buah mangga, oleh rawatan ekstrak etanol buah belimbing buluh berbanding buah kawalan masing-masing. Pengenalpastian sebatian fitokimia menunjukkan kehadiran beberapa kumpulan komponen penting yang menyumbang kepada aktiviti antikulat ekstrak berkenaan. Analisis spektrum UHPLC MS/MS menunjukkan 50 metabolit dalam mod ion negatif, manakala 68 metabolit telah dikenal pasti dalam mod ion positif bagi ekstrak etanolik kulit manggis. Manakala, 59 metabolit dikenal pasti secara tentatif dalam mod ion negatif, manakala 109 metabolit telah dikenal pasti dalam mod ion positif bagi ekstrak etanol buah belimbing buluh. Kesan setara dan lebih besar oleh ekstrak etanol kulit manggis dan buah belimbing buluh berbanding racun kulat carbendazim disebabkan oleh kehadiran sebatian fitokimia yang mempunyai sifat antikulat. Inovasi ini berpotensi untuk diaplikasikan sebagai pendekatan mesra alam dan selamat untuk mengawal penyakit reput buah.

Kata kunci: penyakit reput buah, patogen kulat, ekstrak tumbuhan, TEM, SEM, UHPLC MS/MS

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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:

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| | |
|---|---|
| SEM | Scanning Electron Microscope |
| TEM | Transmission Electron Microscope |
| UHPLC MS | Ultra High-Performance Liquid Chromatography Mass Spectrometry |
| °C | Degree Celcius |
| dai | day after inoculation |
| $ddH_2O$ | double distilled water |
| g | gram |
| g/mL | gram per millilitre |
| mg/mL | milligram per millilitre |
| mL | millilitre |
| L | litre |
| mg | milligram |
| $CaCl_2$ | Calcium Chloride |
| µL | microliter |
| PDA | Potato Dextrose Agar |
| SNA | Spezieller Nahrstoffarmer Agar |
| sp. | species |
| spp. | Species plural |
| t/ha/yr | ton/hectare/year |
| UV | Ultraviolet |
| % | Percent |
| DMSO | Dimethyl sulfoxide |
| CRD | Completely randomized design |
| FG | Fungal Growth |

| | |
|---|---|
| DC | Diameter of control |
| DR | Diameter of test |
| ΔE* | Total colour different |
| % FG | Percentage of inhibition of fungal growth |
| % PS | Percentage of spore germination |
| S | Number of spores germinated |
| B | Number of spores observed |
| C | Germination percentage of spores in negative control |
| T | Germination percentage of spores in treatment |
| CPD | Critical point dryer |
| $O_sO_4$ | Osmium tetroxide |
| RH | Relative humidity |
| LD | Lesion diameter |
| % PR | Percentage of disease severity reduction |
| N | Newton |
| S | Square measure |
| P | Firmness |
| N/kg | Newton per kilogram |
| °Brix | Measurement of dissolved sugar |
| L* | Degree of lightness or darkness |
| a*(+/-) | Degree of greenness or redness |
| b*(+/-) | Degree of blueness or yellowness |
| Yi | Yellowness index |
| μM | micrometer |
| mM | millineter |
| kHz | kilohertz |

| | |
|---|---|
| W | Watt |
| V | Volt |
| ESI | electrospray ionization |
| LC-MS QTOF | Liquid chromatography mass spectrometry quadrupole time of flight |
| FA | Formic acid |
| CID | Collision induced dissociation energy |
| m/z | Mass per charge number of ion |
| psi | Pound per square inch |
| L/min | Litre per minute |
| SE | Standard error |
| RT | Room temperature |
| H | Hot (90°C) |
| PM(L)E | *P. microphylla* (leaf) ethanol |
| PP(L)RT | *P. pellucida* (leaf) aqueous room temperature |
| PO(L)E | *P. odorata* (leaf) ethanol |
| CC(L)H | *C. citratus* (leaf) aqueous hot |
| CC(S)RT | *C. citratus* (stem) aqueous room temperature |
| CC(S)H | *C. citratus* (stem) aqueous hot |
| TI(L)RT | *T. indica* (leaf) aqueous room temperature |
| TI(L)H | *T. indica* (leaf) aqueous hot |
| TI(Pd)H | *T. indica* (pod) aqueous hot |
| TI(Pd)E | *T. indica* (pod) ethanol |
| TI(Pp)RT | *T. indica* (pulp) aqueous room temperature |
| TI(Pp)H | *T. indica* (pulp) aqueous hot |
| TI(Pp)E | *T. indica* (pulp) ethanol |
| GM(L)RT | *G. mangostana* (leaf) aqueous room temperature |

| | |
|---|---|
| GM(L)H | *G. mangostana* (leaf) aqueous hot |
| GM(L)E | *G. mangostana* (leaf) ethanol |
| GM(L)HEX | *G. mangostana* (leaf) hexane |
| GM(P)H | *G. mangostana* (pericarp) aqueous hot |
| GM(P)E | *G. mangostana* (pericarp) ethanol |
| GM(P)HEX | *G. mangostana* (pericarp) hexane |
| AB(L)RT | *A. bilimbi* (leaf) aqueous room temperature |
| AB(F)RT | *A. bilimbi* (fruit) aqueous room temperature |
| AB(F)H | *A. bilimbi* (fruit) aqueous hot |
| AB(F)E | *A. bilimbi* (fruit) ethanol extract |
| UV-Vis | Ultraviolet visible |
| IR | Infrared radiation |
| NMR | Nuclear magnetic resonance |
| nm | nanometre |
| FTIR | Fourier Transform Infrared Spectroscopy |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

Fruit crops are always at high risk of being attacked by various microorganisms. Fungal infections are one of the major causes of post-harvest rots of fresh fruits and vegetables whether in transit or storage with more than 70% of all crop diseases are caused by fungal infection (Satpute & Vanmare, 2017). A considerable amount of work has been carried out all over the world on the effect of plant extracts on quality and shelf life of various fruit and vegetable crops**.** Fruit rot cause significant economic losses in commercialization stage and are rendered unfit for human consumption (Malik et al., 2016).

The yield losses affected by post-harvest diseases are bigger than pre-harvest due to the cost of fresh fruits rising several folds while passing from the field to the market, then consumer (Mohajer et al., 2015). The post-harvest losses are estimated to range from 10% to 50% of the global production of fresh fruits and vegetables per year with approximately 20% of postharvest losses reported for fruits and vegetables in Malaysia (Mahmud, 2017; Singh et al., 2017; Iordăchescu et al., 2019). Reduction in fruit quantity and quality should be expected when estimating post-harvest disease losses as certain diseases may not reduce the unsalable product, but still, it can reduce their value in terms of physical appearance, shelf life and nutritional contents (Brauer et al., 2019).

A lot of chemical-based and synthetic compounds have been used as antimicrobial means to inhibit the phytopathogenic fungi. The fungicides application against fungal plant diseases recovers crop yield, quality, and shelf-life. The chemical fungicides have been used since decades to control plant diseases (Choudhury et al., 2018). Some examples of fungicides widely used in the fields including benzimidazoles, dithiocarbamates, strobilurins, and azoles (Brauer et al., 2019).

Many farmers resort to synthetic chemical fungicide as it promises the fast effect on controlling pathogens. However, excessive and frequent use of fungicide will cause toxic residual in environment, especially soil, water and in the fruit which may affect consumers' health and trigger resistance development in pathogens. Furthermore, the public concerns about food contamination with fungicidal deposits has significantly increased. Considering all these factors, the new, safe and biodegradable alternatives which are both effective and economically reasonable need to be developed (da Cruz Cabral et al., 2013).

## 1.2 Problem statement

Application of the chemical-based fungicides at higher concentration than the allowed one may raise the risk of high-level toxic deposits in the fresh yields, which is mainly serious because fruit and vegetables are consumed in a relatively short time after harvest (Malik et al., 2016). In addition, the pesticide residues on food indicated that fungicides possess more carcinogenic risk than insecticides and herbicides together as reported by The National Academy of Sciences (NAS) in 1986 (El-Khateeb et al., 2013). In addition, many pesticides used in agricultural sector have been banned by World Health Organization (WHO) due to their wide range of toxicity effect against non-target organisms including human and animal as well as causing environmental pollution such as soil and water pollution due to their non-biodegradable nature, thus, affects humans through the food chain (Castillo et al., 2010; Satpute & Vanmare, 2017).

Therefore, the consumers' demand for alternative techniques of controlling post-harvest diseases increases. Additionally, the use of many synthetic fungicides in crop protection that have various degrees of persistence has now been warned due to their carcinogenicity, teratogenicity, hormonal imbalance, spermatotoxicity and other remaining toxicities (Breda et al., 2016). Undoubtedly, the post-harvest care for fresh fruits and vegetables is difficult and complicated in which it is impacted by the location of the different producer economy backgrounds and the projected market either local, regional, national or international (Toivonen et al., 2014).

Besides, the risk of using fungicides during production of fruits and vegetables is assumed as a common issue. Consumption of raw fruits and vegetables increases the chances that contaminated yields may cause illness to the consumers. Numerous recommended strategies for decreasing the risk of fresh yields contamination with pathogens and chemically synthetic fungicides are particularly challenging to practice, especially in the developing countries (Toivonen et al., 2014).

In current years, several alternative approaches such as the use of plant extract has been known to produce an extensive variety of secondary metabolites. Moreover, plant fungal pathogens are using different strategies to attack and enter their host (Eloff & McGaw, 2014). The metabolites produced by plants are a promising alternative due to the presence of various constituents such as alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpens, phenylpropannes, and organic acids (da Cruz Cabral et al., 2013).

In regards to possessing a high variety of bioactive compounds, the plant extracts also have been thought to be able to inhibit the growth of different fungal genera and interrupt the defence system and resistance development of the pathogens as well as protect the hosts against pathogen's attack via different modes of action of the compounds. Nowadays, a lot of research works have been established regarding the application of plant extracts against the growth of several common fungal phytopathogen genera causing fruit rot disease such as *Pythium, Phytophthora,*

*Fusarium, Penicillium, Alternaria, Botrytis, Geotrichum, Sclerotinia,* and *Rhizoctonia* (da Cruz Cabral et al., 2013), *Collectotrichum* (Ye et al., 2020) and *Lasiodiplodia* (Adeniyi & Joseph, 2015). In the previous studies, the most significant disease severity in banana fruits was caused by *Fusarium proliferatum* (Abd Murad et al., 2017), while in tomato fruits was caused by *Fusarium oxysporum* (Abu Bakar et al., 2013) and *Colletotrichum goleosporioides* and *Lasiodiplodia theobroame* were the fungal pathogens that commonly causing anthracnose (Yanpirat & Vajrodaya, 2015) and rot disease (Twumasi et al., 2014) on mango fruits respectively.

The natural plant extracts are usually known as botanical extracts that include an extensive diversity of constituents with different properties and biological activities. The main characteristics of the biological alternatives are easy to be extracted, eco-friendly, biodegradable, possess low toxicity against living things, cheap and are effective against extensive pests (Zaker, 2016). The outcomes from several previous studies had revealed that some of the plant extracts are able to control plant pathogenic pests or at least can be used as a model for construction of new antifungal compounds (Amini et al., 2012).

## 1.3 Objectives of the study

Since Malaysia is among the world's 12 mega biodiversity rich countries and blessed with huge amount of biodiversity of plants with more than 20,000 plant species are found in the wild having almost 2000 or more plants with medicinal properties which are being used in various traditional health care systems, the idea of this study is to utilize plant extracts or plant derived compounds that can be used as commercial controls of phytopathogenic fungi. Therefore, the aim of the study is to explore the use of plant extracts in controlling the growth of fungal phytopathogens *in vitro* and *in vivo* and the mode of action against the pathogens. The framework of the research approach is shown in Figure 1.1.

i) To screen antifungal activity of selected plant extracts against fruit rot pathogens under *in vitro* condition
ii) To investigate morphological and pathological changes of the pathogens treated with selected plant extracts
iii) To evaluate biological effects of selected plant extracts against fruit rot pathogens under *in vivo* condition
iv) To screen and identify phytochemical constituents from selected plant extracts using UHPLC-MS/MS analysis.

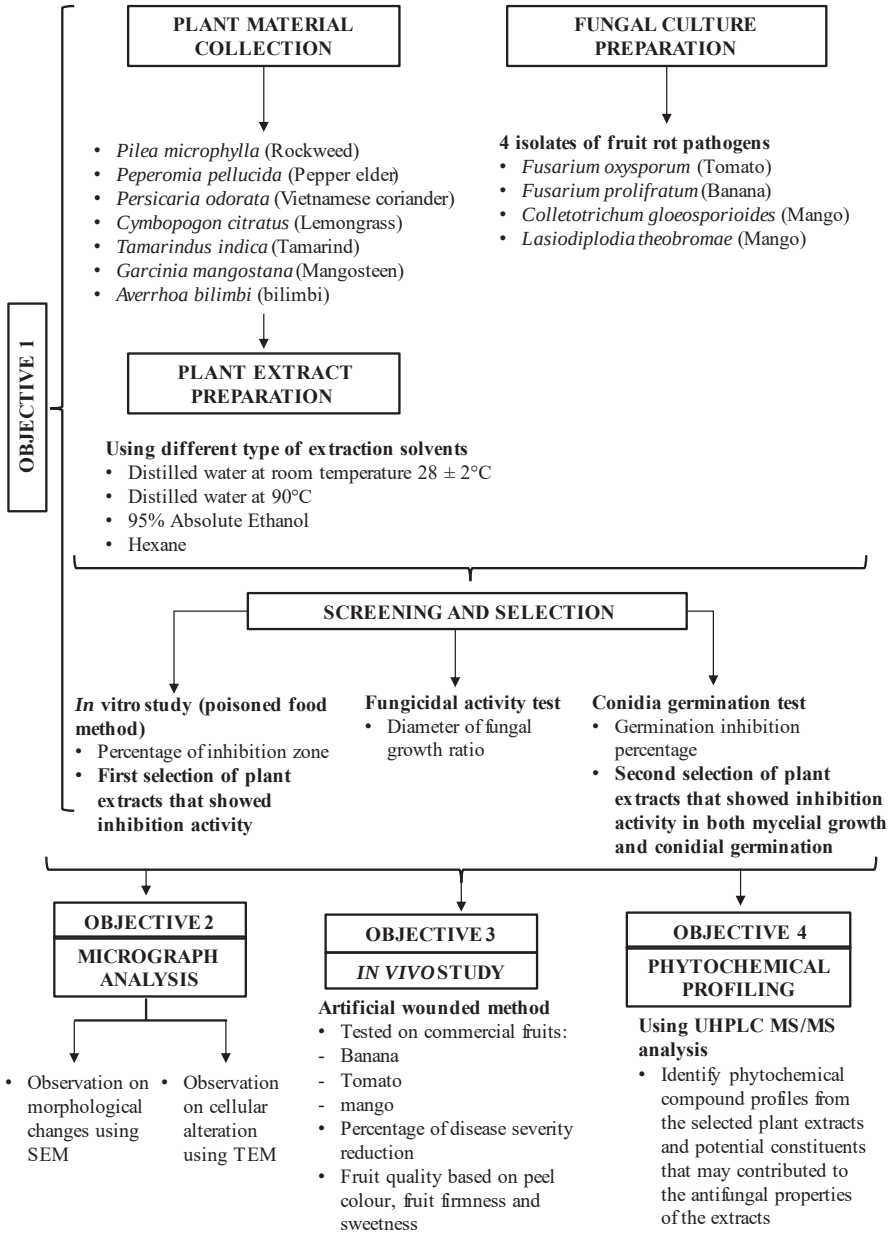


**Figure 1.1: The framework of the research approach and the plan of the study.**

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