

***IN VITRO* AND *IN VIVO* EVALUATION OF ANTIFUNGAL ACTIVITY OF
BOTANICAL EXTRACTS IN THE CONTROL OF LATE BLIGHT**

(*Phytophthora infestans*) OF TOMATO (*Solanum esculentum*)

BY

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DECLARATION

The undersigned certify that they have read and recommended to the Department of Crop Science for acceptance, the thesis entitled:

In vitro and in vivo evaluation of antifungal activity of botanical extracts in the control of late blight (*Phytophthora infestans*) of tomato (*Solanum esculentum*)

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ABSTRACT

Late blight caused by *Phytophthora infestans* establishes quickly in tomato (*Solanum esculentum*) as a result, it is one of the most devastating plant diseases across the world. The control of late blight is not easy because *P. infestans* has an advanced and complicated weaponry such as enzymes and effector molecules coded by avirulence genes. The objective of the study was to evaluate the efficacy of botanical extracts as bio-fungicides on late blight development on tomato. The *in vitro* study evaluated the effect of different concentrations of neem (*Azadirachta indica*), moringa (*Moringa oleifera*), gumtree (*Eucalyptus nigra*) and lantana (*Lantana camara*) on radial growth and percent inhibition of *P. infestans*. The *in vivo* experiment was conducted to evaluate the effectiveness of botanicals to control late blight disease on tomato plants. Data on chlorophyll content, chlorophyll florescence, and plant height, number of nodes, number of flowers, and number of fruits, disease incidence and disease severity were collected and analysed. Increasing concentration of the botanical extracts significantly ($p < 0.05$) reduced radial growth and increased percent inhibition of *P. infestans in vitro*. On the other hand, increasing botanical extract concentration significantly ($p < 0.05$) increased chlorophyll content, chlorophyll fluorescence, activity of defence enzyme peroxidase (POD), Phenylalanine ammonia lyase (PAL) and Polyphenol oxidase (PPO). Disease incidence and severity significantly ($p < 0.05$) decreased as the concentration of the botanical extracts increased. Similarly, plant height, number of nodes, number of flowers and number of fruits significantly ($p < 0.05$) increased with increase in the botanical extract concentration. Gas Chromatography Mass Spectrometry (GCMS) revealed the presence of furfural, 5-Methyl-4- (trifluoromethyl) pyrido, dodecanamide, cyclopentasiloxane and decamethyl. From the research, it can be concluded that neem acetone, neem water, moringa ethyl acetate and lantana water extracts contain antifungal compounds and can therefore be used as bio-fungicides in Integrated Disease Management (IDM) of late blight.

Key words: *Azadirachta indica*, *Moringa oleifera*, *Eucalyptus nigra*, *Lantana camara*, *Phytophthora infestans*, tomato

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DEDICATION

The project is dedicated to my family.

LIST OF ACRONYMS

EMA	Environmental Management Agency
EU	European Union
GC-MS	Gas Chromatography –Mass Spectrometric
IDM	Integrated Disease Management
KEPHIS	Kenya Plant Health Inspectorate Service
MIC	Minimum Inhibition Concentration
MRL	Maximum Residue Limit
PAL	Phenylalanine ammonia lyase
PDA	Potato Dextrose Agar
PPO	Polyphenol oxidase
POD	Peroxidase
ROS	Reactive Oxygen Species
WAT	Weeks after Transplanting

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CHAPTER ONE

INTRODUCTION

1.1 Background

Late blight caused by *Phytophthora infestans* L (Mont.) de Bary, is one of the most devastating diseases of tomatoes (*Solanum esculentum*) (Hubert *et al.*, 2013). The pathogen caused the Irish potato famine in the mid- nineteenth century in Ireland which resulted in the death of approximately a million people and an additional three million emigrated out of the country (O’Boyle, 2006; Nagar *et al.*, 2017). The pathogen affects all stages of plant development. *Phytophthora infestans* causes foliar blight which is characterized by irregular, water-soaked spots, and at advanced stages of disease development, green brown lesions are formed (Hubert *et al.*, 2013). In addition, late blight affects large leaves and stems and causes obvious dark brown spots (Ngadze, 2014).

The most widely used control strategy for the disease is application of fungicides such as metalaxyl, chlorothalonil and mancozeb which are usually applied indiscriminately starting from two weeks after crop emergence (Hubert *et al.*, 2013). Nevertheless, the excessive application of these fungicides is now causing development of fungicide resistant strains of *P.infestans* (Goufo *et al.*, 2010a). Reports from Cameroon, Kenya and Pakistan show that *P.infestans* has developed resistance to chemical fungicides such as metalaxyl (Hubert *et al.*, 2013; Muthomi *et al.*, 2017; Spc, 2017).

There have been numerous reports of up surging concerns on health and environmental risks associated with the use of these chemicals. European markets (European Commission, 2012) have been promoting organic farming by motivating smallholder farmers to employ Integrated Disease Management (IDM) practices that minimise application of synthetic fungicides used in agriculture (Hubert *et al.*, 2013). Therefore, researchers are now advocating for the use of

other alternatives such as living organisms, bio-pesticides, botanicals and compost teas (Mahajan *et al.*, 2015) which are new and effective innovations of disease management that are safe for human health and are environmentally friendly.

Use of botanical extracts is now encouraged as an important tool in IDM programmes to control plant diseases in crops. Biofungicides are also locally available and easily accessible compared to synthetic fungicides, thus lowering the cost of tomato production (Ngadze, 2014). Nonetheless, the evaluation of efficient botanical extracts which originated from African countries can increase their chances of adoption where there is ease of exploitation and adaptation (Goufo *et al.*, 2010a).

1.2 Problem statement

Control of late blight in tomatoes previously relied solely on the application of synthetic fungicides. However, injudicious use of these fungicides containing phenylamide has resulted in excessive environmental pollution and fungicide resistance. Moreover occurrence of phenylamide resistance resulted in the increased virulence and aggressiveness of *P.infestans* (Hubert *et al.*, 2013; Muthomi *et al.*, 2017; Spc, 2017). In addition, the identification of synthetic fungicides residues resulted in rejection of horticultural produce and delisting on the European markets (European Commission, 2012). This is because they also contain copper and other hazardous compounds which cannot be detected directly but their long term effect cannot be ignored.

Botanical extracts could be used as an alternative to synthetic fungicides for the management of late blight. However, most studies working with the botanical extracts concentrated on *in vitro* and paid little attention on *in vivo* studies when investigating antifungal activity of the extract against *P.infestans* in tomatoes. Consequently, this work was designed to evaluate the

efficacy of botanical extracts on *P.infestans* both sporangial germination *in vitro* and disease severity *in vivo*.

1.3 Justification

Botanical extracts contain allelochemicals such as phenols, flavonoids, alkaloids, quinones, saponins, tannins, steroids (Hubert *et al.*, 2013) whose fungicidal properties against *P.infestans* have not been established. These antifungal compounds have direct effect on *P. infestans* or indirectly create a conducive environment for the establishment of antagonistic microbes (Mizubuti *et al.*, 2007).

Botanical plants in Cameroon (Hubert *et al.*, 2013), Malaysia (Sadana and Didwania, 2015), Slovenia (Bohinc *et al.*, 2015), India (Yadav *et al.*, 2017), Kenya (Muthomi *et al.*, 2017) and South Africa (Masangwa *et al.*, 2012.) are now regarded as an important component of IDM. These botanical extracts are environmentally friendly as they are non pollutive, largely non phytotoxic, have no residual effect and are readily biodegradable, renewable and inexhaustible (Muthomi *et al.*, 2017) hence playing a crucial role in smart agriculture.

Botanical extracts of (gumtree) *Eucalyptus nigra*, (lantana) *Lantana camara*, (neem) *Azadirachta indica* and (moringa) *Moringa oleifera* can be used as bio-fungicides to control late blight. Satiating local and international markets and taking care of human health and environmental safety can be achieved by either reducing the usage of synthetic pesticides or by supplementing with bio-fungicides.

1.4 Hypotheses

1. Guntrees, lantana, neem and moringa extracts effectively inhibit radial growth and increase percentage inhibition of late blight *in vitro*.
2. Guntrees, lantana, neem and moringa extracts effectively reduce late blight development and severity of tomato in the greenhouse.
3. Extracts from gumtree, lantana, neem and moringa contain antifungal compounds.

1.5 Objectives

1.5.1 Main objective

To evaluate the efficacy of botanical extracts as bio-fungicides on late blight development of tomato.

1.5.2 Specific objectives

1. To evaluate the antifungal activity of gumtree, lantana, neem and moringa extracts on the radial growth and percent inhibition of *P.infestans in vitro*.
2. To evaluate late blight development and severity on tomato plants treated with gumtree, lantana, neem and moringa extracts in the greenhouse.
3. To identify probable antifungal compounds in the extracts from gumtree, lantana, neem and moringa.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of tomato production

According to Yadav *et al.*, (2017) tomato (*Solanum esculentum* L.) is an essential fruit crop, which belongs to the *Solanaceae* family. Originally, the word tomato was derived from Nantho language word “*tomal*.” Tomato is the second most grown horticultural crop in the world (Spc, 2017). Tomato production can be estimated to be 150 million tonnes per year and the hectares which are cultivated under tomato can be approximated to 5.2 million hectares worldwide (Sadana and Didwania, 2015; Yadav *et al.*, 2017). It is a multipurpose plant that can be used as natural (raw material) or element of other products after value addition. For humans, tomatoes provide an excellent source of vitamin A, B and C (Muthomi *et al.*, 2017).

2.2 Plant diseases affecting tomato

Mizubuti *et al.*, (2007) reported that in developing countries, yield of tomato is low as compared to other developed countries due to several diseases caused by fungi, bacteria, viruses and nematodes. Among the fungal diseases, late blight which is caused by *Phytophthora infestans* L. (Mont.) is one of the most devastating disease in tomato. The pathogen is commonly of greater economic importance in *Solanaceous* crops (Yadav *et al.*, 2017). These *Solanaceous* crops include pepper (*Pipper nigrum*), eggplant (*Solanum melongena*) and potatoes (*Solanum tuberosum*).

2.3 Classification, disease development and economic importance of *P. infestans*

2.3.1 History of *P. infestans*

The pathogen belongs to the kingdom (*Stramenopila*), Phylum (*Oomycota*) (Hubert *et al.*, 2013) and family (*Pythiaceae*). It is an oomycete which is well known for its devastating behaviour (Yanar *et al.*, 2011) due to its explosive development where there is a conducive environment and susceptible hosts such as *Solanaceous* crops (Spc, 2017; Mizubuti *et al.*, 2007). The name of this pathogen was derived from its destructive behaviour, thus the genus *Phytophthora* was derived from a Greek word “*Phyton*” meaning a plant and “*phythora*” meaning a destroyer. It is similar to fungi in terms of morphology but phylogenetically related to brown algae (*Phaeophyceae*) (Haldar *et al.*, 2006).

2.3.2 Economic importance of *P. infestans*

Phytophthora infestans can cause yield loss of up to 100% and an estimated economic loss ranging from USD 3-5 billion annually (Mizubuti *et al.*, 2007). In potatoes, *P. infestans* caused the disastrous Irish potato famine in the 1884 in Ireland where approximately a million people died and three million emigrated out of the country due to starvation (O’Boyle, 2006; Nagar *et al.*, 2017). *Phytophthora infestans* affects the crop at all growth stages and the symptoms appear as irregular, water soaked spots on the leaves, stems and fruit (Kumbar, 2017, Yadav *et al.*, 2017). At advanced stages of disease development, green brown lesions are formed affecting large leaves, stems and fruits and they become dark oblong spots which result in severe damage on plant parts as shown on Fig 2.1 (Hubert *et al.*, 2013).

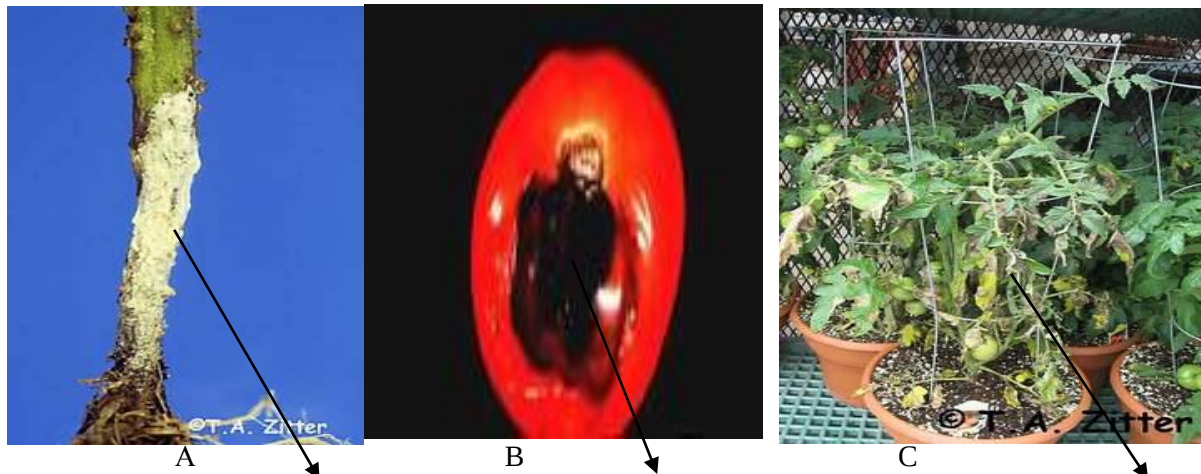


Figure 2. 1(A) damage on stems, (B) dark oblivious spots on tomato fruits and (C) green brown lesions of tomato plant affected by *P. infestans*.

The pathogen has a polycyclic life cycle which lasts for three days from germination to sporulation. During this short time of incubation, a single spore can give rise to thousand spores (Nagar *et al.*, 2017). This is because it has a sophisticated artillery such as effector molecules coded by avirulence genes that allow quick establishment of the disease when the pathogen gets inside the susceptible host tissue. There is activation of specialised enzymes such as cutinases and other proteins required for cell destruction and nutrients converted to inorganic form which can be easily utilised by the pathogen (Mizubuti *et al.*, 2007).

2.3.3 Disease development and epidemiology of *P. infestans*

The pathogen is extremely calamitous to *Solanaceous* crops under conducive weather conditions of 80-100% relative humidity and temperatures ranging from 18-22 °C (Yadav *et al.*, 2017). Germination of oospores and sporangia which is lemon shaped (Goufo *et al.*, 2010b) is the first stage during the pathogenesis process (Mizubuti *et al.*, 2007). The germination tube (a specialised structure) penetrates the plant tissue through cuticle and epidermal cells to extract nutrients from the host cells. Necrosis occurs after invasion and infection. Finally colonisation by *P. infestans* and sporangiophores are formed and then produce sporangia (Goufo *et al.*,

2010b). These sporangia are then dispersed for long distances by wind and short distances by rain. During overwintering the pathogen survives from season to season mostly on infected potato tubers, tomato fruits, crop debris and soil in the form of oospores.

2.3.4 Population dynamics of *P. infestans*

Seasonal reproduction of *P. infestans* generates two main genetic population groups namely panmitic and clonal. The structures of the groups include (1) panmitic population where large populations have distinct genotypes and there is a lot of genetic variation (2) clonal population structure are homogeneous with low genetic variation (Mizubuti *et al.*, 2007). The information of population dynamics is very important as it will influence the control measure which can be employed to manage the pathogen. For panmitic genetic structure, disease management is difficult since the strain can easily adapt to the prevailing climate (Nagar *et al.*, 2017) where as clonal genetic population can be managed easily since there is limited diversity (Grunwald *et al.*, 2006).

2.3.5 Current control measures for *P. infestans*

The pathogen can be efficiently managed by cultural methods (good fertilizer management like Ca and K, optimum plant population, irrigation and use of certified seeds) and physical methods such as solarisation (Seifu, 2017). The most widely used control measure of late blight is the use of fungicides such as metalaxyl, chlorothalonil and mancozeb which are mostly indiscriminately applied (Yadav *et al.*, 2017). Nevertheless, the excessive application of these fungicides is now causing development of fungicide resistance by *P. infestans* (Goufo *et al.*, 2010a, 2010b). Emerging reports in many parts of the world show that *Phytophthora infestans*

is now developing resistance to metalaxyl and several countries have reported cases of resistance, for example Cameroon (Hubert *et al.*, 2013) and Pakistan (Spc, 2017).

2.4 Influence of international markets on use of agrochemicals

For international markets, European Union (EU) has established the maximum residue limit (MRL) of fungicides on all agricultural products at 0.01mg/kg (European Commission, 2012). Some of the synthetic agrochemicals have been banned for use in production of fresh vegetables in the last five years due to detection of high levels of harmful chemical residues (Martínez, 2012). The detection of harmful residues resulted in rejection of fresh produce and loss of access to European niche markets (European Commission, 2012). The MRLs by international markets resulted in decline of crop production and loss of trade for small holder farmers as they are not able to meet these demands (KEPHIS, 2012).

2.5 Use of botanical extracts as bio-fungicides

Botanical extracts are now encouraged as an important component of IDM (Sadana and Didwania, 2015; Yadav *et al.*, 2017). These botanical extracts are environmentally friendly as they are non-pollutive and largely non-phytotoxic. They also have no residual effects, readily biodegradable and inexhaustible (Muthomi *et al.*, 2017). Bio-fungicides are also locally available and easily accessible as compared to synthetic fungicides thus lowering the cost of tomato production (Ngadze, 2014).

Application of botanical extracts exert phytochemical stress to the receptor plant. This will initiate the production of defence enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO) which will detoxify reactive oxygen species

which are produced by stressed plants. As the concentration of the botanical extract increases, physiological processes like respiration, photosynthesis and defence enzyme activity tend to increase. The guiding principle in selection of the botanical plants in this research were the antimicrobial history from published reports by different researchers worldwide (Ngadze, 2014; Muthomi *et al.*, 2017; Nagar *et al.*, 2017).

2.6.1 Gumtree

Eucalyptus nigra which falls under *Myrtaceae* family originated in Australian (Gilles *et al.*, 2010). It is at all, evergreen and superlative plant species which is grown worldwide for oil, gum, pulp, timber, medicine and aesthetic value (Gilles *et al.*, 2010). Essential oils from its leaves are mostly used in food, perfumery and pharmaceutical industry. Furthermore, the oil contains anti-microbial fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematocidal compounds (Inouye *et al.*, 2001; Daizy *et al.*, 2008).

The chemical composition and antimicrobial properties possessed by essential oils of *Eucalyptus* species include piperitone, α -phellandrene, *p*-cymene and terpin-4-ol, 1,8-cineole, neral, geranial, α -phellandrene, methyl geranate and (*E*)-methyl cinnamate (Inouye *et al.*, 2001; Sadana and Didwania, 2015). Ahmad *et al.* (2017) detected flavonoids, alkaloids, saponins, tannins, terpenoids, glycosides and steroids from *Eucalyptus* extract under phytochemical analysis. *Eucalyptus* extract obtained from methanol reduces disease severity (Goufo *et al.*, 2010a) and percentage sporangial germination (Goufo *et al.*, 2010b; Sadana and Didwania, 2015).

2.6.2 Lantana

Lantana camara L. (*Verbanaceae*) is a troublesome, noxious weed, native to temperate, tropical and subtropical regions of world (Patil *et al.*, 2017). In Zimbabwe, it is listed as one of the invasive alien species (Environmental Management Act, 2002). In different regions, it has been used for treatment of different illnesses like respiratory disorders in Nigeria and Senegal (Shriniwas and Subhash, 2018) for jaundice and rheumatism in West Africa and for fever, influenza, chicken pox, measles in Central and South America and Ghana (Patil and Kumbahr, 2017).

Simple, petiolate, ovate, crenate of *L. camara* leaves contain pentacyclic triterpenoids, steroids, flavonoid aglycones, iridoids, polyphenolics and essential oil rich in monoterpenes and sesquiterpenes, majorly included are trans/ β -caryophyllene, bicyclogermacrene, α -curcumene, sabinene, (E)-citral, 1,8-cineole, germacrene D, limonene and α -humulene (Seth *et al.*, 2012), zingeriberene and bisabohene (Nagar *et al.*, 2017). Based on these phytochemicals, *L. camara* leaves have been well accounted for various pharmacological activities like antiulcerogenic, antibacterial and anticancer (Shriniwas and Subhash, 2018). Several researchers have reported antimicrobial activity of leaves and their essential oil (Sulaiman *et al.*, 2013, Patil *et al.*, 2017).

2.6.3 Neem

Neem which belongs to the *Meliaceae* family is a fast-growing evergreen popular tree found commonly in India, Africa and America. Neem, also known as '*aristha*' in Sanskrit, a word that means 'perfect, complete, imperishable and reliever of sicknesses' has been used for more than 4000 years due to its medicinal properties (Biswas *et al.*, 2002). Neem is perhaps the most useful traditional medicinal plant in India. Each part of the neem tree has some medicinal property thus commercially exploitable. It is considered as an important source of valuable

natural ingredients for development of antifungal compounds against both human and crop diseases (Raza *et al.*, 2016).

The importance of the neem tree has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled ‘Neem- a tree for solving global problems’ (Biswas *et al.*, 2002). Fungicidal compounds isolated from neem involve azadirachtin (Mizubuti *et al.*, 2007) tetranortriterpenes, including nimbin, nimbidinin, nimbolide and nimbidic acid (Biswas *et al.*, 2002). According to Mirza *et al.*, (2000) neem products were found highly effective at different life stages of *P.infestans*. Seed and leaf extracts of neem exhibited anti-fungal properties and are also used as an insecticide for controlling agricultural insect pests (Subapriya and Nagini, 2005; Raza *et al.*, 2016).

It was also found that dry neem seed extract completely inhibited the mycelial growth of *Fusarium oxysporum*. According to Mondali *et al.* (2009) the growth of both the *Aspergillus* and *Rhizopus* species was inhibited significantly and controlled with both alcoholic and water extracts of all the concentrations used. Several reports have been made on the fungicidal properties of neem oil. Sitara *et al.*, (2008) reported that in the field *Alternaria alternata*, *Aspergillus niger* and *F. oxysporum* have been completely controlled using 2-10% neem oil.

2.6.4 Moringa

Moringa (*Moringa oleifera* L.) belongs to a single *Moringaceae* family. It is known as Rawag tree in Sudan and is widely cultivated in many countries in tropical as well as in many Asian countries (Fahey, 2005). *M. oleifera* grows well in soils with low fertility. Almost all parts of the tree can be utilized for several purposes that help sustain living conditions (Hasana *et al.*, 2011, Abdalbasit *et al.*, 2017). Many studies showed that aqueous and ethanolic moringa leaf

extracts are the promising potential as the alternative treatment of infections caused by the tested strains (Peixoto *et al.*, 2011).

Mvumi *et al.* (2018) and Abdalbasit *et al.* (2017) reported that spraying moringa leaf extracts on inoculated diseased tomato leaves donates specific quantifiable glucosinolates like sinigrin and tocopherols which may be involved in defence against tomato diseases. Generally, moringa tree is used in alley cropping (biomass production) and used as bio- fertilizer (juice expressed from the leaves) (Hasana *et al.*, 2011), medicine (all plant parts) (Gufta *et al.*, 2013), ornamental plantings, biopesticide (soil incorporation of leaves to prevent seedling damping off), natural coagulant for water purification because of their strong coagulating properties for sedimentation of suspended undesired particles (Abdalbasit *et al.*, 2017), tannin for tanning hides (bark and gum) (Fahey, 2005).

2.7 Use of solvents to extract botanical extract

Ethyl acetate, acetone and water are some of the solvents that are used to extract botanical extracts (Hubert *et al.*, 2013, Ngadze, 2014). Solvents such as ethyl acetate and acetone are normally used because they are less toxic than solvents containing halogens or benzene (Kritzinger, 2005). They also have high volatility so that they can easily evaporate by drying out at room temperature (Inouye *et al.*, 2001). According to Ngadze, (2014), acetone plant extracts showed that they inhibited growth of late blight (*P. infestans*) *in vitro* at all concentration (0.5, 1.0, 2.5, 5 mg/ml) effectively control late blight. However, water extracts showed a poor performance as some of the compounds did not dissolve in water since there are not immiscible (Masoko, 2005).

To avert this, there is need to increase the concentration of the plant extract in water to reach the toxicity level of the compounds. Previous studies showed that water extract had higher quantities of crude extract. (Bandor *et al.* (2013) reported that use of water in extraction increases the amount of impurities and this could impair the quality of extracts. The alcoholic extracts of neem leaf was most effective in comparison to aqueous extract for retarding the growth of *Rhizopus* and *Aspergillus*. The crude aqueous and alcoholic leaf extracts of neem were more effective in inhibiting the growth of *Aspergillus* in comparison to inhibitory effects on *Rhizopus* growth in the artificial culture medium (Mondali *et al.*, 2009).

2.8 Defence enzymes on late blight

Late blight is among plant diseases which cause oxidative stress that result in cytolysis due to production of reactive oxygen species (hydrogen peroxide, oxygen and hydroxyl radicals). To overcome lipid peroxidation, a plant should have a defensive mechanism by synthesising defence enzymes to detoxify these Reactive Oxygen Species (ROS). These defence enzymes include Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL), Catalase (CAT) and Peroxidase (POD) (Siddique, 2013).

2.8.1 Polyphenol oxidase (PPO)

It is a copper metalloprotein which is involved in a number of browning in plants. Furthermore, PPO played a crucial role in tolerance to soft rot in potato tubers (Ngadze *et al.*, 2012). When the plant is infected by the pathogen, the activity of PPO will increase. It was found that the covalent bonding nucleophilic substituents of amino acids and proteins by PPO derived quinones are thought to exert an antinutritive defence against insects and pathogens (Pourcel *et al.*, 2007).

2.8.2 Phenylalanine ammonia lyase (PAL)

It catalyses the conversion of L-phenylalanine to trans-cinnamic acid and ammonia is released.

The reaction catalysed leads to the production of phenolics. Furthermore, the activity of both PAL and PPO lead to build up of antinutritive elements and isoflavanoids thus protect plants against insect and pathogens (Vanitha *et al.*, 2009). According to Ngadze *et al.* (2012), PAL activity increased in wounded potato plants. These reports showed that PAL can play an important role in imparting resistance to infected plants with entomopathogens.

2.8.3 Peroxidase (POD)

It is involved in the oxidative polymerization of hydroxycinnamyl alcohols to yield lignin and crosslinking isodityrosine bridges in cell walls (Ngadze *et al.*, 2012). These compounds act as barriers to pathogen invasion and hence constitute part of host resistance mechanisms. The findings indicate that defence enzymes (PPO, POD, PAL) and antifungal compounds (chlorogenic acid total soluble phenols) play a role in disease resistance against the plethora of entomopathogens. In a related report, Murimwa *et al.* (2019) found that sorghum (*Sorghum bicolor* L. Moench) aqueous extract can increase the POD activity on Sesame (*Sesamum indicum* L.) varieties and selected weeds.

2.9 Antifungal compounds produced by botanical extracts

Antifungal compounds produced by botanical extracts are grouped mainly into two categories namely oils and crude extracts. Botanical extracts are dried and the compounds are extracted by a series of processes which involve filtration, distillation and evaporation. However, oils are liquids extracted through the series of fermentation, extraction and steam distillation (Mizubuti *et al.*, 2007). Different concentrations (0.5, 1.0, 2.5 and 5.0 mg ml⁻¹) are needed for efficacy from different plant extracts and against different pathogens (Ngadze, 2014).

2.9.1 Metabolism of antifungal compounds and their mode of action

Botanical extracts have two major modes of action namely antifungal and antifungistatic effects (Mizubuti *et al.*, 2007). They can create a conducive environment for the establishment of antagonistic micro-organisms which can control the pathogen and host plant (Yadav *et al.*, 2017). These compounds are classified into two major groups namely preformed and inducible preformed compounds.

2.9.1.1 Preformed compounds (preformed resistance in plants)

These are substances naturally forming part of the plant cell composition with antifungal properties. These compounds are present in the plants in their biologically active form. Some preformed compounds such as phenolics, terpenoids and steroids are directly toxic (Inouye *et al.*, 2001), while others appear as conjugates such as glycosides (Hubert *et al.*, 2013) that are not directly toxic but become toxic following disruption of the conjugate but lose toxicity when deglycosylated. The metabolism and mode of action of these botanical extracts is discussed below.

2.9.1.2 Allicin (diallylthiosulfinate; thio2-propene-1-sulfinic acid-S-allyl ester)

These are antifungal extracts of onions (*Allium cepa* L.) and garlic (*Allium sativum* L.) (Masangwa *et al.*, 2012). Some researchers reported that the allicin contain inhibit *Aspergillus flavus*, *A. parasiticus*, *Candida albicans*, *Cryptococcus*, *Penicillium*, *Rhodotorula*, *Saccharomyces*, *Torulopsis* and *Trichosporon species* (Muthomi *et al.*, 2017). This substance is produced by tissues when the cells are damaged and the enzyme (alliin-lyase; E.C. 4.4.1.4) act on the substrate (S-allyl-L-cysteine sulfoxide) because the enzyme and substrate are separated by cellular membranes inside the cells (Martínez, 2012). Basis of its antimicrobial action has been advocated as biofungicide which undertake thioldisulphide exchange reactions with free thiol groups in proteins. Allicin in garlic juice inhibited the germination of sporangia

and cysts and subsequent germ tube growth by *P. infestans* both *in vitro* and *in vivo* on the leaf surface (Masangwa *et al.*, 2012; Ngadze, 2014). However, Bohinc *et al.* (2015) found that garlic (*Allium sativum* L.) did not inhibit the germination of *P. infestans* sporangia.

2.9.1.3 Phenolic compounds

According to Inouye *et al.* (2001) phenolic compounds possess an aromatic ring with one or more hydroxyl groups and can include functional derivatives. Some of the important phenolic compounds include alkyl esters of parabens, phenolic antioxidants and certain of the terpene fraction of the essential oil (Cyprian, 2011). Monophenols (cresol), diphenols (hydroquinone) and triphenols (gallic acid) are some of the simple phenolic compounds. Phenolic compounds can disrupt the cytoplasmic membrane and cause leakage of cells and also directly inhibit cellular proteins (Hubert *et al.*, 2013).

They facilitate plant resistance to pathogens through their antimicrobial properties, which elicit direct effects on the pathogen, or by affecting pathogenicity factors of the pathogen (Mizubuti *et al.*, 2007). However, they may also enhance resistance by contributing to the healing of wounds via lignification of cell walls around wound zones. The cells surrounding the wound can produce and deposit lignin and suberin in their walls (Martínez, 2012). The phenolic compounds also protect the plant host from the action of cell wall degrading enzymes produced by the pathogen. However, some researchers have established that phenolic compounds may have several modes of action and targets which can lead to inhibition of microorganisms (Hubert *et al.*, 2013).

2.9.1.4 Tannins

These are polyphenols, which have been cited by Martínez (2012) as inhibitors of polygalacturonase (PG) activity of *Sclerotinia fructicola* (anamorph of *Monilinia fructicola*) in apples and other pathogen/host combinations. Tannins of young banana (*Musa balbisiana*)

fruits and benzyl isothiocyanate in unripe papaya fruits (Barkai-Golan, 2001) are additional examples of in-fruit toxic compounds.

2.9.1.5 Flavonoids

Flavonoids are a special group of phenolic compounds and some aspects of this group have been described above. The flavonoids consist of catechins and flavones, flavonols and their glycosides can also be extracted from massep grass (*O. grattissium*), billy goat weed (*A. Conyzoides*), black jack (*B. pilosa*) and tchieuc (*T. vogelli*) (Inouye *et al.*, 2001, Hubert *et al.*, 2013). Proanthocyanins or condensed tannins are polymers of flavan-3-ol which are found in wild waterman (*Zanthoxylum gillettii*) (Cyprian, 2011).

2.9.1.6 Steroids

These are terpenes with a ring structure composed of a specific arrangement of four cycloalkane ring that are joined to each other. Saponins are plant glycosylated steroids. The tomatine, (specialised steroids produced in tomato leaves and unripe fruits), is also present in high concentration in the peels of green tomatoes and also bind to 3-hydroxy sterols in fungal membranes (Barkai-Golan, 2001). It has an antifungal and insecticidal activity that react with sterols in membranes. Steroids prevent mycelial growth of *B. cinerea* and other fungal pathogens (Martinez, 2012). Most tomato pathogens, on the other hand, can specifically degrade tomatine and detoxify its effects through the activity of tomatinase (Barkai-Golan, 2001).

2.9.2 Inducible preformed compounds (inducible preformed resistance in plants)

These compounds can be produced due to infection, after association with the pathogen on the surface plant or under an abiotic stress (Martínez, 2012). It can also induce phytoalexins in superficial wounds inoculated with the pathogen. On other hand, heating can also catalyse

biosynthesis of lignin and other compounds in wounds which act on as a physical barrier against hyphae penetration of pathogen. Phytoalexins are considered to be general stress-response compounds, produced after biotic or abiotic stress. The role of phytoalexins shows that disruption of cell membranes is a central factor in their toxicity and that the mechanism is consistent with the lipophilic properties of most phytoalexins (Barkai-Golan, 2001).

2.9.2.1 Sesquiterpenoid compounds

These are substances produced after the infection by microorganisms in *C. domestica* and *P. nigrum* (Yulia, 2005; Inouye *et al.*, 2001). Sesquiterpenoid compounds involve rishitin, solavetivone, lubimin and oxylubimin. Rishitin and solavetivone have been found to be induced in potato tuber discs 24 hours after inoculation with *P. infestans*, *F. sambucinum* and *Erwinia carotovora* in stored potatoes (Barkai- Golan, 2001). Other sesquiterpenoids such as lubimin and oxylubimin that have been extracted in potatoes have the potential to enhance disease resistance in potatoes against *F. sambucinum* (Martínez, 2012).

2.9.2.2 Benzoic acid

It is an example of the phytoalexin compound produced (inducible preformed compounds). Martinez (2012) reported that infection of apples by *Nectria galligena* produce a lot of benzoic acid. The benzoic acid acted as an antifungal compound and was effective at low pH values especially in unripe apples where it reduces the mycelial development of the fungus (Barkai-Golan, 2001).

In conclusion, use of *E.nigra*, *A.indica*, *M.oleifera* and *L. camara* extracts as natural biofungicide is of crucial importance in view of the environmental and toxicological implications of the indiscriminate use of synthetic fungicides and overcoming the problem of increasing fungicide resistance (Daizy *et al.*, 2008).

CHAPTER THREE

METHODOLOGY

3.1 Experimental site

The study was carried out at the University of Zimbabwe. University of Zimbabwe is situated at a longitude of 31° 05'E and latitude of 18° 12'E. The analysis of the soil used in the experiment was done at the Department of Soil Science and Agricultural Engineering. The results from the soil analysis are shown in the Table 3.1,

Table 3.1 Soil analysis report and fertilizer recommendations.

Classification	Clay (%)	pH (CaCl ₂)	Mineral N (mg/kg)	Available P (mg/kg)	Exchangeable cations (%)			
					K	Ca	Mg	Na
Sandy Clay								
Loam	30	5.4	8	4	0.1	8.49	3.44	0.15

3.2 Laboratory experiment: An evaluation the antifungal activity of gumtree, lantana, neem and moringa extracts on percent inhibition of *Phytophthora infestans* in vitro.

3.2.1 Experimental design

The laboratory experiment was laid out as a 3 way factorial experiment with botanical extracts, extraction solvents and extract concentration as the factors. The levels for botanical extract were gum tree, lantana, neem moringa extract and distilled water was used as the control whilst the levels for extract concentration were (0.5, 1.0, 2.5 and 5.0 mg⁻¹). Each botanical extract was evaluated in three solvents (acetone, ethyl acetate and water) to give 13 treatments as shown on Table 3.2. Treatments were arranged in a Completely Randomised Design (CRD) and each treatment was replicated four times. The experiment was repeated twice.

Table 3.2 Treatments and locations where the botanical extracts were collected

Treatment	solvent used	Extract	Common name	Locations where the botanical extracts were collected	
				Geographical area	Climate data
1	Acetone	<i>A. indica</i>	neem	Chiredzi, Masvingo, Zimbabwe	Mean temperature (18-22.5°C)
2	Ethyl acetate	<i>A. indica</i>	neem	21° 30'S 31°40'E	Annual rainfall (566mm)
3	Water	<i>A. indica</i>	neem	Altitude 436m above sea level	
4	Acetone	<i>M. oleifera</i>	Moringa	Africa University, Manicaland, Zimbabwe	Mean temperature (19-25°C)
5	Ethyl acetate	<i>M. oleifera</i>	Moringa	18°97'S 32° 67'E	Annual rainfall (1000 to 1500mm)
6	Water	<i>M. oleifera</i>	Moringa	Altitude- 1116 m above sea level	
7	Acetone	<i>E. nigra</i>	gumtree	Crop Science, UZ, Harare	Mean temperature (15-27°C)
8	Ethyl acetate	<i>E. nigra</i>	gumtree	31° 05'E, 17°48'S	Annual rainfall (800-1000mm)
9	Water	<i>E. nigra</i>	gumtree	Altitude- 1523 m above sea level	
10	Acetone	<i>L.camara</i>	Spanish flag		
11	Ethyl acetate	<i>L.camara</i>	Spanish flag		
12	Water	<i>L.camara</i>	Spanish flag		
13	Distilled water (negative control)				

3.2.3 Extraction of crude extracts from plant samples

Crude extracts from plant samples were extracted using the modified method of Kritzing (2005). The plant samples were washed under running tap water and rinsed in distilled water. All the plant materials were air dried in the shade and ground to fine powder using a hammer mill grinder. Extractions were performed separately on 140g of each plant powder by soaking in 500 ml of acetone (analytical), ethyl acetate (analytical) extracts and sterile distilled water. The mixture was shaken for 24 hours at room temperature on an orbital shaker (Orbital shaker S01, Stuart Scientific Co. Ltd) at 100 rpm. Acetone and ethyl acetate were removed using a rotary evaporator- biobase (Model R-200, Switzerland) at 56 °C and 77 °C, respectively. Water plant filtrates were concentrated to powder using a freeze drier (Lastmark laboratory-biobase). The stock solutions (100mg/100ml) of botanical extracts were prepared and all the extracts were stored in the refrigerator at 4 °C before use. Different concentrations (0.5, 1.0, 2.5 and 5.0 mg ml⁻¹) of each crude extract were added to 20 ml of Potato Dextrose Agar (PDA) into 90 mm diameter of Petri dishes to give final concentrations of 0.5, 1.0, 2.5 and 5.0 mg ml⁻¹.

3.2.4 Isolation of the *P.infestans*

Tomato fruits with late blight symptoms were collected from farms around Harare. The fruits were surface disinfected in 20 % sodium hypochlorite for three minutes and rinsed three times in sterile distilled water, blotted dry with sterile filter papers and then placed on potato dextrose agar (PDA) under continuous ultraviolet light for seven days at 24±2 °C. The PDA was amended with streptomycin (2mg/3 ml of distilled water) to inhibit growth of opportunistic pathogens. *Phytophthora infestans* cultures were morphologically identified using a stereomicroscope, subcultured and stored in a refrigerator at 4 °C prior to use.

3.2.5 Inoculation of pathogen

The PDA containing the different concentrations of botanical extracts was poured into 65 mm diameter Petri dishes with preset diametrical lines drawn at the bottom plate to identify the centre of the plate. Fungal plugs of 5 mm diameter of *P. infestans* from seven day old fungal cultures were placed using a sterilised cork borer at the centre of the Petri dishes containing PDA amended with the different botanical extracts and sterile distilled water. The inoculated Petri dishes were sealed with Parafilm® and incubated at 25 °C for 12 days.

3.2.6 Data collection

3.2.6.1 Radial growth

The diameter of the radial growth inhibition zones was measured after three, six and nine days of incubation (DAI) using vernier calliper.

3.2.6.2 Percent inhibition.

Growth inhibition zones were calculated for 3, 6 and 9 DAI using formula for $\% = (dc - dt / dc) \times 100$ (Masangwa *et al.*, 2012)

Where:

dc is the average diameter of fungal colony of the negative control.

Dt is the average diameter of fungal colonies grown in the presence of the plant extract or the standard fungicides.

3.2.6.3 Crude extracts

The quantities (g) of crude extracts harvested from 140g plant material using acetone, ethyl acetate and water as solvents was weighed using an analytical scale.

Table 3.3 Quantities (g) of crude extract harvested from 140g plant material using acetone, ethyl acetate and water.

Common name	Botanical name	Solvents		
		Acetone	Ethyl acetate	Water
Neem	<i>A.indica</i>	16.93	28.27	20.56
Moringa	<i>M.oleifera</i>	20.65	10.98	26.85
Gumtrees	<i>E.nigra</i>	18.12	12.41	20.81
Wild type Spanish flag	<i>L.camara</i>	12.93	16.54	16.19

3.2.7 Gas chromatography-mass spectrometric (GCMS) analysis of antifungal compounds in botanical extracts

Gas chromatography–mass spectrometry of gumtree, lantana, neem and moringa samples was done at the Pesticides and Phytochemical screening Analysis at Agilent Technologies, Harare, Zimbabwe. GC–MS was used to detect compounds using the relative gas chromatographic retention times ranging from 3.14 to 3.40 minutes and elution patterns of components of a mixture in combination with the mass spectral fragmentation patterns, which is the characteristic of a compound’s chemical structures (Mani *et al.*, 2015).

In general, MS are configured with magnetic sector or quadrupole type mass analyzers. A quadrupole GC–MS had a group of four electromagnets that focus each fragment through a slit into the detector. These were programmed to direct only certain fragments, one at a time (scan), until the complete range of m/z was recovered. This produced a mass spectrum, which was a graph of the signal intensity (relative abundance) versus the m/z ratios (essentially the molecular weight) (Mani *et al.*, 2015). Each compound had a unique fingerprint, and software was equipped with a library of spectra for unknown compounds (Mani *et al.*, 2015). Antifungal compounds with a score of 80% and above were considered for data presentation.

3.3 Greenhouse experiment: Evaluating late blight development and severity on tomato plants treated with gumtrees, lantana, neem and moringa extracts in the greenhouse.

3.3.1 Experimental design

The greenhouse experiment was laid out as a three way factorial experiment with botanical extracts, extraction solvents and extract concentration as the factors. The levels for botanical extract were gum tree, lantana, neem, moringa extract and distilled water was used as the control whilst the levels for extract concentration were 1.0, 2.5 and 5.0 mg ml⁻¹. Each botanical extract was evaluated in three solvents (acetone, ethyl acetate and water) to give 13 treatments as shown on Table 3.2. Treatments were arranged in a Completely Randomised Design (CRD) and each treatment was replicated four times.

3.3.2 Tomato seedlings transplanting

Forty-day-old tomato plants (cultivar rodade) from Emerald seedling company were grown in a green house. Red soil (results from soil analysis shown on Table 3.1) from the Department of Crop Science experimental plots were sterilized in an oven at 100 °C for 24 hours to kill soilborne pathogens. The tomato seedlings were transplanted in polythene plastic pots with dimensions of 21 cm height, 15 cm top diameter and 11.5 cm bottom diameter. A total of 168 pots were filled up to a height of 15 cm with sterilized red soil. The sterilized soil was thoroughly mixed with fungal inoculum adjusted to a concentration of 2.5×10^4 spores ml⁻¹ using a haemocytometer (Neubauer improved double, W. Germany).

Botanical extracts were sprayed with the different concentrations (1.0, 2.5 and 5.0 mg ml⁻¹) of botanical extracts at two weeks after transplanting. Treatments were applied by spraying the extract solution on the tomato plants to runoff, using a handheld sprayer calibrated to discharge at a spray rate of 100 litres ha⁻¹. Pots were watered daily with 500ml of water using a perforated cup. Basal fertilizer was applied at planting at a rate of 40 g pot⁻¹ using compound C (5% N: 15 % P₂O₅: 12% K₂O) to achieve an application rate of 800 kg ha⁻¹. The plants were top dressed with ammonium nitrate (34.5% N) which was split applied at 5 g pot⁻¹ at 3 and 6 weeks after transplanting (WAT) to achieve an application rate of 400 kg ha⁻¹.

3.3.3 Effect of botanical extracts on the activity of Polyphenol oxidase (PPO), Phenylalanine ammonia lyse (PAL) and Peroxidase (POD) on young tomato leaves

PPO, PAL and POD activity assay were carried out in the pathology laboratory. The defence enzyme activity was measured using the procedure described by Ngadze *et al.* (2012). Newly grown leaves of tomatoes were removed from the plant using a scalpel blade every week for six weeks and used in the enzyme assays. The young leaves were ground in liquid nitrogen using a pestle and mortar, and 2.5 g of homogenate was mixed with 5 ml of 0.05 M sodium

phosphate buffer (pH 6.0) containing 5% polyvinylpolypyrrolidone (wt vol⁻¹). The homogenate was filtered through four layers of muslin cloth, and the filtrate was centrifuged at 13000 rpm for 5 min at 4°C. Defence enzymes activity of peroxidase POD (U µl⁻¹ min⁻¹), phenylalanine ammonia lyase PAL (µg PAL g⁻¹) and polyphenol oxidase PPO (U µl⁻¹ min⁻¹) were measured using UV-VIS spectrophotometer (Sahara image).

3.3.3.1 PPO assay

One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer (pH 6.0) and 1 ml of 0.1 M catechol (Sigma). In the control, the extract was replaced with 1 ml of sodium phosphate buffer. The enzyme activity was measured at an absorbance of 540 nm at 20 second intervals for four minutes, and values were calculated per minute.

3.3.3.2 POD assay

One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer (pH 6.0), 1 ml guaiacol (Sigma) and 1 ml of 2% H₂O₂ (vol vol⁻¹). The resultant mixture was partitioned into three and absorbance was measured at 540nm at 20 second intervals for 4 minutes.

3.3.3.3 PAL assay

One ml of supernatant was mixed with 2 ml of 0.05 M borate buffer (pH 8.8) and 1 ml of 0.02 M L-phenylalanine. The samples were incubated at 30°C for one hour. In the control, the extract was replaced with 1 ml of borate buffer (pH 8.8). The reaction was stopped by adding 0.2 ml of 6 M trichloroacetic acid (TCA). The supernatant was aliquoted into three portions for measurement. One activity unit was defined as a change in absorbance of 0.01 at 540 nm h⁻¹ g⁻¹ protein.

3.4 Data collection

3.3.1 Disease severity

Late blight severity (the proportion of leaf area infected) was estimated visually on each plant with the aid of the modified Horsfall-Barrat rating scale of 1 to 12 (1=0%, 12_=100% disease severity).

Table 3.4 Modified Horshall Barrat rating scale of late blight disease

Index	1	2	3	4	5	6	7	8	9	10	11	12
% leaf area affected	0	0-3	3-6	6-12	12-25	25-50	50-75	75-87	87-94	94-97	97-100	100

3.4.2 Disease incidence

Disease incidence was calculated using the following formula:

$$DI (\%) = \frac{n \times \text{Number of infected plants}}{\text{Total number of plants in the field}} \times 100$$

3.4.3 Plant height

Plant height was recorded from base of the plant up to the youngest leaf at the top of the tomato plant using a metre rule up to week 8.

3.4.4 Number of nodes

Number of nodes emerged were recorded per plant visually every week from 2 to 8 weeks after transplanting.

3.4.5 Number of flowers

Number of flowers were recorded visually per plant every week from four to ten weeks after transplanting.

3.4.6 Number of fruits

Number of fruits were recorded visually per plant every week from 8 to 1 WAT.

3.4.7 Chlorophyll content and chlorophyll fluorescence

Chlorophyll content and chlorophyll fluorescence were measured from 2 to 6 WAT using a spadmeter (Spectrum Technologies, Inc 502) and chlorophyll fluorometer (Opti-sciences OS 30 pt), respectively. The recordings were taken between 10:00 am and 15:00 pm where the photosynthetic activity of the plant was at its maximum.

3.5 Data analysis

The data collected were subjected to analysis of variance (ANOVA). Repeated measurements using Genstat 18th was used on plant height, number of fruits, number of flowers, chlorophyll content and chlorophyll fluorescence. Mean separation was done using the least significant difference (LSD) at the 5% significance level. Analysis of late blight disease severity data was done using Kruskal-Wallis test.

CHAPTER FOUR

RESULTS

4.1 Laboratory experiment: Effect of botanical extracts on radial growth and percent inhibition of *P. infestans*

4.1.1 Effect of botanical extracts on radial growth of *P. infestans*

There was a significant ($p < .005$) interaction among time x concentration x botanical extract on radial growth (cm) of *P. infestans* (Figure 4.1). *Azadirachta indica* ethyl acetate, *Andica. indica* water, *Moringa oleifera* and *Lantana camara* water extracts showed a concentration dependent reduction of radial growth of *P. infestans*. There was a significant increase of radial growth as time increased from 3 to 12 days after incubation. Maximal reduction of radial growth was obtained at 2.5 and 5 mg ml⁻¹ botanical extract concentration on *A. indica* water and *M. Oleifera* ethyl acetate respectively. *L. camara* water extract significantly elicited the lowest radial growth as compared to *M. oleifera* water, *E. nigra* water and *E. nigra* ethyl acetate extracts. Complete inhibition of *P. infestans* was observed on *L. camara* water extracts where 5 mg ml⁻¹ of botanical concentration was used.

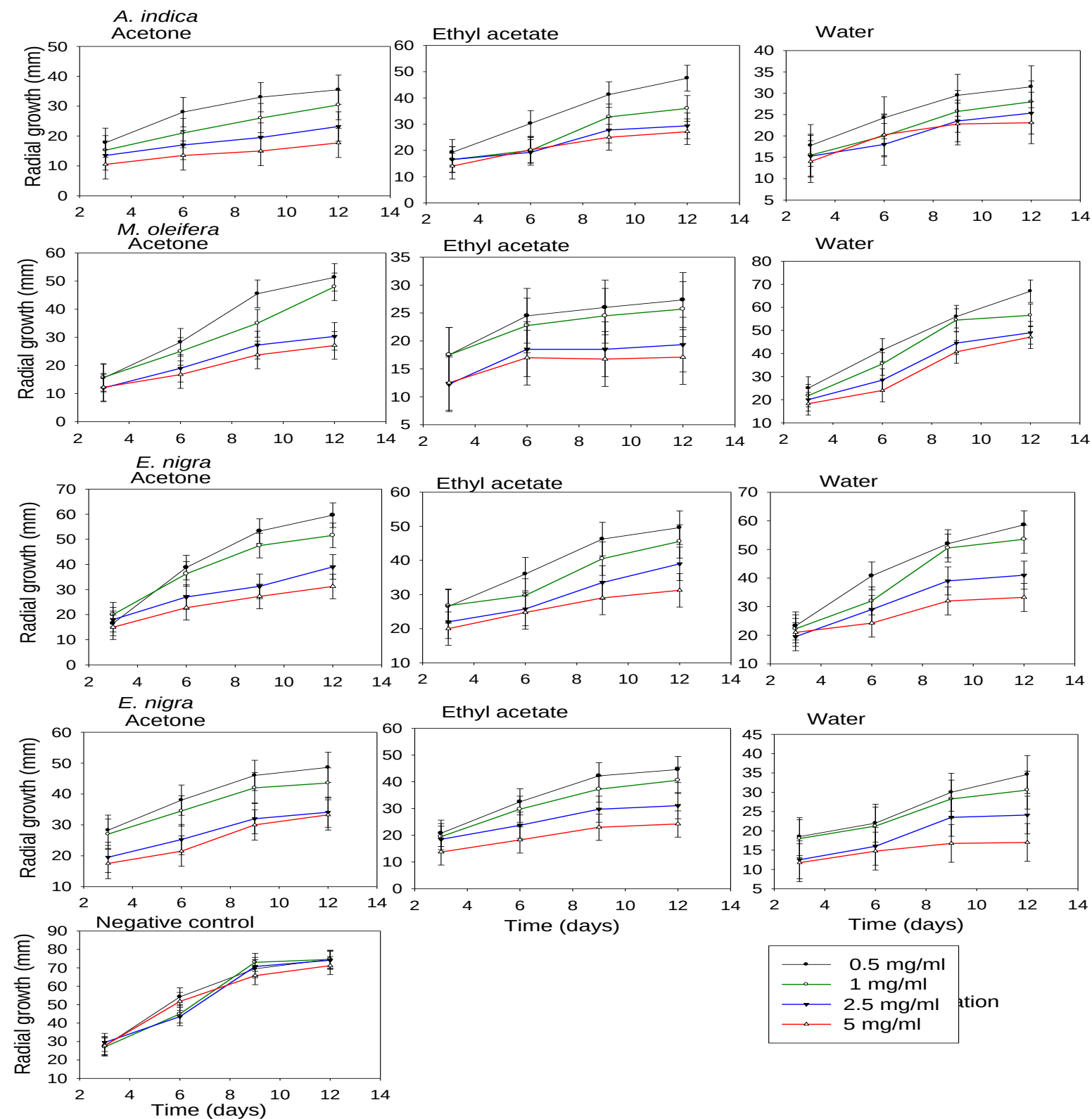


Figure 4. 1 Effect of crude botanical extracts on radial growth of *P. infestans* after 3, 6, 9 and 12 days of incubation (DOI). Error bars represent least significant difference (LSD) at $p < 0.05$ of a mean pooled from four replications.

4.1.2 Effect of crude botanical extracts on percent inhibition of *P. infestans*

The interaction between botanical extract x extraction solvent x extract concentration on the percent inhibition of *P. infestans* was significant ($p < 0.05$). Figure 4.2 shows that percent inhibition was significantly lower in *E. nigra* water extracts at 12 days of incubation compared to the other botanical extracts. The % inhibition was significantly reduced as the days of incubation (DOI) increased. On the other hand, the extract concentration of 5 mgml⁻¹ significantly increased the % inhibition of *P. infestans*.

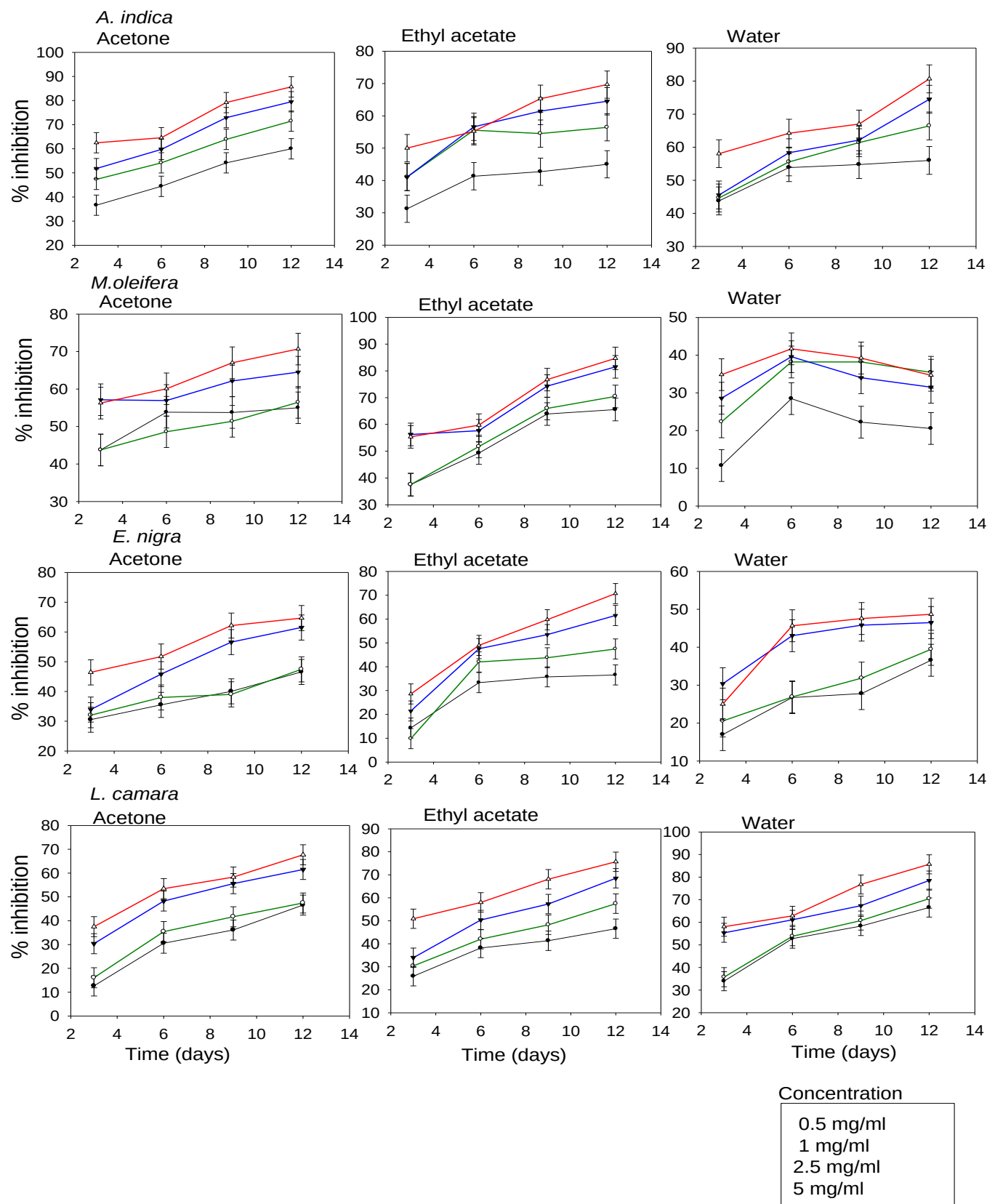


Figure 4. 2 Effect of crude botanical extracts on % inhibition of *P. infestans* *in vitro*. Error bars represent LSD at $p < 0.05$ of a mean pooled from three replications.

4.2 Greenhouse experiment: Effect of botanical extracts on physiological, vegetative and yield parameters of tomato

4.2.1 Chlorophyll content

The interaction amongst botanical extract x extraction solvent x extract concentration on chlorophyll (millimoles m^{-2}) content of tomato was significant ($p < 0.05$) (Figure 4.3). Chlorophyll content significantly increased on tomato plants treated with *L. camara* water, *M. oleifera* ethyl acetate, *A. Indica* water and *A. Indica* acetone extracts. Chlorophyll content was significantly increased as the concentration increased from 2.5 to 5 mg ml^{-1} . However, chlorophyll content was significantly reduced in tomato plants treated with *M. oleifera* water, *E. nigra* water extracts and negative control at six weeks after transplanting compared to the other botanical extracts.

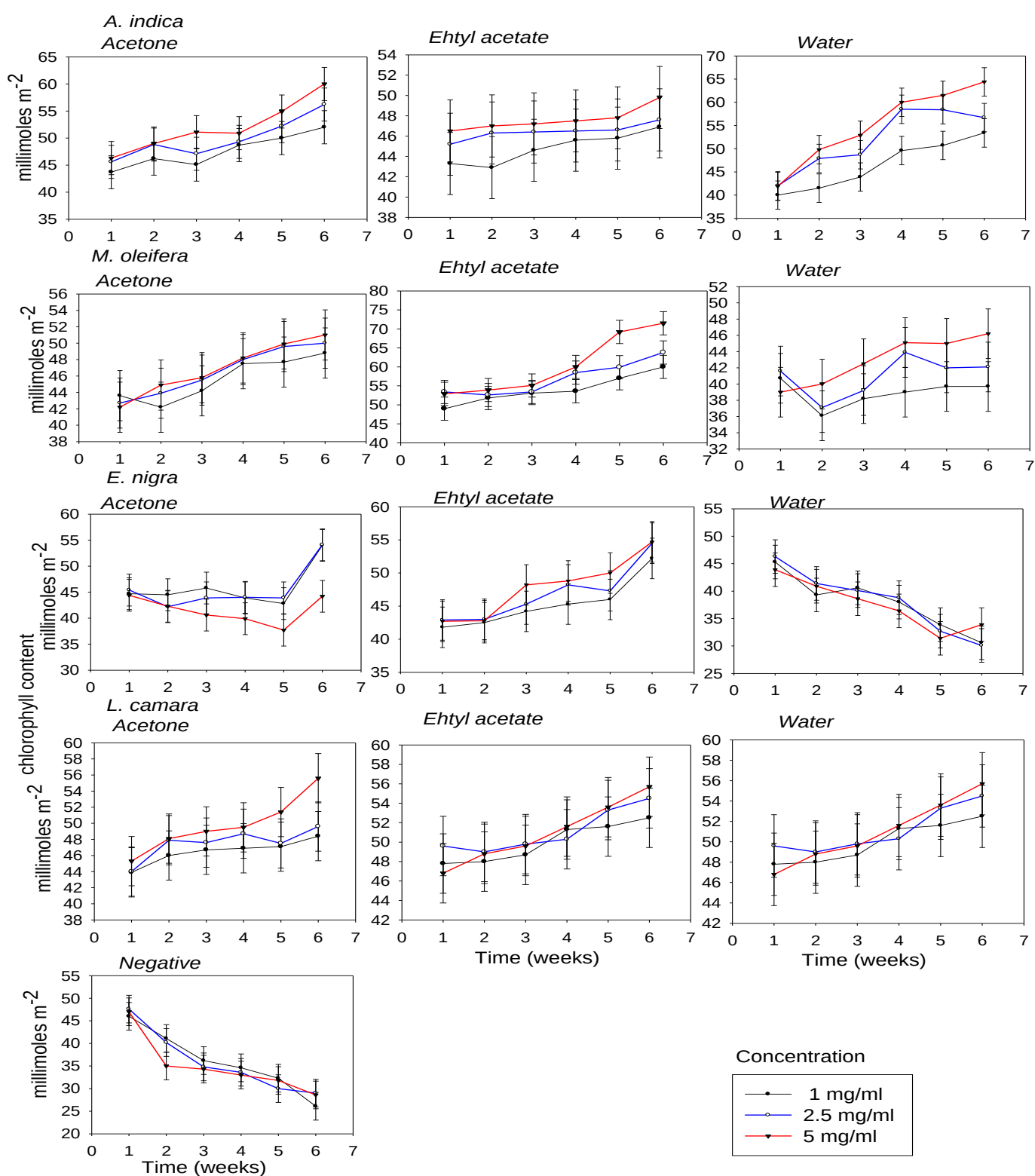


Figure 4.3 Effect of botanical extracts on chlorophyll content of tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars represent LSD at $p < 0.05$ of a mean pooled from three replications.

4.2.2 Chlorophyll fluorescence

The interaction amongst botanical extract x extraction solvent x extract concentration on the chlorophyll fluorescence was significant ($p < 0.05$) (Figure 4.4). Chlorophyll fluorescence ($\text{mmol m}^{-2} \text{s}^{-2}$) was significantly higher in plants treated with *L. camara* water, *M. oleifera* ethyl acetate, *A. Indica* water and *A. Indica* acetone extracts at extract concentration 2.5 to 5mg ml^{-1} . Chlorophyll fluorescence was significantly lower in plants treated with *M. oleifera* water extracts and negative control at 6 WAT compared to the other botanical extracts. Chlorophyll fluorescence significantly decreased as the concentration of *E. nigra* water and *L. camara* extracts increased from 0 to 2.5mg ml^{-1} . Conversely, chlorophyll fluorescence significantly increased in plants that were sprayed with 5mg ml^{-1} of *E. nigra* ethyl acetate extracts.

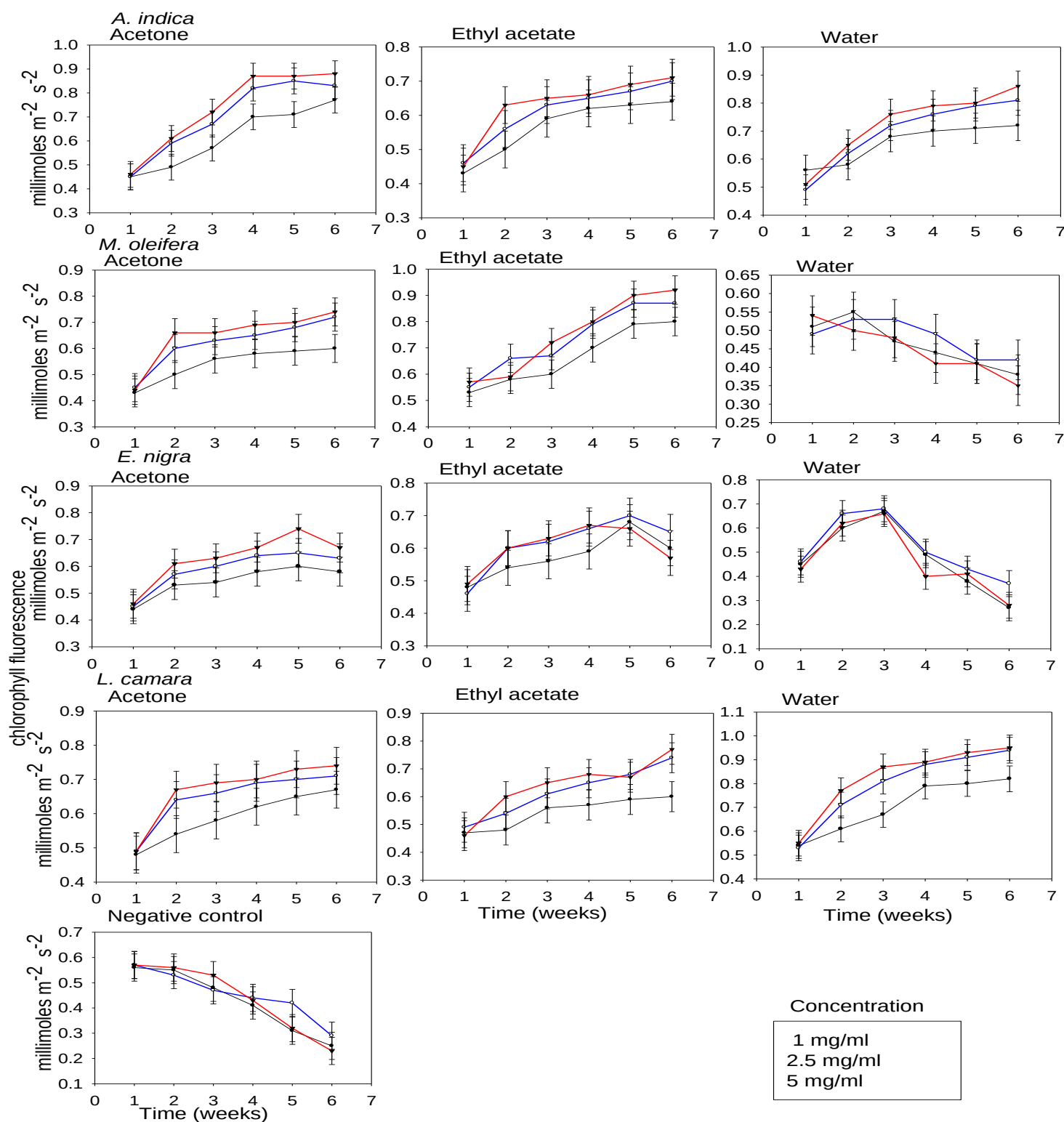


Figure 4.4 Effect of botanical extracts on chlorophyll fluorescence of tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars represent LSD at $p < 0.05$ of a mean pooled from three replications.

4.2.3 Effect of botanical extracts on defence enzymes

4.2.3.1 POD

There was a significant ($p < 0.05$) interaction among botanical extract x concentration x time on the activity of POD ($\text{U } \mu\text{l}^{-1} \text{ min}^{-1}$) in tomato plants infested with late blight (Figure 4.5). The POD activity significantly increased as the time progressed from 1 to 6 WAT. There was a significant difference in POD activity as the concentration of botanical extracts increased from 1.0 to 5.0 mg ml^{-1} in *A. Indica* acetone, *A. indica* water and *M. oleifera* ethyl acetate extracts. However, *E. nigra* and *M. oleifera* water extracts showed a concentration dependent reduction in the activity of POD.

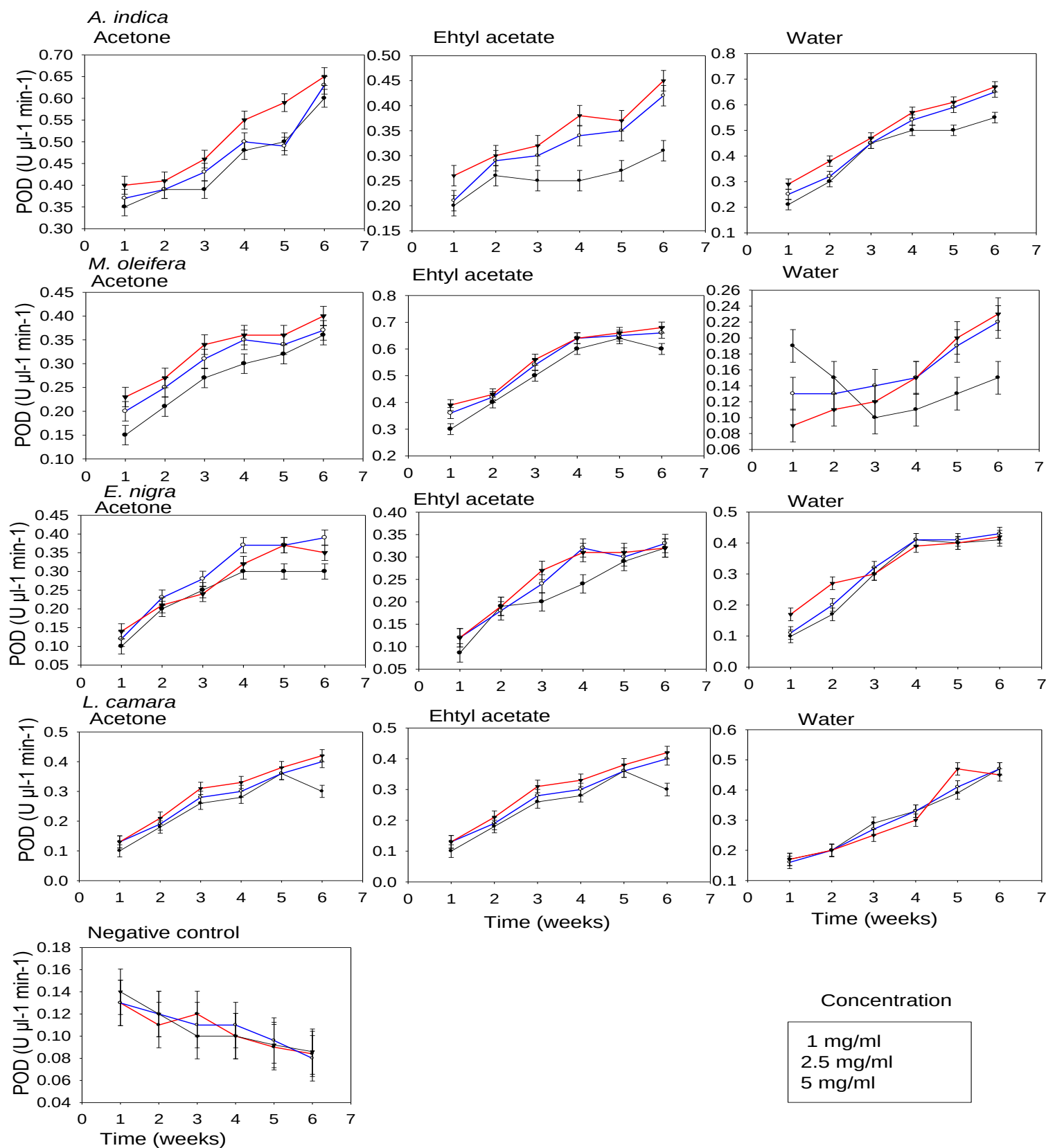


Figure 4.5 Effect of botanical extracts on POD activity in tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate LSD at $p<0.05$.

4.2.2.2 PPO

There was a significant ($p < 0.05$) interaction between botanical extracts x concentration x time on the activity of PPO ($\text{U } \mu\text{l}^{-1} \text{ min}^{-1}$) in tomato plants infected with *P. infestans* (Figure 4.6). *A. indica* acetone, *A. indica* water and *M. oleifera* ethyl acetate extracts at 1 mg ml^{-1} significantly reduced the activity of PPO. As the botanical extract concentration increased from 1 to 2.5 mg ml^{-1} , there was no significant difference from 5.0 mg ml^{-1} on the activity of PPO. For most (except *M. oleifera* and *E. nigr*a water) extracts, there was a concentration dependent increase in PPO activity. However, in negative control, there was significant decrease on PPO activity.

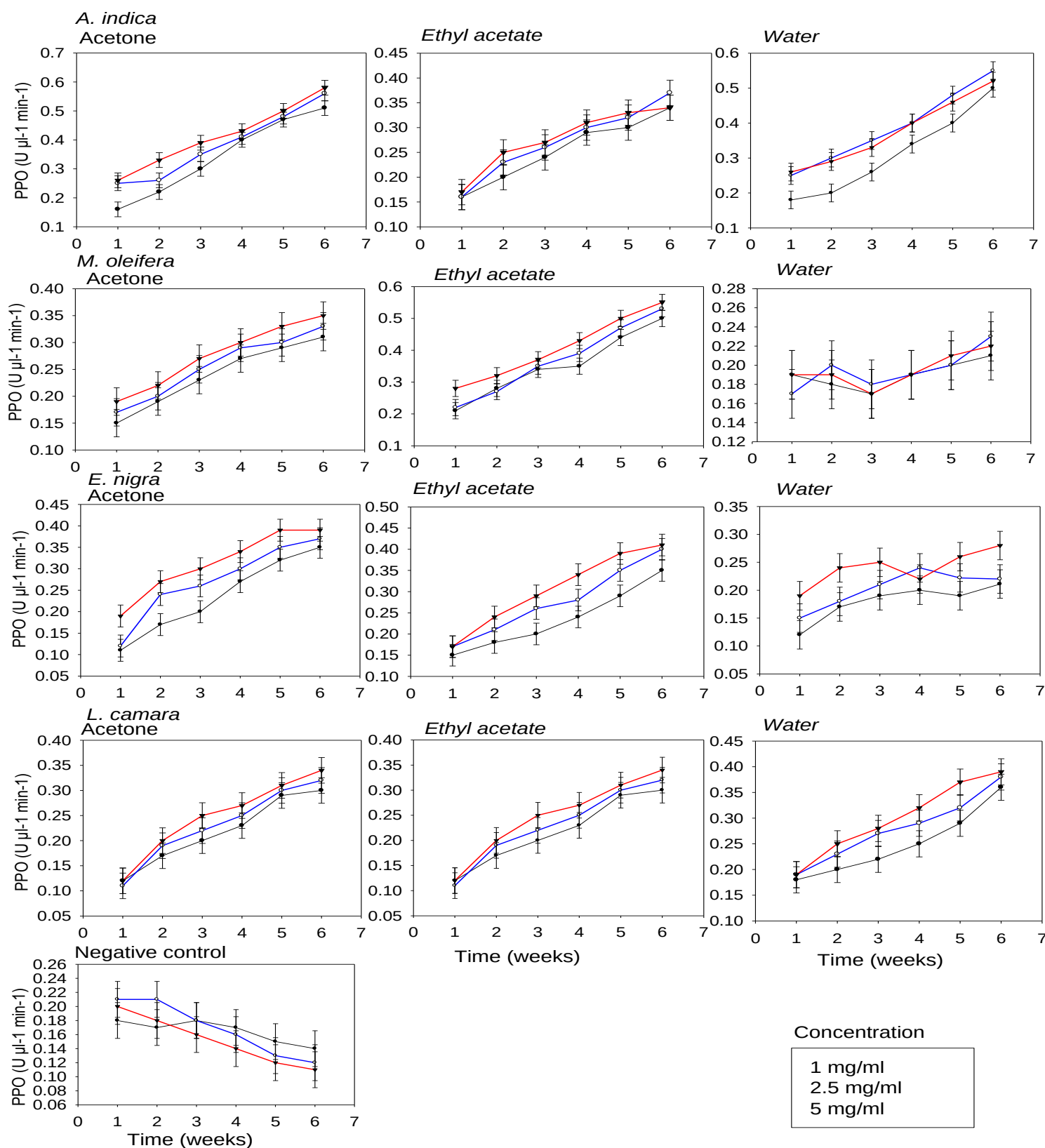


Figure 4. 6 Effect of different botanical extracts on PPO of tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate LSDat $p < 0.05$.

4.2.2.3 PAL

The interaction among botanical extract x concentration x time was significant ($p < 0.05$) on PAL activity ($\mu\text{g PAL g}^{-1}$). Figure 4.7 showed that increasing the extract concentration from 1 to 5mg ml^{-1} significantly increased the PAL activity of plants treated with *A. indica* acetone, *A. indica* ethyl acetate, *A. indica* water and *M. oleifera* ethyl acetate extracts. Figure 4.7 shows that activity significantly increased as the time increased from 1 to 6 WAT.

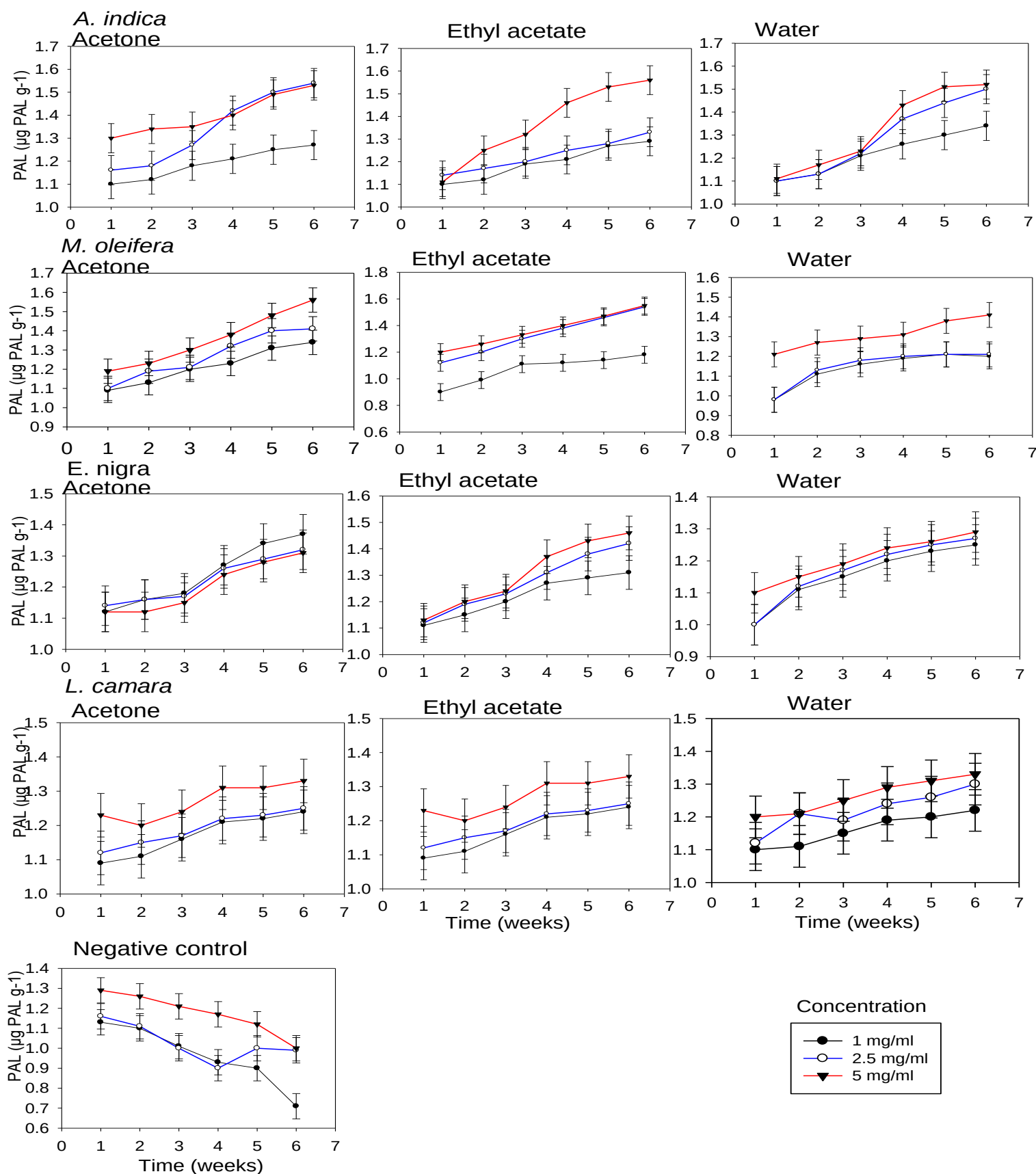


Figure 4. 7 Effect of botanical extracts on the PAL of tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate LSD at $p < 0.05$.

4.3 Morphological parameters

4.3.1 Plant height

There was significant ($p < 0.05$) interaction between botanical extracts x concentration x time on the plant height of tomato plants infected with *P. infestans* (Figure 4.8). Height significantly increased from week 5 to week 9 (Figure 4.8). Plant height was significantly ($p < 0.05$) higher on 2.5 and 5 mg ml⁻¹ as compared to the 1 mg ml⁻¹ extract concentration on tomato plants treated with *A. indica* acetone, *A. indica* ethyl acetate, *A. indica* water and *M. oleifera* ethyl acetate extracts (Figure 4.8).

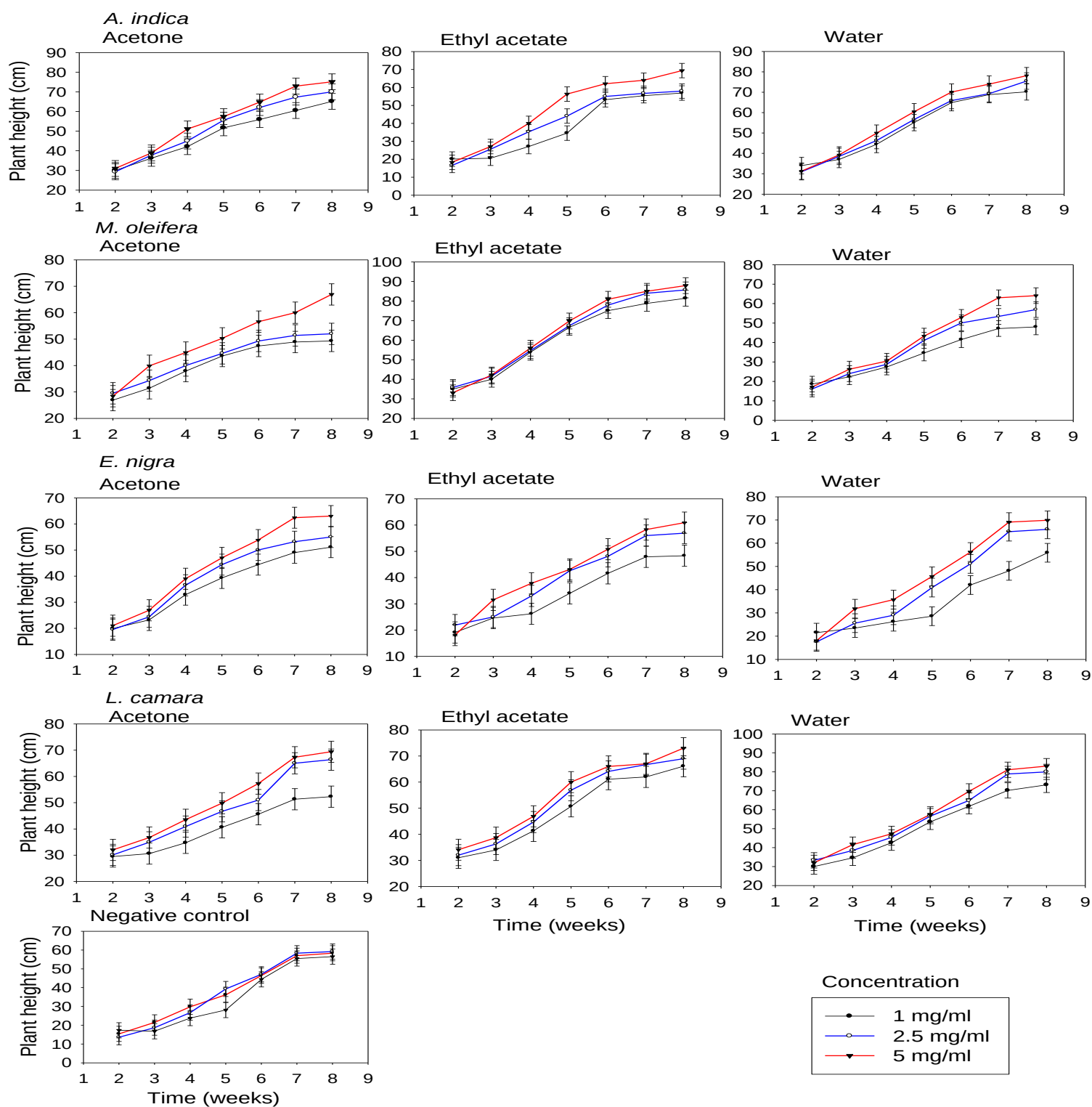


Figure 4.8 Effect of botanical extracts on plant height of tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate LSD at $p < 0.05$.

3.3.2 Number of nodes

There was a significant ($p < 0.05$) interaction among botanical extracts x concentration x time on the number of nodes on tomato plants infected with *P. infestans* (Figure 4.9). The number of nodes significantly increased with time. Figure 4.9 showed that increasing the extract concentration from 1 - 5 mg ml⁻¹ significantly increased the number of nodes on tomato plants that were treated with *A. indica* acetone, *A. indica* ethyl acetate, *A. indica* water and *M. oleifera* ethyl acetate extracts.

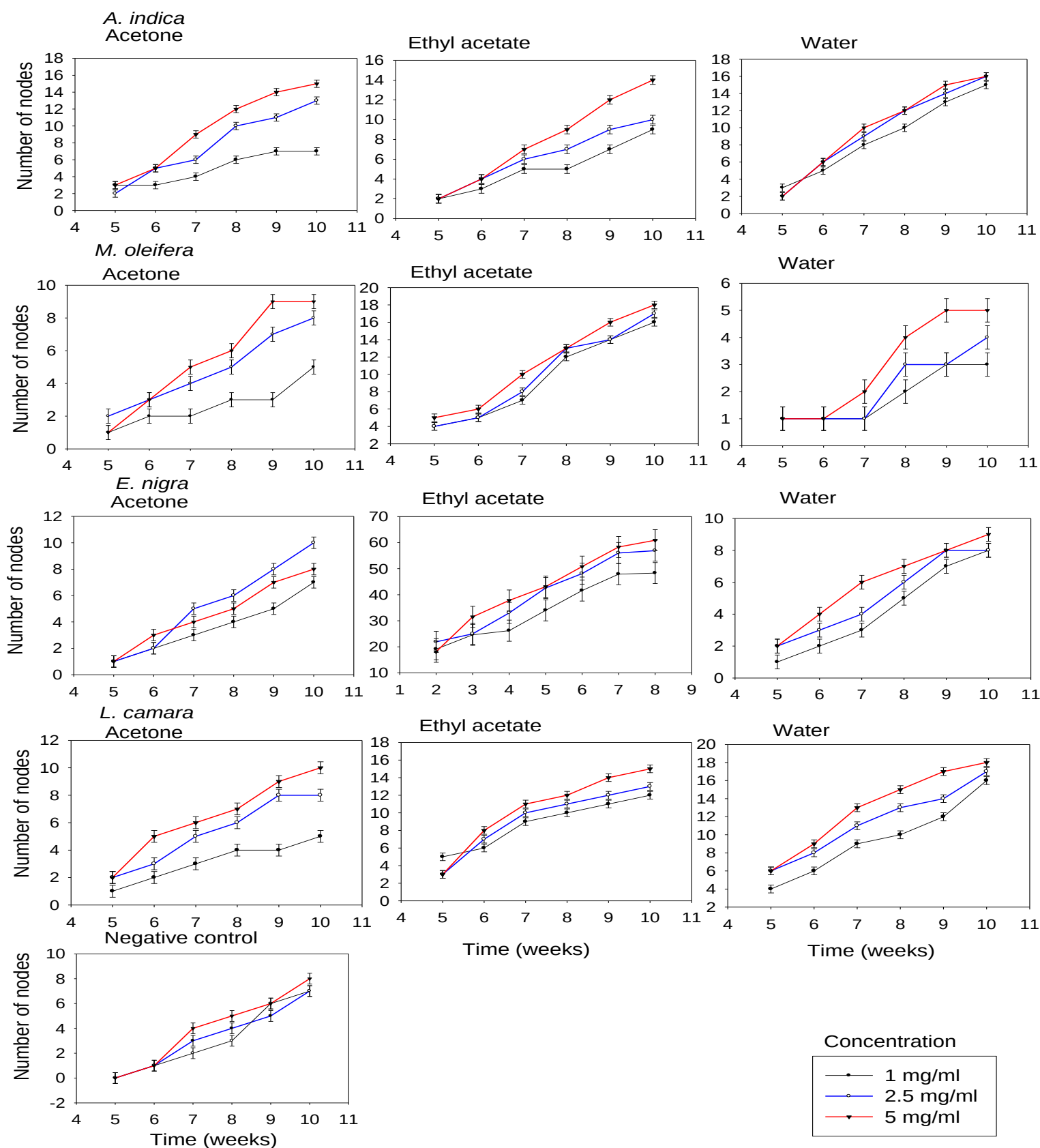


Figure 4.9 Effect of botanical extracts on the number of nodes on tomato plants inoculated with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars represent LSD at $p < 0.05$.

4.3.3 Number of flowers

There was a significant ($p < 0.05$) interaction among botanical extracts x concentration x time on the number of flowers in tomato plants infected with *P. infestans* (Figure 4.10). However, there was no significant difference between the concentration and time. Increasing extract concentration from 1 to 5 mg ml⁻¹, significantly increased the number of flowers on plants treated with *A. indica* acetone, *A. indica* ethyl acetate, *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts.

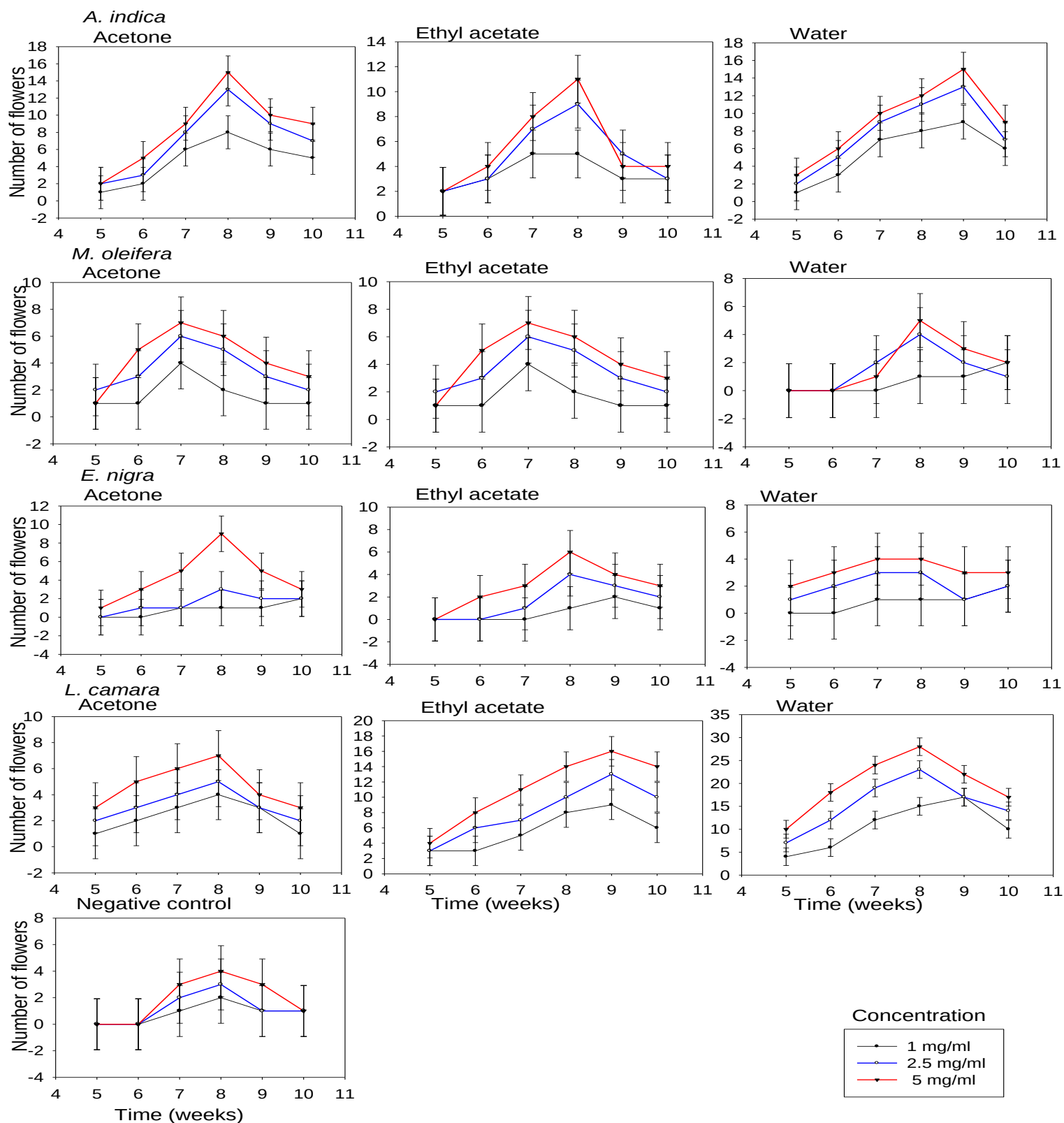


Figure 4.10 Effect of botanical extracts on the number of flowers on tomato plants inoculated with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate the LSD at $p < 0.05$.

4.3.4 Number of fruits

The interaction between botanical extracts x concentration x time was significant ($p < 0.05$) on the number of fruits on tomato plants infected with *P. infestans* (Figure 4.11). The number of fruits significantly increased on tomato plants treated with *A. indica* acetone, *A. indica* water, *M. oleifera* and *L. camara* water extracts. However, the numbers of fruits were significantly reduced on tomato plants treated with *M. oleifera* water, *E. nigra* ethyl acetate and *L. camara* acetone extracts. The number of fruits significantly increased from week 10 to week 13 across all the treatments evaluated.

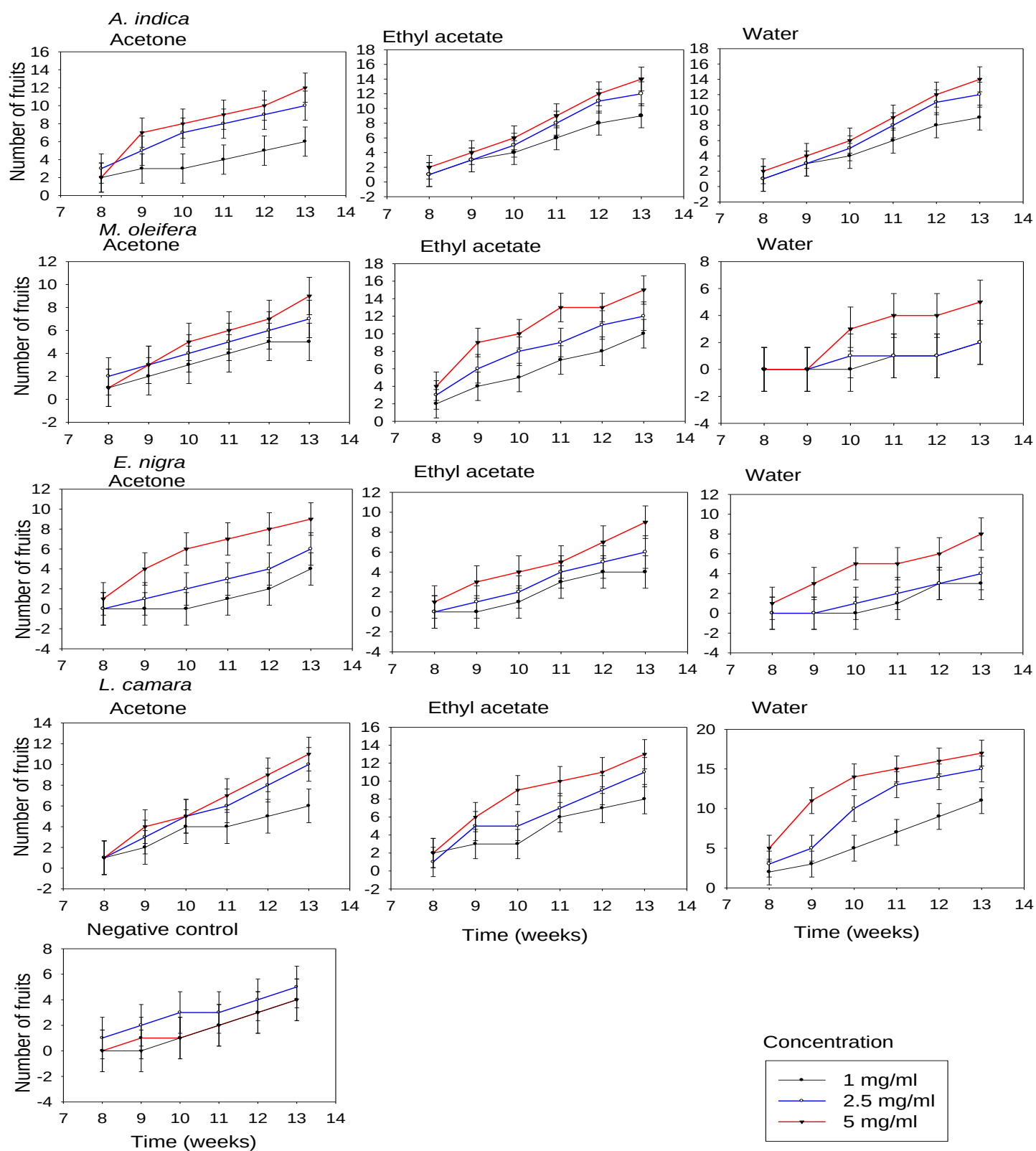


Figure 4. 11 Effect of botanical extracts on the number of fruits on tomato plants with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate the LSD at $p < 0.05$.

4.4 Leaves, stem and root biomass

There was a significant ($p < 0.05$) concentration x botanical extract interaction on the leaves, stem and roots biomass of tomato plants infested with *P. infestans*. Extracts from *A. Indica* acetone, *A. Indica* water, *M. oleifera* and *L. camara* water showed a concentration dependent increase on leaf, stem and root biomass. Maximum biomass on leaves, stems and roots was obtained at 2.5 and 5mg ml⁻¹ botanical extract concentration of *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extract. Leaf, stem and roots biomass was significantly increased on *M. oleifera* ethyl acetate and *A. indica* acetone, *A. indica* water and *L. camara* water extracts. However, *M. oleifera* water, *E. nigra* water, *L. camara* acetone extracts and the negative control significantly reduced leaf, stem and root biomass.

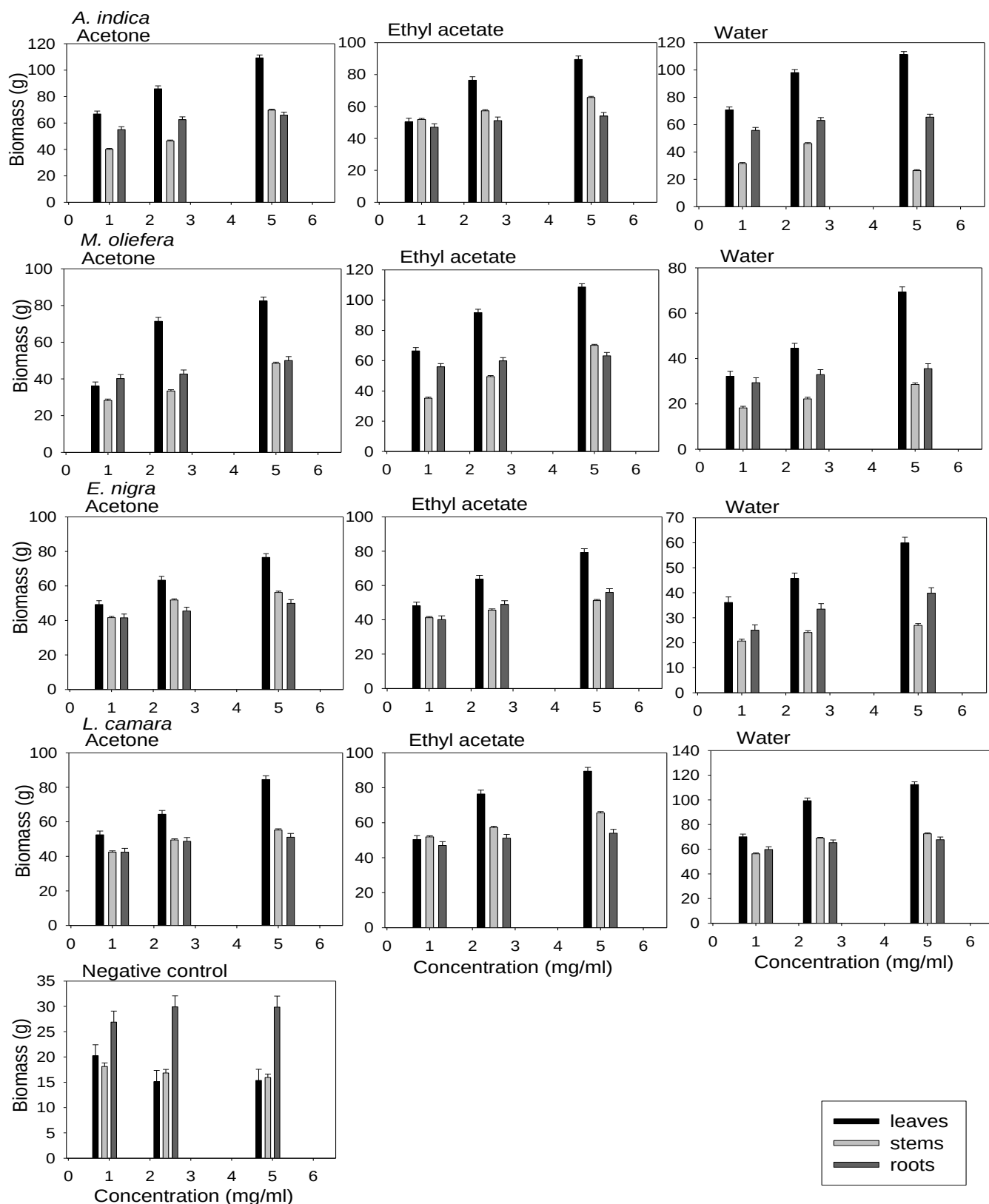


Fig 4.16 Effect of botanical extracts on leaf, stem and root biomass of tomato plants inoculated with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate the LSD at $p < 0.05$.

4.5 Yield parameters

There was a significant ($p < 0.05$) concentration x botanical extract interaction on the total and marketable yield of tomato plants infested with *P. infestans* (Figure 4.16). Extracts from *A. Indica* acetone, *A. Indica* water, *M. oleifera* and *L. camara* water extracts showed a concentration dependent increase on the yield of tomatoes. Maximum total and marketable yield was obtained at 2.5 and 5mg ml⁻¹ botanical extract concentration in plants treated with *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extract (Figure 4.16 and Figure 4.17). There was no marketable tomato fruit obtained from the the negative control.

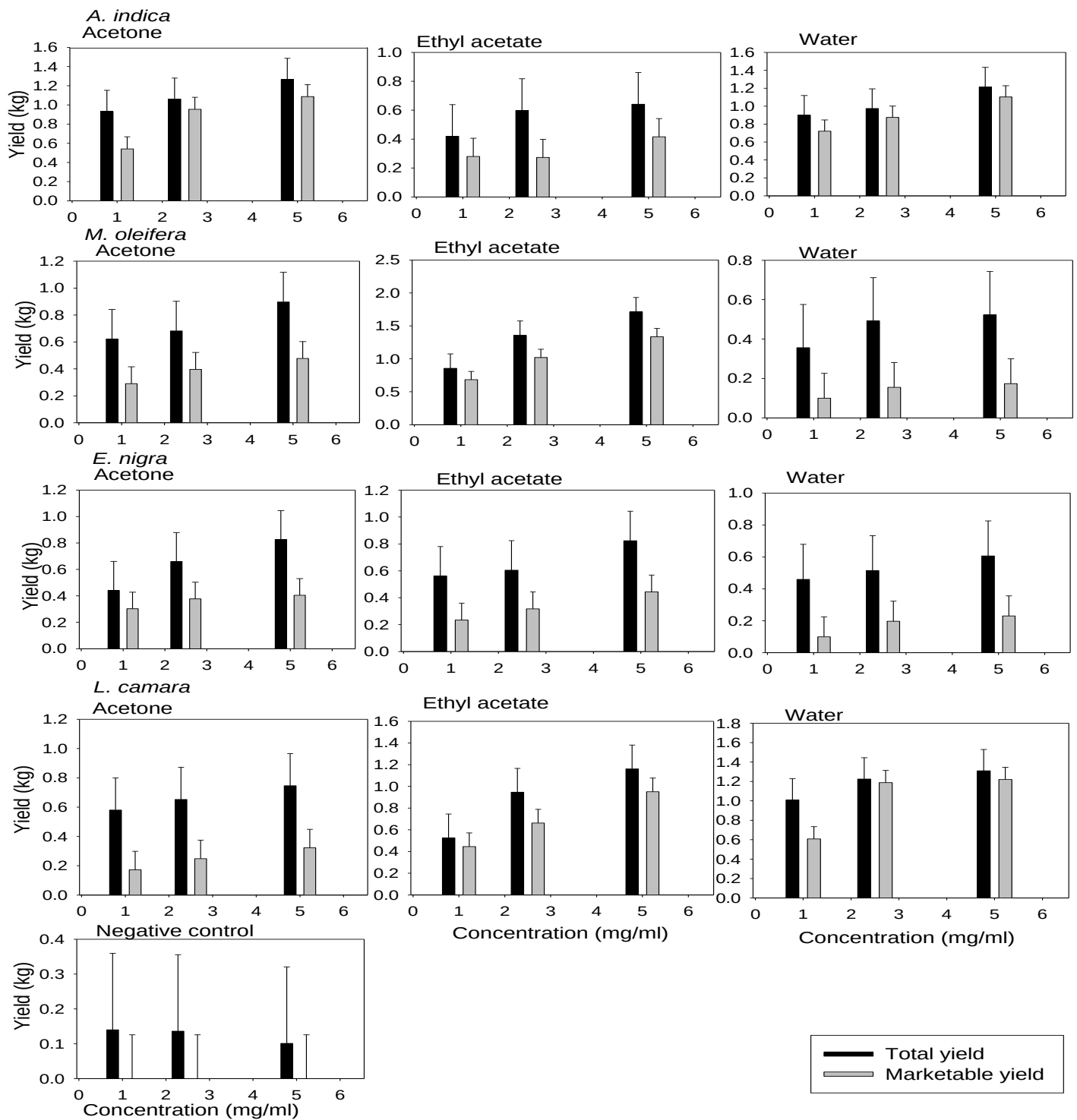


Fig 4.16 Effect of botanical extracts on the yield on tomato plants after inoculated with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicate the LSD at $p < 0.05$.

Non marketable tomatoes



E.nigra water extract



L.camara acetone
extract



Negative control



M.oleifera water extract

Marketable tomatoes



M.oleifera ethyl acetate
extract



A. indica water
extract



A.indica acetone
extract



L. camara water extract

Fig 4.17 Effect of botanical extracts on the marketable yield from tomato plants after inoculation with *P. infestans* under glasshouse conditions at the University of Zimbabwe.

4.6 Disease incidence

The interaction between botanical extract x concentration x time was significant ($p < 0.05$) on the disease incidence (%) of tomato plant infected with *P. infestans*. Figure 4.12 showed that *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts significantly reduced the disease incidence of late blight. However, disease incidence was significantly higher on tomato plants treated with *M. oleifera* water and *E. nigra* water extracts compared to the other treatments. As the number of weeks increased from 5 to 11 weeks after transplanting, *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts significantly reduced the disease incidence of late blight. On the other hand, increasing extract concentration from 1 to 2.5 mg ml⁻¹ significantly reduced disease incidence. However, extract concentration of 2.5 mg ml⁻¹ was statistically similar to 5.0 mg ml⁻¹ on tomato plants treated with *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts.

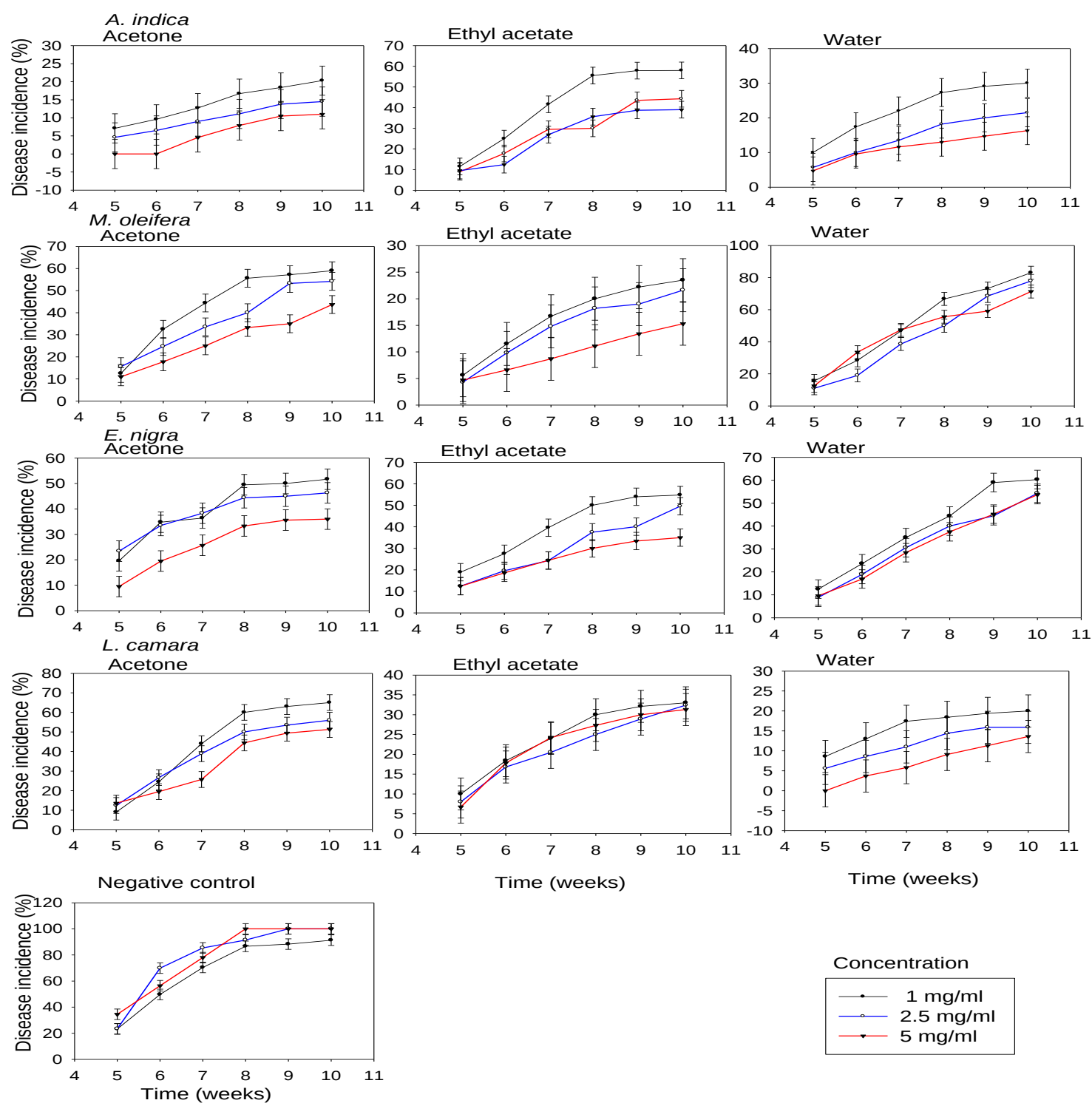


Figure 4.12 Effect of botanical extracts on the disease incidence on tomato plants inoculated with *P. infestans* under glasshouse conditions at the University of Zimbabwe. Error bars indicates LSD at $p < 0.05$.

4.7 Disease severity

There was a significant ($p < 0.05$) time x concentration x botanical extract interaction on the disease severity of *P. infestans* (Figure 4.14). Extracts from *A. Indica* acetone, *A. Indica* water, *M. oleifera* ethyl acetate and *L. camara* water showed a concentration dependent reduction on disease severity of *P. infestans*. Disease severity was significantly reduced at 2.5 and 5mg ml⁻¹ botanical extract concentration on plants treated with *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts. Disease severity was significantly reduced on *M. oleifera* ethyl acetate and *A. indica* acetone, *A. indica* water and *L. camara* water extracts. However, *M. oleifera* water, *E. nigra* water, *L. camara* acetone extracts and the negative control significantly increased disease severity.

Treatments with high disease severity



E.nigra + water
(7.67)



L.camara+ acetone
(8.67)



Negative control
(11)



M.oleifera+ water
(9.33)

**Low disease
severity**



M.oleifera + ethyl acetate
(2.63)



A. indica + water
(3)



A.ndica + acetone
(3.667)



L. camara+ water
(1.33)

Figure 4. 13 Effect of botanical extracts on disease severity on tomato plants infected with *P. infestans* over a period of 10 weeks

4.8 Gas chromatography-mass spectrometric (GCMS) analysis of botanical extracts of *M. oleifera*, *A. indica*, *E. nigra* and *L. camara*

GC-MS analysis of *M. oleifera*, *A. indica*, *E. nigra* and *L. camara* extracts was done using acetone, ethyl acetate and water as extraction solvents. It showed major molecular peaks at different m/z values of the possible antifungal compounds as shown in the Tables 4.1, 4.2, 4.3 and 4.4.

Table 4. 1 Mass spectra data and tentative identification of the antifungal compounds in neem extract

Solvent	Compound Name	Formula	Match Score
Acetone	Heptane, 4-methyl-	C ₈ H ₁₈	85.9
	Furfural	C ₅ H ₄ O ₂	89.4
	Octanal	C ₈ H ₁₆ O	84.7
	(1-Ethyl-2-methylpropyl) methylamine	C ₇ H ₁₇ N	80.8
	Hydroxymethyl 2-hydroxy-2-methylpropionate	C ₆ H ₁₂ O ₄	80.7
	Isopropyl Alcohol	C ₃ H ₈ O	81
	1H-Benzimidazole, 1,2-diphenyl-	C ₁₉ H ₁₄ N ₂	81.8
	Silane, diethylpentadecyloxytetradecyloxy-	C ₃₃ H ₇₀ O ₂ Si	83.2
	Silane, diethyldecyloxyoctyloxy-	C ₂₂ H ₄₈ O ₂ Si	81.9
Ethyl Acetate	Furfural	C ₅ H ₄ O ₂	86.1
	Piperazine, 1,4-dinitro-	C ₄ H ₈ N ₄ O ₄	83.4
	Pyrrolidine, 1-nitroso-	C ₄ H ₈ N ₂ O	96.8
	2-Butanol	C ₄ H ₁₀ O	83.5
	2-Butanol	C ₄ H ₁₀ O	83.5
Water	Furfural	C ₅ H ₄ O ₂	80.2
	1-Heptene	C ₇ H ₁₄	86.4
	1-Pentanol	C ₅ H ₁₂ O	90.7
	2,2'-Trimethylenebis-1,3-dioxolane	C ₉ H ₁₆ O ₄	80.1
	3-Butenoic acid, ethyl ester	C ₆ H ₁₀ O ₂	83

Table 4.2 Mass spectra data and tentative identification of the antifungal compounds in moringa extract.

Solvent	Compound Name	Formula	Match Score
acetone	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	90.1
	Cyclobutanone, 2-methyl-	C5H8O	86
	1H-1,2,4-Triazol-3-amine, 5-methyl-	C3H6N4	81.4
	Nonanal	C9H18O	82.9
	Propanal, 2-methyl-	C4H8O	81.8
Ehtyl acetate	Ethanol, 2-ethoxy-	C4H10O2	85.7
	5-Methyl -4-(trifluoromethyl)pyrido[4,3-1H-Tetrazole	C9H6F3N3	80.9
	4-Aminoheptane	CH2N4	80.1
	9-(2-(5-Methyl-2-pyridyl)ethyl)-6-methyl-2-Ethyl-2-phenyl-1,3-benzodioxole	C7H17N	81.2
	3,4-Dihydroxyphenylglycol, 4TMS derivative	C21H24N2	81.8
		C15H14O2	81.4
		C20H42O4Si4	89.7
Water	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	87.9
	Octanal	C8H16O	84
	Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6	86.5
	Nonanal	C9H18O	83.2
	Isopropylcyclobutane	C7H14	87.3
	Oxalic acid, butyl cyclobutyl ester	C10H16O4	89.7
	Acenaphthene	C12H10	80.1
	Biphenyl	C12H10	90.8
	Acenaphthene	C12H10	91.5
	Ethanol, 2-ethoxy-	C4H10O2	82.5
	Ethyl diazoacetate	C4H6N2O2	86.6

Table 4.3 **Mass spectra data and tentative identification of the antifungal compounds in guntree extract**

Solvent	Compound Name	Formula	Match Score
Acetone	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	82.1
	1,2,4,5-Tetrazine, 3,6-bis(1,1-dimethylethyl)-	C10H18N4	82.2
	Nonanal	C9H18O	83.8
	Benzene, nitro-	C6H5NO2	85.4
	Dodecanamide	C12H25NO	80.4
	1H-Benzimidazole, 1,2-diphenyl-	C19H14N2	86.4
	3-Pyridinecarboxamide, N-phenyl-	C12H10N2O	83.4
	1H-1,3-Diazepine, 4,5,6,7-tetrahydro-2-methyl-	C6H12N2	81.1
	Silane, diethylpentadecyloxytetradecyloxy-	C33H70O2Si	83
Ethyl acetate	Furfural	C5H4O2	90.1
	Eucalyptol	C10H18O	93
	Ethanone, 1-(2-furanyl)-	C6H6O2	83
	1-(3H-Imidazol-4-yl)-ethanone	C5H6N2O	80.9
	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-	C10H18O2	81.8
	1,3-Cyclobutanedione, 2,2,4,4-tetramethyl-	C8H12O2	82.6
	1,2,5-Thiadiazole	C2H2N2S	80.7
	Dodecanamide	C12H25NO	81.8
Water	1-Hexen-3-one	C6H10O	87.3
	3-Furaldehyde	C5H4O2	98.3
	Propane, 1-isocyanato-	C4H7NO	83.1
	Dodecanamide	C12H25NO	81.5
	Isopropyl Alcohol	C3H8O	83.7
	4-(2-Methoxyethyl)-2-methylphenol	C10H14O2	81

Table 4.4 **Mass spectra data and tentative identification of the antifungal compounds in lantana extract**

Solvent	Compound Name	Formula	Match Score
Acetone	Furfural	C5H4O2	92.9
	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	90.3
	Octanal	C8H16O	82.3
	Piperazine, 1,4-dinitro-	C4H8N4O4	92.4
	Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6	93.4
	Nonanal	C9H18O	86.6
	Dodecanamide	C12H25NO	82.9
	Guanidine	CH5N3	90
	Ethanol, 2-ethoxy-	C4H10O2	81.5
	Ethanol, 2-ethoxy-	C4H10O2	81.7
	Isolan	C10H17N3O2	88.8
Ethyl	1-n-Butoxy-2,3-dimethyldiaziridine	C8H17NO	81.3
Acetate	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	90.1
	Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6	87.4
	2,3,4-Trihydroxybenzaldehyde	C7H6O4	80.6
	1-Propene, 2-methoxy-	C4H8O	80.2
	2-Butanol	C4H10O	84.8
	Dodecanamide	C12H25NO	84.5
	Acetaldoxime	C2H5NO	83.7
	Ethyl diazoacetate	C4H6N2O2	85.4
	Ethanol, 2-ethoxy-	C4H10O2	85.1
	Ethanol, 2-ethoxy-	C4H10O2	81.4
Water	2-Pentanone, 4-hydroxy-4-methyl-	C6H12O2	95.9
	Cyclopentasiloxane, decamethyl-	C10H30O5Si5	91.1
	Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6	88.9
	Nonanal	C9H18O	86
	Ethanol, 2-ethoxy-	C4H10O2	86
	Dodecanamide	C12H25NO	82.5
	Formic acid, ethenyl ester	C3H4O2	85.4
	Pentanamide	C5H11NO	82.7
	(S)-(+)-2-Amino-3-methyl-1-butanol	C5H13NO	84.6

CHAPTER FIVE

DISCUSSION

5.1 Effect of botanical extracts on radial growth and percentage inhibition of *P. infestans* *in vitro*

Fungal growth is measured using radial growth and percentage inhibition. The findings obtained from this study showed that botanical extracts of *Azadirachta indica*, *Moringa oleifera*, *Eucalyptus nigra* and *Lantana camara* contain antifungal compounds which reduced radial growth and increased percent inhibition of *P. infestans*. High percentage inhibition was recorded in *L. camara* water, *M. oleifera* ethyl acetate, *A. indica* water and *A. ndica* acetone extracts.

These results concur with the findings by Ngadze *et al.* (2014) who reported inhibition of mycelial growth of the same pathogen using *A. indica* water and acetone extracts. Reduced mycelial growth of *P. infestans* by *L. camara* and *A. indica* seed and leaf extracts was also reported by Raza *et al.* (2016). Reduced development of *P. infestans* on tomato plants inoculated with the pathogen could be attributed to presence of antifungal compounds in the botanical extracts. Nagar *et al.* (2017) reported that these antifungal compounds bind to and penetrate the surface of *P. infestans* resulting in denaturation of enzymes and proteins. This also concurs with the work done by Ghannoum and Rice (1999) on imidazole derivatives (such as miconazole, econazole, and ketoconazole) which had a complex mode of action, inhibiting synthesis of several membrane-bound enzymes as well as membrane lipid biosynthesis of pathogens.

In contrast, *M. oleifera* water, *E. nigra* water and *L. camara* acetone extracts recorded low mycelium inhibition. Lower inhibition by aqueous extracts reported in their study can be attributed to the fact that oil antifungal compounds do not dissolve in water. This result is in line with the findings of Bandor *et al.* (2013) where Labanese weed (*Urtica dioica*) water extracts recorded low mycelium inhibition. The maximal percent inhibition of *P. infestans* was observed on 5mg ml⁻¹ botanical concentration. It is postulated that high extract concentration of 5 mg ml⁻¹ effectively inhibited mycelial growth and development of *P. infestans*. The findings concur with those of Nagar *et al.* (2017) who reported that willow (*Salix* species) extracts at high concentration effectively increased percent inhibition on *P. infestans*.

5.2 Effect of botanical extracts on the vegetative and reproductive parameters

The application of the botanical extracts significantly increased chlorophyll content and chlorophyll fluorescence of tomato plants. This effect showed that the botanical extracts did not affect the photosynthetic pigment system and consequently photosynthesis. However, chlorophyll content and chlorophyll fluorescence were low in plants treated with botanical extracts from *M. oleifera* water, *E. nigra* water, *L. camara* acetone. These findings complemented results from the laboratory experiment from the current study, where the same extracts caused lower inhibition of *P. infestans*. This could be attributed to their low efficacy in suppressing the development of the disease which resulted in quick termination of biosynthesis of chlorophyll. Kaur and Sharma (2016) reported that some botanical extracts can initiate termination of chlorophyll biosynthesis and lead to fast chlorophyll degradation.

The *in vivo* experiment showed that *L. camara* water, *M. oleifera* ethyl acetate, *A. indica* water, *A.indica* acetone and *E.nigra* ethyl acetate extracts were significantly superior over other

treatments in terms of increasing plant height, number of nodes and number of flowers. The increase in plant height and yield parameters could be attributed to the stimulatory effect of antifungal compounds produced by these botanical extracts. In a related report, Yadav *et al.* (2017) reported that *A. indica* increased root length, plant height and shoot weight by hindering the growth of *P. infestans* and ultimately resulting in good health of the tomato plants. However, *M. oleifera* water, *E. nigra* water, *L. camara* acetone extracts produced low number of nodes, number of flowers and number of fruits. This could be due to these botanical extracts producing compounds that had a negative impact on other physiological processes like chlorophyll content, chlorophyll fluorescence and enzyme activity (Cheng and Cheng, 2015). These physiological parameters are very crucial for the vegetative growth and development of tomato plants. These results concur with the findings of Romero-Romero *et al.* (2005) who reported a similar trend when leachates of *S. deppci* caused a strong allelochemicals stress on tomato growth.

The application of the botanical extracts significantly increased leaves, stem and root biomass of tomato plants. However, the leaf, stem and root biomass were low in plants treated with *M. oleifera* water, *E. nigra* water, *L. camara* acetone extracts. This concurs with the findings of Yadav *et al.* (2017) where *A. indica* and other botanical extracts increased shoot and root biomass of tomato. This can be attributed to the fact that compounds in these botanical extracts quickly inhibit growth and development of late blight.

5.3 Effect of botanical extracts on the defence enzymes

There was a significant increase in defence enzyme activity of PPO, PAL and POD in plants treated with *M. oleifera* ethyl acetate, *A. indica* water and *A. indica* acetone extracts. The increase in enzyme activity could be explained by the fact that *M. oleifera* ethyl acetate, *A.*

indica water and *A. indica* acetone extracts contained compounds that had a stimulatory effect on the activity of PPO, PAL and POD which can protect plant cells from lipid peroxidation (Nunes *et al.*, 2015). These findings concur with the work of Singh and Sunaina (2014) who reported increased defence enzyme activity after application of bitter melon (*M. charantia*) extracts in tomatoes.

Moreover, PPO, PAL and POD activity was also reported by Ngadze *et al.* (2012) as having the ability to impart resistance to soft rot in potatoes (*S. tuberosum*). The increase in POD activity was very crucial as the first line of defence because it produces lignin and isodilyrosine from oxidation of hydroxycinnamyl alcohol and PAL enhances production of cinnamic acid (Vanitha *et al.*, 2009). These compounds cement the cell walls of host plant so as to inhibit the entry of the pathogen. Even though the tomato plants were inoculated with *P. infestans*, these defence enzymes had the ability to detoxify the reactive oxygen species (ROS) which were synthesised when the plant were stressed by *P. infestans*. Although *L. camara* water extract reduced growth and development of *P. infestans* both *in vitro* and *in vivo*, there was low enzyme activity for all the defence enzymes evaluated. Defence enzyme activity was lower compared to *M. oleifera* ethyl acetate, *A. indica* water and *A. indica* acetone extracts.

5.4 Effect of botanical extracts on disease incidence and late blight disease severity on tomatoes

Four botanical extracts *L. camara* water, *M. oleifera* ethyl acetate, *A. indica* water and *A. indica* acetone extracts reduced disease incidence and severity under greenhouse conditions after ten weeks. The possible reason for these findings may be due to the activation of plant defence

enzymes by the four botanical extracts which detoxify the ROS produced by the tomato plants infected by *P. infestans*.

Moreover, tomato can produce defence compounds after application of botanical extracts as reported by Mvumi *et al.* (2018) where *M. oleifera* extract resulted in increased amount of sinigrin which is important in defence system against late blight on tomato. These results concur with the findings of Goufo *et al.* (2010a) where floss flower (*A. haustonianum*), horsewood (*C. anisata*) and fish poison (*T. vogelli*) inhibited *P. infestans* development.

Highest disease incidence and severity were recorded in *M. oleifera* water, *E. nigra* water, *L. camara* acetone extracts. These botanical extracts had less inhibitory effects on late blight. Interestingly, all the extracts tested reduced disease incidence and disease severity compared to negative control.

5.5 Effect of botanical extracts on yield parameters.

The application of the botanical extracts significantly increased marketable yield and total yield of tomato fruits. This effect showed that botanical extracts did not cause chemical phytotoxicity to the tomato fruits. This can be attributed to the fact that, compounds in these botanical extracts quickly inhibit growth and development of late blight. However, low marketable and total yield obtained in tomato plants treated with *M. oleifera* water, *E. nigra* water and *L. camara* acetone extracts.

5.5 Gas Chromatography-Mass Spectrometric analysis of antifungal compounds in botanical extracts

The antifungal compounds in *A. indica*, *M. oleifera*, *E. nigra* and *L. camara* using acetone, ethyl acetate and water as extraction solvents were tentatively identified using GC-MS. These

antifungal compounds were isolated and eluted at a retention time interval from 0 to 3.4 minutes. Interestingly, most of the antifungal compounds found in the study have not yet been documented especially on *M. oleifera* and *A. indica*. From the research, the main antifungal compound found in neem across all the solvent was furfural, which is one of the furan derivative formed from the portion of lignicellulosis. Mondali *et al.* (2009) previously reported that the active ingredients of neem water extracts constituted mostly of triterpenoides, such as Nimbin, Nimbidine, Azadirachtin. The difference in the type of compounds isolated could be attributed to the differences in extraction methods that were used.

Cyclopentasiloxane and decamethyl were found mostly in *E. nigra* and *L. camara* extracts across all the solvents. In contrast, Patil *et al.* (2017a); Shriniwas and Subhash, (2018) reported that *Eucalyptus* species and *L.camara* had a higher constituency of terpenes. The variation in different antifungal compounds found in botanical extract can be influenced by extraction solvent and method of extraction. According to Rouhallah and Choong (2018), proficiency in sensing and responding to these antifungal compounds increases plant ecological advantage and ability to survive in competitive environments, with respect to plant-pathogen interactions. So from the antifungal compounds which were extracted from these botanical extracts using different solvents, the antifungal compounds had a lethal effect on the growth and establishment of *P. infestans* both *in vivo* and *in vitro* at all the parameters tested.

These botanical extracts are environmentally friendly as they are non pollutive, largely non phytotoxic and have no residual effects thus being readily biodegradable, renewable and inexhaustible (Muthomi *et al.*, 2017) hence playing a crucial role in smart agriculture as it can be used as a management tool on late blight resistance. For the reserchers, there is still need to

establish the antifungal properties using pure standards of these antifungal compounds that were detected in this study.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the study it can be confirmed that:

1. *A. indica* acetone, *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts effectively increased percent mycelial inhibition and reduced radial growth of the *P. infestans in vitro*. The study revealed that extract concentration of 5 mg ml⁻¹ was more effective as compared to 0.5 mg ml⁻¹.
2. *A. indica* acetone, *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water extracts increased chlorophyll content and chlorophyll fluorescence. This effect showed that the botanical extracts did not affect the photosynthetic pigment system and consequently photosynthesis.
3. Extracts from *A. indica* acetone, *A. indica* water, *M. oleifera* ethyl acetate and *L. camara* water effectively increase plant height, number of nodes, number of flowers, number of fruits, and reduce disease incidence and severity in the greenhouse experiment. The reduced late blight disease incidence and disease severity could be attributed to the inhibitory effect of antifungal compounds produced by these botanical extracts.
4. The PPO, PAL and POD enzymatic activity increased after the application of botanical extracts on tomato plants. This showed that botanical extracts have a potential to increase the defence enzymes activity which have the capacity to detoxify toxic compounds like ROS which are produced after the plant is infested by late blight.

5. Neem acetone, neem water, moringa ethyl acetate and lantana water extracts can produce antifungal compounds such as furfural, 5-Methyl-4- (trifluoromethyl) pyrido, dodecanamide, cyclopentasiloxane and decamethyl and can therefore be used as bio-fungicides in Intergrated Disease Management (IDM) of late blight.

6.2 Recommendations

The study showed that *L. camara* water, *M. oleifera* ethyl acetate, *A. indica* acetone and *A. indica* water extracts reduced development of late blight in all the parameters evaluated. If the trial is repeated under field conditions in sufficient replications and produce the same results, small scale farmers may substitute synthetic fungicides for these botanical extracts in controlling late blight. This is because they are easily accessible, non pollutive, non-phytotoxic, biodegradable and cheap.

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APPENDICES

Appendix 1 Analysis of variance for the effect of botanical extracts on the radial growth of *P. infestans*

Variate: 3, 6 and 9 days of incubation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	3	6.4198	2.1399	1.82	
Extract	12	377.1096	31.4258	26.71	<.001
Concetration_	3	116.4584	38.8195	32.99	<.001
ExtractxConcetration	36	95.2727	2.6465	2.25	<.001
Residual	153	180.0460	1.1768	2.28	
Time	2	482.0818	241.0409	467.95	<.001
TimexExtract	24	198.5378	8.2724	16.06	<.001
Time xConcetration	6	35.9148	5.9858	11.62	<.001
TimexExtractxConcetration_	72	100.0272	1.3893	2.70	0.005
Residual	312	160.7117	0.5151		
Total	623	175	2.5799		

Appendix 2 Analysis of variance for the effect of botanical extracts on the % inhibition of *P. infestans*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	3	563.5	187.8	0.57	
Concentration	3	3687.6	1229.2	3.76	0.012
Extract	3	14460.0	4820.0	14.73	<.001
Solvent	2	3900.2	1950.1	5.96	0.003
Concentration x Extract	9	2794.5	310.5	0.95	0.485
Concentration x Solvent	6	3979.2	663.2	2.03	0.066
Extract x Solvent	6	24501.7	4083.6	12.48	<.001
Conc x Extract x Solvent	18	3791.6	210.6	0.64	0.859
Residual	141	46129.1	327.2	1.70	
Time	2	77850.1	38925.1	202.21	<.001
Time x Concentration	6	591.8	98.6	0.51	0.787
Time x Extract	6	15718.5	2619.7	13.61	<.001
Time x Solvent	4	14975.3	3743.8	19.45	<.001
Time x Concentration x Extract	18	4588.4	254.9	1.32	0.178
Time x Concentration x Solvent	12	4517.6	376.5	1.96	0.032
Time x Extract x Solvent	12	12754.8	1062.9	5.52	<.001
Time x Conc x Extract x Solvent	36	11513.4	319.8	1.66	0.016
Residual	288	55439.3	192.5		
Total	575	301756.5			

Appendix 3 Analysis of variance for the effect of the botanical extracts on chlorophyll content (millimoles m⁻²) of tomato plants infected with late blight period of 6 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Botanical extract	12	1.758883	0.146574	16.26	<.001
Concentration	2	0.027166	0.013583	1.34	<.001
Botanical extract x concentration	24	0.212867	0.008869	6.43	<.001
Time	5	0.171870	0.034374	11.51	<.001
Time x Botanical extract	60	1.321636	0.022027	4.23	<.001
Time x concentration	10	0.040830	0.004083	7.12	<.001
Time x Botanical extract x conc	120	0.395737	0.003298		
Total	233	3.928988			

Appendix 4 Analysis of variance for the effect of botanical extracts on chlorophyll fluorescence (millimoles m⁻² s⁻²) of tomato plants infected with late blight over a period of 6 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Botanical_extract	12	1.75888	0.147	16.26	<.001
Concentration	2	0.02717	0.014	1.34	<.001
Botanical extract x concentration	24	0.21287	0.0089	6.43	<.001
Time	5	0.17187	0.03437	11.51	<.001
Time x Botanical_extract	60	1.32164	0.02203	4.23	<.001
Time x Botanical x concentration	10	0.04083	0.00408	7.12	<.001
Residual	120	0.39574	0.0033		
Total	233	3.92899			

Appendix 5 Analysis of variance for the effect of botanical extracts on PAL activity in young leaves of tomatoes for 6 weeks after crop emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Botanical_extract	12	1.979807	0.164984	18.49	<.001
concentration	2	0.035327	0.017663	1.74	<.001
Botanical extract x concentration	24	0.231583	0.009649	2.09	<.001
Time	4	0.116068	0.029017	77.05	<.001
Time x Botanical extract	48	1.063938	0.022165	19.24	<.001
Time x concentration	8	0.032018	0.004002	2.16	<.001
Time x Botanical extract x concentration	96	0.338413	0.003525	0.02	<.001
Total	194	3.797153			

Appendix 6 Analysis of variance for the effect of botanical extracts on PPO activity in young leaves of tomatoes for 6 weeks after crop emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Botanical_extract	12	1.758883	0.146574	15.16	<.001
concentration	2	0.027166	0.013583	1.41	0.057
Botanicalextractxconcentration	24	0.212867	0.008869	20.75	<.001
Time	5	0.17187	0.034374	19.5	<.001
TimexBotanical extract	60	1.321636	0.022027	11.56	<.001
Timexconcentration	10	0.04083	0.004083	6.5	0.051
TimexBotanicalextractx concentration	120	0.395737	0.003298	11.9	0.031
Total	233	3.928988			

Appendix 7 Analysis of variance for the effect of botanical extracts on POD activity in young leaves of tomatoes for 6 weeks after crop emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Botanical_extract	12	2.584212	0.215351	332.54	<.001
concentration	2	0.000851	0.000425	0.2	<.001
Botanical extractxconcentration	24	0.033139	0.001381	1.63	<.001
Time	5	4.747953	0.949591	648.96	<.001
TimexBotanical extract	60	0.70694	0.011782	12	<.001
Timexconcentration	10	0.004385	0.000439	6.45	0.065
Time x Botanical extractxconcentration	78	0.122015	0.001017	15.12	0.030
Residual	156	0.0456			
Total	233	8.199495			

Appendix 8 Analysis of variance for the effect botanical extracts on plant height (cm) of tomato plants at different botanical extracts over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	980.53	490.26	6.4	<.001
Botanical_extract	13	17984.42	1383.42	30.95	<.001
Residual	26	1162.31	44.70	3.46	
Time	6	60524.70	10087.45	781.12	<.001
Timex Concentrtion	12	464.80	38.73	3.00	0.051
TimexBotanical_extractx Concentration	78	2424.15	31.08	2.41	0.005
Residual	156	2014.60	12.91		
Total	293	85555.51			

Appendix 9 Analysis of variance for the effect botanical extractson number of nodes of the tomato infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	2.5918	1.2959	8.5	<.001
Botanical extract	13	483.5918	37.1994	15.97	<.001
Residual	26	60.5510	2.3289	5.19	
Time	6	2290.6395	381.7732	849.98	<.001
TimexConcentration	12	4.7891	0.3991	0.89	0.533
Time x Botanical x extract	78	102.2177	1.3105	2.92	<.001
Residual	156	70.0680	0.4492		
Total	293	3014.4490			

Appendix 10 Analysis of variance for the effect of botanical extracts on number of flowers of tomato plants infected with late blight over a period of 10 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	38.170	19.085	3.35	<.001
Botanical extract	13	3983.946	306.457	19.00	<.001
Residual	26	419.259	16.125	6.89	
Time	6	2085.041	347.507	148.57	<.001
Time x Concentration	12	35.020	2.918	1.25	0.295
Time x Botanical extract x Concentration	78	1271.054	16.296	6.97	<.001
Residual	156	364.884	2.339		
Total	293	8197.374			

Appendix 11 Analysis of variance for the effect botanical extracts on number of fruits of tomato plants infected with late blight over a period of 13 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	70.7857	35.3929	3.78	<.001
Botanical extract	13	1702.7976	130.9844	8.69	<.001
Residual	26	391.8810	15.0723	19.59	
Time	5	1454.7976	290.9595	378.16	<.001
Time x Concentration	10	13.3095	1.3310	1.73	0.138
Time x Botanical extract x Concentration	65	287.3690	4.4211	5.75	<.001
Residual	130	100.0238	0.7694		
Total	251	4020.9643			

Appendix 12 Analysis of variance for the effect of botanical extracts on disease incidence (%) of tomato plants infected with late blight over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	624.60	312.30	21.34	<.001
Botanical extract	13	62796.26	4830.48	17.30	<.001
Residual	26	7259.57	279.21	23.14	
Time	5	36549.35	7309.87	605.73	<.001
Time x Concentration	10	138.49	13.85	1.15	0.063
Time x Botanical extract x Concentration	65	10895.98	167.63	13.89	<.001
Residual	130	1568.81	12.07		
Total	251	119833.06			

Appendix 13 Analysis of variance for the effect of botanical extracts on disease severity of tomato plants infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	6.5000	3.2500	3.44	<.001
Botanical extract	13	860.6230	66.2018	16.44	<.001
Residual	26	104.7222	4.0278	12.94	
Time	5	799.1786	159.8357	513.46	<.001
Time x concentration	10	3.6429	0.3643	1.17	0.029
Time x Botanical extract x Concentration	65	163.5437	2.5161	8.08	0.034
Residual	130	40.4683	0.3113		
Total	251	1978.6786			

Appendix 14 Analysis of variance for the effect of botanical extracts on leaves biomass of tomato plants infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	16375.689	8187.844	1726.61	<.001
Botanical extract	12	35090.071	2924.173	616.63	<.001
Concentration x Botanical extract	24	2784.944	116.039	24.47	<.001
Residual	38	180.202	4.742		
Total	77	54452.135			

Appendix 15 Analysis of variance for the effect of botanical extracts on stem biomass of tomato plants infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	2557.1310	1278.5655	2657.62	<.001
Botanical extract	12	15313.7516	1276.1460	2652.59	<.001
Concentration x Botanical extract	24	2133.7200	88.9050	184.80	<.001
Residual	39	18.7626	0.4811		
Total	77	20023.3653			

Appendix 16 Analysis of variance for the effect of botanical extracts on root biomass of tomato plants infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r	F pr.
Concentration	2	1096.608	548.304	117.72	<.001
Botanical extract	12	9939.575	828.298	177.83	<.001
Concentration x Botanical extract	24	169.550	7.065	1.52	0.121
Residual	39	181.657	4.658		
Total	77	11387.390			

Appendix 17 Analysis of variance for the effect of botanical extracts on marketable yield of tomato plants infected with over a period of 8 weeks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	0.17550	0.08775	5.69	0.007
Botanical extract	12	2.04250	0.17021	11.03	<.001
Concentration x Botanical extract	24	1.71500	0.07146	4.63	<.001
Residual	38	0.58624	0.01543		
Total	77	4.64180			

Appendix 18 Analysis of variance for the effect of botanical extracts on total yield yield of tomato plants infected with over a period of 8 weeks.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Concentration	2	1.16037	0.58019	12.32	<.001
Botanical extract	12	8.22102	0.68508	14.55	<.001
Concentration x Botanical extract	24	2.45409	0.10225	2.17	0.016
Residual	38	1.78881	0.04707		
Total	77	13.68083			