Analysis of phenolic compounds in *Carica papaya*, *Zingiber officinale*, *Ipomoea batatas* and *Myrothamnus flabellifolius* using RP-HPLC-UV/VIS-DAD, and *in vitro* evaluation of antifungal activity on plant pathogenic fungi of economic importance

By

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A thesis submitted in partial fulfilment of the requirements for the Master of Science Degree in Crop Protection

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THESIS APPROVAL

The undersigned certify that they have read and recommend to the Department of Horticulture, the thesis entitled: "*Analysis of phenolic compounds in Carica papaya, Zingiber officinale, Ipomoea batatas and Myrothamnus flabellifolius* using RP-HPLC-UV/VIS-DAD, and *in vitro* evaluation of antifungal activity on plant pathogenic fungi of economic importance".

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DECLARATION

I **Phumelela Peace Mwelasi** declare that the work presented in this thesis is from my own research and has not been submitted for the award of a degree in any university globally. Where other sources have been used, they have been fully acknowledged.

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ABSTRACT

Agricultural pesticides use is rising in crop protection against plant invaders of which fungi are chief in reducing both yield quantity and quality. *Botrytis cinerea, Rhizoctonia solani, Macrophomina phaseolina* and *Alternaria alternata* are polyphagous fungi of economic importance with severe yield and quality losses worldwide. Environmental concerns on pesticide use and their resistance now on a worldwide scale. Extensive studies of plant secondary metabolites have not exhausted their potential use as natural crop proctetants. The antifungal activity of plant phenolic extracts: *Carica papaya* leaves and bark; *Zingiber officinale* rhizomes; *Myrothamnus flabellifolius* above ground parts; and *Ipomoea batatas* leaves were investigated on the plant pathogenic fungi. Methanol (80%) was used to extract phenolic compounds from dried samples. Qualitative and quantitative analyses of extracts were done through Reversed-Phase High Performance Liquid Chromatograph, Ultraviolet-Visible light Diode Array Detector (RP-HPLC-UV/VIS-DAD) analyses. In antifungal *in vitro* assays, Potato Glucose Agar (PGA) in petri dishes was incorporated with three concentrations 250 ppm,500 ppm and1000 ppm) of sample plant phenolic compounds and active ingredients of fungicides (as positive controls); the negative controls were incorporated with diluent used to reconstitute plant phenolic compounds. The study was laid in a 7 x 3 factorial treatment in a completely randomized design replicated three times with the two factors being plant phenolic extracts and extract concentration. A 6mm diameter disc of the 7-day old pure fungi culture cultivated on PGA was aseptically inoculated at the centre of the petri dish for each treatment and petri dishes incubated at 20±2°C *(B. cinerea*) and 26±2°C (*R. solani*, *M. phaseolina* and *A. alternata*). Mycelial growth (diameter) measurements (in mm) were taken at 24hour exposure and stopped upon full colony diameter (85mm) observation in the negative control plates. Quantitative RP-HPLC-UV/VIS-DAD analyses yielded a strong peak at a Retention Time (RT) =1.01min for *Ipomoea batatas* however this could not be elucidated. Other extracts produced nondescript chromatograms that were not similar to standard phenolic compounds RTs. There were significant differences ($\rho < 0.05$) in Total Phenolic Content of plant phenolic extracts yields (mgGAE/g), 55.7±2.2, 7.0±3.3, 106.1±12.0, 33.5±3.4, and 41.9±1.5 for *C. papaya* leaves, bark, *Z. officinale*, *M. flabellifolius* and *I. batatas* respectively. Phytochemical test results were varied amongst plant phenolic extracts, mostly being positive. Antifungal *in vitro* assays, plant phenolic extract and extract concentraction interacted significantly (all $\rho < 0.05$) at all the time intervals for all the species except for *B. cinerea* 96 hours interval where only the plant phenolic extract were significant ($\rho < 0.05$) whilst there was no significant interaction of the two factors. The interaction of the two factors showed varied results in their inhibition of mycelial growth with differences amongst plant phenolic extracts and fungi species noted. Percentage Mycelial Reduction (PMR) varied amongst phenolic extracts and phytopathogenic fungi. Generally, the phenolic extracts showed less antifungal activity compared to the positive control treatments (fungicides). *Carica papaya* bark and *Z. officinale* rhizomes showed the highest PMR against *M. phaseolina* ($30\% \leq PMR \leq 40\%$) at the 1000 ppm concentration. *Z. officinale* showed the highest PMR ($45.3\% \leq PMR \leq 54.7\%$) for all the concentrations on *A. alternaria*. *M. flabellifolius* showed the highest PMR ($26.5\% \leq PMR \leq 53.9\%$) for all the three concentrations on *R. solani*. Only *M. flabellifolius* had limited PMR of 0-5.9%, whilst other plant phenolic extracts showed a 0% PMR agaist *B. cinerea*. The result of PMRs shows that plant phenolic extracts have potential use *in vivo* as crop protectants however further research may consider reduced fungicide dosages by using both synthetic and plant phenolic extract as mixtures.

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DEDICATION

This work is hereby dedicated to my wife Longane Mwelase Manyathela (nee Bhebhe) and my beloved daughter Nobenkosi Stacey Mwelase Manyathela, my parents Mrs. S. Manyathela (nee Ncube) and Mr. P.E. Manyathela, my in-laws Mrs. P. Bhebhe (nee Ndlovu) and Mr. A. Bhebhe, for the great support, encouragement, prayers and inspiration they gave me throughout this piece of work. God bless you all, you are the greatest.

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CHAPTER 1: INTRODUCTION

1.1 Background of the Study

The global population currently stands at 7.1 billion (PRB, 2013) and this shows a 24.6% increase from the population levels in 1993 which were 5.7 billion (Oerke, *et al*., 1999). This global increase in population is subsequently coupled with an increase in food consumption levels. The Millennium Development Goals (MDGs), particularly MDG-1 which is at the core of eliminating poverty and eradication of food hunger by 2015 is worth the taking (UN, 2013). Crop losses due to insect pests, weeds and diseases have been estimated to be between 31-42% of total worldwide crop production and this has had huge attachments to economic income and for plant disease alone per year, US$220billion in losses (Agrios, 2005). Global crop losses due to disease alone are enormous (10-16%) (Chakraborty and Newton, 2011) beside postharvest losses due to pathogens which are 6-12% (Agrios, 2005). Strange and Scott (2005) attributes crop losses due to plant disease being at least 10%. Deacon, (2006) asserts fungi to be chief causes of diseases. Therefore efforts to emancipate potential famines due to food starvation must be coupled with elaborate crop protection measures which are environmentally friendly.

Population of fungi are estimated to be 1.5 million with only 5% having been characterized (Deacon, 2006; Lattanzio, *et al*., 2006) being different species and about 250 000 crop species (Lattanzio, *et al*., 2006) to 420 000 seed plants which are potential hosts for fungi (Hawksworth 2001, 2002 in Deacon, 2006).

Historical records in the United States showed cost of controlling plant pathogens being second after controlling of weeds with figures of US$500 million and US$3 billion respectively

(Pimentel, *et al*., 2000). Deacon (2006) attributes losses due to plant diseases being 70% of fungal origin and the phylum Oomycota being the chief perpetrators in disease severity and Reddy (2013) also attributes fungi as the major perpetrators of crop losses.The extent of losses due to diseases depends on crops and the pathogen involved, for example the well documented Irish potato famine (1845-1846) caused by *Phytophthora infestans* (late blight of potato) emaciated the whole potato crop resulting in death of approximately 1 million due to starvation and migration of an estimated 500 million people to Europe (Ristaino, 2002; Agrios, 2005; Clarkson and Crawford, 2007; Curran and Fröling, 2010). Worldwide losses due to disease in various crops (major producing countries) in the period 1988-1990 were 15.1% (rice), 12.4% (wheat), fungal diseases in barley (10.1%), maize (10.9%), potato (16.3%), soyabean (9.0%), cotton (8.8%) and coffee (14.8%) (Oerke, *et al*., 1999). *Phytophthora infestans* is reported to have caused 5.2 billion Euros per annum globally (Haverkort, *et al*., 2009). Moreover, late blight disease has been shown to be a global threat and most destructive to potato production worldwide (Hussain, *et al*., 2014).

This study sought the investigation of the antifungal potential of four phenolic-rich plant extracts using aqueous methanolic extracts *in vitro* tests against phytopathogenic fungi of economic importance *viz. Botrytis cinerea, Rhizoctonia solani, Macrophomina phaseolina* and *Alternaria alternata*. The four phenolic-rich plants species evaluated were *Carica papaya* (pawpaw) leaves and bark, *Ipomoea batatas* (sweet potato) leaves, *Myrothamnus flabellifolius* (resurrection plant) above ground parts and *Zingiber officinale* (ginger) rhizomes.

1.2 Statement of the Problem

There is an increased use of agricultural pesticides in crop protection (De, *et al*., 2014; Parrón, et al., 2014; Zhang, *et al*., 2011) against plant invaders of which fungi are chief in reducing both quantity and quality (Deacon, 2006). In the current milieu, there has been increased use of synthetic fungicides against fungal pathogens (Prüss-Ustün, *et al*., 2011). Environmental protection laws internationally on one hand are greatly concerned on the negative effects of the chemical fungicides to the environment, human intoxication and severe illnesses that have resulted due to their use (Havelaar, *et al*., 2010; Kalia and Gosal, 2011). On the other hand, agriculturalists are concerned about decreased yields due to fungal pathogens. They are also concerned about the increased resistance of fungal pathogens to conventional chemical fungicides on the market (Hahn, 2014; Reddy , 2013).

1.3 Justification of the study

There is a compelling need to find alternative plant pathogenic fungi control measures other than commercial synthetic fungicides. Zimbabwean farmers in the country produce fruits and vegetables for both subsistence and commercial purposes (Nyamupingidza and Machakaire, 2005). Nursery diseases such as damping-off caused by for example *Botrytis cinerea* (Elad, *et al*., 2007) and *Rhizoctonia solani* (Islam and Faruq, 2012) are widespread globally. *B. cinerea* for example is a serious field and postharvest pathogen. Coates and Johnson (1997) attest that almost all postharvest diseases of fruits and vegetables are of fungal origin. Research on plant phenolic compounds in conferring resistance against phytopathogenic fungi continues to advance and offers a potential trajectory towards curbing against losses (Lattanzio, *et al*., 2006; Bhattacharya, *et al*., 2010; Mazid, *et al*., 2011; Singh, *et al*., 2011; Lattanzio, 2013).

The use of synthetic pesticide in crop protection on one hand has unequivocally led to increased yields and/or suppression of plant invaders. However, on the other hand, environmental concerns and impact on human health such as occupational pesticide intoxications, residues in vegetables and fruits, contamination of baby food, water bodies has raised an alarm (Jørs, *et al*., 2006; Bjørling-Poulsen, *et al*., 2008; Lo, 2010; Kalia and Gosal, 2011; Prüss-Ustün, *et al*., 2011). A dithiocarbamate fungicide for example Mancozeb which has a contact effect and is used in the control of Frogeye Leaf Spot (FELS) and also *Botrytis cinerea* among other plant pathogens was reported to be carcinogenic (Axelstad, *et al*., 2011). Furthermore, resistance of plant pathogens on commercial synthetic fungicides have been reported (Vallejo, *et al*., 2003; Myresiotis, *et al*., 2007; Sun, *et al*., 2010; Chapman, *et al*., 2011; Karaoglanidis, *et al*., 2011; Weber, 2011; Hahn, 2014), and accumulation in soils and vegetables (Apladasarlis, *et al*., 1994). Safeties of natural compounds have been exalted over synthetic ones due to their exhibited biodegradability and low mammalian toxicity (Tedeschi, *et al*., 2007; Zabka and Pavela, 2013). Research on the new generation of natural antifungal agents is expected to focus on toxicity to the target organism and should be non-toxic to the human populace and/or environment (Komarek, *et al*., 2009). The use of phenolic-rich plant extracts is expected to at least meet the just stated principle of focus. Plant phenolic compounds are natural constituents of plant and they are not expected to cause serious challenges to the environment and/or consumers.

1.4 Main Objective

To investigate the antifungal potential of five aqueous methanolic plant phenolic extracts *in vitro* tests against phytopathogenic fungi of economic importance.

1.5 Specific Objectives

i. To analyse the quality and quantity of phenolic compounds in aqueous methanolic extracts of *Zingiber officinale* rhizomes*; Carica papaya bark, leaves; Ipomoea batatas* leaves and *Myrothamnus flabellifolius* above ground parts through RP-HPLC-UV/VIS-DAD analysis.

ii. To determine Total Phenolic Content (TPC) of aqueous methanolic extracts of the test plant species through Folin-Denis' reagent for determination of total phenolic content.

iii. To determinethe phytochemistry of plant phenolic extracts using standard methods to qualitatively show the presence of some important secondary metabolites

iv. To assess the activity of aqueous methanolic plant phenolic extracts on mycelial growth of plant pathogenic fungi of economic importance *in vitro*.

1.6 Research Hypotheses

i. Aqueous methanolic extracts of *Carica papaya, Zingiber officinale, Ipomoea batatas* and *Myrothamnus flabellifolius* contain phenolic compounds which can be quantified using RP-HPLC-UV/VIS-DAD.

ii. Different plant extracts quantitatively show different yield in their Total Phenolic Content (TPC).

iii. Plant phenolic extract show presence of some other plant secondary metabolites.

iv. Plant phenolic extracts show significant antifungal activity in reduction of mycelial growth in vitro tests.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The exploration of plant phenolic compounds or plant phenolic-rich extracts exists and there are growing interests and literature towards sustainable crop protection against crop invaders using natural products as alternatives to synthetic pesticides. Synthetic pesticides (insecticides, fungicides, herbicides, nematicides, mollicides) have been shown to have negative impact on the environment (Kalia and Gosal, 2011; Podio, *et al*., 2008). Triadimefon and propiconazole which are fungicide applied against fungal diseases of orchards were found to be persistent in the soil. The use of natural products and/or plant extracts as antimicrobial agents are gaining significance and pragmatism on one hand (Tedeschi, *et al*., 2007) and on the other, food safety concern is on the increase (Havelaar, *et al*., 2010).

2.2 Plant Defence Mechanism

The global economic losses due to plant pathogens and pests are quite notable and more often with devastating ramifications such as famines and plant biodiversity reduction (Anderson, *et al*., 2010), reduction of both yield and quality (Park and Paek , 2007). Plants also show extensive irritability another indispensable characteristic of a living organism that often leads to perception, signalling and response to both internal and external stimuli (Hopkins and Hüner, 2009).

Plants are either resistant or susceptible to potential attackers such as insects and microbial agents. Resistance herein denotes the ability of a plant to evade or avoid and/or reduce potential injury caused by attackers (Walters, 2011). Susceptibility is displayed by no or low degree of

resistance such that the plant is unable to overcome the effects of invasion by insect pests or microbial invaders. Resistance and susceptibility are thus relative, low or no degree of resistance means high susceptibility and the converse is true (Walters, 2011). Defence signifies the inherent (constitutive) or systemic acquired immunity (inducible or elicited by aggressors) ability of plants to guard or shield against deleterious effects that may be caused by successful attackers (Dangl and Jones, 2001; Jones and Dangl, 2006; Spiteller, 2008; Stamp, 2008; Walling, 2009; Mithofer and Boland, 2012). Every plant species has some degree of resistance towards potential attackers, a phenomenon known as non-host resistance (Heath, 2000).

Plant defence mechanism is on one hand passive wherein physical barriers together with chemical barriers exist and on the other hand, the defence mechanism is considered active with variations of rapid and delayed (Brown and Ogle, 1997; Agrios, 2005; Spiteller, 2008; Walters, 2011; Mithofer and Boland, 2012). Earlier research showed plants for example Irish potato (*Solanum tuberosum*) plant cells exhbiting an oxidative burst (relaease and accumulation of superoxides) upon attack by non-compartible races of *Phtyophthora infestans*. Morever, it was shown thatoxidative burst occurs before hypersensative cell death and phytoalexin production and thus confering a defencive role against attack by pathogens (Doke, *et al*., 1996). Some pathogens for example fungi excrete a myriad of potent extracellular enzymes that are capable of degrading some of the most resilient materials in the cell walls of plants such as cutin, lignin, cellulose, pectin and using these as a source of nutrition (Ramawat and Mérillon, 2013; Deacon, 2006). *Fusarium solani* f. sp. *pisi* secretes cutinolytic enzymes that are capable of penetrating the cell walls of its hosts for example pea stems leading to disease development (Podila, *et al*., 1995; Kwon , *et al*., 2009; Halonen, *et al*., 2009; Morid, *et al*., 2009). However, not all pathogens are capable of overcoming the passive defence exhibited by the plant due to its variation from one

species to another. Physical barriers such as cuticle, wax, the cell wall and natural apertures present an impediment to potential invaders and some pathogens have to be facilitated entry through wounds. Structural components provided by large biochemical polymers such as cellulose in the cell walls preclude potential attackers to the vulnerable meristematic tissues and lignification processes further reinforce protection (Spiteller, 2008; Ramawat and Mérillon, 2013).

Chemical passive barriers that pathogens must contend with include plant defensins, phytoanticipins, pH, phenolic compounds, and nutrient deprivation amongst many (Brown and Ogle, 1997). Active defence underpins the passive defence and in both mechanisms plant-pathogenic interactions are essential. Irritability is an indispensable characteristic of living organisms, recognition or perception of signals involves complex signal transduction pathways (STPs) which provide the basis of any meaningful interaction (Campbell and Reece, 2005;; Hopkins and Hüner, 2009). Exudates by the plant play an important role either as antimicrobial secretions, anti-herbivory compounds or actually expedite compatible plant-pathogenic interactions which ultimately lead to disease development (Konno, 2011). Exudates are also known to contain a lot of secondary metabolites, proteins and defence chemicals (Konno, 2011) and the some phenolic compounds for example, phenylpropanoids have been found in the rhizosphere as a consequence of root exudates (Baetz and Martinoia, 2014).

Inducible chemical defence mechanism involves innumerable complex biochemical reactions and these are often amplified upon invasion (Heil and Ton, 2008). A research on the penetration of sorghum epidermal cells by *Colletotrichum sublineolum* after 42 hours of inoculation showed encapsulation of the infection peg through deposition of papilla. Papilla can be composed of various compounds for example phenolic compounds, callose, enzymes such as peroxidases, cell

wall material, reactive oxygen species (ROS). A notable example is that of *Blumeria graminis f.sp.hordei* which when it attacks barley resulting in powdery mildew, the papillae have been shown to be composed of phenolics compounds (with exception of lignin), callose, peroxidases and cell wall material (Huang, 2001; Walters, *et al*., 2007). Callose upon deposition after infection forms a structural physical and also chemical barrier to microbe invasion (Wu, *et al*., 2014).

Secondary metabolites produced by plants during their normal growth and development have been found to beeffective antimicrobial compounds. Phytoanticipins either released into the rhizosphere or phylloplane have been found as first line chemical defence that microbes have to contend with (Mazid, *et al*., 2011). Simple to complex phenolic phytoalexins, phenolic phytoanticipins have been shown to inhibit the growth of soil-borne fungal pathogens (Bhattacharya, et al., 2010). The spore germination of the neck rot disease of onion caused by *Botrytis cinerea* is known to be inhibited by phenolic phytoanticipins catechol and protocatechuic acid; *Phytophthora cinnamomi* that causes avocado root rot is inhibited by borbinol (phenolic compound) secreted into rhizosphere by resistant avocado cultivars rootstocks (Brown and Guest, 1997).

2.2.1 Plant-Pathogenic interactions

2.2.1.1 Possible Relationships between Plants and Potential Pathogens

Plant-Pathogenic interactions can be defined as an interface between the plant and the pathogen and thus forming an ‘interaction’ where there is exchange of information (Boyd, *et al*., 2013). Plant response therefore can be due to abiotic or biotic tress factors such pathogenic microbes (Hopkins and Hüner, 2009). The disease triangle or pyramid/tetrahedron tries to explain a

delicate relationship of the pathogen, host plant, environment and time which influence plant-pathogenic interactions. In cultivated plants the human component is added as it contributes to the host species grown at any given time and this largely influenced by the environmental cues that promote the host and ultimately the pathogenic species as well (Agrios, 2005).

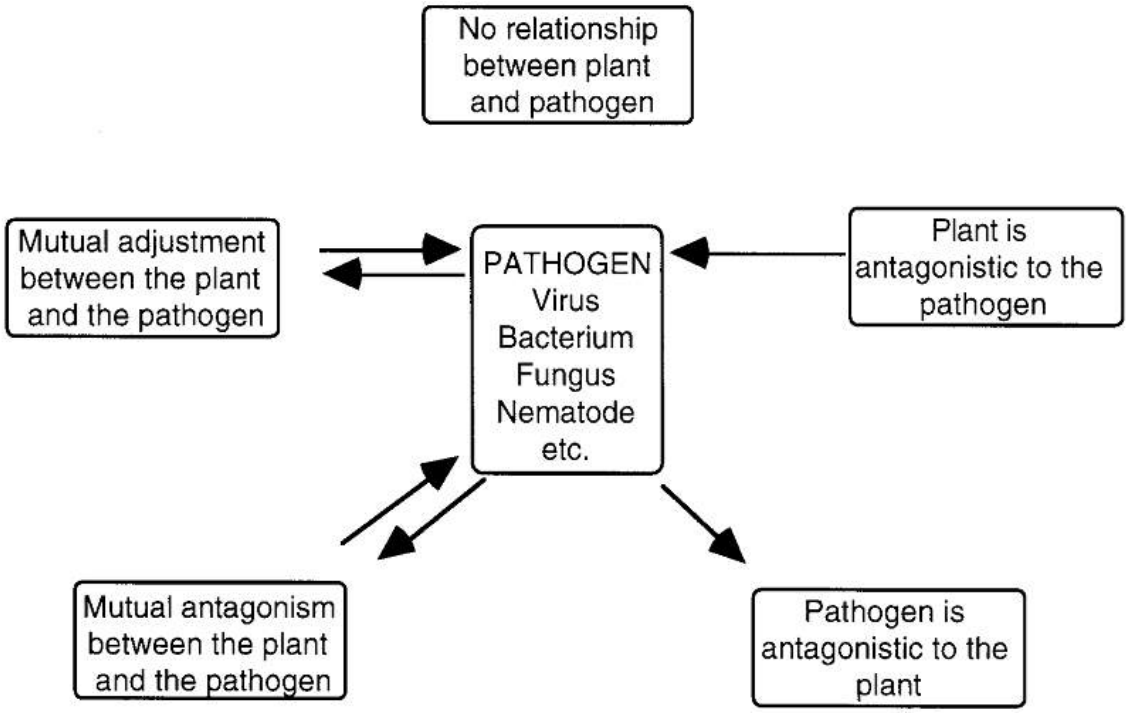


Figure 1.0 Five possible relationships between plants and potential pathogens
(Source: Brown and Ogle, 1997 pg.265)

In the interplay of the host plant species and the pathogen Brown and Ogle (1997) (in Figure 1.0 above) shows five possible relationships deep rooted in plant-pathogenic interactions and imperative in understanding disease proliferation. Non-host plant-pathogenic interactions fail to establish a parasitic relationship between the pathogen and invaded plant despite the presence of favourable environmental conditions (Brown and Ogle, 1997).

Plants may secrete root exudates that inhibit root-infecting fungi, egg hatching of nematodes and *Brassica* and *Alliaceae* species have been implicated in the release of 'biofumigants' (; Barile, *et*

al., 2007; Björkman, *et al*., 2011; Mowlick, *et al*., 2013). *Meloidogyne incognita* (nematodes) that affect tomato roots were effectively reduced with raw garlic straw in vitro experiments (Gong, *et al*., 2013). *Brassicas* and *Alliaceae* release sulphur containing volatiles (glucosinolates) which on their breakdown act as biofumigants (Larkin and Griffin, 2007). Another trial for three successive years elsewhere showed disease incidence reduction (43, 44 and 47% reduction) of sugar beet rot caused by *Rhizoctonia solani* after incorporation of crushed *Brassica juncea* (brown mustard) into the soil (Motisi, *et al*., 2009). Related research showed reduction of damping-off disease caused by *R. solani* after soil amendments with Brassica species and as well as increase in Actinomycetes population ultimately inhancing disease control (Ascencion, *et al*., 2015).

Plant secondary metabolites such as phenolics are produced by plants as chemical warfare against pathogens for example *Colletotrichum circinans* which causes onion smudge disease is suppressed by sufficient accumulation of the phenolic compounds catechol and protocatechuic acid (Lattanzio, *et al*., 2006; Mazid, *et al*., 2011).

2.3 Biosynthesis of Plant Phenolic Compounds

Primary metabolism of plants includes biochemical processes such as glycolysis, the oxidative pentose phosphate pathway (PPP), the tricarboxylic acid pathway (TCA) which apart from production of energy also produce precursor molecules which are fed into secondary metabolism (Hopkins and Hüner, 2009; Taiz and Zeiger, 2002; Walters, 2011). The Figure 2.0 below tries to show the summarised link of plant primary metabolism and secondary metabolism. Secondary metabolites comprise of chiefly terpenes (55%), alkaloids (27%) and phenolic compounds (18%) (Shahzad, *et al*., 2013).

The Shikimic acid pathway in plant secondary metabolism is responsible for the biosynthesis of aromatic amino acids for example tyrosine, phenylalanine and tryptophan. These are precursors in the production of phenolics such as ρ-Hydroxy-cinnamic acid (ρ-coumaric acid) from tyrosine; production of cinnamic acid and then ρ-coumaric or flavonoids and further biosynthesis from these lead to other phenolics such a ferulic acid, lignans or condensed tannins from flavonoids. Protocatechuic acid, gallic acid, hydrolysable tannins are all products from the Shikimic acid pathway (Hopkins and Hüner, 2009).

Plant phenols and phenolic compounds are thus produced naturally. They make the natural phytochemistry of plants and are also referred to as secondary metabolites (as shown in Figure 3.0). Secondary metabolites are involved in a myriad of physiological biochemical activities together with conferring resistance or defence against phytopathogens, attack by herbivores/predators and competing plants (Swain, *et al*., 1979; Harborne, 1989; Lattanzio, *et al*., 2006; Hopkins and Hüner, 2009; Bhattacharya, *et al*., 2010; Mazid, *et al*., 2011; Ramawat and Mérillon, 2013; Walters, 2011). Plants unlike animals are non-motile and often have to bear the brunt of both biotic and abiotic factors that are deleterious to their normal physiology such as generation of free radicals and/or reactive oxidative species (ROS) and cannot evade attack (Ramawat and Mérillon, 2013; Mayer, 2004). Resistance to these factors is imperative to the survival of the plants and successful production of both biological and economic yield.

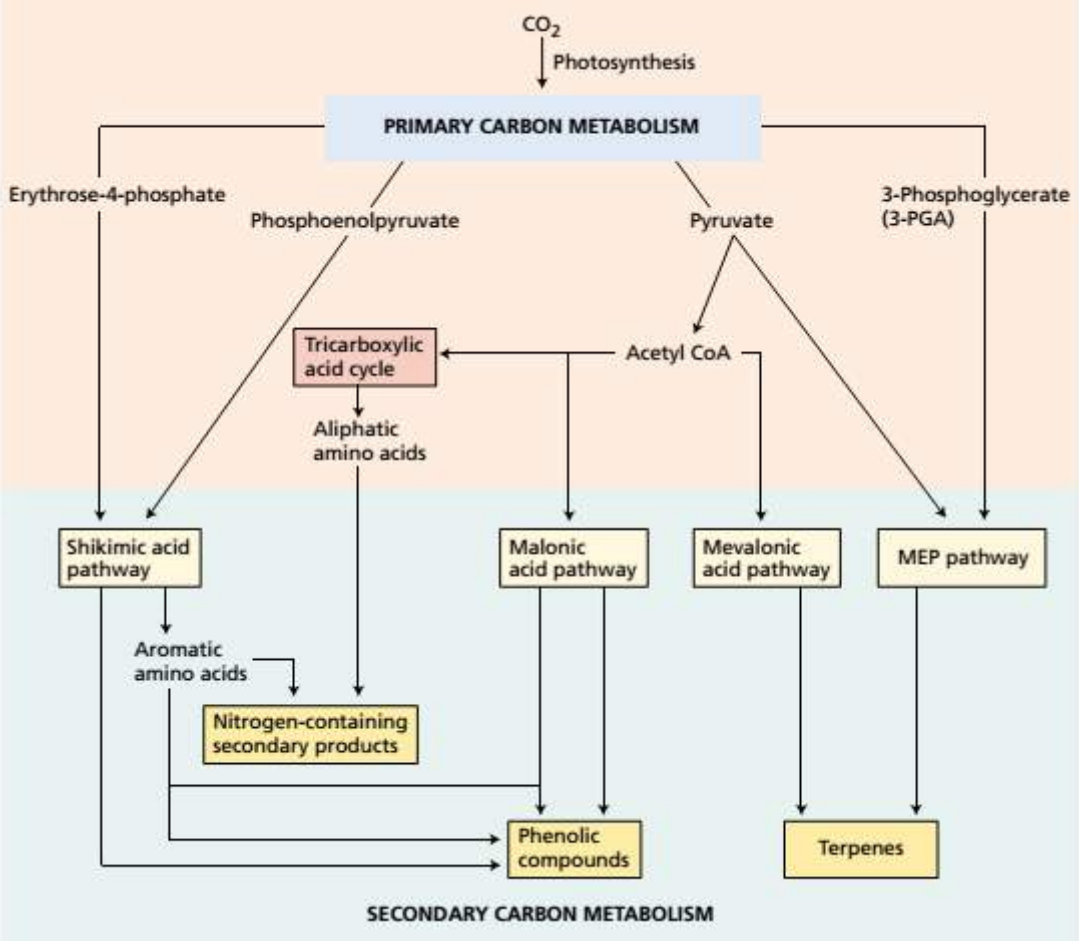


Figure 2.0 A simplified view of the major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism
(Source Taiz and Zeiger, 2004 pg.286**)**

Natural biosynthesis of secondary metabolites has been shown to occur in at least three pathways: the Shikimate/chorimate or succinylbenzoate pathway, acetate/malonate or polyketide pathway and the acetate/mevalonate pathway, plant phenols and phenolic compounds are a consequence of these complex pathways and thus by definition many commentators would show unanimity in mention of biogenetical pathways rather than just making mention of the benzene ring attached to a hydroxyl group(s) or other functional groups associated with plant phenolic compounds (Waksmundzka-Hajnos and Sherma, 2011; Walters, 2011; Ramawat and Mérillon, 2013). Plant phenolic classification is generally refined into two groups (flavonoids and non-

flavonoids) and they range from simple to complex (polyphenols) (Harborne, 1989); examples of some plant phenolics are: caffeic acid, *p*-coumaric acid, gallic acid, salicylic acid, vanillin, thymol, gingerol, cyanidin, catechin, anthocyanins, ellagic acid, pelargonidin (Lattanzio, *et al*., 2006; Chung, *et al*., 2011; Waksmundzka-Hajnos and Sherma, 2011;Ramawat and Mérillon, 2013; Zabka and Pavela, 2013). Flavonoids (examples of phenylpropanoid) characteristically bear the C_6–C_3–C_6 structure and account for more than 50% of more than 8,000 different phenolic compounds (Balasundram, *et al*., 2006). Classes of plant phenolic compounds range from simple phenols (C_6), simple phenolic acids such as gallic acid, caffeic acid, salicin, and getting more complex, hydroxybenzoic acids (C_6-C_1), Acethophenones, phenylacetic acids (C_6-C_2), Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes) (C_6–C_3) to more complex ones such as lignans, neolignans $(C_6\text{-}C_3)_n$ or condensed tannins $(C_6\text{-}C_3\text{-}C_6)_n$ (Harborne, 1989; Wink, 2010; Ramawat and Mérillon, 2013). Phenolic compounds can be grouped into a first level of eight (8) classes viz. hydroxycinnamic acids, hydroxybenzoic acids, chalcones, coumarins, lignans, flavonoids, xanthones and lignins which can further be divided as subclasses (with different levels) of the first level classes. Hyrdoxycinnamic acids constitute examples of common phenolic compounds such as ρ-coumaric acid, ferulic acid, caffeic acid, sinapic acid whilst hydroxybenzoic acids have popular phenolic compounds such as protocatechuic acid, gallic acid, vanillic acid, salicylic acid (Martins, *et al*., 2015).

Plant phenolics biosynthesis or in general, secondary metabolites largely depend on a variety of factors *inter alia*, plant species, different stages of growth and development in plants, and growth season (Lynn and Chang, 1990). Moreover, concentration in plant tissues of for example plant phenolics apart from the already mentioned factors may also be varied due to age, and for

example, actively growing parts exhibit higher content (Ozyigit, *et al*., 2007; Naz, *et al*., 2008). According to Kefeli, *et al*., (2003) biosynthesis of plant phenolics have been shown experimentally using *Salix spp.* (willows) to increase in the chloroplasts as a result of exposure to light; and moreover growth and development of the plant is marked with accumulation of phenolic compounds in the vacuoles as well polymerization of phenolics into lignin to reinforce secondary cell walls. Tissue culture technology shows high accumulation of phenolic compounds in callus (Ngomuo, *et al*., 2014) and is often perturbed by phenolic compounds oxidation (browning) resulting in high mortality of tissues (Ngomuo, *et al*., 2014) and production of quinones (Naz, *et al*., 2008) which are highly reactive and toxic (Ko, *et al*., 2009). Several derivatives of cinnamic acid (phenylpropanoids) and other phenolic compounds can be produced as a result of photoinhibition, wounding, pathogen attack and deficiency in nutrients (N, P, S, K, Mg, Bo, Fe) (Dixon and Paiva, 1995). In a greenhouse experiment (Ibrahim, *et al*., 2011) were able to show a decrease in total phenolics (TP) and total flavonoids (TF) with increase of nitrogen from 0-270KgNha^{-1}. A myriad of responses by the plant in production of phenolics has been shown by various commentators for example low iron deficiency show high phenolic compounds production in roots (Dixon and Paiva, 1995; Ramakrishna and Ravishankar, 2011), salt stress induces production of anthocyanins (members flavonoids) (Parida and Das, 2005). Heavy metal stress on plants have been shown to induce production phenolic compounds believed to act as metal chelators and also scavenge free radicals or reactive oxygen species (ROS) that accumulate in plants due to physiological stress (Michalak, 2006). Various functions are played by root exudates which contain phenolic compounds (mainly flavonoids) and this include rhizobial nod gene expression(stimulation or inhibition) , rhizobia are attracted towards the root (chemoattration), inhibition of pathogen in the rhizosphere, mediation of quorum

sensing, mycorrhizal spore germination and hyphal branching, mediate allelopathic interactions amongst other functions (Hassan and Mathesius, 2012).

At molecular level, biosynthesis of plant phenolic compounds happens upon recognition of potential plant invaders (insects and microbes) by PAMPS (recognition molecules with conserved regions in the pathogen genome) (Newman, *et al*., 2007; Boyd, *et al*., 2013). Ultimately, PAMP triggered immunity (PTI) becomes a consequence (Yokoyama and Colonna, 2008) and normally the first arsenal of defence to plant pathogenic ingress (Li, *et al*., 2013). After PTI, progression of infection is truncated before full proliferation by the pathogen (Bittel and Robatzek, 2007).

2.3.1 Analysis of Phenolic Compounds and their Role in Resistance against Phytopathogens

The plant kingdom is endowed with natural production of diverse chemical constituents with phenolics as one of the most abundant phytochemicals (Lattanzio, 2013). Phenolic compounds have been shown to play different roles in plant metabolism and plant defence for example as signalling molecules (salicylic acid in quorum sensing in *Agrobacterium*) or as precursors (Chlorogenic acid in lignin and suberin synthesis in plants) (Bhattacharya, *et al*., 2010). Plant phenolic compounds have been found acting as antifungal agents, protective agents (Bhattacharya, *et al*., 2010) or fungal deterrents or repellents (Bhattacharya, *et al*., 2010; Lattanzio, *et al*., 2006; Singh, *et al*., 2011), prohibitins/phytoanticipins (Mazid, *et al*., 2011), phenolic phytoalexins, nematicides for pathogens in the soil, repellents of herbivorous insect pests and various phenolics function as either chemical defence warfare or as communication molecules involved in defence or pathogenesis, as functional components or involved as

allelochemicals and so on (Bhattacharya, *et al*., 2010). Coumarins (these are simple phenolics) and its derivatives show both antifungal and antibacterial activity (Mazid, *et al*., 2011).

Tannins which are complex polyphenols are known to bind fungal proteins hence prevent disease proliferation and have also been known to deter fungal spore germination and also prevent other fungal growth (Mazid, *et al*., 2011). Chlorogenic acid (III) confers resistance of Irish potato tubers against *Streptomyces* scabies, *Verticillium alboatrum* which causes yellowing and wilting in kiwi fruits, and shows antifungal activity on *Phytophthora infestans* an attendant to solanaceous crops with devastating consequences (Lattanzio, *et al*., 2006). An *in vitro* research done in Germany on olive pomace (by-product of olive oil production) showed antifungal activity of phenolics present in the extracts and these were effective in inhibiting growth of *Alternaria solani*, *Botyris cinerea* and *Fusarium culmorum* (Winkelhausen, *et al*., 2005). An earlier *in vitro* research of olive plants phenolic compounds (tyrosol, cathechin, oleuropein) showed resistance against *Phytophthora* spp. with increase in effectiveness in the order oleuropein, catechol and tyrasol respectively (Rio, *et al*., 2003).

Zabka and Pavela (2013) in their in vitro tests of 21 natural phenolic compounds against six (6) filamentous fungi were able to show antifungal activity (some compounds showing 100% inhibition of fungal mycelial growth); and antifungal activity dependence on complexity of the phenolic compound (molecular structure) and different sensitivity amongst species. *Carica papaya* L. seed extracts successfully inhibited the growth of *Rhizopus* spp, *Aspergillus* spp and *Mucor* spp *in vitro* tests (Chukwuemeka and Anthonia, 2010). Ngadze, *et al*. (2012) were able to show increase in the activity of enzymes associated with defence against phytopathogens as well as increased concentrations of chlorogenic acid in the Zimbabwean and South African potato (*Solanum tuberosum*) varieties used; they were able to also show increase in total soluble

phenols and all these activities indicated an important role these play in conveying resistance against potato soft rot pathogens. Another study on sweet and chilli pepper (*Capsicum annuum*) was able to show that phenolic compounds such as quercitrin, isoquercitrin, chlorogenic acid accumulate at the site of infection compared to health tissue when the peppers are attacked by anthracnose causing fungi such as *Colletotrichum coccodes* (Mikulic-Petkovsek, *et al.*, 2013). Furthermore, it has been shown than phenylalanine ammonia-lyase (PAL) a key enzyme in biosynthesis of phenolic compounds accumulates at the site of infection (Mikulic-Petkovsek, *et al*., 2013). Plant phenolic compounds have also been implicated in aiding subsistence and infection of host plants; for example through quorum sensing *Agrobacterium* and *Rhizobium* have been found to utilize phenolics in pathogenesis (Bhattacharya, *et al*., 2010).

As early as 1955 some phenolics (catechol and protocatechuic acid) in onion scales (*Allium cepa*) particularly the pigmented varieties (red/purple) showed resistance to *Colletotrichum circinans* (smudge fungus). Field trials showed a considerable resistance to onion smudge disease by coloured onion varieties compared to white varieties (Walker and Stahmann, 1955; Agrios, 2005). High Performance Liquid Chromatography (HPLC) analysis of methanolic crude extracts of *Acorus calamus, Tinospora cordifolia* and *Celestrus paniculatus* produced varying amounts of six phenolic compounds (benzoic acid, cinnamic acid, caffeic acid, ferulic acid, gallic acid and tannic acid). These showed varying antifungal activity against *Alternaria solani, Curvularia lunata, Fusarium* spp., *Bipolaris* spp. and *Helminthosporium* spp. for example 5000 mg/mL, *C. paniculatus* was overall best against *Alternaria solani* and *Helminthosporium spp*. *T. cordifolia* was overall best on *Helminthosporium* spp. (Singh, et al., 2011). Twenty-one (21) pure phenolic compounds from plant essential oils were evaluated on their antifungal activity against three fungal genera (*Fusarium, Aspergillus and Penicillium*). Minimum inhibitory concentration

(MIC_{50} and MIC_{100}), 15 of the 21 phenolic compounds by probit analysis, carvacrol and thymol proved superior against the test fungi (Zabka and Pavela, 2013).

Malformed inflorescence ethanolic extract of *Mangifera indica L.*(mango) was analysed by HPLC. Its antifungal activity against various fungi genera and species (*Ustilago cynodontis*, *Cercospora cajani*, *Sphaerotheca* spp., *Cercospora* spp., *Alternaria solani*, *Bipolaris* spp., *Helminthosporium* sp., *Curvularia* spp., *Fusarium udum* and *Alternaria cajani*) were evaluated. Varied results (species specific) were exhibited with strong antifungal activity with increasing concentrations particularly 3000-5000µL/mL showing a range of over 50% to 100% inhibition in some test fungi (Singh, *et al*., 2010). *Nuerospora crassa* fungal conidial germination was completely inhibited by quercetin 3-methyl ether and its glycosides in an experiment to investigate quercetin (a phenolic compound) and its seven derivatives on the growth of the test fungi. Other derivatives did not show any fungal conidial inhibitory activity. The experiment also showed that methyl groups in the nucleus of phenolic compounds (flavonoids) play an important role on inhibition (Parvez, *et al*., 2004). Flavanoids (quercetin, dihydroquercetin, kaempferol and naringenin) were tested on their antimicrobial activity against rice pathogenic fungi (*Pyricularia oryzae* and *Rhizoctonia solani*) and six strains of the rice bacterial blight pathogen (*Xanthomonas oryzae* pv. *oryzae*). Spore germination inhibition agianst *Pyricularia oryzae* at concentrations of 7µg/mL and 14 µg/mL was significant for naringenin (90.4% and 98.27%, respectively), and kaempferol (53% and 65%, respectively). The other flavonoids showed insignificant to no activity against *P. oryzae*. All flavonoids showed no activity on *Rhizoctonia solani* (Padmavati, *et al*., 1997).

The Reversed-Phase High Liquid Chromatography (RP-HPLC) C_{18} column is regarded as traditional, most appropriate and efficient in separating most major phenolic acids in an

optimized isocratic or gradient mobile phase program (Xu, 2012). Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a RP-C_{18} column, 250mm in length, with an internal diameter of 4.6mm and particle size of 5µm have been employed by various researchers in similar plant phenolic extraction experiments (Linares, *et al*., 2011; Singh, *et al*., 2011; Dai and Mumper, 2010). Other shorter C_{18} columns for example 100mm and 3.9µm in internal diameter are quite common in phenolic acid analysis (Xu, 2012). Various methods in phytochemistry analysis of phenolic compounds reinforce the application of RP-HPLC as a popular method and use of 5µm particle size as key in attaining of clear resolutions (Ramawat and Mérillon, 2013; Waksmundzka-Hajnos and Sherma, 2011; Harborne, 1989; Sticher, 2008). The C_{18} column is nonpolar packing material and most phenolic acids have quite similar molecular weights; and the most polar phenolic acids exhibit shorter retention times in the reversed-phase C_{18} column (Xu, 2012). The reversed-phase C_{18} column separation of phenolic compounds have shown varied runtimes and most often lengthy separation times (>45minutes). The advent of ultra-pressure liquid chromatography (UPLC) has seen use of shorter columns with quite small internal diameters (≤2.5µm) and very shorter separation times achieved (as much as 5 times less than the HPLC normal systems) (Xu and Howard, 2012).

The Solid Phase Extraction (SPE) achieves purification of crude sample extracts and fractional separation which is normally done prior to HPLC analysis (Waksmundzka-Hajnos and Sherma, 2011; Sticher, 2008; Khoddami, *et al*., 2013). It also achieves removal of interferences together with an advantage of high reproducibility (Waksmundzka-Hajnos and Sherma, 2011) and thus part of the sample clean-up procedure prior to HPLC analysis. Different solvents: methanol, ethanol, acetone, acetonitrile, 2-propanol, water have been known to give different yields of phenolic compounds and have been used for solvent extraction of simple phenols to polyphenols

(Azmir, *et al*., 2013; Ramawat and Mérillon, 2013; Tiwari, *et al*., 2011; Harborne, 1989). Various extraction techniques (conventional and modern) flexible with solvent use have been employed and these differ in yield output, solvent use, complexity in laboratory apparatus, instrumentation or equipment use and also cost (Gupta, *et al*., 2012; Khoddami, *et al*., 2013).

The RP-HPLC analysis of plant phenolic compounds often involves use of different acidified/buffered mobile phases: Methanol/4% acetic acid; 2-Propanol/acetic acid/methanol/0.018M ammonium acetate; 4% (v/v) THF in ACN and 0.4% (v/v) phosphoric acid (Xu and Howard, 2012). Plant phenolic compounds are good absorbers of light and ultraviolet-visible light (UV-VIS detectors) are normally coupled with the HPLC instrumentation and detection done at multiple wavelengths to enable detection of various phenolics which normally have a varied UV light absorbance. Furthermore, elucidation of compounds is often done through nuclear magnetic resonance (NMR), a combination of detection techniques such as electrospray ionization mass spectroscopy (ESI-MS), liquid chromatography (LC) is often coupled with other detectors such as Mass spectrometer (MS) for identification of compounds through molecular weight (Fang, *et al*., 2015; Zhang, *et al*., 2015).

It has been shown that phenolic compounds have a complex (multifunctional) role they play than meets the eye, they are involved in process such as: wood tree statics, resistance defence, cell wall suberin, interaction with proteins/enzymes, signal gene expression, antioxidants free radical scavengers, interaction with phytohormones, taste astringency, fruit colours amongst many functions (Treutter, 2010). There are many articles that have reviewed the role of phenolic compounds and/or secondary metabolites in plant defence, notably, Lattanzio, *et al*. (2006), Lattanzio, (2013), Mazid, *et al*. (2011), Bhattacharya, *et al*. (2010) and is still a growing interest

in isolating the main effects of phenolic compounds as the major secondary metabolites involved in defence.

Having noted the multiple roles depicted above, phenolics have been found to be potent natural antioxidants and have extensive abilities of scavenging free radicals and/or ROS. Free radicals are connected to the causal of diseases such as cardiovascular diseases, cancers, allergens, ulcers, diabetes, hypertension and various perturbations in the body immune system and many physiological functions in the body (Grassmann, *et al*., 2007; Chaira, *et al*., 2009; Kim, et al., 2009; Jaime, *et al*., 2010; Henning, *et al*., 2011;; Park, 2011). Undoubtedly, plant phenolic compounds are important in human well-being or health.

2.3.2 Mode of action of Fungicides and Phenolic Compounds

Plant disease due to fungi can lead to as much as 90% losses or more (Khan and Nasreen, 2010). Agricultural crop production has heavily depended on crop protectant agents (pesticides) for a time immemorial. "*The worldwide consumption of pesticides is about two million tonnes per year: Out of which 45 % is used by Europe alone, 25 % is consumed in the USA, and 25% in the rest of the world*" (De, *et al*., 2014 pg.5). Fungal diseases have been managed through use of either synthetic or natural bioactive plant extracts apart from biological control efforts (Copping and Hewitt, 1998; Zhang, *et al*., 2011; De, *et al*., 2014; Parrón, *et al*., 2014). Fungicides alone command 17.5% of the world pesticide (insecticides, herbicides, fungicides, other pesticides) consumption (De, *et al*., 2014). The mechanism of action and mode of action are often used interchangeably although in strict sense they do not mean the same thing. The mechanism of action of a substance/chemical describes the sum total of the biochemical events that lead to its toxicity (Aliferis and Jabaji, 2011). On the other hand, the mode of action of a substance/chemical in biological system refers to the actual or specific biochemical interaction

(specific enzyme/protein/biochemical step) (Khambay, *et al*., 2003) in which the substance or chemical produces a notable physiological perturbation (Connon, *et al*., 2012) or interruption (its bioactivity) often accompanied by severe malfunction or death of the affected part/organism (Aliferis and Jabaji, 2011). It is imperative to know the mode of action of bioactive constituents so as to be able to develop sustainable crop protection agents on one hand applying bioactive agents with different mode of action (reduce resistance), on the other hand, it is important to understand the physiology of the target organism as well as to compare them with the already known mode of action of pesticides (El-Wakeil, 2013). The other trust is the increase in demand of bioactive constituents with low or no mammalian toxicity whilst at the same time effective in controlling targeted plant invaders (Aliferis and Jabaji, 2011).

There are over 85 modes of action of pesticides (herbicides, fungicides and insecticides) whilst for particularly fungicides, over 40 modes of action are in existence. Aliferis and Jabaji, (2011) in their review article enumerate the mode of action of fungicies/bactericides showing columns of mode of action (categorized by targetted biochemical system); representative active ingredients and chemical group. The functional categories of targeted biochemical systems are : Amino acid, protein, and nucleic acid synthesis; Biosyntheses/cell metabolism; Melanin biosynthesis in cell wall; Sterol biosynthesis in membranes; Host defense induction, Mitosis/cell division; Respiration; Signal transduction; Multi-site action. The above are aslo elaborated in Copping and Hewitt, (1998); Yang, *et al*., (2011), and Stenersen, (2004). Examples of popular mutli-site action fungicides include Captan, Chlorothalonil, Maneb, Sulfur, copper oxychloride amongst others.

The available literature on the antimicrobial and/or antifungal activity of plant phenolic compounds is not often accompanied by the proposed or suggested mode or mechanism of action

and this therefore presents a lot of opportunities towards such a trajectory in scientific inquisition. The mode of action of some natural phenols on fungi is shown by Figure 4.0 below. This figure was taken from studies fungi of medical importance. Some medically important fungi are also notable plant pathogenic fungi for example *Aspergillus* spp, *Alternaria* spp, *Fusarium oxysporum*, *Rhizopus* spp amongst many.

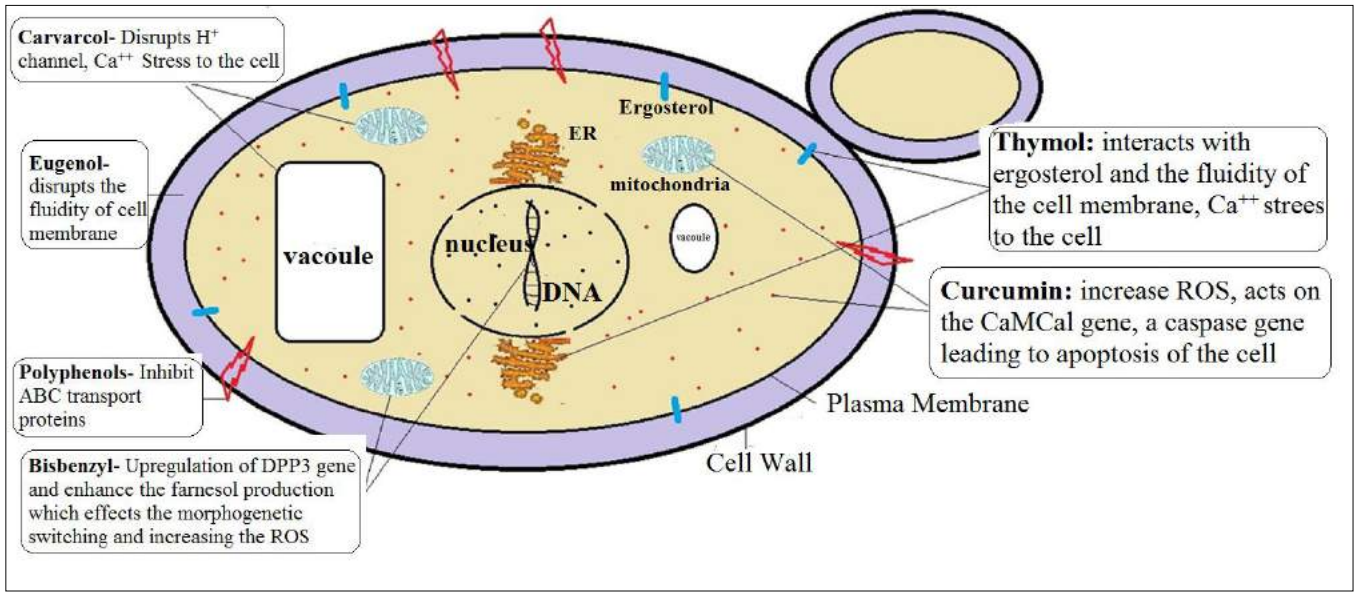


Figure 3.0 Different antifungal phenols and their mode of action on fungi
(Source: Ansari, *et al*., 2013)

From the Figure 3.0 above, eugenol, carvacrol and thymol (notable phenolic compounds) have been shown to disrupt the fluidity of cell membrane and inhibit mycelia growth of Aspergillus spp. Moreover, thymol is shown to disrupt fluidity of the cell membrane of *Rhizopus* spp by interacting with ergosterol (Ansari, *et al*., 2013).

Mechanisms of action of plant phenolic compounds have been reported in literature much more generally and very much varied as either perceived function or through some *in vitro* tests. Tannins for example have been reported to bind fungal proteins as one mechanism that gives it its antifungal properties (Mazid, *et al*., 2011). Fungi excrete a battery of extracellular of enzymes

(pectinases, cellulases, xylanases, ligninases, chitinases, proteolytic, lipases and so on) capable of degrading a variety of substrates (Deacon, 2006). Historical work on tannin mechanism of action suggested a possible inhibition of fungal extracellular hydrolases, a possibility of nutrient deprivation through for example metal complexation and protein insolubilization (Harborne, 1989), thus preservation of heartwood of temperate trees from decay is majorly attributed to their tannin content.

Plants upon infection have been shown to increase in phenolic content especially the pre-existing antifungal phenolic compounds owing to increasing levels of PAL and Chalcone synthase enzymes which act as elicitors (Dixon and Paiva, 1995; Pallas, *et al*., 1996). The increased antifungal phenolic compounds are oxidized by PPO and/or peroxidases leading to production of fungitoxic quinones, and the environment harsh for fungal/pathogen development.

2.4 Phytochemistry and ethnobotany of Study Plants

2.4.1 *Carica papaya*

Carica papaya Linnaeus, (pawpaw/papaya), belongs to the family of Caricaceae (Spínola, *et al*., 2015) and is regarded as a medicinal plant (Banala, *et al*., 2015). Papaya is a perennial herbaceous, succulent plants that possess self-supporting stems (Ayoola and Adeyeye, 2010) characterized by rapid growth rate. The papaya lifespan can be at most 20years (Banala, *et al*., 2015) in which it is capable of fruiting and is widely distributed in tropics and subtropics (Canini, *et al*., 2007). In Zimbabwe, *Carica papaya* is an exotic plant widely cultivated by individuals and at a very small scale commercially.

Carica papaya (leaves, stem, roots, seeds) are known to be rich in bioactive constituents with varied ethnobotanical uses (Ekaiko, *et al*., 2015). Papain (active constituent in milky juice from

all parts of the plant), is known for clarification of beer, remedy for dyspepsia (digestive disorders) (Aravind, *et al*., 2013). Indigestion, overweight/obesity, arteriosclerosis, high blood pressure and weakening of the heart are relieved or treated through tea preparations from green leaves (Aravind, *et al*., 2013). Rheumatism can be soothed through oral taking of a mixture of sugarcane alcohol and fresh root extracts (Ekaiko, *et al*., 2015).

HPLC-MS analysis of phenolic compounds showed (ferulic acid, caffeic acid and rutin) as most abundant in fruit whilst other phenolic compounds found in both fruits and mesocarp tissue included inter alia, caffeoyl-hexoside, gallic acid-deoxyheside, protocatechuic acid-hexoside, caffeoyly hexose-deoxyhexoside, quercetin 3-O-rutinoside, quercetin, myricetin, isorhamnetin and kaemperol (Rivera-Pastrana, *et al.*, 2010). Similar result and more other phenolic constituents were obtained by (Spínola, *et al*., 2015) through HPLC-DAD–ESI-MS^n and UPLC-ESI–MS verified the presence of significant metabolites in papaya extract (Gogna, *et al*., 2015). Total Phenolic Content (TPC) was found to 1263.70 mgGAE/100g in papaya (Silva, *et al*., 2014) and values differ in various reported literature. Other phytochemical qualitative tests have indicated the presence of saponin, tannins, steroids and terpenoids (Wahyuni, 2015).

2.4.2 *Zingiber officinale*

Zingiber officinale Roscoe (ginger) belongs to the family Zingiberaceae (Kumar, *et al*., 2011) and is a well-known plant (particularly) its rhizomes or underground stems used both in medicinal and gastronomic purposes (Kumar, *et al*., 2011). Ginger is a tropical plant which is produced by many throughout the world. India and China are the main producers of ginger and it is on record that India produced the highest tonnage in 2009. The powdered ginger rhizome contain fatty oils (3-6%), proteins (9%) , carbohydrates (60-70%), crude fibre (3-8%), ash (8%), water (9-12%) and volatile oils (2-3%) (Ghosh, *et al*., 2011). Ginger is well known food spice

(Ghosh, *et al*., 2011; Ashraf, *et al*., 2014) and its ethnobotanical, pharmacological and other biological uses which range from relief of many conditions viz. nausea, vomiting, asthma, cough, palpitation, inflammation, dyspepsia, loss of appetite, constipation, indigestion (Omoya and Akharaiyi, 2011).

Pharmacologically as shown in Figure 4.0 ginger is rich in medicinal constituents and more literature supports this: ginger is reported for antimicrobial activity (Ayse, *et al*., 2008), anticancer activity, antioxidant activity (Al-Tahtawy , *et al*., 2011), antidiabetic activity (Akhani, *et al*., 2004) nephroprotective activity, hepatoprotective activity (Abdullah, *et al*., 2004), larvicidal activity (Lin, *et al*., 2010), analgesic activity (Raji, *et al*., 2002), anti-inflammatory activity (Grzanna, *et al*., 2005), and immunomodulatory activities (Carrasco, *et al*., 2009).

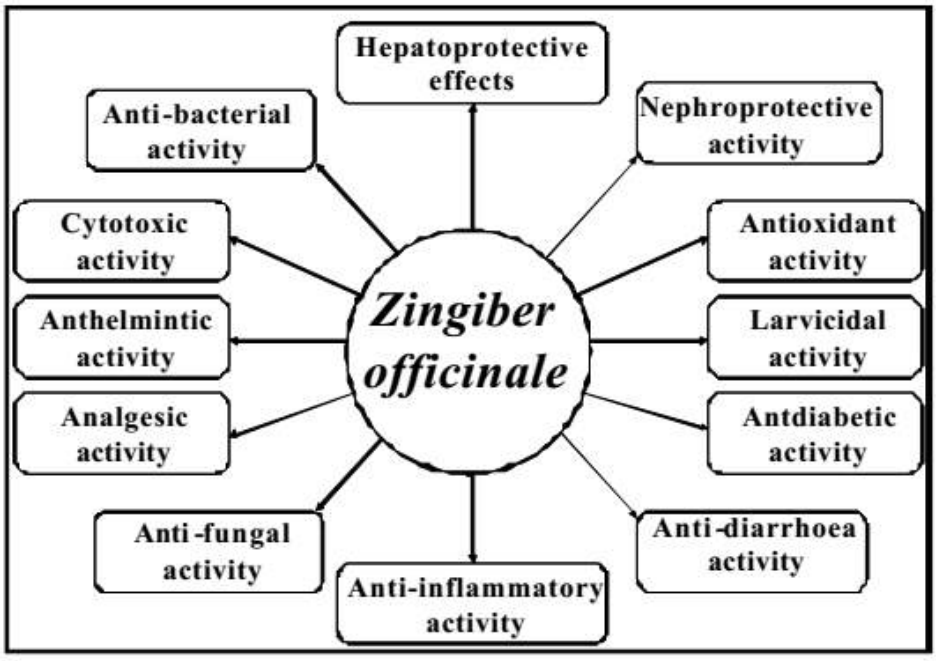


Figure 4.0 Medicinal properties of Zingiber officinale
Source : (Kumar , *et al*., 2014)

Qualitative phytochemical tests have revealed presence of phenols, alkaloids, tannins, glycosides, saponins, flavonoids, terpenoids, phlobotannins, steroids (Bhargava, *et al*., 2012;

Kumar, *et al*., 2011). Some studies have isolated nine phenolic compounds from ginger rhizomes viz. 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, 10-shogaol, dehydro-6-gingerdione, dehydro-10-gingerdione and 6-paradol compounds (Lee, *et al*., 2011; Mishra, *et al*., 2013; Pawar, *et al*., 2015). Phenolic compounds (6-gingerol, 6-shogaol and zingerone) were reported to exhibit quorum sensing inhibitory activity against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* (Kumar, *et al*., 2014). Ginger powder was reported as effective as a protectant for stored groundnuts against the Khapra beetle (*Trogoderma granarium* Everts) (Asawalam and Onu, 2014).

2.4.3 *Myrothamnus flabellifolius*

Myrothamnus flabellifolius (Welw.) belongs to the family Myrothamnaceae and is one of the documented resurrection plants (Moore, *et al*., 2007; Mabona and Vuuren, 2013) and shows wide spread distribution in Southern Africa and central southern Zimbabwe (Moore, *et al*., 2005; Cheikhyoussef, *et al*., 2015). The species has a growth range of 0.5-1.5m in height depending on region (Moore, *et al*., 2007) and the only woody species of the resurrection plant family (Moore, *et al*., 2007). The plant has characteristically aromatic leaves which are fan-shaped and conspicuously toothed on the upper leaf margin and they fold up upon desiccation (Viljoen, *et al*., 2002). *Myrothamnus flabellifolius* is used as a herbal tea in Zimbabwe and was found to exhibit antioxidant and total phenolic content comparable to commercial herbal teas such as Roobois^{TM} (*Aspalathus linearis*) (Bhebhe, *et al*., 2015).

African tradition medicine uses are varied (Moore, *et al*., 2007) for example, a survey done by Semenya and Maroyi, (2013) in South Africa (three Districts of the Limpopo Province) showed 11,5% respondents using it for tuberculosis (TB) treatment amongst some of the already mentioned medicinal uses. A promising outcome in an investigation showed *M. flabellifolius*

having an activity as a cancer (Acute myeloid leukemia) drug (Dhillon, *et al*., 2014). Furthermore, other commentators have found *M. flabellifolius* showing anti-viral, anti-microbial and anti-inflammatory properties (Dhillon, *et al*., 2014) whilst its tea and decoctions are widely used for other disorders (kidney, influenza, hemorrhoids) (; Moore, *et al*., 2007), and antioxidant properties have also been reported (Baratto, *et al*., 2003).

There are many other medicinal uses of *M. flabellifolius* reported elsewhere in literature and still growing. Hutchings, *et al*., (1996) documented oral take up of decoctions to alleviate for example backache, kidney problems, haemorrhoids, menstrual pains; moreover, the smoke from burning the plant when directed into the vagina could treat infections and uterus pains. A comparative study on five indigenous oils including *Myrothamnus flabellifolius* compared also with five popular commercial oils, showed M. flabellifolius' antimicrobial activity in the MIC assay coming second after *Thymus vulgaris*; whilst with the time-kill assay, *M. flabellifolius* came first on its activity on the three test pathogens (*Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans*) (van Vuuren, 2008).

There is a wealth of knowledge that still has to be gained from the study of *Myrothamnus flabellifolius* as 85 compounds were identified by GC-MS in the hydro-distilled essential oils, which also revealed pinocarvone and trans-pinocarveol as the chief terpenoids (Viljoen, *et al*., 2002). Qualitatibe phytochemical tests on *Myrothamnus flabellifolius* have shown the presence of alkaloids, flavonoids, gylcosides, phenols and tannins, saponins and steroids (Maroyi, 2013; Molefe-Khamanga, *et al*., 2012).

2.4.4 *Ipomoea batatas*

Ipomoea batatas (L.) Lam., (sweet potato) belongs to the family of Convolvulaceae and is a dicotyledonous a perennial vegetable which is widely cultivated in the tropics and subtropics. China commands about 90% (Sun, *et al*., 2014) of the world production and ranks 7th most important crop in the world and the 5th in developing countries (Jung, *et al*., 2011). *Ipomoea batatas* is a major contributor to energy and phytochemical source of nutrition for human populations, animal feed and industrial raw materials (Shekhar, *et al*., 2015). Sweet potatoes are rich in antioxidants, such as phenolic compounds (Fidrianny, *et al*., 2013). Sweet potato leaves are rich in protein, fibre and minerals (K, P, Ca, Mg, Fe, Mn, and Cu), although tubers are widely consumed, leaves are much better compared to other leafy vegetables (Sun, *et al*., 2010). Moreover, the sweet potato leaves can be harvested several times through the year and have been shown to have high levels of resistance to insect pests and diseases even under high moisture conditions. The sweet potato tuber is an important source of nutrition in the diet (high quality carbohydrates, dietary fibre, Vitamins A, B_6, and C) and minerals as shown above (Pochapski, *et al*., 2011).

Traditionally, *Ipomoea batatas* is reported to show various ethnobotanical uses for example a review by (Meira, *et al*., 2012) reported uses such as treatment of tumours (mouth and throat), decoctions of leaves with many applications (aphrodisiac, astringent, bactericide, demulcent, fungicide, laxative and tonic). A list of other uses are recorded from literature by (Sani, *et al*., 2014) and this include treatment of asthma, bug bites, burns, catarrh, ciguatera, convalescence, diarrhea, dyslactea, fever, nausea, renosis, splenosis, stomach distress, tumors and whitlows amongst others. In Japan raw sweet potatoes (a white variety) is eaten raw as treatment for anemia, diabetes and hypertension (Ludvik, *et al*., 2004). In some study, in vitro simulations of

digestion showed that sweet potato leaf extracts inhibit gastrointestinal digestion of native starch which is a major contribution to hyperglycaemia in diabetes patients. It was concluded that the sweet potato leaf extracts could be an effective starch digestion modulator, and thus might manage hyperglycaemia in the long term (Jeng, *et al*., 2015). In Brazil, sweet potato leaves are reportedly used as treatment for inflammatory and/or infectious oral diseases (Pochapski, *et al*., 2011).

Sweet potato has been shown to possess high amounts of polyphenols, especially derivatives of caffeoylquinic acid (CQA), such as 5-CQA, 3,4-diCQA, 3,5-diCQA,and 4,5-diCQA (IP01). Further studies in analyses of sweet potato leaf extracts through HPLC–DAD and HPLC–ESI–MS/MS reported 29 phenolic compounds (Luo, *et al*., 2013). In another study, HPLC-QTOF-MS^2 analysis of the ethyl acetate fraction of the sweet potato leaf extracts resulted in characterization of 37 compounds including 20 phenolic acids, 12 flavonoids amongst others (Zhang, *et al*., 2015).

Sweet potato leaf extracts yielded 452mgGAE/g TPC according to (Jeng, *et al*., 2015) and in another study, the *n*-butanol, and ethyl acetate fraction yielded 569.38 mg GAE/g TPC (Zhang, *et al*., 2015) whilst (Fidrianny, *et al*., 2013) in a study of five different varieties of sweet potato, one of the variety yielded high TPC of 19.64g GAE/100g and (Pochapski, *et al*., 2011) showed a yield of 662mgGAE/100g TPC. Qualitative phytochemical tests in sweet potato leaf extracts showed the presence of triterpenes/steroids, alkaloids, anthraquinones, coumarins, saponins, tannins and phenolic acids (Pochapski, *et al*., 2011).

2.5 Plant pathogenic fungi

2.5.1 Identification, Biology, Epidemiology and Economic importance of *Botrytis cinerea*

Botrytis cinerea Pers. Fr., is an ascomycete, subphylum Pezizomycotina, class Leotiomycetes, order Helotiales, and family Sclerotiniaceae (Beever and Weeds, 2007). Macroscopically on infected host, for example fruits, appears as a mass of grey mycelia covering the fruit due to profuse sporulation. On growth media (PGA/PDA) various morphological types exists. Mycelial masses can appear short without sporulation, or aerial mycelium with sporulation can be observed, thick and woolly mycelium can also be seen and sclerotia can appear at the edge of the petri dish or arranged in a circle, or large sclerotia can be placed irregularly, and some the sclerotia can be small and scattered (Tanović, *et al*., 2014). *Botrytis cinerea* can grow at a wide optimal temperature range (15-25°C) (Janisiewicz and Korsten, 2002). Source of inoculum is usually sclerotia, chlamydospores, conidia or mycelium in dead plant tissues, mulch, and weeds where overwintering occurs (Beever and Weeds, 2007). Conidium germination and mycelial growth can occur at temperatures as low as 0°C (pathogen active at lower temperatures). Complete infection cycle can occur in 3 to 4 days when conditions are favourable also dependent on the type of host tissue being infected. *B. cinerea* requires a relative humidity of >95% for disease development (high humidity is required for germination of conidia and infection of the host) (Barnes and Shaw, 2002).

Botrytis cinerea Pers. Fr., is a necrotrophic fungus (Elad, *et al*., 2007; Agrios, 2005) that causes grey mold, a global threat to crop production (Hahn, et al., 2008) due to its wide host range of over 200 crop species (Li, *et al*., 2012). Economic crops such as tomatoes, potatoes, onions, strawberries, kiwi fruits, table grapes, and in general, vegetables, fruits and ornamentals are

vulnerable to *Botrytis cinerea* which ultimately causes a variety of diseases on numerous plant species; the greatest disease-causing pathogen ever known to man with deleterious consequences pathogen (Elad, *et al*., 2007). Elsewhere *Botrytis cinerea* yield losses have been reported to be significant each year (Li, *et al*., 2012), causes huge economic losses in wide variety of crops (Rodríguez-García, *et al*., 2014; Myresiotis, *et al*., 2007), and losses in vineyards are significant worldwide (Elmer and Reglinski, 2006). In Serbia, losses have been reported to be over 50% in berry production (Tanović , *et al*., 2009). Zimbabwean farmers produce for example potatoes, tomatoes and onions which are some of the chief hosts of *Botrytis cinerea*.

Botrytis cinerea cultural management practices are varied and normally plants must be kept dry and crop residues and weeds must be controlled to eliminate sources of inoculum. *Trichoderma, Gliocladium, Ulocladium* (filamentous fungi), *Bacillus, Pseudomonas* (bacteria), and *Pichia, Candida* (yeasts) as biological control agents, have shown great potential against *B.cinerea* (Elad, *et al*., 2007; Kovach, *et al*., 2000; Lima, *et al*., 1997). Chemical control and/or management have been found much effective. Fungicide treatment is done through site-specific fungicides (seven classes) which include anilinopyrimidines (APs), dicarboximides (DCs), hydroxyanilides (HAs), methyl benzimidazole carbamates (MBCs), phenylpyrroles (PPs), quinone outside inhibitors (QoIs; disease suppression only) and succinate dehydrogenase inhibitors (SDHIs). Despite the availability of fungicides as shown above, resistance has been shown to build up because *B. cinerea* is genetically variable (Fernández, *et al*., 2014).

2.5.2 Identification, Biology, Epidemiology and Economic importance *of Alternaria alternata*

Alternaria alternata (Fr.) Keissl., belongs to Phylum: Ascomycota; Class: Dothideomycetes ; Order: Pleosporales , and Family: Pleosporaceae. *A. alternata* has a species complex, complex

taxonomy and poses a challenge in identification. Colonies on PGA media appear flat, downy to woolly and covered with greyish, short aerial hyphae, becoming in time greenish black or olive brown with a light border.

Alternaria alternata lives saprophytically on numerous substrates. Wet conditions, long dew periods with temperatures (>18ºC) favour the development of *A. alternata* (Kirk and Wharton, 2012). *A. alternata* can overwinter in plant debris, soil and weeds.

Alternaria alternata is a cosmopolitan polyphagous fungi affecting over 380 plant species. In potatoes losses due to A. alternata are typically around 20% (Soleimani and Kirk, 2012). When pathogen is not managed losses can reach staggering levels of 70-80%.

2.5.3 Identification, Biology, Epidemiology and Economic importance of *Macrophomina phaseolina*

Macrophomina phaseolina (Tassi) Goid., belongs to the Phylum: Ascomycota ; Class: Dothideomycetes ; Order: Botryosphaeriales, and Family: Botryosphaeriaceae (Schinke and Germani, 2012). The fungus produced both producing both sclerotia and pycnidia. It is highly variable, with isolates differing in microsclerotial size and presence or absence of pycnidia. Pycnida are initially immersed in host tissue, then erumpent at maturity. They are 100-200 µm in diameter; dark to grayish, becoming black with age; globose or flattened globose; membranous to subcarbonaceous with an inconspicuous or definite truncate ostiole. The pycnida bear simple, rod-shaped conidiophores, 10-15 µm long. Conidia (14-33 x 6-12 µm) are single celled, hyaline, and elliptic or oval (Partridge, 2003).

Macrophomina phaseolina survives as microsclerotia in the soil or infected plant debris which serves as a source of primary inoculum. *M. phaseolina* persist under water stress conditions and

high temperatures (>30°C) and thus it is widely distributed in tropical and subtropical climates and in semi-arid and arid regions (Mayek-Pérez, *et al*., 2002; Schinke and Germani, 2012). Proliferation of microsclerotia persists throughout the growing seasons particularly where temperature ranges are 28-35°C (Bressano, *et al*., 2010). Hot, dry weather promotes infection and development of charcoal rot. Host penetration is though mechanical pressure and enzymatic digestion or through natural openings. Rate of growth is highly correlated to climatic conditions; low soil moisture aggravates *M. phaseolina* severity.

Macrophomina phaseolina (Tassi) Goid., is a polyphagous plant pathogenic fungus of economic importance causing the phenomenal charcoal rot disease on more than 500 species worldwide (Su, *et al*., 2001). Crops such as groundnuts (Chatterjee, *et al*., 2014), sorghum in semi-arid and arid regions (Tonin, *et al*., 2013), cotton (Abd-Elsalam, 2010), sunflower (Aboshosha, *et al*., 2007), and even common bean (*Phaseolus vulgaris*) is not spared (Hernández-Delgado, *et al*., 2009; Mayek-Pérez, *et al*., 2002). *M. phaseolina* attacks other hosts of economic importance such as fruits, vegetables and fibre crops (Bressano, *et al*., 2010). Yield losses due to charcoal rot for example in soybean have been demonstrated elsewhere to be 60-93% (Argentina), up to 80% in drier areas (Perez-Brandán, *et al*., 2012), 30-50% for some growers (Missouri, USA) (Gupta, *et al*., 2012). *M. phaseolina* can incite 100% yield losses in various crops instigated by degree of severity (Iqbal, *et al*., 2010; Mayek-Pérez, *et al*., 2002).

Seed and oil content in Sunflower is severely affected (Aboshosha, *et al*., 2007).

Conventional cultural practices are done to manage the fungi. *M. phaseolina* would not normally persist in wet soil conditions. Crop rotation and high moisture soil content would drastically

reduce the incidence of *M. phaseolina*. Chemical control has not been effective due to non-availability of specific fungicides.

2.5.4 Identification, Biology, Epidemiology and Economic importance of *Rhizoctonia solani*

Rhizoctonia solani Kühn belongs to the phylum basidiomycota, order, ceratobasidiales and family, ceratobasidiaceae. Macroscopically, colony morphology is brown to dark with aerial mycelium and sclerotia. Infected host for example bean, pod rots are brown to greenish-brown, mostly circular, and sunken (Uchida, 2015). Microscopically, R. *Solani* colonies show a wide range of morphological variation. *Rhizoctonia solani* produces thread-like hyphae (mycelium) which show some characteristics such as a shade of brown, dolipore septum, multinucleate cells, right angled branches and no asexual spores are formed by the mycelium. *Rhizoctonia solani* is a species complex, subdivided into anastomosis groups (AGs) based on an isolate's ability to anastomose with known tester or type isolates. AGs are further subdivided into intraspecific groups (ISGs) (Budge, *et al*., 2009; Taheri and Tarighi, 2012). According to (Nikraftar, *et al*., 2013), 14 anastomosis groups (AGs) are known.

Upon invasion of a host by *Rhizoctonia solani*, sexual spores are formed on basidia. Four spores are produced on each basidium. Moist environments facilitate proliferation of Basidia. Basidiospores are wind-dispersed and germinate with moisture. Each basidiospore has a single nucleus. The hyphae produced by germinating spores will fuse (anastamose), forming new hyphae with a mixture of different types of nuclei. *R. solani* is soilborne and can persist in the soil for several years as sclerotia where upon growth of crops on infested soils results in damping off diseases.

Rhizoctonia solani Kühn is a cosmopolitan pathogenic fungi causing widespread of crop losses in many crop species, Uchida, (2015), shows literaure reporting over 500species in the USA alone. The pathogen causes widespread losses in tomato due to tomato foot and root rot (Nikraftar, *et al*., 2013) and a worldwide problem in tuber and root crops (Castillo, *et al*., 2010). Agrios (2005) attest damping-off disease caused by *R. solani* as a serious disease complex worldwide. Other authors attribute *R. solani* as a devasting soil-borne fungal pathogen (Pascual, et al., 2000; Cao, *et al*., 2004; Huang, *et al*., 2012; Solanki, *et al*., 2012; Goudjal, *et al*., 2014). Yield losses yield losses up to 20-30% (Shailbala and Tripathi, 2007) have been reported in *Vigna mungo* (Urd bean).

The literature on control of *R. solani* through biological and chemical means is overwhelming and varied in scope and results obtained. A list for attempts to control *R. solani* includes, inter alia, seed treatments, soil fungicides/fumigants, cultural practices and their modification, soil amendments, inducing changes in soil microflora, direct introduction of biological antagonist into the soil and the list goes on. Despite this vast literature, *R. solani* total control has proved to be insurmountable as no single remedy is definitive (Henis, *et al*., 1978).

Chemical control of *R. solani* is practiced. A study by (Kumar , *et al*., 2014) showed the effect of six (6) fungicides on R. solani both *in vitro* and *in vivo* tests. The six (6) fungicides (Tilt, Contaf, Bevesten, Captof, Mancozeb and Sulphure) showed variability in their effectiveness of control of *R. solani* in vitro and field conditions.

CHAPTER 3: RP-HPLC-UV/VIS-DAD Analysis of Phenolic Compounds in *Carica Papaya* bark, *Carica Papaya* leaves, *Zingiber Officinale*, *Myrothamnus Flabellifolius* above ground parts and *Ipomoea Batatas* leaves

3.1 Introduction

Plants are endowed by natural production of chemical constituents in their primary and secondary metabolism (Harborne, 1989; Hopkins and Hüner, 2009; Ramawat and Mérillon, 2013). Plant phenolic compounds are one of the chief products of plant secondary metabolism (Hopkins and Hüner, 2009; Waksmundzka-Hajnos and Sherma, 2011). Plant phenols have been implicated in plant defence against potential invaders such as pathogens and insect pests (Bhattacharya, *et al*., 2010; Lattanzio, *et al*., 2006; Mazid, *et al*., 2011). *Carica papaya* apart from its popular fruit has been found rich in phytochemical ingredients (including phenolic compounds) that have rendered the plant medicinal in many parts of the world, where the unripe seed, the leaves, roots and bark are utilized for various purposes (Canini, *et al*., 2007; Ifesan, *et al*., 2013; Ocloo, *et al*., 2012) for example as an antimalarial remedy (Iyawe and Azih, 2011). *Zingiber officinale* rhizomes apart from being ground into powder and used to produce beverages considered high in antioxidant activity (Ghasemzadeh and Ghasemzadeh, 2011). It contains plant phenolic compounds thought to be responsible for warding off pests, for example seed treatment of soybean with *Zingber officinale* totally inhibited the charcoal rot fungi (*Macrophomina phaseolina*) (Gupta, *et al*., 2012). *Ipomoea batatas* also contains phytochemicals with high antioxidant activities (Zhang, *et al*., 2015) and leaves have often been utilised as relish. The leaves also contain high amount of phenolic compounds (Jeng, *et al*., 2015; Jung, *et al*., 2011).

The resurrection plants are popular in dry climates and *Myrothamnus flabellifolius* powder from leaves and twigs is used for wound healing (Mabona and Vuuren, 2013), treating colds, menstrual pain, epilepsy, schizophrenia (Bhebhe, *et al*., 2015) and has been shown to contain phenolic compounds (Bhebhe, *et al*., 2015; Maroyi, 2013).

Plant extracts present complex mixtures of naturally biosynthesized organic compounds and the use of High Performance Liquid Chromatography (HPLC) as an analytic tool in both qualitative and quantitative analyses have found much pragmatism (Khoddami, *et al*., 2013; Ramawat and Mérillon, 2013; Waksmundzka-Hajnos and Sherma, 2011) since the invention of the HPLC system in the early 1960s (Xu, 2012).

Considerable research on plant or botanical extracts is present in Zimbabwe and increasing, however there is a dearth of information on the active constituents that make plant extract act the way they do against pathogens or insect pests. It is therefore imperative to consider the phytochemical constituents of the plant extracts under study, and if possible, isolate the active ingredients that ward off against pathogens or other pests for possible development of natural, safer, better and cheaper bio-pesticides compared to synthetics.

3.2 Objectives

1. To determine the individual phenolic standard retention time in the RP-HPLC method adapted and optimized for analysis.
2. To determine the presence of phenolic compounds in *Carica papaya*, *Zingiber officinale*, *Ipomoea batatas* and *Myrothamnus flabellifolius* extracts using RP-HPLC-UV/VIS-DAD analysis.

3.3 Experimental Materials and Methods

3.3.1 Study Site Description

The research was conducted at the University of Botswana (24° 40′ 0″ S, 25° 56′ 0″ E) main campus in Gaborone. Extraction and determination of phenolic compounds was done at the Chemistry Department whilst in vitro bioassays of phenolic extracts were done at the Department of Biological Sciences (University of Botswana) .Gaborone is located in the southern east part of Botswana (24^0 40′ 0″ S, $25^0$55′0″ E) and approximately 100km South of the Tropic of Capricorn with altitude reaching approximately 1,000m above sea level in the capital (Gaborone). Gaborone is situated in one of the semi-arid to arid regions of the world with average rainfall of 538mm and average temperature of 20.7^0C (Jonsson, 2004).

3.3.2 Collection, Drying and Plant Sample Preparation

Ginger (*Zingiber officinale*) rhizomes were bought from the River Walk Mall (Pick n Pay Supermarket) near University of Botswana. The leaves and bark for paw-paw (*Carica papaya*) and sweet potato leaves (*Ipomoea batatas*) were collected from communities near the University of Botswana (Tlokweng Village). The aboveground parts of the resurrection plant (*Myrothamnus flabellifolius*) were obtained from a faculty member (Mr. T. Kwape) in the Department of Biological Sciences at the University of Botswana. All species were botanically confirmed at the botany laboratory at the Department of Biological Sciences at the University of Botswana. The materials were brought to the laboratory (leaves for and bark for *Carica papaya*, *Ipomoea batatas* leaves, aboveground parts of *Myrothamnus flabellifolius* and *Zingiber officinale* rhizomes). Collected leaf material were all surface sterilized with 1% Sodium Hypochlorite (NaHOCl), rinsed with running distilled sterile water, blotted with filter paper, and then freeze-dried for 24 hours at temperatures between -50^0C to -54^0C (Hussin, *et al*., 2009). Ginger

rhizomes were thoroughly washed to remove any soil and/or foreign material traces, surface sterilized with 1% NaHOCl, and then rinsed with running sterile distilled water, then blotted with filter paper. The rhizomes were cut into minute pieces with a sterile scalpel blade to facilitate rapid shade-drying in the laboratory. The resurrection plant aboveground parts were shred into5-10 mm pieces using an ergonomic pruning shear. After drying, all the plant materials were ground into powder (2 mm particle diameter) using a laboratory electric powered mill, and then passed through a 500 µm sieve. The fine powders were sieved through a 500 µm sieve and stored in air-tight, opaque sterile bottles at 5ºC in the dark ready for solvent maceration. The procedure for drying plant materials in preparation for solvent extraction of plant phenolic compounds was done according to Ramawat and Mérillon (2013).

3.3.3 Ultrasound Sound Assisted Solvent Extraction (USAE)

Ultrasound Assisted Solvent Extraction (USAE) was performed in an ultrasonic cleaner (model 704, SCIENTECH, South Africa) with a maximum of 12L ("LO", "HI" frequencies, 300W) following methodologies of (Tabaraki, *et al*., 2012; Wang, *et al*., 2013; Ali and Kumar, 2014; Rezaie, *et al*., 2015) with modifications on ultrasonication temperature, extraction time, filtration and centrifugation parameters. Plant dry powders (500µm particle diameter), *Carica papaya* bark (60 g) and the rest 40 g of each (*Carica papaya* leaves, *Ipomoea batatas* leaves, *Zingiber officinale* rhizomes and the aboveground parts of *Myrothamnus flabellifolius*) were quantitatively transferred into 500 mL conical flask. HPLC grade solvent (methanol) prepared to make 80% methanol (v/v) with ultra-distilled water was mixed with powdered extracts (240 mL for *Carica papaya* bark) and 160 mL for other plant extracts. Ultrasound equipment was operated at the "LO" frequency (considered aggressive) and power of 300W with temperature regulation at 45-50°C (Ali and Kumar, 2014). Extractions were done for 45minutes (Rezaie, *et al*., 2015) and

three times using the same amount of solvent and then pooled. The conical flasks were immersed into the ultrasonic bath, with the liquid level in the conical flasks kept lower than that of the cleaner tank (Ali and Kumar, 2014). Pooled extracts of each plant extract were thoroughly mixed in a shaker and centrifuged for 15 minutes at 6000 rpm for deposit suspension particle (Wang, *et al*., 2013), after which the supernatants were filtered through Whatman™ 1 filter paper (pore size of 11 μm) (Rezaie, *et al*., 2015). The filtrates of the extracts were concentrated by rotary evaporation at 40°C under vacuum to dryness (Tabaraki, *et al*., 2012). After rotary evaporation, the plant extracts yielded 5.80 g (*Ipomoea batatas*), 7.93 g (*Carica papaya* bark), 3.79 g (*Carica papaya* leaves), 7.98 g (*Myrothamnus flabellifolius*) and 6.08 g (*Zingiber officinale*).

3.3.4 Plant extract Pre-treatment for HPLC analysis

One gram (1.000 g) of each dry plant extract was weighed and reconstituted in 5 mL absolute methanol (HPLC grade) in a 15 mL centrifugal vial followed by vortexing till dissolution following a method by (Oniszczuk, *et al*., 2014).. The reconstituted plant extracts were quantitatively transferred to a 25 mL volumetric flask with 92% aqueous acetonitrile (HPLC grade) and filled to the 25 mL mark. Activated charcoal treatment (preconditioned with HPLC grade methanol) was done according to the method by Chee, *et al*., (2013). . Sep-Pak SPE, Waters C18 cartridges (6 mL) were emptied of their contents, ultrasonicated in methanol to remove all residues and using a disc of Whatman™ 1 filter paper as a plug on the outlet end packed the cartridges with 4-6 g of activated charcoal. A vacuum manifold was employed to precondition the activated charcoal prior to passing the reconstituted plant extracts which gave clear filtrates after the treatment. Biotage Solid Phase Extraction (SPE) ISOLUTE® C4 200 mg/3 mL cartridges were employed in a reverse phase mode to further clean-up unwanted constituencies according to (Oniszczuk, *et al*., 2014). Using a vacuum manifold, the cartridges

were conditioned with 4 mL HPLC grade methanol followed by 4mL deionized water. Loading and elution of the filtrates from the activated charcoal treatment were done for each plant sample. The SPE cleaned filtrates were further subjected to filtration using Whatman^{TM} syringe disc filter (0.45 µm) into brown HPLC vials which were stored in the dark at 5°C prior to HPLC analyses.

3.3.5 HPLC solvents and Preparation of Phenolic Acids Standards

High Performance Liquid Chromatograph (HPLC) grade (>98%) phenolic standards (Ferulic acid, ρ-Coumaric acid, Gallic acid, Vanillic acid, Syringic acid, Catechol and Protocatechuic acid) were procured from Sigma–Aldrich (Steinheim, Germany). Formic acid, methanol and acetonitrile (>99%) HPLC grade were obtained from Department of Chemistry (University of Botswana). The method by (Mradu, *et al*., 2012) was adapted with some modifications. Briefly, 5 mg of each phenolic standard was weighed using an analytical balance on a weighing boat and quantitatively transferred to a 50 mL volumetric flask using 5 mL HPLC grade methanol, gentle shaking employed to entirely dissolve the solids. Dilution was done by 92% aqueous acetonitrile to the 50 mL mark to make 100 ppm of each phenolic standard stock solution and stored at 5°C in the dark till further use. Using a 1000 µm micropitte, 2 mL of each standard stock solution were pipetted into 20 mL volumetric flasks and diluted to the mark using 92% aqueous acetonitrile to make up 10ppm of each phenolic standard. The 10ppm standard solutions were passed through Whatman^{TM} HPLC-certified Acrodisc® syringe filter (0.45 µm) into brown HPLC vials for HPLC analysis and stored at 5°C in the dark till analysis. All HPLC analysis solvents (formic acid in ultra-distilled water (pH 3.0) (Santos, *et al*., 2011), methanol and

acetonitrile) were filtered through a Millipore® HNWP02500 Nylon Membrane Filters and thoroughly degassed through ultrasound treatment.

3.3.6 Analysis of phenolic compounds by Reversed-Phase High Performance Liquid Chromatography UV/VIS-DAD

3.3.6.1 RP-HPLC-UV/VIS-DAD of Standard Phenolic Compounds

The RP-HPLC analysis was done according to the method by (Mradu, et al., 2012) with modifications. The chromatographic separations of the phenolic standards were carried out on a reversed-phase Waters Nova-Pak C_{18} Column (100 mm x 4.6 mm i.d, 3.9 µm particle size) fitted with a guard column and thermostated at 25°C±1°C. HPLC-UV/VIS analyses were carried out using a Hewlett-Packard HP 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Germany) equipped with an Agilent UV/VIS-diode array detector (DAD), operated using an Agilent 3D ChemStation. The HPLC instrument was also equipped with a quaternary gradient pump, a membrane degasser, a thermostated column compartment, an injection valve with a 20µL loop. The mobile phase of the HPLC system consisted of (A) water/formic acid (pH 3.0) (92%) and (B) acetonitrile (8%) in isocratic elution. The flow rate of 1.0mL/min was maintained throughout the runtime of 15minutes with the UV/VIS-DAD scanning at 254 nm, 260 nm, 270 nm, 280 nm, 290 nm, 300 nm, 310 nm and 324 nm. All phenolic standards were injected manually using an analytical syringe at a volume of 20 µL (as set by the instrument) and in triplicate determinations.

3.3.6.2 RP-HPLC-UV/VIS-DAD of *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves

The RP-HPLC analysis was done according to the method by (Mradu, *et al*., 2012) with modifications. The chromatographic separations of the samples (plant extracts) were carried out on a reversed-phase Waters Nova-Pak C_{18} Column (100mm x 4.6mm i.d, 3.9µm particle size) fitted with a guard column and thermostated at $25^0C \pm 1^0C$. HPLC-UV/VIS analyses were carried out using a Hewlett-Packard HP 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Germany) equipped with an Agilent UV/VIS-diode array detector (DAD), operated using an Agilent 3D ChemStation. The HPLC instrument was also equipped with a quaternary gradient pump, a membrane degasser, a thermostated column compartment, an injection valve with a 20 µL loop. The mobile phase of the HPLC system consisted of (A) water/formic acid (pH 3.0) (92%) and (B) acetonitrile (8%) in isocratic elution. The flow rate of 1.0 mL/min was maintained throughout the runtime of 15minutes with the UV/VIS-DAD scanning at 254 nm, 260 nm, 270 nm, 280 nm, 290 nm, 300 nm, 310 nm and 324 nm. All samples were injected manually using and analytical syringe at a volume of 20 µL (as set by the instrument) and in triplicate determinations.

3.3.7 Data Collection and Data Analysis

The HPLC method adapted and modified from (Mradu, *et al*., 2012) managed to separate 7 Phenolic Compounds (Standards) with retention times (RT) of peaks noted and presented with their standard deviations. Representative phenolic standards chromatograms for each phenolic standard were presented as shown in Appendix I for other replicates. The RTs were used to

qualitatively pick a peak from the analyte with equal or similar RT with the standard mixture RTs. The HPLC instrument an analytical instrument was able to detect the presence of phenolic compounds which lie within the runtime (15 minutes) of the developed method or exhibit properties similar to the standards, and thus eluted at the same RT or close. Triplicate injections were done for each phenolic standard or sample (plant extract) during the HPLC analyses. Retention times for triplicate determinations on phenolic standards and samples (plant extracts) were exported to Microsoft Office Excel (2010) where averages and standard deviations were computed.

3.4 Results

3.4.1 Plant Phenolic Standards HPLC Results

The seven (7) phenolic compounds in the standard mixture were separated in a total run time of 15 minutes in the order: Gallic acid (GA), Protocatechuic acid (PA), Catechol (Cat), Vanillic acid (VA), Syringic acid (SA), ρ-Coumaric acid (ρ-CA), and Ferulic acid (FA) according to their polarity. The table below (Table 1.0) shows the seven (7) phenolic standard mixture separations and their retention times (RTs). The figure (Figure 5.0) also shows a representative chromatogram of the standard phenolic mixture HPLC separation. The Figure 6.0 shows representative chromatograms of each separate phenolic standard while enlarged separate chromatograms for each phenolic standard are shown in Appendix I.

Table 1.0 Phenolic Standard Mixture RTs and their standards deviations at 270nm

Phenolic Standard	Retention Time (RT) mins
Gallic Acid	1.523 ± 0.001
Protocatechuic Acid	2.150 ± 0.002
Catechol	2.675 ± 0.002
Vanillic Acid	4.231 ± 0.006
Syringic Acid	5.264 ± 0.006
ρ-Coumaric Acid	8.858 ± 0.006
Ferrulic Acid	12.755 ± 0.004

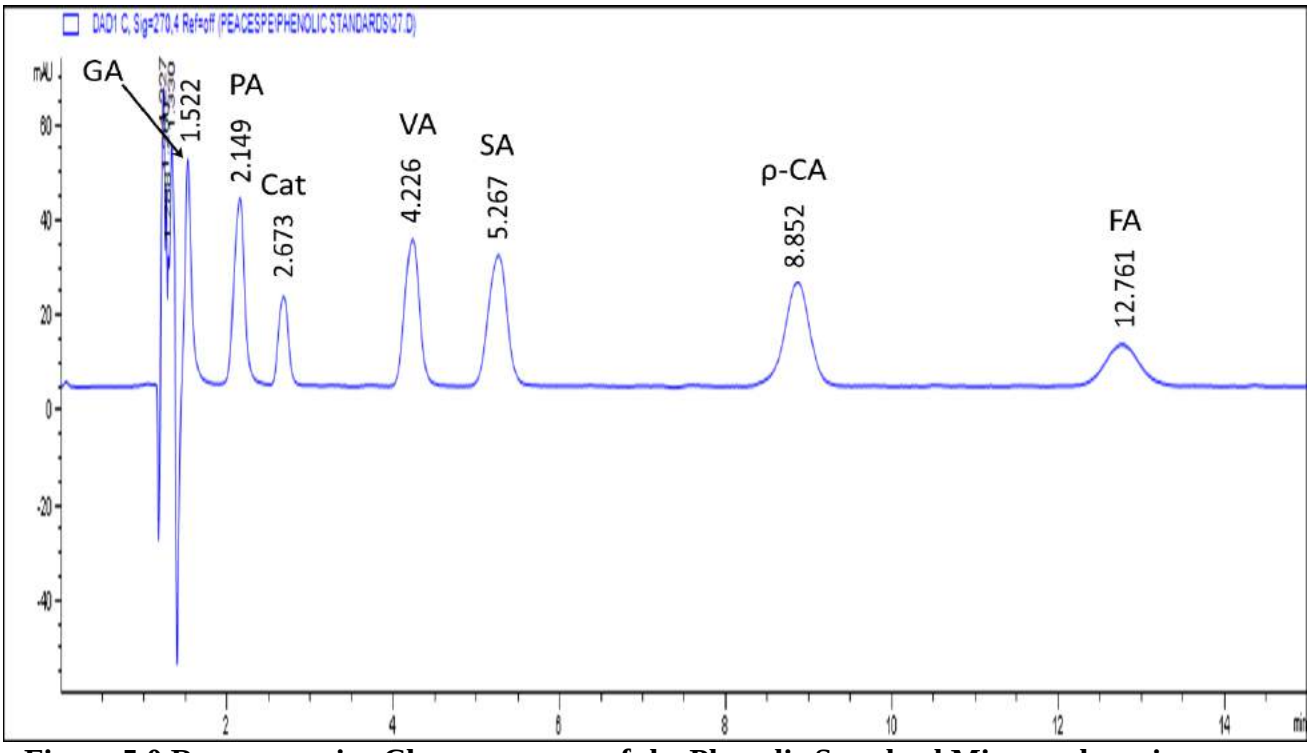


Figure 5.0 Representative Chromatogram of the Phenolic Standard Mixture detection at 270nm

GA-Gallic Acid; PA-Protocatechuic Acid; Cat-Catechol; VA-Vanillic Acid; SA-Syringic Acid; ρ-CA-ρ-Coumaric Acid; FA-Ferulic Acid

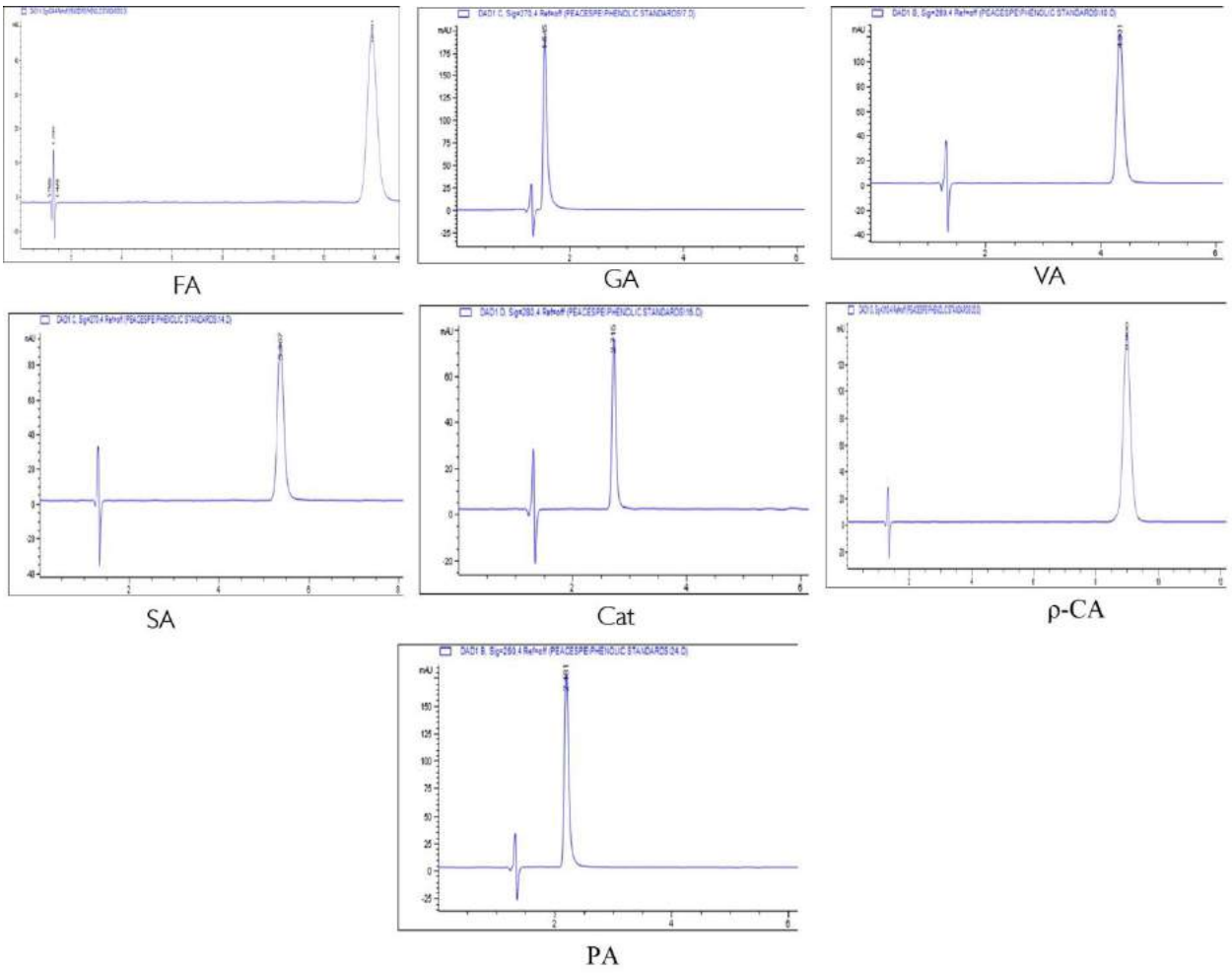


Figure 6.0 Representative Chromatograms of Phenolic Standards

Standards: FA-Ferulic Acid, GA-Gallic Acid, VA-Vanillic Acid, SA-Syringic Acid, Cat-Catechol, ρ-CA- ρ-Coumaric Acid and PA-Protocatechuic Acid at 270nm

3.4.2 Plant Phenolic Extracts HPLC Results

The HPLC analyses of the samples (plant extracts) did not generate chromatograms that were congruent to the observed retention times (RTs) shown in the standard mixture of phenolic compounds. However, some indistinct peaks were observed for all plant extract samples with the strongest peak shown by *Ipomoea batatas* at an RT of 1.101 minutes. All the peaks produced

could not be elucidated. The following figure (Figure 7.0) shows representative chromatograms of the sample plant extracts HPLC analyses.

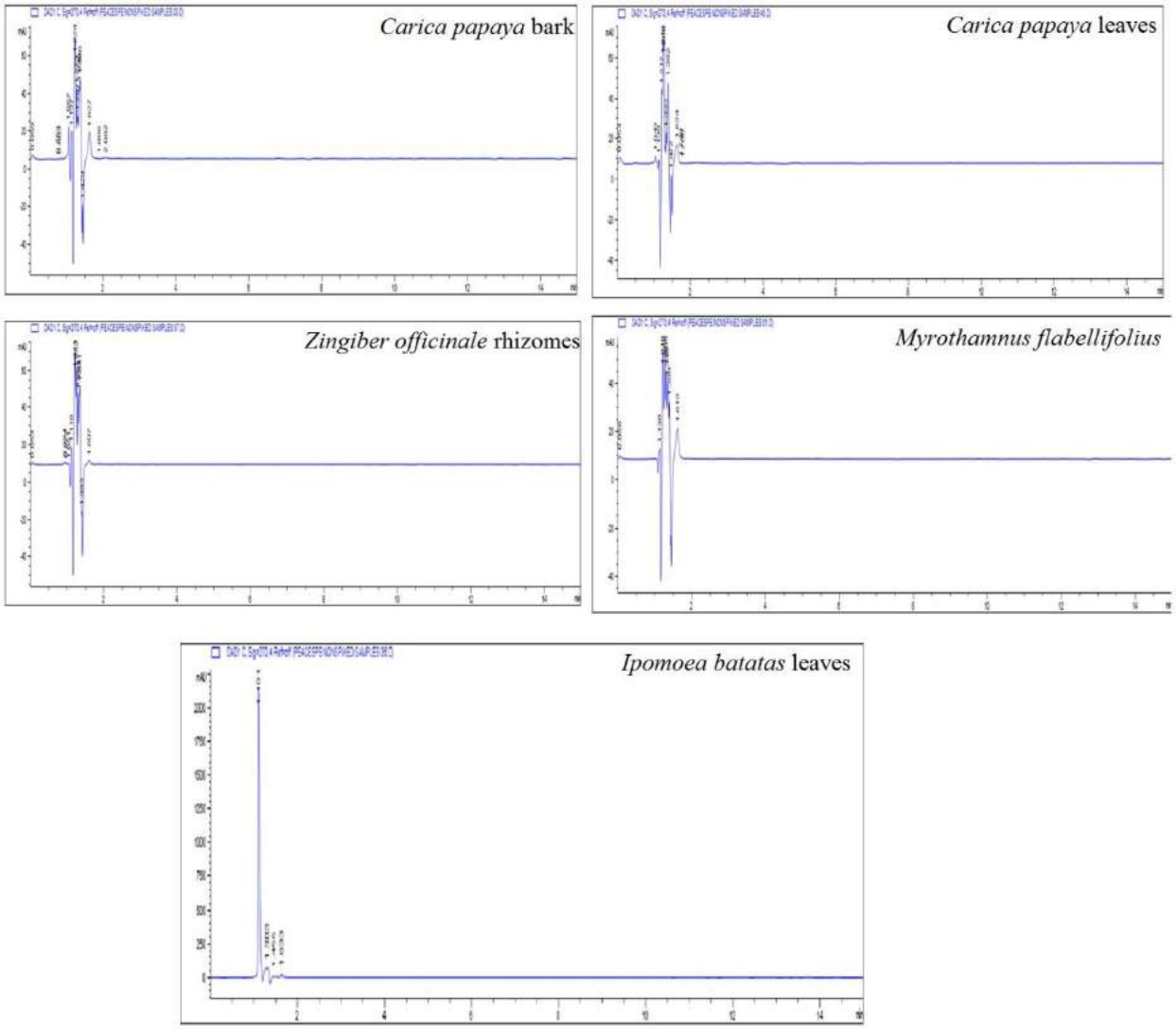


Figure 7.0 Representative Chromatograms of Plant Extract Samples at 270nm

3.5 Discussion

3.5.1 Plant Phenolic Standards and Plant Extract HPLC Results

The phenolic standards results yielded retention times (RTs) in accordance to polarity of the phenolic compounds (Mradu, *et al*., 2012). Representative chromatograms of plant phenolic

extracts did not show any similarities with standard phenolic chromatograms.Similarities occur when the samples under analyses have some or all of the phenolic compounds composition in their plant matrices. Moreover, sample preparation may contribute to different results (Gupta, *et al.*, 2012). RP-HPLC analysis of 6-gingerol (a phenolic compound) in *Zingiber officinale* cultivars was successfully employed by Pawar, *et al*.,(2015). The current method did not have 6-gingerol as a standard. In other studies, *Zingiber officinale* has been found to posses phenolic compounds such as 6-gingerol, 8-gingerol, 10-gingerol amongst 9 that were detected (Lee, *et al*., 2011). Lee, *et al*., (2011) employed a multistep procedure involving fractionation using different solvents and various preperative HPLC steps. Other studies in rhizomes showed presence of phenolic flavanoids (quercetin, apigenin, luteolin, myricetin), phenolic acids such as gallic acid, vanillic acid, ferulic acid and caffeic acid (Ghasemzadeh and Ghasemzadeh, 2011).Apparently, these analyses differed considerably with the sample preparation methods done in the current study. Extraction and analysis of plant phenolic compounds vary and there is no standardized protocol in their determination from different plant matrices although there could be some general procedures in sample preparation (Waksmundzka-Hajnos and Sherma, 2011).

Carica papaya leaves have been shown to contain phenolic compounds such as protocatechuic acid, ρ-coumaric acid, 5,7-Dimethoxycoumarin (derivitized coumarin compound), caffeic acid, kaempferol, quercetin, chlorogenic acid (Canini, *et al*., 2007; Gogna, *et al*., 2015). The sample preparations from the indicated scholars significantly differed from the present study and one from another hence different in their output.

The current study yielded a very strong peak in *Ipomoea batatas* which could not be elucidated. HPLC-MS, HPLC–DAD and HPLC–ESI–MS/MS studies on *Ipomoea batatas* leaves revealed the presence of phenolic compounds such as neochlorogenic acid, chlorogenic acid,

cryptochloregenic acid, isochlorogenic acids (A, B and C) (Jung, *et al*., 2011; Xu, *et al*., 2010). Luo, *et al*., (2013) were able to further reveal other phenolic compounds such as quercetin, kaempferol, ρ-coumaric acid among the 17 phenolics elucidated in *Ipomoea batatas* leaves extracts. The different results could be explained by the fact that detection is dependent on many factors such as solvents, sample preparation (Kowalski and Wolski , 2003; Stalikas, 2007) and the conditions or parameters used in the HPLC instrument as well as standards (Waksmundzka-Hajnos and Sherma, 2011). The strongly detected peak in *Ipomoea batatas* leaves shows a more polar phenolic compound than gallic acid and its true identity could be done for example through coupling the HPLC instrument with a mass spectrometer detector (Robbins, 2003; Stalikas, 2007; Khoddami, *et al*., 2013).

The current study did not yield any phenolic compounds comparable to standards in *Myrothamnus flabellifolius* despite that it is known to contain phenolic compounds that attribute to its medicinal properties (Maroyi, 2013). Gallotannins and 3,4,5-tri-O-galloylquinic acids (hydrolysable tannins) have been found to be some constituents of the polyphenols in *Myrothamnus flabellifolius* (Molefe-Khamanga, *et al*., 2012). Different plant matrices may require different sample preparation procedures which are coupled with many costs and an investment of ample time. Majors (2013) showed that sample preparation takes approximately 61% of the total analysis. The results thus obtained could be much related to sample preparation procedures employed.

Sample cleaning was done through the use of activated charcoal (carbon) and whilst removal of chlorophylls is achieved by this method, some studies have revealed that some phenolic compounds can be adsorbed to the charcoal used and thus remain adsorbed to the charcoal (Chee, *et al*., 2013; Scheepers, *et al*., 2011). The non-detection of some of the phenolic

compounds despite being reported in literature for the same plant extracts could have been mitigated by the application of activated charcoal. Application of Solid Phase Extraction (SPE) cartridges (in their variety) is a novel method in sample preparation prior to HPLC analyses and has been employed by many, in both small and commercial laboratories (Oniszczuk, *et al*., 2014; Waksmundzka-Hajnos and Sherma, 2011; Khoddami, *et al*., 2013).

3.6 Conclusion and Recommendations

3.6.1 Conclusion

The method employed in HPLC analysis was able to convincingly separate and quantify the seven (7) pure phenolic standards (ferulic acid, gallic acid, vanillic acid, syringic acid, catechol, ρ-coumaric acid and protocatechuic acid) and their mixture. Maceration of the different plant extracts with aqueous methanol, ultrasound assisted extraction and HPLC analysis of *Carica papaya* bark and leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts, did not yield chromatograms comparable or congruent in Retention Times (RTs) with the phenolic standard mixture. *Ipomoea batatas* leaves extract gave a very notable strong peak that could not be elucidated. All the nondescript chromatograms obtained in all samples within the first 3 minutes of the runtime (15 ninutes) of HPLC analysis could not be elucidated since the HPLC was not coupled to a mass spectrometer or any other detetctor beside the Diode Array Detector (DAD). Despite the results obtained in the current study, the HPLC analyses is still a crucial technique in the analysis of plant phenolic compounds for researchers wishing to qualitatively and quantitate plant phenolic compounds in the plant species of interest.

3.6.2 Recommendations

It would be of huge interest to use different extraction methods and as well as increase the plant phenolic standards for an HPLC method to be developed for analysis. Literature as shown above

has revealed presence of plant phenolic compounds in all the plant species in the current study. Noteworthy too, is the fact that different plant matrices are complex in nature and thus the sample preparation methods could be varied for each plant extract noting the difference amongst leaves, rhizomes/stems and bark. There is need to probably apply other sample preparation solvents and/or chemicals to help hydrolyse plant phenolic compounds that could be bound in the different plant parts. Sample cleaning procedures could also be explored upon realization of the compromise caused by use of activated charcoal (carbon). Whilst the activated charcoal could be used, more work has to be done in its optimization so that analytes are not retained. Mass spectrometry and other techniques could be used to help elucidate unknown peaks such as found in *Ipomoea batatas* leaves extract.

CHAPTER 4: Quantitative Determination of Total Phenolic Content and qualitative phytochemical tests on *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* aboveground parts and *Ipomoea batatas* leaves

4.1 Introduction

The Total Phenolic Content (TPC) quantitation is traditionally done through the most popular Folin–Ciocalteu method, a somewhat standard UV/VIS spectrophotometry method that was developed by Otto Folin and Vintila Ciocalteu in the 1920s. Much scientific research has tended to apply this method for TPC analyses (Yang, *et al*., 2011; Blainski, *et al*., 2013). This study however applied the Folin-Denis method for TPC analysis as it was shown to give better yields compared to the Folin–Ciocalteu method (Araújo, *et al*., 2013). The Folin–Ciocalteu method and Folin-Denis method show a lot of similarities as far as their protocols are concerned, thus the Folin-Denis method has also been applied in TPC analyses. Other methods for TPC are the permanganate titration, UV absorbance and colorimetry with iron salts. All the mentioned methods are considered not perfect owing to interferences in plant extracts, for example the presence of other readily oxidized substances in the extract (Dai and Mumper, 2010).

Studies in the antioxidant properties of phenolic compounds in fruits revealed that there is more antioxidant activity shown by the whole fruit compared to individual phenolic compounds which ultimately suggest synergistic interaction of the total phenolic content or presence of other not yet unidentified chemical constituents (Reber, *et al*., 2011). Moreover, increase in total phenolic content has been shown to be highly correlated with increase in antioxidant activity (Grassmann, *et al*., 2007; Saxena, *et al*., 2007; Jaime, *et al*., 2010; Jiang, *et al*., 2011).

Various studies have shown the application or use of gallic acid (GA), tannic acid, coumaric acid, quercetin in the standard calibration curve of the standard phenolic compound selected for use (Khoddami, *et al*., 2013). Gallic acid as a standard phenolic acid/compound is the most popularly used phenolic standard with regards to construction of the calibration curve for the quantification of total phenolic content (TPC) of plant extracts (Chaira, *et al*., 2009; Dai and Mumper, 2010; Araújo, *et al*., 2013; Blainski, *et al*., 2013; Luo, *et al*., 2013; Wang, *et al*., 2013; Bhebhe, *et al*., 2015). Total phenolic content (TPC) is expressed as Gallic Acid Equivalent per gram (GAE/g) of dry weight (DW) or fresh weight (FW) depending on method of extraction.

4.2 Objectives

1. To quantitatively determine the Total Phenolic Content (TPC) in *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves extracts.
2. To qualitatively test for the presence of some important phytochemicals in *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves extracts.

4.3 Experimental Materials and Methods

4.3.1 Study Site Description

The research was conducted at the University of Botswana (24° 40′ 0″ S, 25° 56′ 0″ E) main campus in Gaborone. Extraction and determination of phenolic compounds was done at the Chemistry Department whilst in vitro bioassays of phenolic extracts were done at the Department of Biological Sciences (University of Botswana) .Gaborone is located in the southern east part of Botswana (24^0 40′ 0″ S, $25^0$55′0″ E) and approximately 100km South of the

Tropic of Capricorn with altitude reaching approximately 1,000m above sea level in the capital (Gaborone). Gaborone is situated in one of the semi-arid to arid regions of the world with average rainfall of 538mm and average temperature of 20.7^{0}C (Jonsson, 2004).

4.3.2 Collection, Drying and Plant Sample Preparation

Ginger (*Zingiber officinale*) rhizomes were bought from the River Walk Mall (Pick n Pay Supermarket) near University of Botswana. The leaves and bark for paw-paw (*Carica papaya*) and sweet potato leaves (*Ipomoea batatas*) were collected from communities near the University of Botswana (Tlokweng Village). The aboveground parts of the resurrection plant (*Myrothamnus flabellifolius*) were obtained from a faculty member (Mr. T. Kwape) in the Department of Biological Sciences at the University of Botswana. All species were botanically confirmed at the botany laboratory at the Department of Biological Sciences at the University of Botswana. The materials were brought to the laboratory (leaves for and bark for *Carica papaya*, *Ipomoea batatas* leaves, aboveground parts of *Myrothamnus flabellifolius* and *Zingiber officinale* rhizomes). Collected leaf material were all surface sterilized with 1% Sodium Hypochlorite (NaHOCl), rinsed with running distilled sterile water, blotted with filter paper, and then freeze-dried for 24 hours at temperatures between -50^{0}C to -54^{0}C (Hussin, *et al*., 2009). Ginger rhizomes were thoroughly washed to remove any soil and/or foreign material traces, surface sterilized with 1% NaHOCl, and then rinsed with running sterile distilled water, then blotted with filter paper. The rhizomes were cut into minute pieces with a sterile scalpel blade to facilitate rapid shade-drying in the laboratory. The resurrection plant aboveground parts were shred into5-10 mm pieces using an ergonomic pruning shear. After drying, all the plant materials were ground into powder (2 mm particle diameter) using a laboratory electric powered mill, and then passed through a 500 µm sieve. The fine powders were sieved through a 500 µm sieve and stored

in air-tight, opaque sterile bottles at 5^{o}C in the dark ready for solvent maceration. The procedure for drying plant materials in preparation for solvent extraction of plant phenolic compounds was done according to Ramawat and Mérillon (2013).

4.3.3 Ultrasound Sound Assisted Solvent Extraction (USAE)

Ultrasound Assisted Solvent Extraction (USAE) was performed in an ultrasonic cleaner (model 704, SCIENTECH, South Africa) with a maximum of 12L ("LO", "HI" frequencies, 300W) following methodologies of (Wang, *et al*., 2013; Tabaraki, *et al*., 2012; Ali and Kumar, 2014; Rezaie, *et al*., 2015) with modifications on ultrasonication temperature, extraction time, filtration and centrifugation parameters. Plant dry powders (500 μm particle diameter), *Carica papaya* bark (60 g) and the rest 40 g of each (*Carica papaya* leaves, *Ipomoea batatas* leaves, *Zingiber officinale* rhizomes and the aboveground parts of *Myrothamnus flabellifolius*) were quantitatively transferred into 500 mL conical flask. HPLC grade solvent (methanol) prepared to make 80% methanol (v/v) with ultra-distilled water was mixed with powdered extracts (240mL for *Carica papaya* bark) and 160mL for other plant extracts. Ultrasound equipment was operated at the "LO" frequency (considered aggressive) and power of 300W with temperature regulation at 45-50^{o}C (Ali and Kumar, 2014). Extractions were done for 45minutes (Rezaie, *et al*., 2015) and three times using the same amount of solvent and then pooled. The conical flasks were immersed into the ultrasonic bath, with the liquid level in the conical flasks kept lower than that of the cleaner tank (Ali and Kumar, 2014). Pooled extracts of each plant extract were thoroughly mixed in a shaker and centrifuged for 15minutes at 6000rpm for deposit suspension particle (Wang, *et al*., 2013), after which the supernatants were filtered through Whatman^{TM} 1 filter paper (pore size of 11μm) (Rezaie, *et al*., 2015). The filtrates of the extracts were concentrated by rotary evaporation at 40^{o}C under vacuum to dryness (Tabaraki, *et al*., 2012). After rotary evaporation,

the plant extracts yielded 5.80g (*Ipomoea batatas*), 7.93g (*Carica papaya* bark), 3.79g (*Carica papaya* leaves), 7.98g (*Myrothamnus flabellifolius*) and 6.08g (*Zingiber officinale*).

4.3.4 Quantitative Determination of Total Phenolic Content in *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves using Folin-Denis Standard Method

4.3.4.1 Sample Pre-treatment for Total Phenolic Content Analysis

Half a gram (0.5000g) of each dry plant extract was weighed and reconstituted in 5mL absolute methanol (HPLC grade) in a 50mL centrifugal vial followed by vortexing till dissolution following a method by (Oniszczuk, *et al*., 2014) with modifications. The dissolved extracts were further diluted to the 25mL mark using absolute HPLC grade methanol, further vortexed to homogeneity. Activated charcoal treatment (preconditioned with HPLC grade methanol) was done according to the method by (Chee, *et al*., 2013) with modifications. Sep-Pak SPE, Waters C18 cartridges (6mL) were emptied of their contents, ultrasonicated in methanol to remove all residues and using a disc of Whatman™ 1 filter paper as a plug on the outlet end packed the cartridges with 4-6g of activated charcoal. A vacuum manifold was employed to precondition the activated charcoal prior to passing the reconstituted plant extracts which gave clear filtrates after the treatment. Biotage Solid Phase Extraction (SPE) ISOLUTE® C4 200 mg/3mL cartridges were employed in a reverse phase mode to further clean-up unwanted constituencies according to (Oniszczuk, *et al*., 2014) with modifications. Using a vacuum manifold, the cartridges were conditioned with 4mL HPLC grade methanol followed by 4mL deionized water. Loading and elution of the filtrates from the activated charcoal treatment were done for each plant sample to give stock solutions which were stored at 5°C for further analysis.

4.3.4.2 Sample Total Phenolic Content Analysis using Folin-Denis Method

The Total Phenolic Content (TPC) was determined by means of spectrophotometry as previously described by (Araújo, *et al*., 2013) with modifications. Briefly, 1mL of each stock solution from each plant extract (sample) described above was pipetted into 50mL centrifugal vials and 9mL absolute HPLC grade methanol added to further dilute the plant extracts. One millilitre (1mL) of Folin-Denis reagent was pipetted into each sample vial and briefly vortexed to homogeneity and the mixture allowed standing. After 5minutes, 10mL of 7% Na_2CO_3 followed by 4mL ultra-distilled water were added and the resultant mixture of the total 25mL homogenized through vortexing for 2minutes and allowed standing in the dark. After 2hours, the mixture was centrifuged at 4000rpm for 5minutes and the supernatant absorbance data were registered at 725nm using a previously conditioned UV/VIS spectrophotometer (UV/VIS spectrophotometer, Model UV-9100, Biotech Engineering Management, Co., Ltd., United Kingdom). The gallic acid (GA) standard calibration curve was done by dissolution of 20mg in 20mL absolute HPLC grade methanol to make a stock solution of 1000ppm. Serial dilutions (1ppm, 5ppm, 10ppm, 25ppm, 50ppm, 100ppm, 200ppm, 300ppm, 400ppm, 600ppm, 800ppm) of GA were done in 50mL centrifugal vials and 1mL of each dilution and a blank (ultra-distilled water) were subjected to the sample treatment. Absorbance readings were done against a blank (all reagents without the plant sample extract or GA and in place of the plant extract or GA, 1mL ultra-distilled water added). The quantitation of TPC was done through the GA standard curve and expressed as mg of gallic acid equivalent/g (mgGAE/g). Triplicate determinations were carried out for all experiments.

4.3.5 Qualitative determination of selected plant secondary metabolites in *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves using standard phytochemical analysis protocols

Nine (9) qualitative phytochemical tests were carried out according to standardized phytochemical tests without any modifications.

Table 2.0 Standardized Phytochemical Protocols showing methods and expected results

Phytochemical Test For :	Method	Expected Results	Reference
Alkaloids	1 mL of methanol extract was added to 5 mL of Hager's reagent	Yellow precipitate	Tiwari, *et al*., 2011
Anthocyanins	Added few drops of 10% NaOH to 1 mL of extract	Blue colour solution	Lalitha, *et al*., 2012
Coumarins	3mL of plant extract taken in a test tube. Mouth of the test tube covered with filter paper treated with 1M NaOH solution. Test tube placed for few minutes in boiling water and then filter paper removed and examined under the UV light.	Yellow fluorescence	Kumar, *et al*., 2013
Flavanoids	Added 5 mL of plant extract to concentrated H_2SO_4 (1 mL) and 0.5g of Magnesium	A pink or red colouration that disappear on standing within few minutes	Zohra, *et al*., 2012
Saponins	1 mL of plant extract put in a test tube and diluted with 20 mL distilled water and shaken for 15 minutes	Foam layer (1cm) on the top of the solution in the test tube	Tiwari, *et al*., 2011
Phenolic Compounds (phenols)	To 1 mL of plant extract 3-4 drops of ferric chloride solution added	Formation of bluish black colour	Tiwari, *et al*., 2011
Quinones	1 mL of each of the various extracts was treated separately with alcoholic potassium hydroxide solution	Coloration ranging from red to blue	Kumar, *et al*., 2013

Terpenoids	5mL of plant extract mixed in 2mL of chloroform followed by addition of 3 mL concentrated sulphuric acid	Layer of reddish brown colour	Kumar, *et al*., 2013
Tannins	To 3mL of plant extract in test tube diluted with chloroform and added acetyl acetate followed by addition of 1mL concentrated sulphuric acid	Green colour formed	Hossain, *et al*., 2013

4.3.6 Data Collection and Data Analysis

Total Phenolic Content (TPC) measurements were recorded as absorbance units from the UV/VIS spectrophotometer readings in triplicate determination and replicated three times. Data was entered into Microsoft Excel 2010 and then imported into Genstat 14th Ed. (2011). Residual tests were done to check for compliance with ANOVA assumptions in Genstat 14th Ed. and data was transformed through Square Root Transformation ($\sqrt{(x+c)}$, $x = mgGAE/g\ and$) $c = 0.5$, $a\ constant\ factor$. Data were subjected to One-way ANOVA analysis, wherein upon significant differences in TPC content of plant phenolic extracts Fisher's protected Least Significant Difference (LSD) was used to separate means for the plant phenolic extracts. TPC expressed as mgGAE/g dry weight ± standard deviation of triplicate trials. Gallic Acid calibration curve was produced through an average of the three independent trails and a linear graph with the regression equation, and R^2 value done through Microsoft Excel 2010. Back calculations through the calibration curve were done for the three trials of TPC and results expressed as mgGAE/g of dry plant extract (dilution taken into account). Qualitative phytochemical tests results for three independent trials were reconcilled into a table to show the presence or absence of the particular phytochemical(s).

4.4 Results

4.4.1 Quantitative Total Phenolic Content of plat phenolic extracts

Figure 8.0 shows the standard calibration curve of gallic acid produced from the experiment and used for calculation of sample Total Phenolic Content (TPC).

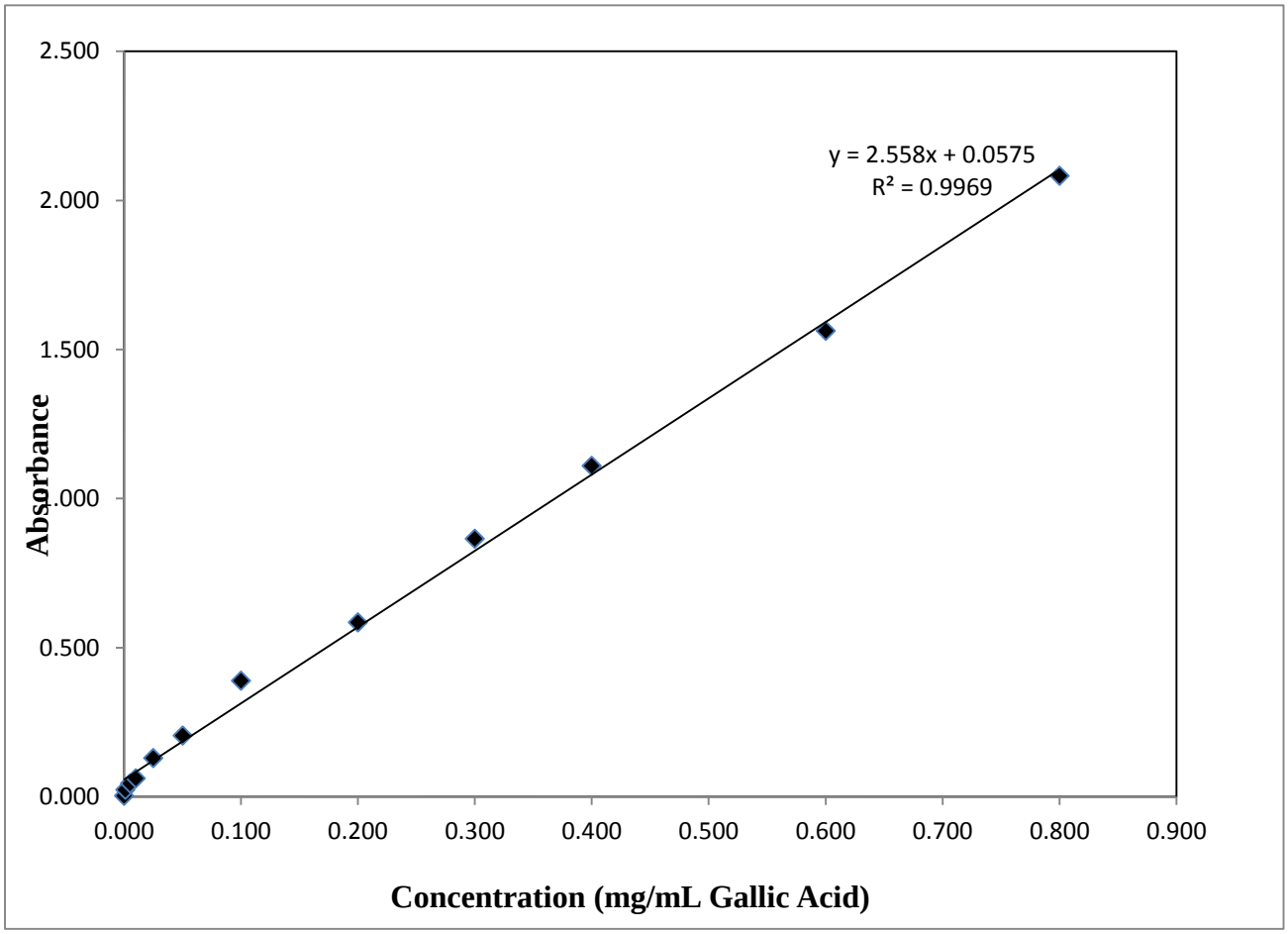


Figure 8.0 Standard Calibration Curve of Gallic Acid for TPC Quantitation

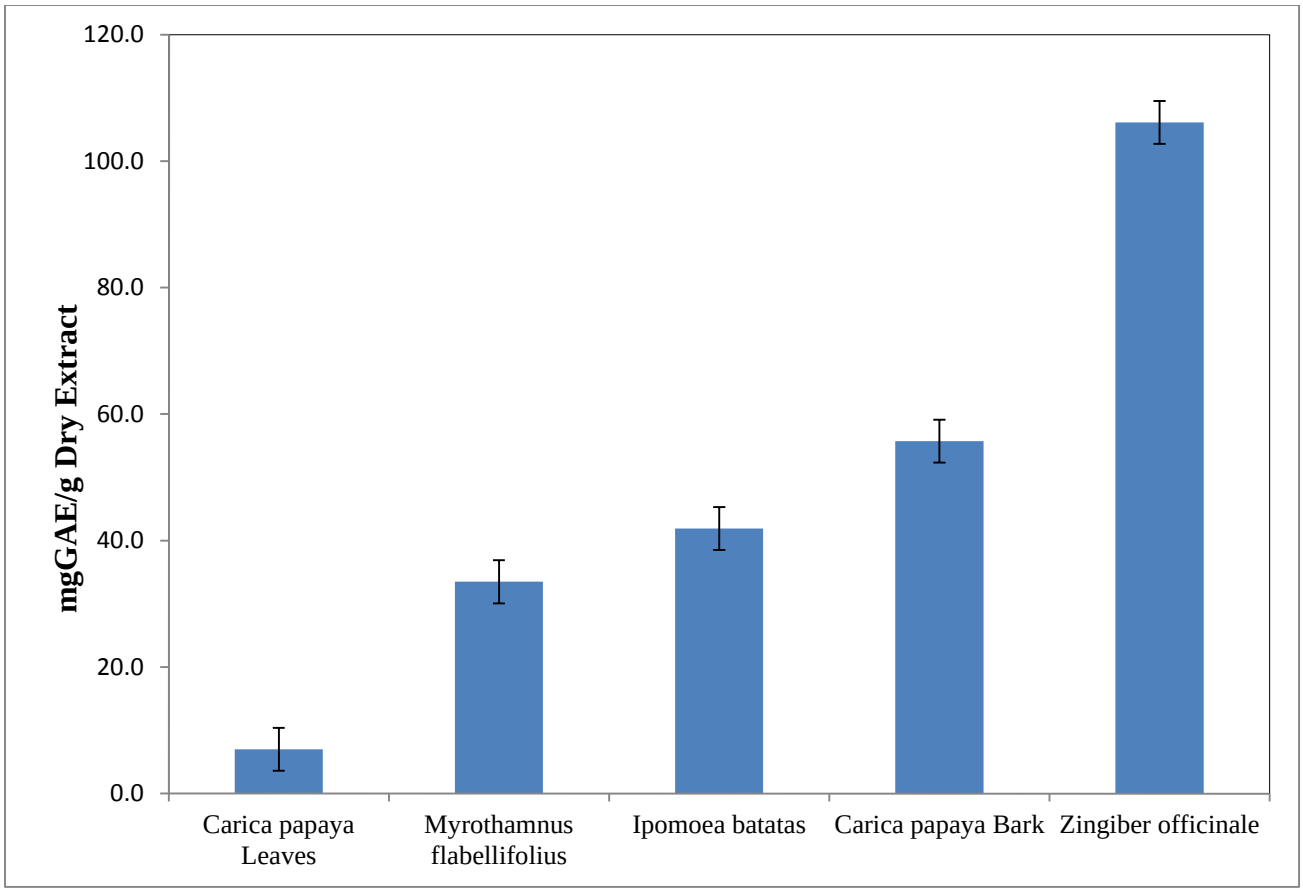


Figure 9.0 TPC in plant extracts in mgGAE/g dry extract

The Figure (9.0) above shows that the Total Phenolic Content (TPC) were significantly ($\rho < 0.05$) different amongst plant extracts with *Zingiber officinale* rhizome extract as highest, followed by *Carica papaya* bark extract however significantly different from another. *Ipomoea batatas* leaves extract and *Myrothamnus flabellifolius* above ground parts extracts TPC were not significantly different. *Carica papaya* leaves extract TPC was the least and significantly different from the rest. *Carica papaya* leaves extract and *Carica papaya* bark were significantly different in their TPC content.

4.4.2 Qualitative Phytochemical Analysis of plant phenolic extracts

The following table shows the selected qualitative phytochemical tests results for all the plant phenolic extracts. Qualitative tests showed some similarities and differences in the presence of the tested secondary metabolites. All plant extract showed the presence of phenolic compounds with *Zingiber officinale* showing abundant presence. *Carica papaya* bark and leaves showed fair presence of phenolic compounds whilst *Ipomoea batatas* and *Myrothamnus flabellifolius* had presence of phenolic compounds in moderate amounts. All samples axcept *Zingiber officinale* showed presence of coumarins. Saponins were present in all plant extracts. Quinones were also present in all samples except *Ipomoea batatas*. Alkaloids were also present in all samples except *Carica papaya* leaves.

Table 3.0 Showing Qualitative Phytochemical test results for the plant phenolic extracts

Phytochemical Test	***Ipomoea batatas***	***Myrothamnus flabellifolius***	***Carica papaya* bark**	***Carica papaya* leaves**	***Zingiber officinale***
Saponins	+	++	++	+++	++
Flavanoids	++	-	-	+	++
Alkaloids	+	+	+	-	+
Quinones	-	+	+++	+	++
Terpenoids	+	-	-	+	+
Anthocynanins	++	-	+	++	-
Tannins	++	+++	-	++	-
Phenolic Compounds (Phenols)	++	++	+	+	+++
Coumarins	+	++	+	+	-
Key: + = fairly present; ++ = moderately present; +++ = abundantly present; - = not detected					

4.5 Discussion

4.5.1 Quantitative Total Phenolic Content of plant phenolic extracts

The results of this experiment show the presence of plant phenolic compounds in all the plant samples under study, however there were differences in quantities. Phenolic compounds are rich in many natural plant products (Ramawat and Mérillon, 2013). Many studies in organic chemistry or phytochemistry and food chemistry show varied literature on the quantitation of total plant phenolic content (TPC) as well as their antioxidant abilities (Zhang, *et al*., 2013). It has been shown that consumption of natural products rich in plant phenolic content prevents oxidative stress and related diseases (Vichapong, *et al*., 2010; Yang, *et al*., 2011; Iswaldi, *et al*., 2012; Meneses, *et al*., 2013).

There is considerable literature on Total Phenolic Content (TPC) of the studied plant extracts, some with comparable results with the ones obtained in this study whilst some with varied results mainly due to the differences in methods of extraction or experimental procedures.This study used old leaves (*Carica papaya* and *Ipomoea batatas*). Scholarship studies have revealed that TPC varied in young and old leaves of *Carica papaya* with quantities of 3.18mgGAE/g DW and 2.81mgGAE/g DW respectively (Gogna, *et al*., 2015); 4.25 mg GAE /g DW (Maisarah , *et al*., 2014); Elsewhere it was shown to be 21.8mgGAE/g DW (Iyawe and Azih, 2011). *Zingiber officinale* TPC was very high as observed in one research (871 mgGAE/g DW (Stoilova, *et al*., 2007) also 870.1mgGAE/g DW (Kumar, *et al*., 2011); In contrast very low 13.5mgGAE/g DW (Ghasemzadeh, *et al*., 2010); low also 15.7mgGAE/g DW (Pawar, *et al*., 2015); varietal differences were noted together with method of extraction.

Total Phenolic Content (TPC) in *Myrothamnus flabellifolius* was found to be 47.5mgGAE/g DW (Bhebhe, *et al*., 2015) which is comparable to the research results of 33.5mgGAE/g DW; was elsewhere found high, 372.42-375.14mgGAE/g DW (Cheikhyoussef, *et al*., 2015). *Ipomoea batatas* leaves TPC was found to be variable depending on method of extraction and variety, for example, 16.35mgGAE/g DW, also observed to be 569.38mg GAE/g DW (Zhang, *et al*., 2015). Five varieties in another research showed varied TPC amounts of their ethanolic extracts (52.5, 118.5, 138, 157.4 and 196.4mgGAE/g DW); also, different methods of extraction varied greatly with some solvents showing no detection (Fidrianny, *et al*., 2013). Total phenolic content (TPC) has also been shown to vary with varietal differences and also largely due to the method/technique employed in extraction (Azmir, *et al*., 2013; Khoddami, *et al*., 2013).

4.5.2 Qualitative Phytochemical Analysis of plant phenolic extracts

The presence of other secondary metabolites as qualitatively revealed by the phytochemical tests was quite anticipated given that in plant metabolism, plant phenolics are the third largest group (18%), with terpenes (55%) as first, and alkaloids (27%) on the second position (Shahzad, *et al*., 2013). Phytochemical tests did reveal the presence of various secondary metabolites in the plant extracts. Some plants for example, *Petroselinum crispum* and *Ammi maju* have been shown to accumulate coumarins as their defence strategy against potential pathogens (Matern, 1991).The terpenoid test was affirmative in *Ipomoea batatas*, *Carica papaya* leaf and *Zingiber officinale*.Terpenes have been shown to constitute the highest percentage of constituents in plant secondary metabolism and they also play a role in plant defence (Mazid, *et al*., 2011). Plant phenolic compounds are implicated in chemical defence weaponry of many plants (Crozier, et al., 2006). The TPC of apple leaves was found to increase upon infection by *Venturia inaequalis* an attendant pathogenic ascomycete fungus that causes apple scab disease (Petkovšek, *et al*.,

2008). Another study showed increase of phenolic compounds upon infection of red raspberry (*Rubus idaeus*) by two pathogenic fungi, *Didymella applanata* and *Leptosphaeria coniothyrium* which causes spur blight and cane blight respectively (Mikulic-Petkovsek, *et al*., 2014). It has also been shown *in vitro* tests that increase in phenolic content increased the antifungal activity of the tested plant extracts (Singh, *et al*., 2011). It is also noteworthy that although plant phenolics compound have been shown to have a major role in plant defence, other secondary metabolites such as terpenes also have their contribution (Mazid, *et al*., 2011).

A research investigating the phytochemical constituents of some medicinal plants revealed presence of tannins, flavanoids, alkaloids amongst other phytochemicals tested in *Zingiber officinale* rhizomes and *Carica papaya* leaf extracts (Mbadianya, *et al*., 2013). These results reported in literature show some similarities and differences with the ones obtained in the current study. Alkaloids were not present in *Carica papaya* leaves extract whilst the ones reported in literature above showed moderately high concentrations; morever, in the current study *Zingiber officinale* showed no presence of tannin content versus the small concentration reported in literature. Flavanoid and tannin content for *Carica papaya* showed some similar trend as reported in literature above. The differences noted, could be attributed to different extraction methods and sample treatment. Different solvents yielded different results for *Carica papaya* leaves extracts phytochemical tests (Anjum, *et al*., 2013). Activated charcoal treatment whilst it can successfully remove chlorophyll from plant extracts, it has been shown to adsorb tightly some plant active constituents (Scheepers, *et al*., 2011) and can also remove some phenolic compounds (Chee, *et al*., 2013); the literature reported above did not carry out any activated charcoal treatments.

Alkaloid, saponin, flavanoid and tannin presence in *Ipomoea batata* leaves extract in the current study was found somewhat consistent with some report in literature although different solvents and sample treatements were applied (Mbaeyi-Nwaoha and Emejulu, 2012). Another similar research on *Ipomoea batatas* leaves extract reported different outcomes with application of different solvents. Much of the phytochemicals were not detected in most of the solvents except for aqueous ethanolic extract which showed presence of alkaloids, coumarins, flavanoids, saponins,anthraquinones, tannins and phenolic acids (Pochapski, *et al*., 2011), a somewhat consistence with the current study observations.

Qualitative phytochemical tests for *Myrothamnus flabellifolius* in the current study showed the presence of six out of the nine phytochemical tests with flavanoids, terpenoids and anthocynanins not being detected. Some authors (Cheikhyoussef, *et al*., 2015; Molefe-Khamanga, *et al*., 2012) have reported results with some similarities and differences between themselves and with the current study and the major points deference emanate to the different sample preparation, parts of the plant used and solvent used for extraction.

4.6 Conclusion and Recommendations

4.6.1 Conclusion

The experiment was able to quantify the total phenolic content (TPC) of the plant extracts, a very useful result in studies of contribution of phenolic compounds to plant defence. This is quite significant in trying to understand the behaviour and subsequent chemical constituent linked to plant defence. This study was able to show that different plant species contain plant phenolic compounds in varying amount. It was aslo noted that different plant parts may contain different levels of plant phenolic compounds as shown by *Carica papaya* leaves and bark. It is therefore

imperative to analyse different plant parts in seeking for active constituents against plant pathogens.

4.6.2 Recommendations

The studies of total phenolic content (TPC) with regards to plant defence however a good pointer they do not give the exact plant phenolic compounds implicated in plant defence. Neither do they give the specific phenolic compounds that increase in concentration upon infection. It would beneficial to do *in vivo* analysis of the individual plant phenolic compounds present in a plant prior to infection and post infection so as to establish a general trend upon which inquisitions can then be made. Other studies have through the HPLC analysis coupled with detectors capable of aiding in characterization of plant phenolic compounds managed to show the behaviour of individual plant phenolic compounds. There is also room in exploring different solvents in extraction and/or techniques so as to establish optimized conditions that give the highest quantities of total phenolic compounds. A further development could be done where the total phenolic content is further divided into total simple phenolic compounds and also total polyphenols of which some like tannic acid can be quantified on their own given the availability of such assays. Into the same bargain, different sample preparation with regards to cleaning or removal of chlorophylls from the plant extracts need to be explored since activated charcoal (carbon) has been shown to also retain analytes of interest.

CHAPTER 5: Antifungal evaluation of phenolic extracts of *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* aboveground plant parts and *Ipomoea batatas* leaves on pathogenic fungi of economic importance

5.1 Introduction

A decade ago more than 800million people had inadequate food globally and more than 1.3 billion lived on less than a dollar (US$) a day (Christou and Twyman, 2004; FAO, 2000). Prevalence of undernourishment in the Sub-Saharan Africa is still reported high 23.8% (in the period 2012-14), a decrease of approximately 10% from the 1990s (FAO, *et al*., 2014). Worsening the food insecurity is undoubtedly loss of yields (10-16%) due to plant pathogens (Agrios, 2005; Strange and Scott, 2005; Chakraborty and Newton, 2011). Fungi decimate harvests, often leading to widespread malnutrition and starvation, for example *Magnaporthe oryzae* (rust blight fungus) claims 10-30% rice losses annually, yet over 3 billion people use rice as a staple food (Skamnioti and Gurr, 2009). Plant disease therefore is certainly one of the major threats (Gurr, *et al*., 2011) towards global food security and undeniably, needs to be curbed. All crop protection efforts are set toward a goal of increasing yields and thus profitability in the agribusiness enterprises; without them, losses would rise to an estimate of 70% and undermine global food supply by more than 50% (Oerke, *et al*., 1999).

Plants are known to produce a myriad of natural bioactive constituents of which secondary metabolites alone constitute 18% as phenolic compounds (Shahzad, *et al*., 2013). Lattanzio, *et al*., (2006) have extensively reviewed the role of plant phenolic compounds ranging from simple phenols to polyphenols, on plant defence. Lattanzio, *et al*., (2006) further show a list of phenolic

compounds for example chlorogenic acid, rutin, ρ-coumaric acid, cynidin amongst others, which exhibit antifungal activity on some fungi such as *Fusarium oyxporum, Gleosporum perennas, Penicillium glabrum* and *Cladosporium herbarum*. Phenolic compounds (carvacol and thymol) are reported to be the chief compounds in *Origanum syriacum* and responsible for their antifungal on *Aspergillus* spp. (El Gendy, *et al*., 2015).

The present study sought to evaluate the phenolic extracts of *Carica papaya* (leaves and bark), *Ipomoea batatas* leaves, *Zingiber officinale* rhizomes and *Myrothamnus flabellifolius* above ground parts; these plants have been shown to have some enthnobotanical uses whilst some are used as food. Their chemical composition from previous studies added impetus on their investigation as potential natural products that could be used to control phytopathogenic fungi.

5.2 Objectives

1. To assess the aqueous methanolic plant phenolic extracts on their activity on mycelial growth of *Macrophomina phaseolina, Alternaria alternata, Rhizoctonia solani* and *Botrytis cinerea, in vitro* tests.
2. To evaluate the effectiveness of the aqueous methanolic plant phenolic extracts on their percentage reduction of mycelial growth of *Macrophomina phaseolina, Alternaria alternata, Rhizoctonia solani* and *Botrytis cinerea, in vitro* tests.

5.3 Experimental Materials and Methods

5.3.1 Study Site Description

The research was conducted at the University of Botswana (24° 40′ 0″ S, 25° 56′ 0″ E) main campus in Gaborone. Extraction and determination of phenolic compounds was done at the Chemistry Department whilst in vitro bioassays of phenolic extracts were done at the

Department of Biological Sciences (University of Botswana) .Gaborone is located in the southern east part of Botswana (24^0 40′ 0″ S, $25^0$55′0″ E) and approximately 100km South of the Tropic of Capricorn with altitude reaching approximately 1,000m above sea level in the capital (Gaborone). Gaborone is situated in one of the semi-arid to arid regions of the world with average rainfall of 538mm and average temperature of 20.7^{0}C (Jonsson, 2004).

5.3.2 Collection, Drying and Plant Sample Preparation

Ginger (*Zingiber officinale*) rhizomes were bought from the River Walk Mall (Pick n Pay Supermarket) near University of Botswana. The leaves and bark for paw-paw (*Carica papaya*) and sweet potato leaves (*Ipomoea batatas*) were collected from communities near the University of Botswana (Tlokweng Village). The aboveground parts of the resurrection plant (*Myrothamnus flabellifolius*) were obtained from a faculty member (Mr. T. Kwape) in the Department of Biological Sciences at the University of Botswana. All species were botanically confirmed at the botany laboratory at the Department of Biological Sciences at the University of Botswana. The materials were brought to the laboratory (leaves for and bark for *Carica papaya*, *Ipomoea batatas* leaves, aboveground parts of *Myrothamnus flabellifolius* and *Zingiber officinale* rhizomes). Collected leaf material were all surface sterilized with 1% Sodium Hypochlorite (NaHOCl), rinsed with running distilled sterile water, blotted with filter paper, and then freeze-dried for 24 hours at temperatures between -50^{0}C to -54^{0}C (Hussin, *et al*., 2009). Ginger rhizomes were thoroughly washed to remove any soil and/or foreign material traces, surface sterilized with 1% NaHOCl, and then rinsed with running sterile distilled water, then blotted with filter paper. The rhizomes were cut into minute pieces with a sterile scalpel blade to facilitate rapid shade-drying in the laboratory. The resurrection plant aboveground parts were shred into5-10 mm pieces using an ergonomic pruning shear. After drying, all the plant materials were

ground into powder (2 mm particle diameter) using a laboratory electric powered mill, and then passed through a 500 µm sieve. The fine powders were sieved through a 500 µm sieve and stored in air-tight, opaque sterile bottles at 5ºC in the dark ready for solvent maceration. The procedure for drying plant materials in preparation for solvent extraction of plant phenolic compounds was done according to Ramawat and Mérillon (2013).

5.3.3 Ultrasound Sound Assisted Solvent Extraction (USAE)

Ultrasound Assisted Solvent Extraction (USAE) was performed in an ultrasonic cleaner (model 704, SCIENTECH, South Africa) with a maximum of 12L ("LO", "HI" frequencies, 300W) following methodologies of (Tabaraki, *et al*., 2012; Wang, *et al*., 2013; Ali and Kumar, 2014; Rezaie, *et al*., 2015) with modifications on ultrasonication temperature, extraction time, filtration and centrifugation parameters. Plant dry powders (500 µm particle diameter), *Carica papaya* bark (60 g) and the rest 40 g of each (*Carica papaya* leaves, *Ipomoea batatas* leaves, *Zingiber officinale* rhizomes and the aboveground parts of *Myrothamnus flabellifolius*) were quantitatively transferred into 500 mL conical flask. HPLC grade solvent (methanol) prepared to make 80% methanol (v/v) with ultra-distilled water was mixed with powdered extracts (240 mL for *Carica papaya* bark) and 160 mL for other plant extracts. Ultrasound equipment was operated at the "LO" frequency (considered aggressive) and power of 300W with temperature regulation at 45-50ºC (Ali and Kumar, 2014). Extractions were done for 45 minutes (Rezaie, *et al*., 2015) and three times using the same amount of solvent and then pooled. The conical flasks were immersed into the ultrasonic bath, with the liquid level in the conical flasks kept lower than that of the cleaner tank (Ali and Kumar, 2014). Pooled extracts of each plant extract were thoroughly mixed in a shaker and centrifuged for 15 minutes at 6000 rpm for deposit suspension particle (Wang, *et al*., 2013), after which the supernatants were filtered through Whatman™ 1 filter paper (pore

size of 11μm) (Rezaie, *et al*., 2015). The filtrates of the extracts were concentrated by rotary evaporation at 40°C under vacuum to dryness (Tabaraki, *et al*., 2012). After rotary evaporation, the plant extracts yielded 5.80 g (*Ipomoea batatas*), 7.93 g (*Carica papaya* bark), 3.79 g (*Carica papaya* leaves), 7.98 g (*Myrothamnus flabellifolius*) and 6.08 g (*Zingiber officinale*).

5.3.4 In vitro antifungal evaluation of plant phenolic extracts of *Carica papaya* bark, *Carica papaya* leaves, *Zingiber officinale* rhizomes, *Myrothamnus flabellifolius* above ground parts and *Ipomoea batatas* leaves on the mycelial growth of plant fungi of economic importance

5.3.4.1 Experimental Design and Description of Treatments

The experiment had 7 treatments (plant phenolic compounds/fungicides) with plant phenolic extracts/fungicides applied at three concentrations/levels, C1= 250ppm, C2 = 500ppm and C3 =1000ppm. A 7 x 3 Factorial experiment in a Completely Randomized Design (CRD) replicated 3 times was used for the evaluation of the antifungal activity of plant phenolic extract on mycelial growth of the plant pathogenic fungi. Randomization of experimental units was achieved through use of www.randomization.com as recommended by (Suresh, 2011). Treatments were labelled as follows: TR1= *Carica papaya* Bark; TR2 = *Carica papaya* leaves; TR3 = *Zingiber officinale*; TR4 = *Myrothamnus flabellifolius;* TR5 = *Ipomoea batatas*; TR6 = Fungicide (positive control); TR7 = Negative Control.

The plant phenolic extract incorporated petri dishes for each fungi (9cm top lid diameter) were aseptically inoculated at the centre with a 5mm disc of a 7days old culture media of each respective test fungi. *Botrytis cinerea* petri dishes were incubated at 20 ± 2^{0}C in the dark whilst

Macrophomina phaseolina, *Alternaria aternata* and *Rhizoctonia solani* were incubated at 26±2^{o}C in a temperature room.

5.3.4.2 Preparation of Plant Phenolic Extracts

Plant phenolic extracts stock solution were prepared according to the methods by (Singh, *et al*., 2011) and (Hussin, et al., 2009) with modification. Briefly, 1.000g dry extract of each plant sample obtained after rotary evaporation was quantitatively transferred to a 50mL centrifugal vial and reconstituted in 5mL absolute methanol, vortexed to dissolution. Sterile distilled water (45mL) was added to the reconstituted extract, vortexed to uniformity to give a concentration of 20,000ppm (2%) stock solution. Serial dilutions were obtained by pipetting 25mL of this solution into another 50mL centrifugal vial followed by addition of 25mL 90% aqueous methanol as diluent and vortexed to homogeneity to obtain a 10,000ppm (1%). Using the 10,000ppm solution another dilution was done to obtain a 5,000ppm (0.05%) concentration. All the stock solutions were wrapped with aluminium foil and stored at 4°C till further use.

5.4.3.3 Preparation of Fungicides and Media Preparation

Fungicide stock solutions and media were prepared according to the method by Oniango, *et al*., (2005) with modifications. Briefly, 1388.89 mg Chemlaxyl 72WP, 1250 mg Mancozeb 80WP, 1428.57 mg Antracol 70WP and 1176.47 mg Copper Oxychloride 85WP (1000 mg of Active ingredient) were weighed through an analytical balance in a weighing boat and quantitatively transferred using 25 mL sterile distilled water to 50 mL centrifugal vials and vortexed to uniformity. Sterile distilled water (25 mL) was added and vortexed to uniformity resulting in 20,000 ppm (2%) stock solution. Serial dilutions were obtained by pipetting 25 mL of this solution into another 50 mL centrifugal vial followed by addition of 25 mL sterile distilled water as diluent and vortexed to homogeneity to obtain a 10,000 ppm (1%). Using the 10,000 ppm

solution another dilution was done to obtain a 5,000 ppm (0.05%) concentration. All the stock solutions stored at 4°C till further use. Potato Glucose Agar (PGA) or Potato Dextrose Agar (PDA) media (composition: 20 g Dextrose, 15 g Agar and 4 g Potato Extract) were brought to boil and autoclaved at 121°C for 15minutes. Using a 1000 µL pipette, 1 mL of thoroughly shaked/vortexed plant phenolic extracts/fungicide (at the 3 concentrations) was measured into 9cm appropriately labelled petri dishes. The diluent used for plant phenolic extract preparations (1mL 90% aqueous methanol) were pipetted into labelled negative control petri dishes. 19 mL of media adjusting stock solution concentration of fungicide/plant phenolic extracts to 250 ppm (C1), 500 ppm (C2) and 1000 ppm (C3) were poured into respective treatment petri dishes aseptically with media temperature ranging from 65-70 °C, allowed to cool and set.

5.3.4.4 Plant Pathogenic Fungi Pure Cultures

Pure cultures of plant pathogenic fungi (*Macrophomina phaseolina, Alternaria alternata, Botrytis cinerea* and *Rhizoctonia solan*i) were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), Universite Catholique de Louvain, Earth and Life Institute (ELI)-Applied Microbiology-Mycology Laboratory (ELIM), Belgium. The active cultures on slants were aseptically cultivated on Potato Glucose/Dextrose Agar in 9 cm petri dishes upon arrival from Belgium according to fungi culture preparation standard protocols in laboratory manual or textbooks (Trigiano, *et al*., 2004; Agrios, 2005). Petri dishes were sealed with Parafilm® and incubated at recommended temperatures for the test fungi. *Macrophomina phaseolina, Alternaria alternata,* and *Rhizoctonia solan*i were incubated at 26±2°C and *Botrytis cinerea* at 20±2°C. All cultures were incubated in the dark and allowed to grow radial from the centre of the petri dish where inoculum was deposited.

5.3.5 Data Collection and Data Analysis

Mycelial growth colony diameter (mm) was measured in 2 dimensions, diagonally across the origin form where the 5mm disc inoculum was deposited and the value divided by 2 for each measurement time interval until the test fungi reached the negative control petri dish edges (85mm diameter). Data were subjected to residual tests to check for compliance with ANOVA assumptions. Data not meeting the ANOVA assumption were transformed through Square Root Transformation ($\sqrt{(x+c)}$, where x= colony diameter value and c=0.5, a constant factorData meeting ANOVA assumptions or transformed data were subjected to Two-way ANOVA analysis, wherein upon significant interactions, interaction effects were shown graphically. Upon significant treatment effects and non significant interactions, Fisher's protected LSD was used to separate treatment means. All statistical analyses were done through Genstat 14th Edition (2011). Using the means for each concentration level, Percentage Mycelial Reduction (PMR) was calculated according to the formula (Baka, 2014):

$$Percentage\ Mycelial\ Reduction(\%) = \left(\frac{C-P}{C}\right) \text{x } 100$$

$Where$:

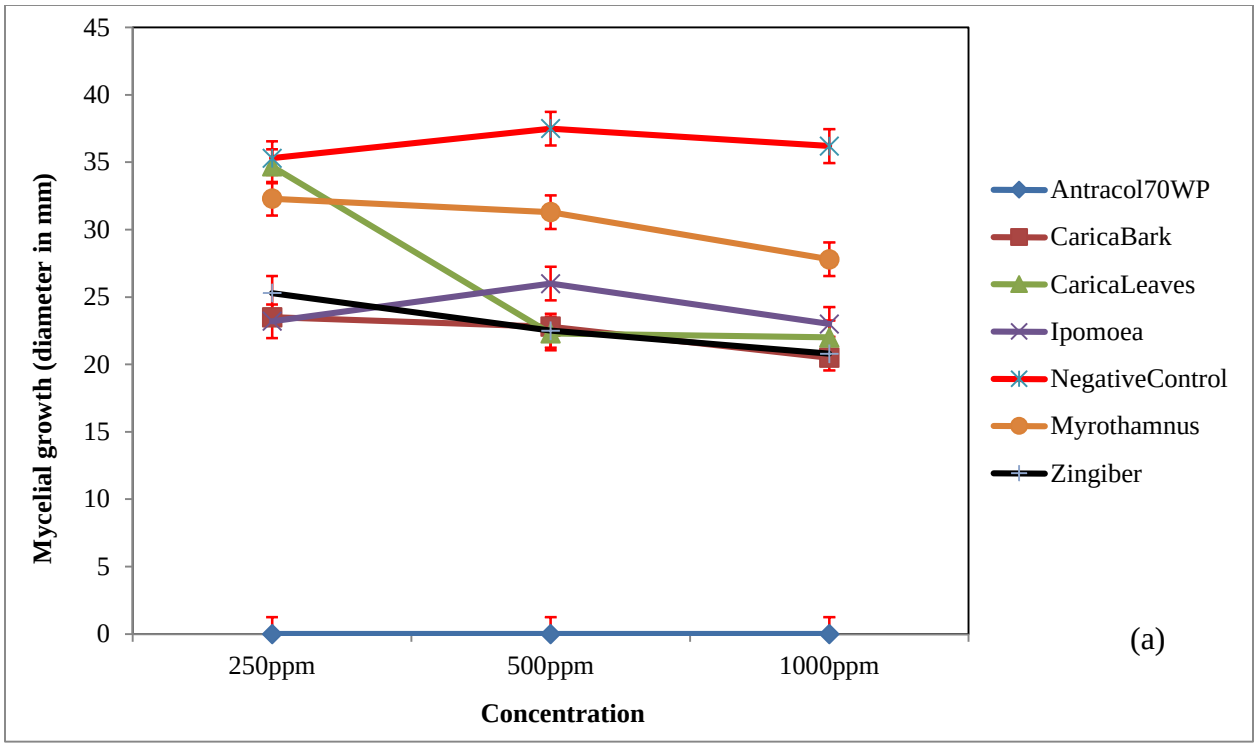
C = Mean Colony Diameter of Negative Control plates

P = Mean Colony Diameter of test fungi

5.4 Results

5.4.1 *In vitro* antifungal activity of plant phenolic extracts on the mycelial growth of *Macrophomina phaseolina*

The main effects (concentration and plant extract) and interaction effects (plant extract●concentration) in analysis of variance (ANOVA) tests were significant (all $\rho - values < \alpha(0.05)$ at 5% level of significance) after 24, 48 and 72 hours of incubation.The figures below (Figure 10.0a and 10.0b) show graphical interaction effects of plant extracts and concentration on the mycelial growth of *M. phaseolina* after 24 and 72 hours of incubation respectively. Fungal growth for the negative control treatment was the highest, and the fungicide (Antracol 70WP) treatment (positive control) totally inhibited *M. phaseolina* mycelial growth at all concentrations at 24 hours however at 72 hours only the 500 ppm and 1000 ppm totally inhibited mycelial growth. *M. flabellifolius* and *I. batatas* generally showed the least antifungal activity throughout the experiment. *C. papaya* leaves showed better antifungal activity at the 500 ppm and 1000 ppm and least at 250 ppm throughout the experiment. *Z. officinale* and *C. papaya* bark had the best antifungal activities from 250 ppm to 1000 ppm amongst plant phenolic extracts. A similar level of antifungal activity for *Z. officinale*, *C. papaya* leaves and bark was observed at 500 ppm. A similar trend in mycelial growth was observed at 48 hours of incubation for all the phenolic extracts.



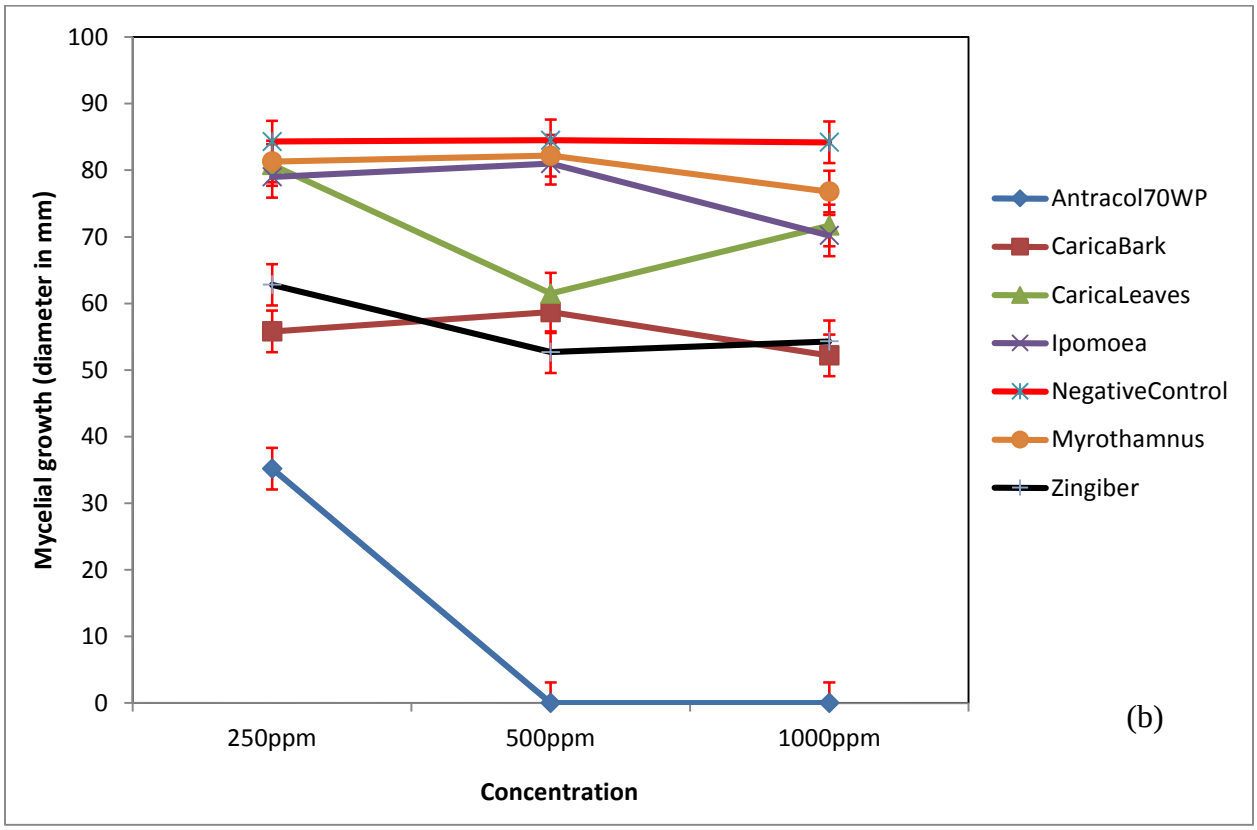
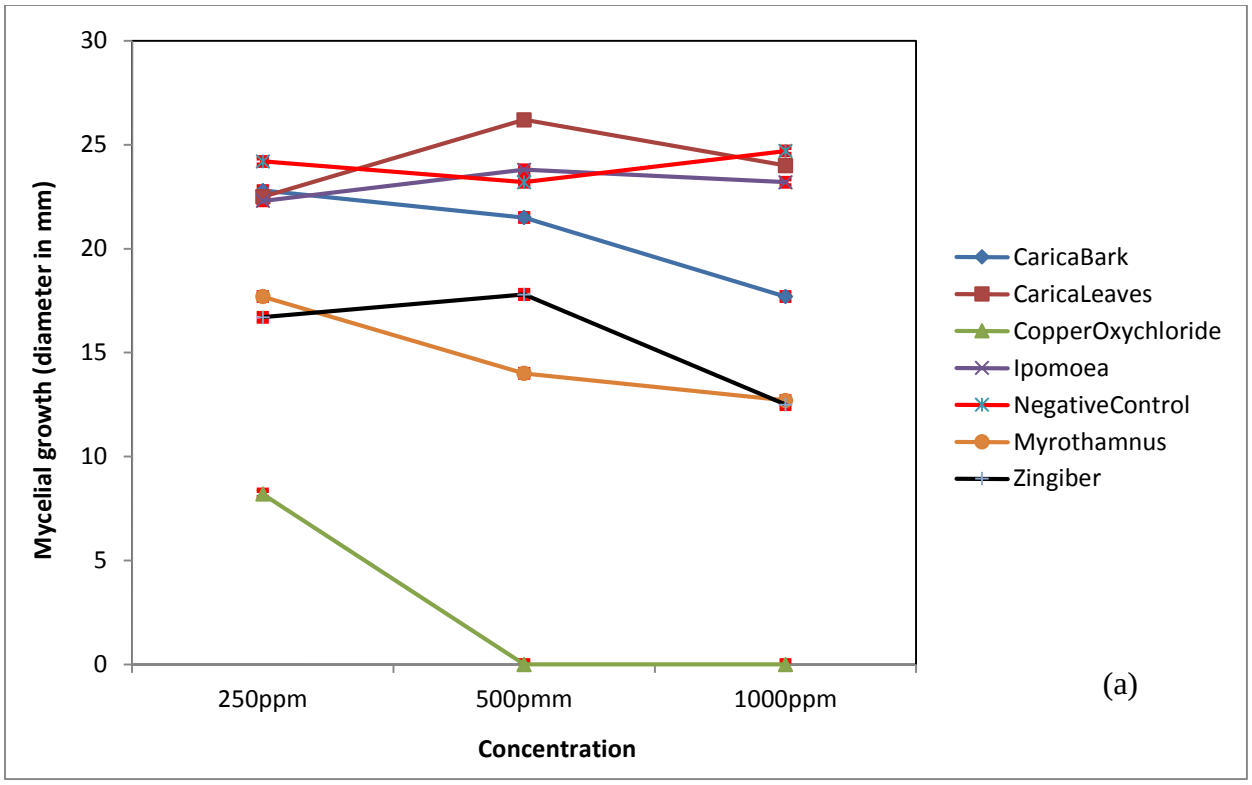


Figure 10.0a and 10.0b Effect plant phenolic extracts on *M. phaseolina* at 24 and 72 hours of incubation

Figure 11.0 Representatives pictograms showing antifungal activity of phenolic extracts on *M. phaseolina* at different concentrations

5.4.2 *In vitro* antifungal activity of plant phenolic extracts on the mycelial growth of *Alternaria alternata*

The main effects (concentration and plant extract) and interaction effects (plant extract●concentration) in analysis of variance (ANOVA) tests were significant (all $\rho - values < \alpha(0.05)$ at 5% level of significance) after 48, 144 and 168 hours of incubation. At 96 hours concentration effects were not significant, $\rho - value = 0.121 > \alpha(0.05)$. The figures below (Figure 12.0a and Figure 12.0b) show mycelial growth for *Alternaria alternata* after 48 and 168 hours of incubation respectively. Fungal growth for the negative control was the highest throughout the experiment reaching 85mm colony diameter at 168 hours. Copper Oxychloride totally inhibited mycelial growth at 500 ppm and 1000 ppm whilst at 250ppm, growth was less than 10mm at 24 hour whilst at 168 hours, limited mycelial growth was observed at all concentrations. At 24 hours, *Carica papaya* extract seemed to promote fungal growth whilst *Myrothamnus flabellifolius* had the best antifungal activity at 500 ppm and 1000 ppm amongst all phenolic extracts. *Zingiber officinale*'s activity at 1000 ppm was the same with that of *M. flabellifolius*. At 168 hours, *Carica papaya* leaves showed the least antifungal activity at the 500ppm and 1000ppm amongst the phenolic extracts, the 250ppm activity was congruent with that of *Carica papaya* bark. *Carica papaya* bark, *Myrothamnus flabellifolius* and *Zingiber officinale* exhibited a similar trend in antifungal activity at all concentrations with *Zingiber officinale* emerging the best amongst all the phenolic extracts. A generally similar trend of phenolic extract activity was observed at the 96 and 144 hours of incubation for all the extracts.



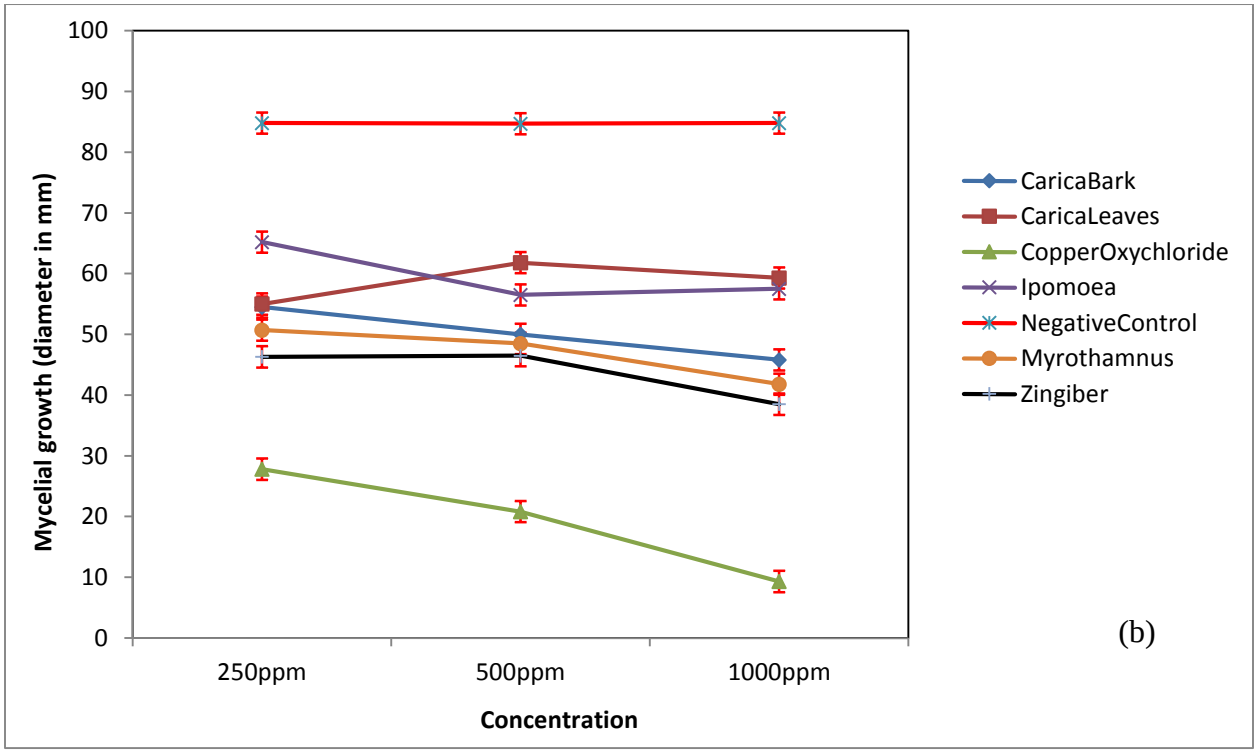


Figure 12.0a and 12.0b Effect plant phenolic extracts on *A. Alternata* at 48 and 168 hours of incubation

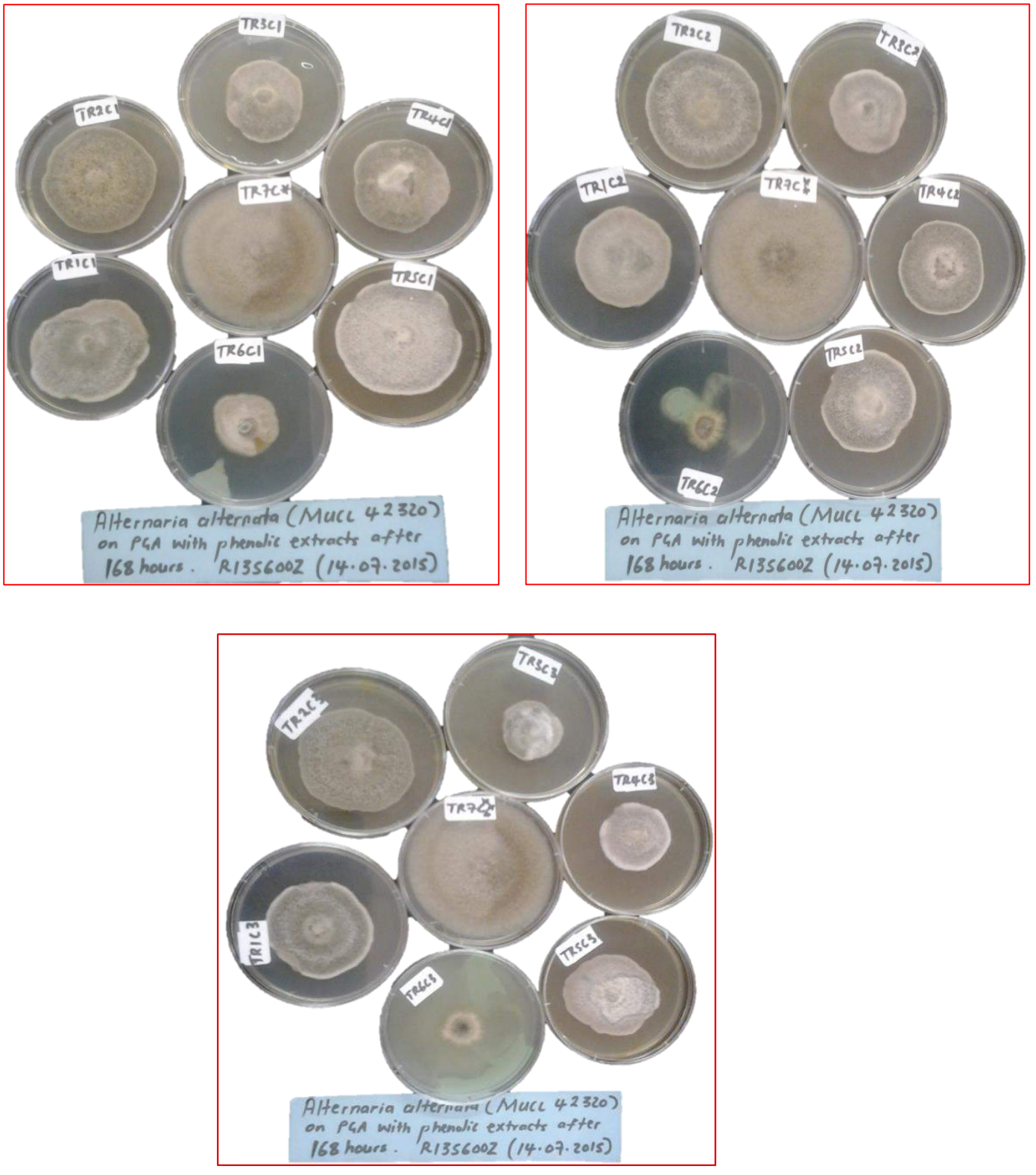
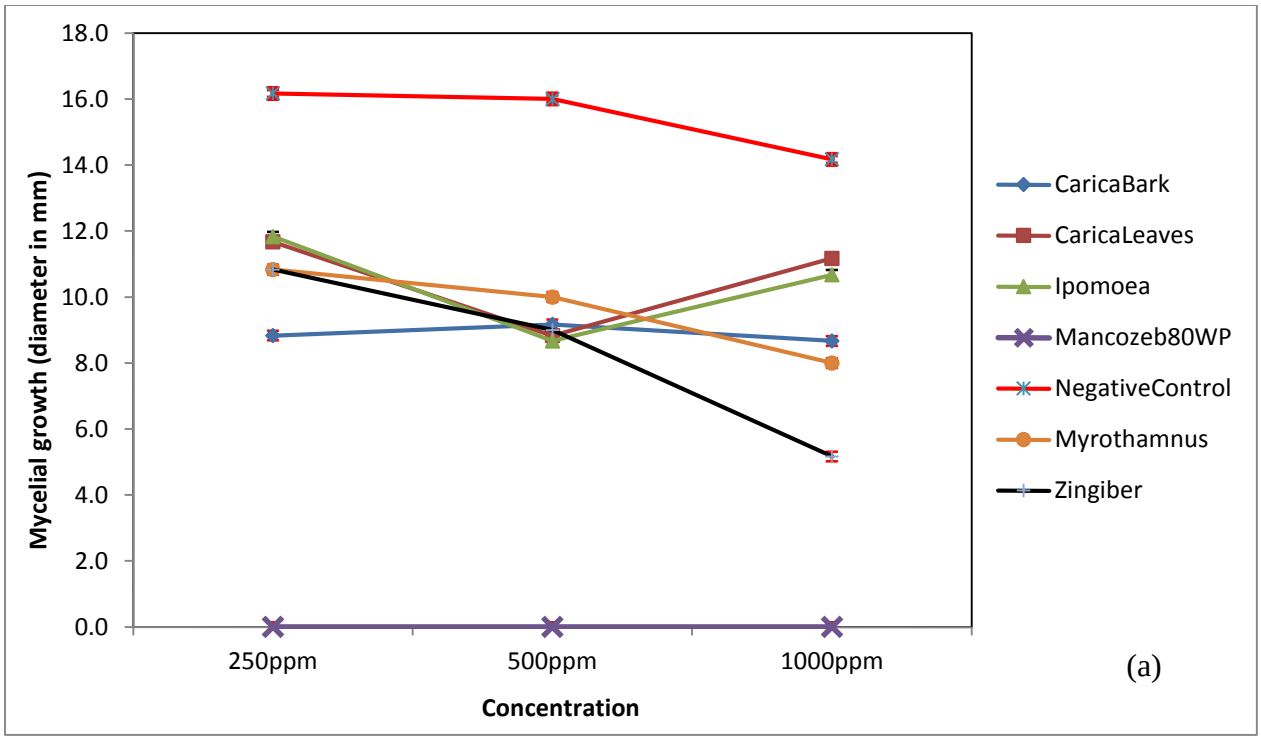


Figure 13.0 Representatives pictograms showing antifungal activity of phenolic extracts on *Alternaria alternata* at different concentrations

5.4.3 *In vitro* antifungal activity of plant phenolic extracts on the mycelial growth of *Rhizoctonia solani*

The main effects (concentration and plant extract) and interaction effects (plant extract●concentration) in analysis of variance (ANOVA) tests were significant (all $\rho - values < \alpha(0.05)$ at 5% level of significance) after 24, 48, 72 and 96 hours of incubation. The figures below (Figure 14.0a and Figure 14.0b) show mycelial growth for *Rhizoctonia solani* after 24 and 96 hours of incubation respectively. Fungal growth for the negative control treatment was the highest, and Mancozeb 80WP totally inhibited *Rhizoctonia solani* mycelial growth at all concentrations throughout the experiment. At 24 hours, *Zingiber officinale* showed a marked increase in antifungal activity from the 250ppm to 1000ppm. *Carica papaya* bark's antifungal activity was almost the same at all the concentrations. *Carica papaya* leaves seemed to promote fungal growth with increase in concentration. *Myrothamnus flabellifolius* showed the least antifungal activity compared to other phenolic extracts and comparable to *Zingiber officinale* at 250ppm. *Ipomoea batatas* although with less mycelial growth at 250ppm compared to other phenolic extracts, seemed to promote mycelial growth with increase in concentration. At 96 ours, *Carica papaya* bark showed the least antifungal activity. *Carica papaya* leaves and Bark had the same antifungal activity at 250ppm, with the former showing better activity at other concentrations. *Myrothamnus flabellifolius* showed an increased antifungal activity with increased concentration. *Ipomoea batatas* emerged the best phenolic extract showing better antifungal activity against *R. solani* at the 500ppm and 1000ppm concentrations. *Zingiber officinale* exhibited an increased antifungal activity with increase in concentration.



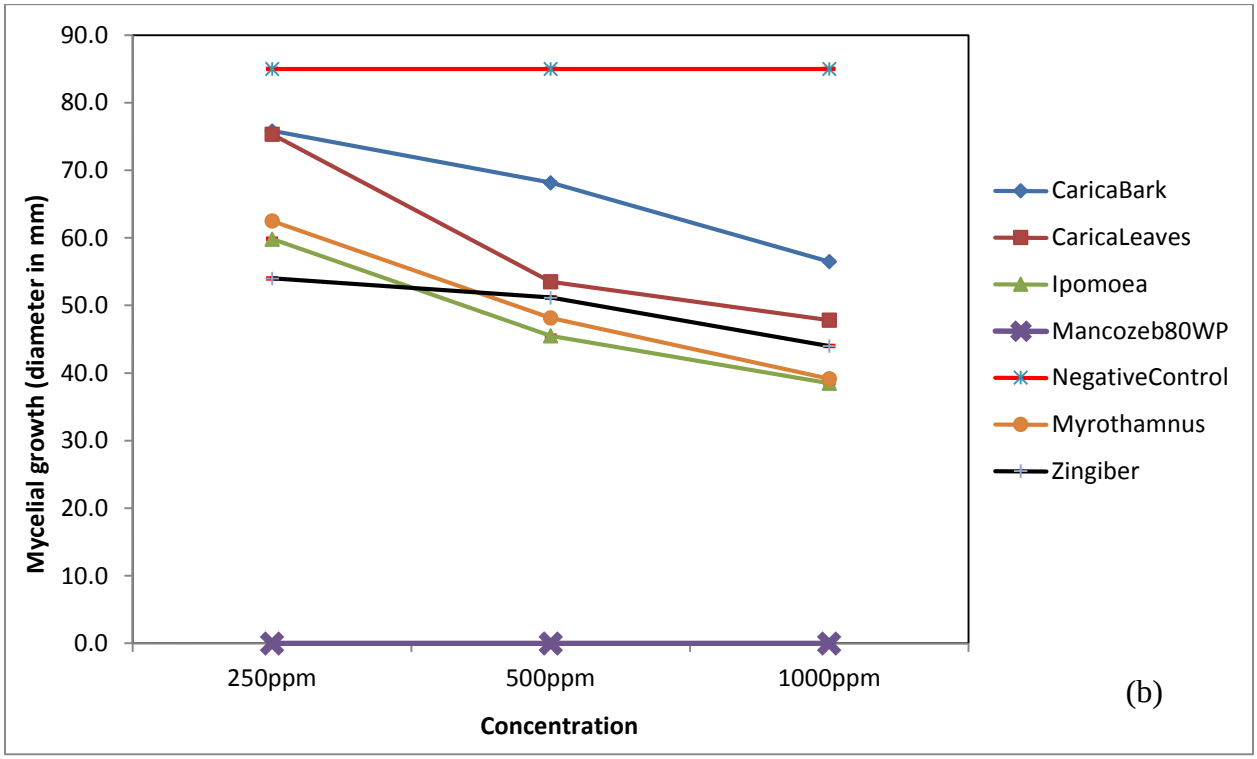


Figure 14.0a and Figure 14.0b Effect of Plant Phenolic Extracts on *Rhizoctonia solani* growth

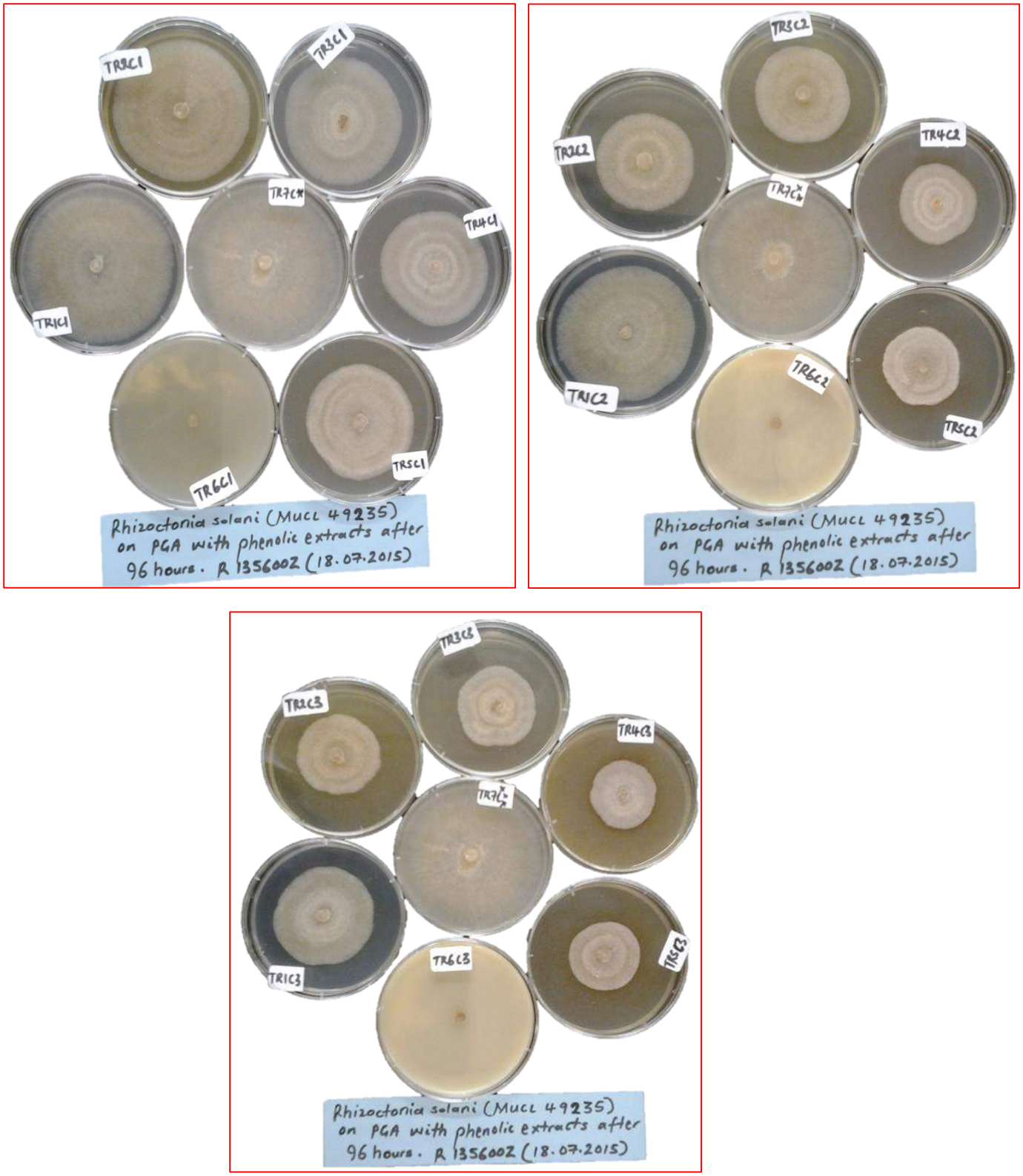
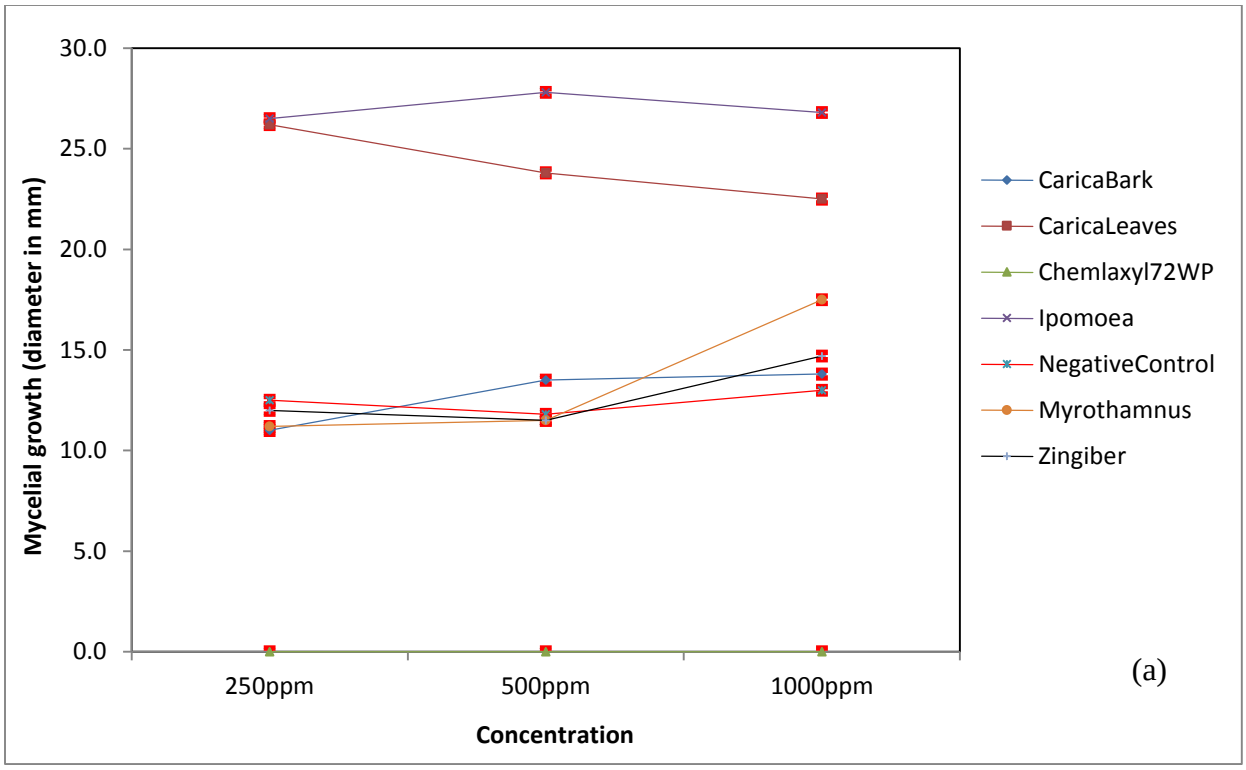


Figure 15.0 Representatives pictograms showing antifungal activity of phenolic extracts on *Rhizoctonia solani* at different concentrations

5.4.4 *In vitro* antifungal activity of plant phenolic extracts on the mycelial growth of *Botrytis cinerea*

Main effects (concentration and plant extract) and interaction effects (plant extract●concentration) in analysis of variance (ANOVA) tests were significant (all $\rho - values < \alpha(0.05)$ at 5% level of significance) after 24, 48 and 72 hours of incubation except concentration effects at 48 hours $\rho - value = 0.077 > \alpha(0.05)$ which were not significant. Treatment (plant phenolic extracts) in ANOVA tests were significant ($\rho - value < \alpha(0.05)$ at 5% level of significance) after 96hours whilst interaction effects (plant extract●concentration) effects and concentration $\rho - values > \alpha(0.05)$, $\rho - value = 0.466$; $\rho - value = 0.376$ respectively, hence not significant. The figures below (Figure 16.0a and Figure 16.0b) show mycelial growth for *Botrytis cinerea* after 24 and 96 hours of incubation respectively. At 24 hours, fungal growth for *Ipomoea batatas* was the highest amongst all phenolic extracts Chemlaxyl 72 WP totally inhibited *Botrytis cinerea* mycelial growth at all concentrations throughout the experiment. *Carica papaya* leaves at 250ppm showed the same growth with that of *Ipomoea batatas* and the two actually showed a notable mycelial growth more than that of the negative control treatment. *Carica papaya* bark, *Myrothamnus flabellifolius* and *Zingiber officinale* showed a comparable mycelial growth to the negative control treatment, with all having a somewhat mycelial growth promotion other than antifungal activity. All phenolic extracts (main effects) did not show any antifungal activity against *Botrytis cinerea* at end of the experiment and hence not significantly different from each other and negative control, save for the positive control (Chemlaxyl 72WP) which was significantly different from the rest.



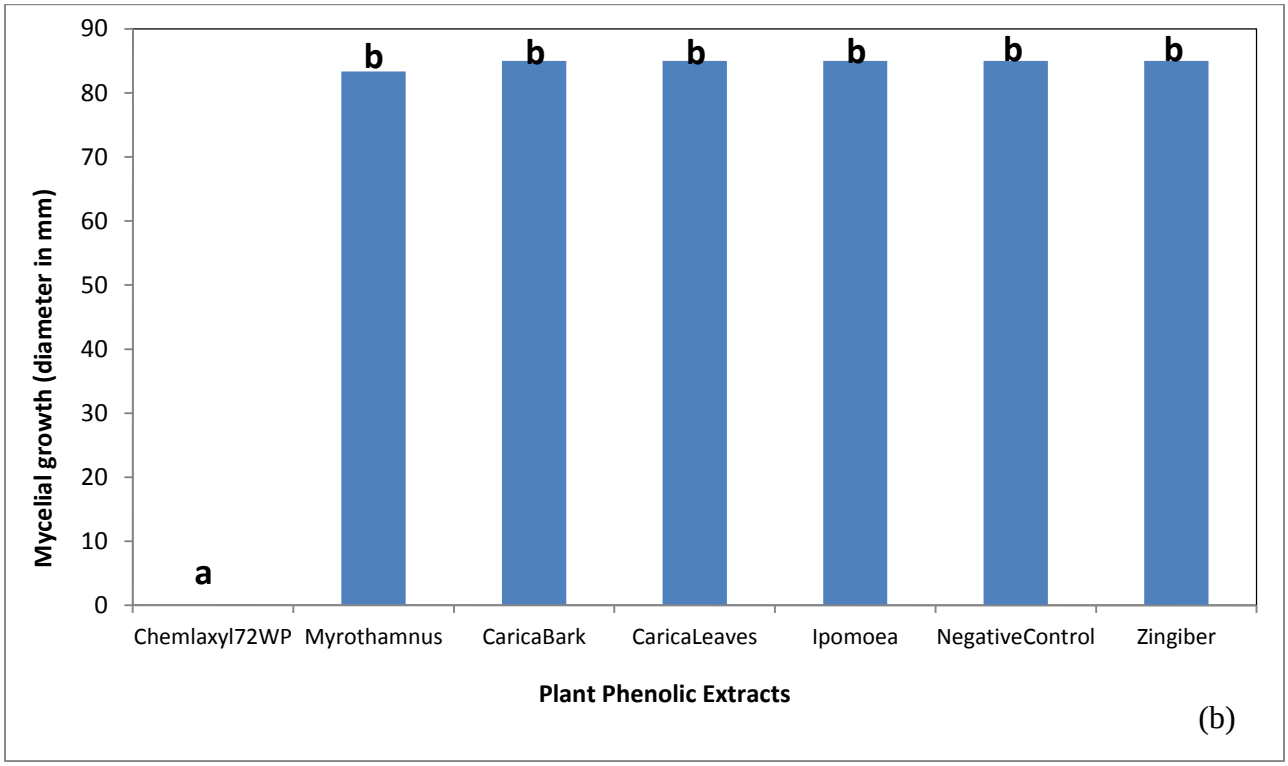


Figure 16.0a and Figure 16.0b Effect of plant phenolic extracts on *Botrytis cinerea*

(**NB:** Bars with the same letter not significantly different at 5% level of significance)

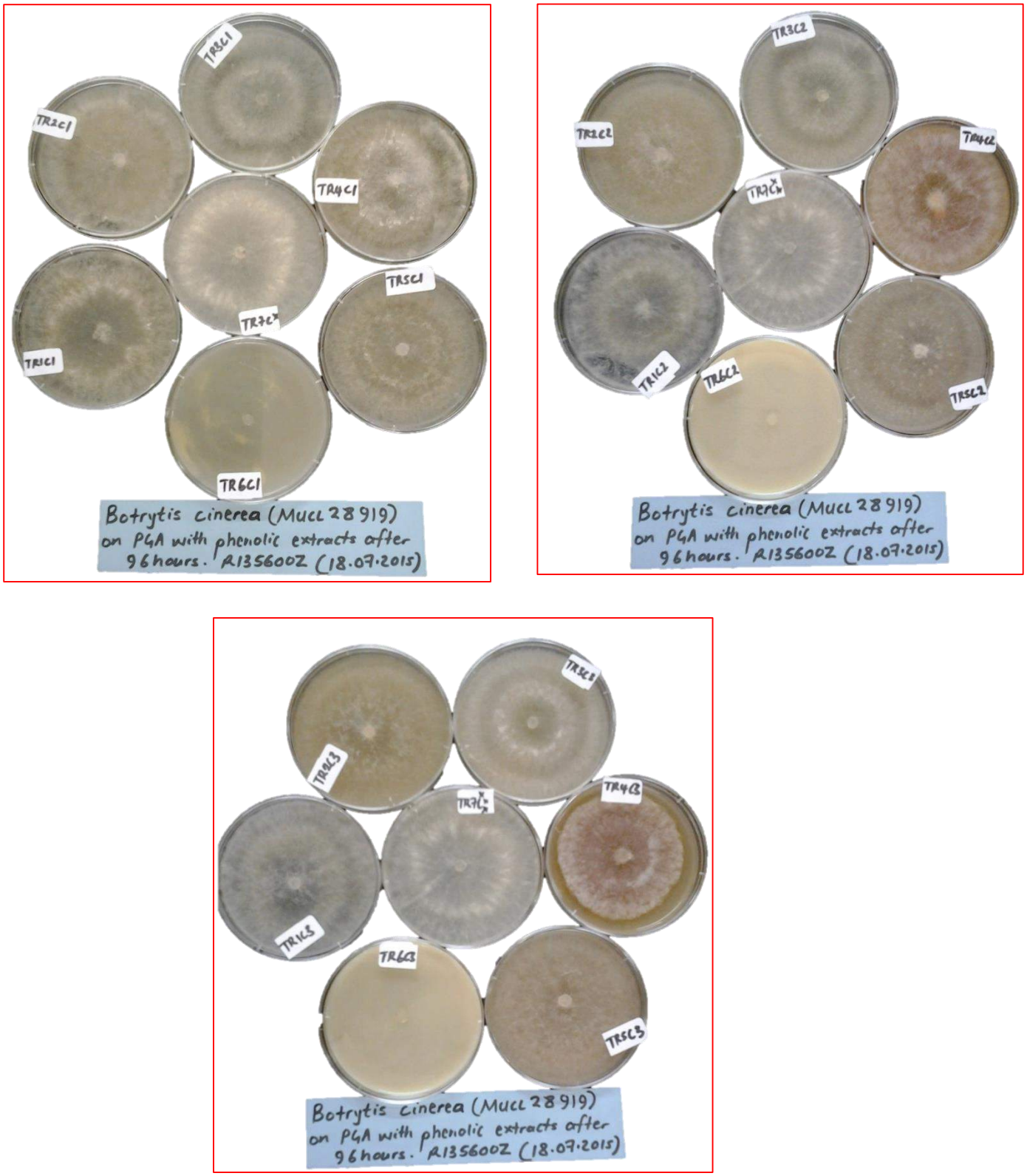


Figure 17.0 Representatives pictogramss showing antifungal activity of phenolic extracts on *Botrytis cinerea* at different concentration

5.4.5 Percentage Mycelial Reduction (PMR) for test fungi in vitro tests

The Table 4.0 shows Percentage Mycelial Reduction (PMR) for plant phenolic extract evaluation on the test fungi. At the end of the experiment, *M. phaseolina* was totally inhibited by Antracol 70WP at the 500ppm and 1000ppm concentrations only, whilst the 250ppm showed a 58.1% inhibition. *C. papaya bark* and *Z. officinale* showed more than 30% but less than 40% inhibition of *M. phaseolina* at 1000ppm. The rest of phenolic extracts showed less than 10% mycelial reduction, save for *C. papaya* leaves (26.8% at 500ppm and 14.7% at 1000ppm), *I. batatas* 16.4% at 1000ppm. No treatment totally inhibited *A. alternata;* however percentage mycelial reduction was highest on Copper Oxychloride 89%, 75.5% and 67.3% for the three concentrations. *Z. officinale* mycelial percentage reduction ranged from 45.3-54.7%. *M. flabellifolius* mycelial percentage reduction ranged from 40.4-50.8%. *C. papaya* bark mycelial percentage reduction ranged from 35.9-46.1%. *C. papaya* leaves mycelial percentage reduction ranged from 27.3-35.3%, and *I. batatas* mycelial percentage reduction ranged from 23.3-33.5%. Mancozeb 80WP exhibited a 100% mycelial inhibition of *Rhizoctonia solani* at all concentrations. *I. batatas* mycelial percentage reduction ranged from 29.6-54.7%. *M. flabellifolius* mycelial percentage reduction ranged from 26.5-53.9%. *Z. officinale* mycelial percentage reduction ranged from 36.5-48.2%. *C. papaya* bark mycelial % reduction ranged from 10.8-33.5%. *C. papaya leaves* mycelial % reduction ranged from 11.4-43.7%. *Botrytis cinerea* was totally inhibited (100%) by Chemlaxyl 72 WP at all concentrations. *M. flabellifolius* antifungal activity ranged from 0-5.9%. Other phenolic extracts (*C. papaya* Bark, Leaves, *Z. officinale* and *I. batatas* showed no antifungal activity against *B. cinerea* as they exhibited 0% mycelial reduction.

Table 4.0 Showing Percentage Mycelial Reduction (PMR) of plant phenolic extracts

Colony Diameter (D) in mm and Percentage Mycelial Inhibition (PMR) (%) by Phenolic Extracts Treatments at Different Concentrations

		M. phaseolina				*A. alternaria*				*R. Solani*				*B. Cinerea*			
		24 Hrs		72 Hrs		48 Hrs		168 Hrs		24 Hrs		96 Hrs		24 Hrs		96 Hrs	
	Conc.	D	%	D	%	D	%	D	%	D	%	D	%	D	%	D	%
Carica papaya Bark (TR1)	250ppm	24	34.7	56	33.5	23	4.9	55	35.9	9	41.1	76	10.8	11	8.3	85	0.0
Carica papaya Bark (TR1)	500ppm	23	36.6	59	30.2	22	10.4	50	41.2	9	38.9	68	19.8	14	-12.5	85	0.0
Carica papaya Bark (TR1)	1000ppm	21	43.1	52	37.9	18	26.4	46	46.1	9	42.2	57	33.5	14	-15.3	85	0.0
Carica papaya Leaves (TR2)	250ppm	35	3.7	81	3.8	23	6.3	55	35.3	12	22.2	75	11.4	26	-118.1	85	0.0
Carica papaya Leaves (TR2)	500ppm	22	38.0	62	26.8	26	-9.0	62	27.3	9	41.1	54	37.1	24	-98.6	85	0.0
Carica papaya Leaves (TR2)	1000ppm	22	38.9	72	14.7	24	0.0	59	30.2	11	25.6	48	43.7	23	-87.5	85	0.0
Zingiber officinale (TR3)	250ppm	25	29.6	63	25.2	17	30.6	46	45.5	11	27.8	54	36.5	12	0.0	85	0.0
Zingiber officinale (TR3)	500ppm	23	37.5	53	37.3	18	25.7	47	45.3	9	40.0	51	39.8	12	4.2	85	0.0
Zingiber officinale (TR3)	1000ppm	21	42.1	54	35.3	13	47.9	39	54.7	5	65.6	44	48.2	15	-22.2	85	0.0
Myrothamnus flabellifolius (TR4)	250ppm	32	10.2	81	3.2	18	26.4	51	40.4	11	27.8	63	26.5	11	6.9	85	0.0
Myrothamnus flabellifolius (TR4)	500ppm	33	7.4	82	2.2	14	41.7	49	42.9	10	33.3	48	43.3	12	4.2	85	0.0
Myrothamnus flabellifolius (TR4)	1000ppm	28	22.7	77	8.5	13	47.2	42	50.8	8	46.7	39	53.9	18	-45.8	80	5.9
Ipomoea batatas (TR5)	250ppm	23	35.6	79	6.0	22	6.9	65	23.3	12	21.1	60	29.6	27	-120.8	85	0.0
Ipomoea batatas (TR5)	500ppm	26	27.8	81	3.6	24	0.7	57	33.5	9	42.2	46	46.5	28	-131.9	85	0.0
Ipomoea batatas (TR5)	1000ppm	23	36.1	70	16.4	23	3.5	58	32.4	11	28.9	39	54.7	27	-123.6	85	0.0
Fungicide* (TR6)	250ppm	0	100.0	35	58.1	8	66.0	28	67.3	0	100.0	0	100.0	0	100.0	0	100.0
Fungicide* (TR6)	500ppm	0	100.0	0	100.0	0	100.0	21	75.5	0	100.0	0	100.0	0	100.0	0	100.0
Fungicide* (TR6)	1000ppm	0	100.0	0	100.0	0	100.0	9	89.0	0	100.0	0	100.0	0	100.0	0	100.0

*Fungicides: Antracol 70WP (*M. phaseolina*); Copper Oxychloride (*A. alternata*); Mancozeb 80WP (*R. solani*); Chemlaxyl 72WP (*B. cinerea*)

5.5 Discussions

5.5.1 *In vitro* antifungal activity and Percentage Mycelial Reduction of plant phenolic extracts on plant pathogenic fungi of economic importance

The results obtained show varied effects of the plant phenolic extracts depending on the plant pathogenic fungi, the type of plant pehnolic extracts and concentration thereof. This is expected due to the species differences in growth and development. *Zingiber officinale* rhizome, *Myrothamnus flabellifolius* and *Carica papaya* bark showed some moderate degree of antifungal properties. A research by Singh, *et al.*, (2011) implicated phenolic compounds in their antifungal activities against 6 plant pathogenic fungi showing increased fungal activity with increase in total phenolic content, which has somewhat been observed in the current study.

A study of phenolic compounds in a by-product obtained during olive oil extraction (olive pomace) against *A. solani*, *B. cinerea* and *F. culmorum* also observed an increase in antifungal activity with the increase in the phenolic content (Winkelhausen, *et al*., 2005). Some phenolic compounds (thymol, carvcrol, isoeugenol, eugenol, 2-ethylphenol, 4-ethylphenol, Salicylic acid) at 1000ppm managed to totally inhibit some filamentous fungi of agricultural importance (*F. oxysporum, F. verticillioides, A. flavus, A. fumigatus*) in vitro studies investigating 21 pure phenolic compounds antifungal activity against six fungi (Zabka and Pavela, 2013). The minimal inhibitory concentrations (MIC_{50} *and* MIC_{100}) were also done for the 21 phenolic compounds and they showed different efficacies. Such studies help isolate effects of phenolic compounds although in nature the phenolic compounds occur in minute concentrations and as a mixture, for example an HPLC-DAD analysis detected a mixture of phenolics (chlorogenic acid, caffeic acid, ferulic acid, ρ-coumaric acidphloridzin, epicatechin, rutin, catechin, quercetin-3-rhamnoside and phloretin) in apple tree leaves (Petkovšek, *et al*., 2008).

Tannins were present in *Ipomoea batatas*, *Myrothamnus flabellifolius* and *Carica papaya* leaves. Tannins are known to bind fungal proteins which aid in their antifungal activity Mazid, *et al*., (2011), also known to cause membrane disruption, enzyme inhibition, substrate deprivation among others effects in their antimicrobial activity (Tiwari, *et al*., 2011). Perhaps the tannins present in the current research plant extract can to some extent explain their antifungal activity. The TPC and qualitative phytochemical studies indeed show the presence of phenolic compounds in the plant extracts however, it was not possible through the HPLC method used for analysis to detect any of the selected standards despite their detection in other experiments (Canini, *et al*., 2007; Xu, 2012; Luo, *et al*., 2013; Sani, *et al*., 2014; Gogna, *et al*., 2015; Pawar, *et al*., 2015).

Caffeic acid has been for example held responsible for its antifungal effects it has on the sugarcane smut fungi (*Sporisorium scitamineum)* Santiago, et al., (2010) and probably this attribute may have played a similar role in the observed *Ipomoea batatas* antifungal activity. Gas Chromatography-Mass Spectrometry analysis of *Carica papaya* leaf extracts reported the presence of eight phenolic compounds (protocatechuic acid, ρ-coumaric acid, 5,7-Dimethixycoumarin, caffeic acid, kaempferol, quercetin and chlorogenic acid (Canini, *et al*., 2007). Previous research has shown that there is a correlation between disease resistance in plant and accumulation of phenolic compounds (Chérif, *et al*., 2007; Chavan and Kamble, 2014), perhaps *Ipomoea batatas* leaves are able to resist many fungal attack due to their phenolic content constituents.

In the present study, the antifungal activity of Antracol 70WP at increased dosage proved effective with the 250ppm achieving 58.1% percentage mycelial reduction whilst both 500ppm and 1000ppm achieved 100% mycelial growth inhibition compared to the previous studies where

50ppm, 100ppm and 200ppm achieved 0%, 11.1% and 27.8% respectively percentage mycelial reduction (Iqbal, et al., 2004).The 1000ppm antifungal activities of *Carica papaya bark and Zingiber officinale* which showed an antifungal activity in the range 30-40% could compare well with the 200ppm of Antracol 70WP used by Iqbal, *et al*., (2004) in their studies. In the current studies of Copper Oxychloride 85WP 250ppm, 500ppm and 1000ppm had 67.3%, 75.5% and 89% mycelial reduction of *Alternaria alternata* over the negative control treatment, this varied with studies done by Sharma , et al., (2013) where they used 0.2% (2000ppm) of Copper Oxychloride (50% WP) using the poisoned-food technique and achieved 81.4% mycelial inhibition. Another study by Gohel and Solanky, (2012) used Copper Oxychloride (50 % WP) in 1000ppm, 1500ppm and 2000ppm and achieved 100% mycelial inhibition at all concentrations. Differences could probably be accounted due largely different methodologies in incorporating fungicide into the growth media.

Botrytis cinerea was totally inhibited (100%) by Chemlaxyl 72 WP at all the three concentrations. Chemlaxyl 72 WP is a combination of Mancozeb (64%) and Metalaxyl (8%) and Agromil 72 WP has similar composition. In some studies, 500ppm (61.35%), 1000ppm (67.85%), 1500ppm (75.46%) and 2000ppm (84.42%), mycelial percentage reduction in parentheses were achieved through use of Agromil 72 WP (Hosen, et al., 2010). Differences may be due to technique in incorporating fungicide into growth media or the strain of *Botrytis cinerea.* Mancozeb 80WP successfully inhibited *Rhizoctonia solani* growth at all concentrations. Overall, in the current research, fungicides showed different antifungal activity against a particular plant pathogen with most being effective in 100% mycelial reduction at relatively higher concentrations. Although some plant phenolic extracts showed noteworthy antifungal activity, none was 100% effective against all the plant pathogens.

5.6 Conclusion and Recommendations

5.6.1 Conclusion

The present study based on total phenolic content and phytochemical tests, managed to show the presence of phenolic compounds and their activity to some degree, increasing with increasing content. Phytopathogenic fungi of economic importance are indeed affected by plant extracts containing phenolic compounds to different degrees depending on phenolic quantity and sensitivity of the pathogen. Percentage Mycelial Reduction (PMR) varied with concentration, phenolic extract and phytopathogenic fungi. Generally, positive control treatments (fungicides) showed higher PMRs compared to phenolic extracts. *Carica papaya* bark and *Z. officinale* rhizomes showed the highest PMR against *M. phaseolina* (30%≤PMR≤40%) at the 1000ppm concentration. For *A. alternaria*, *Z. officinale* showed the highest PMR (45.3%≤PMR≤ 54.7%) for all the concentrations. For *R.* solani, *M. flabellifolius* showed the highest PMR (26.5%≤PMR≤53.9%) for all the three concentrations. For *B. cinerra*, only *M. flabellifolius* had limited PMR of 0-5.9% for all concentrations whilst all other plant phenolic extracts showed a 0% PMR. The plant phenolic extracts have a potential use as antifungal phytochemicals.

5.6.2 Recommendations

The study of plant phenolic compounds presents a very huge opportunity of further studies particularly their isolation and characterization to single out the effects caused by a particular compound and/or combination of the compounds. There is need for a collaborative effort by both agriculturalists and chemists in coming up with novel methods of extraction of bioactive compounds. Whilst some of the plant phenolic extracts showed interesting antifungal activity *in vitro* tests, it would be imperative to do *in vivo* tests and screen for the most effective concentration. There is a notable opportunity for reduced dosages of fungicide use through a

combination of the plant phenolic extracts and commercial fungicides. This may help lower the environmental burden of pesticide application.

The extractions methods used are still beyond the reach of the proletariat farmers and use of toxic substance such as methanol still brings the vicious cycle of the toxic burden to the environment, therefore there is need for exploration for more ‘safer’ or ‘green chemistry’ and environmentally friendly extraction techniques. Lastly but not least, plant pathogenic fungi of economic importance occur in nature, therefore there is need for a deliberate profiling of plants with antimicrobial activityfocus in the very nature to provide solutions against warding off fungal pests.

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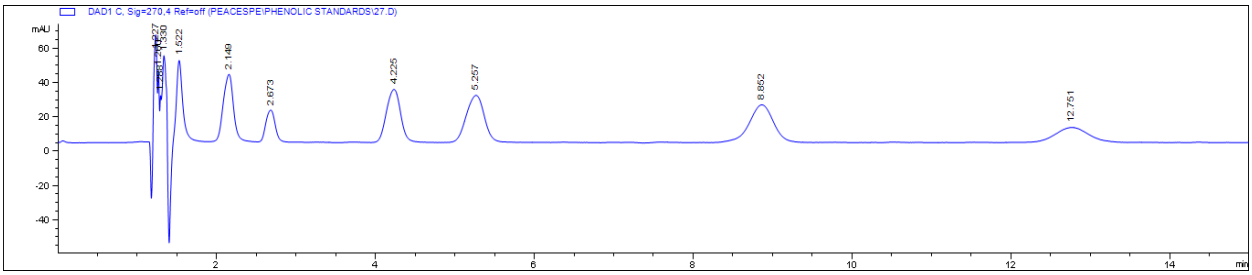
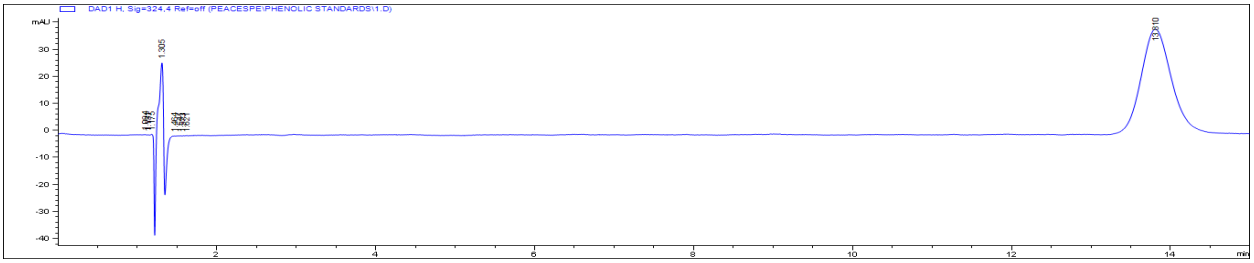
APPENDICES

Appendix I: Representative Phenolic Standards Chromatograms

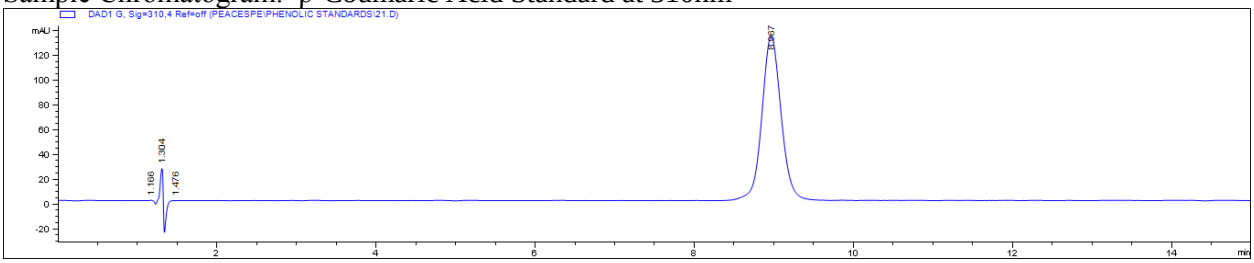
Sample Chromatogram Standard Mixture Phenolic Compounds at 270nm



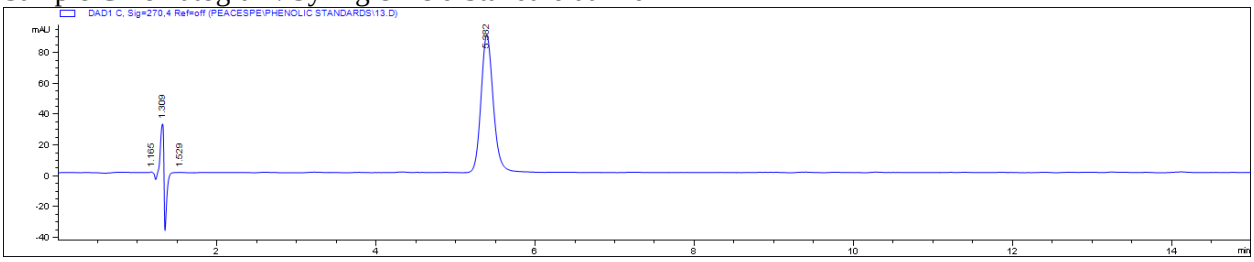
Sample Chromatogram: Ferulic Acid Standard at 324nm



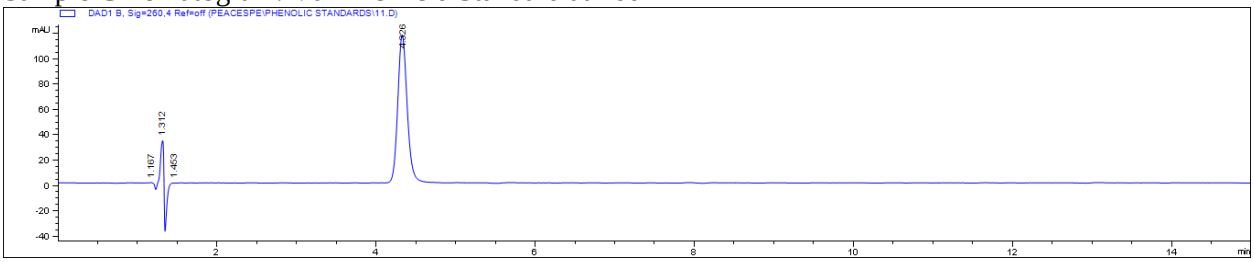
Sample Chromatogram: ρ-Coumaric Acid Standard at 310nm



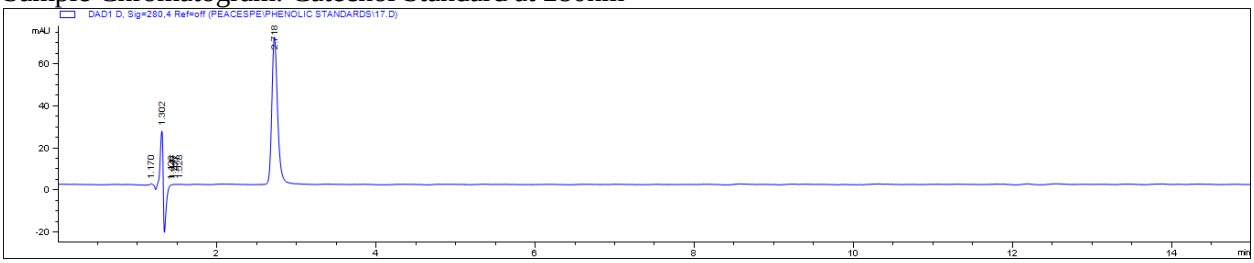
Sample Chromatogram: Syringic Acid Standard at 270nm



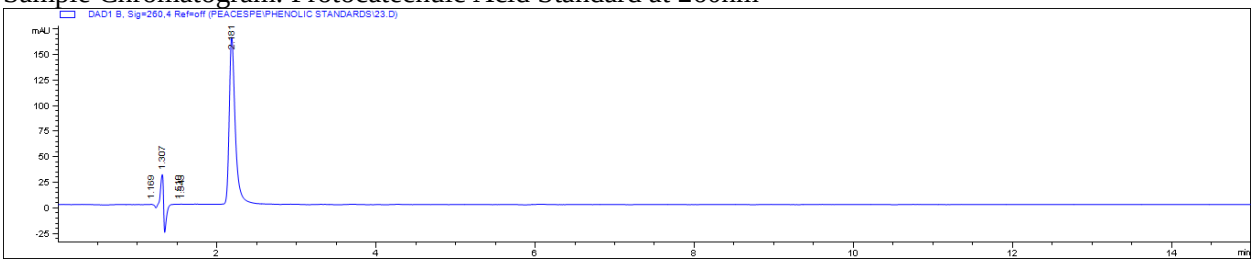
Sample Chromatogram: Vanillic Acid Standard at 260nm



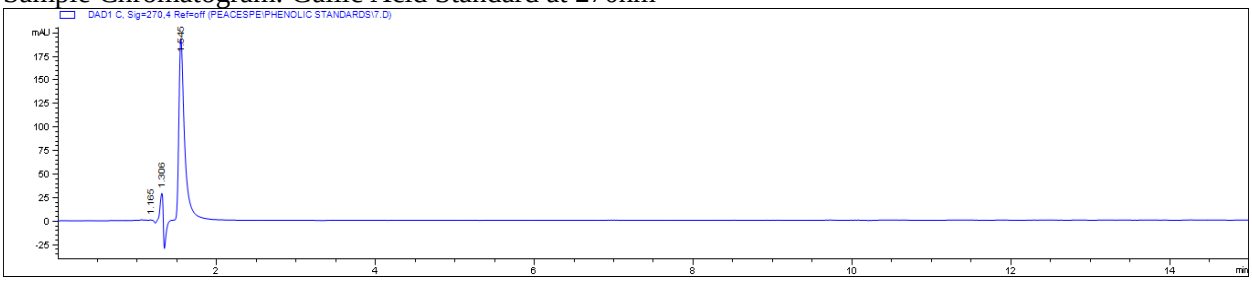
Sample Chromatogram: Catechol Standard at 280nm



Sample Chromatogram: Protocatechuic Acid Standard at 260nm



Sample Chromatogram: Gallic Acid Standard at 270nm



Appendix II: Analysis of Variance (ANOVA) GenStat14 Outputs

Analysis of variance (*Macrophomina phaseolina* at 24hrs)

Variate: CDmm_24hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	6979.603	1163.267	248.22	<.001
Concentration	2	123.437	61.718	13.17	<.001
Treatment.Concentration	12	292.897	24.408	5.21	<.001
Residual	42	196.833	4.687		
Total	62	7592.770			

Analysis of variance (*Macrophomina phaseolina* at 48hrs)

Variate: CDmm_48hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	29322.63	4887.11	118.77	<.001
Concentration	2	860.17	430.08	10.45	<.001
Treatment.Concentration	12	1062.89	88.57	2.15	0.034
Residual	42	1728.17	41.15		
Total	62	32973.86			

Analysis of variance (Macrophomina phaseolina at 72hrs)

Variate: CDmm_72hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	33560.06	5593.34	191.31	<.001
Concentration	2	1210.75	605.37	20.71	<.001
Treatment.Concentration	12	2312.25	192.69	6.59	<.001
Residual	42	1227.96	29.24		
Total	62	38311.02			

Analysis of variance (*Alternaria alternata* at 48hrs)

Variate: CDmm_48hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	84.12595	14.02099	148.39	<.001
Concentration	2	2.87114	1.43557	15.19	<.001
Treatment.Concentration	12	9.24358	0.77030	8.15	<.001
Residual	42	3.96842	0.09449		
Total	62	100.20908			

Analysis of variance (*Alternaria alternata* at 96hrs)

Variate: CDmm_96hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	7101.270	1183.545	141.02	<.001
Concentration	2	37.294	18.647	2.22	0.121
Treatment.Concentration	12	639.206	53.267	6.35	<.001
Residual	42	352.500	8.393		
Total	62	8130.270			

Analysis of variance (*Alternaria alternata* at 144hrs)

Variate: CDmm_144hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	13098.604	2183.101	228.02	<.001
Concentration	2	249.743	124.872	13.04	<.001
Treatment.Concentration	12	552.370	46.031	4.81	<.001
Residual	42	402.107	9.574		
Total	62	14302.824			

Analysis of variance (*Alternaria alternata* at 168hrs)

Variate: CDmm_168hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	21081.992	3513.665	381.66	<.001
Concentration	2	495.389	247.694	26.90	<.001
Treatment.Concentration	12	600.056	50.005	5.43	<.001
Residual	42	386.667	9.206		
Total	62	22564.103			

Analysis of variance (*Rhizoctonia solani* at 24hrs)

Variate: CDmm_24hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	57.59019	9.59836	150.81	<.001
Concentration	2	0.86281	0.43141	6.78	0.003
Treatment.Concentration	12	1.86864	0.15572	2.45	0.016
Residual	42	2.67307	0.06364		
Total	62	62.99471			

Analysis of variance (*Rhizoctonia solani* at 48hrs)

Variate: CDmm_48hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	161.44443	26.90741	538.51	<.001
Concentration	2	0.62620	0.31310	6.27	0.004
Treatment.Concentration	12	11.59601	0.96633	19.34	<.001
Residual	42	2.09859	0.04997		
Total	62	175.76523			

Analysis of variance (*Rhizoctonia solani* at 72hrs)

Variate: CDmm_72hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	286.51390	47.75232	553.25	<.001
Concentration	2	1.11251	0.55626	6.44	0.004
Treatment.Concentration	12	11.93686	0.99474	11.52	<.001
Residual	42	3.62513	0.08631		
Total	62	303.18841			

Analysis of variance (*Rhizoctonia solani* at 96hrs)

Variate: CDmm_96hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	412.91660	68.81943	910.47	<.001
Concentration	2	9.87122	4.93561	65.30	<.001
Treatment.Concentration	12	5.74982	0.47915	6.34	<.001
Residual	42	3.17463	0.07559		
Total	62	431.71226			

Analysis of variance (*Botrytis cinerea* at 24hrs)

Variate: CDmm_24hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	116.28629	19.38105	371.85	<.001
Concentration	2	0.43209	0.21605	4.15	0.023
Treatment.Concentration	12	1.70493	0.14208	2.73	0.008
Residual	42	2.18905	0.05212		
Total	62	120.61236			

Analysis of variance(*Botrytis cinerea* at 48hrs)

Variate: CDmm_48hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	288.23696	48.03949	1477.28	<.001
Concentration	2	0.17750	0.08875	2.73	0.077
Treatment.Concentration	12	8.60806	0.71734	22.06	<.001
Residual	42	1.36579	0.03252		
Total	62	298.38831			

Analysis of variance (*Botrytis cinerea* at 72hrs)

Variate: CDmm_72hrs_Transform

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	451.42130	75.23688	1928.56	<.001
Concentration	2	0.62524	0.31262	8.01	0.001
Treatment.Concentration	12	1.35572	0.11298	2.90	0.005
Residual	42	1.63851	0.03901		
Total	62	455.04076			

Analysis of variance(*Botrytis cinerea* at 96hrs)

Variate: CDmm_96hrs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	55392.857	9232.143	2585.00	<.001
Concentration	2	7.143	3.571	1.00	0.376
Treatment.Concentration	12	42.857	3.571	1.00	0.466
Residual	42	150.000	3.571		
Total	62	55592.857			

Analysis of variance (Total Phenolic Content of Plant Extracts)

Variate: mg_GAE_g_transform2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant_Phenolic_Extracts	4	91.4497	22.8624	139.32	<.001
Residual	10	1.6410	0.1641		
Total	14	93.0906			

Appendix III: Turnitin Originality Report

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- Percentage: %

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Document Viewer

Turnitin Originality Report

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- ID: 592579005
- Word Count: 33740
- Submitted: 1

Analysis of phenolic compounds in Carica papa... ***By Phumelela Mwelasi***

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Appendix IV: Basic Information Relative Test Fungi Pure Cultures

BASIC INFORMATION RELATIVE TO THE BIOLOGICAL MATERIAL

Scientific name:	***Alternaria alternata (Fries : Fries) von Keissler***		
MUCL number:	MUCL 042320		
Country of origin:	South Africa		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	25°C		
	potato carrot extract agar (PCA)		
	potatoe dextrose agar (PDA)		

Scientific name:	***Alternaria solani (Ellis & Martin) Sorauer***		
MUCL number:	MUCL 028928		
Country of origin:	United States		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	20°C		
	malt agar 2% (MA2)		
	potatoe dextrose agar (PDA)		

Scientific name:	***Botrytis cinerea***		
MUCL number:	MUCL 028919		
Country of origin:	Belgium		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	20°C		
	dextrose 1% yeast extract asparagine agar (DYAA)		
	potato carrot extract agar (PCA)		
	potatoe dextrose agar (PDA)		

Scientific name:	***Cercospora beticola Saccardo***		
MUCL number:	MUCL 016495		
Country of origin:	Netherlands		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	20°C		
	dextrose 1% yeast extract asparagine agar (DYAA)		
	potatoe dextrose agar (PDA)		

Scientific name:	***Macrophomina phaseolina (Tassi) Goidanich***		
MUCL number:	MUCL 053604		
Country of origin:	Argentina		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	25°C		
	potatoe dextrose agar (PDA)		

Scientific name:	***Rhizoctonia solani Kühn***		
MUCL number:	MUCL 049235		
Country of origin:			
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	25°C		
	potatoe dextrose agar (PDA)		

Scientific name:	***Athelia rolfsii (Curzi) Tu & Kimbrough***		
MUCL number:	MUCL 042861		
Country of origin:	Morocco		
Results of the outgoing controls:	Viability:	OK	27/04/2015
	Purity:	OK	27/04/2015
	Identity:	OK	30/04/2015
Recommended culture conditions:	25°C		
	potatoe dextrose agar (PDA)		

Appendix V: Media Recipes for Fungi Culture

MEDIA RECIPES

dextrose 1% yeast extract asparagine agar (DYAA)	dextrose - 10.0 g yeast extract - 1.0 g L-asparagine - 0.5 g K2HPO4.3H2O - 0.5 g MgSO4.7H2O - 0.25 g FeCl3 (10% solution) - 0.5 ml agar - 20 g water - 1 L
malt 2% yeast extract agar (MYA2)	malt extract - 20.0 g yeast extract - 1.0 g agar - 20 g water - 1 L
malt agar 2% (MA2)	malt extract - 20.0 g agar - 15 g water - 1 L
oat meal agar (OA)	rolled-oats - 10.0 g glycerine - 5.0 ml lactic acid - 0.2 ml agar - 12 g water - 1 L
potato carrot extract agar (PCA)	potatoes (unpeeled, sliced) - 300 g carrots (peeled, sliced) - 25 g agar - 15 g water - 1 L
potatoe dextrose agar (PDA)	potatoes (peeled, sliced) - 200 g dextrose - 20.0 g agar - 20 g

Appendix VI: Belgian Coordinated Collections of Microorganisms letter

BELGIAN CO-ORDINATED COLLECTIONS OF MICRO-ORGANISMS

BCCM · MUCL

GREAT AT SMALL THINGS

Dr David Tanyala Takuwa
University of Botswana
Chemistry Department
Private Bag 00704
Gaborone
Botswana

Louvain-la-Neuve, 04 May 2015

Your ref.: -
Our ref.: 2015-0874
Customer nbr: 135617

Dear customer,

Please find enclosed the basic information relative to the biological material ordered. Prior to sending, the viability, purity and identity of this biological material have been verified according to specific criteria established in the BCCM/MUCL quality management system. We suggest you to subculture the biological material as soon as possible after reception, referring to the modalities recommended by BCCM/MUCL and as listed on the following pages.

BCCM/MUCL has always used the most adequate techniques for long-term preservation of its biological material, with the aim of minimizing the risk of degeneration or loss of the biological material intrinsic properties. Therefore, BCCM/MUCL cannot take further responsibility for the possible degeneration or loss of any properties expected from the biological material provided.

Any claim on the biological material should be submitted within a period of thirty days after sending. We wish to remind you also that payment should be effected at maturity.

For additional information on the biological material provided, do not hesitate to contact our secretary at the address mentioned below.

We wish to thank you for your confidence in BCCM/MUCL.

Yours sincerely,

Dr C. Decock,

Curator

BCCM DCG
BCCM IHEM
BCCM ITM
BCCM LMBP
BCCM LMG
BCCM ULC
BCCM CC

Université catholique de Louvain
Earth and Life Institute (ELI) - Applied Microbiology (ELIM) - Laboratory of Mycology
Croix du Sud 2 box L7.05.06 - B-1348 Louvain-la-Neuve - Belgium
T +32 (0)10 47 37 42 - bccm-mucl@uclouvain.be

UCL Université catholique de Louvain

Belgian Science Policy Office .be

Please find enclosed the following BCCM strains:

MUCL number	Scientific name	Batch number	Qty	Form supplied
MUCL 016495	*Cercospora beticola Saccardo*	FRT-2000-2201	1	Active
MUCL 028919	*Botrytis cinerea*	FRT-1997-3386	1	Active
MUCL 028928	*Alternaria solani (Ellis & Martin) Sorauer*	OIL-2015-0139	1	Active
MUCL 042320	*Alternaria alternata (Fries : Fries) von Keissler*	FRT-1999-1584	1	Active
MUCL 042861	*Athelia rolfsii (Curzi) Tu & Kimbrough*	FRT-2000-1633	1	Active
MUCL 049235	*Rhizoctonia solani Kühn*	FRT-2013-0670	1	Active
MUCL 053604	*Macrophomina phaseolina (Tassi) Goidanich*	FRT-2011-0337	1	Active

Appendix VII: Import Permit for Plant Pathogenic Fungi

REPUBLIC OF BOTSWANA

Plant Diseases and Pests Act, CAP 35:02

Permit No.: 09909/15

PERMIT AUTHORIZING THE IMPORTATION OF PLANTS

Permission is granted to (Address of Importer) **University of Botswana**

Private bag 0074 Gaborone, Botswana

to import in single consignment (s), within **30** days of the date of this permit, by post/rail/road **Air**

from (address of consignor) **Belgium**

through (Port of Entry) **SSKA**

the following

20 g of each of the following

MUCL 42320	*Alternaria alternata*
MUCL 28928	*Alternaria solani*
MUCL 28919	*Botrytis cinerea*
MUCL 16495	*Cercospora beticola*
MUCL 53604	*Macrophomina phaseolina*
MUCL 49235	*Rhizoctonia solani*
MUCL 42861	*Sclerotium rolfsii*

Subject to the following conditions

Each consignment must be accompanied by an original Phytosanitary certificate with additional declaration that it is free from contaminants especially the hosts from the source country. The material safety sheet must accompany the consignment. The consignment must be sealed in properly labeled containers indicating the name and country of origin.

CHIEF PLANT PROTECTION OFFICER 2015 18 PRIVATE BAG 0091 GABORONE BOTSWANA

N.B.: ***This permit does not exempt the holder from the provisions of any other Act or Statutory Instrument***

Date: 18 May 2015

(This Permit must accompany the consignment above)

CHIEF PLANT PROTECTION OFFICER

Enquiries: Telephone No: (+) 267 3928745/6 **Fascimile: (+) 267 3928768**

Appendix VIII: Letter of Co-supervision at the Department of Chemistry (UB)

MIDLANDS STATE UNIVERSITY

P. Bag 9055
Gweru
Zimbabwe

Tel: (263) 054 260404/260464 Ext. 344
Fax: (263) 054 260233
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smuzemus@gmail.com
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FACULTY OF NATURAL RESOURCES MANAGEMENT AND AGRICULTURE
Department of Horticulture

20 November 2014

MIDLANDS STATE UNIVERSITY
NATURAL RESOURCES MANAGEMENT
HORTICULTURE
20 NOV 2014
PRIVATE BAG 9055,GWERU
ZIMBABWE TEL/FAX: 054 - 260233

Dr. R. Mapitse (H.O.D Chemistry Department)
C/O Dr. D.T. Takuwa (Analytical Chemistry)
University of Bostwana
4775 Notwane Road
Gaborone, Botswana
Tel: +267 3552 491

Dear Sir/Madam

RE: LETTER OF INTRODUCTION OF MSC CROP PROTECTION STUDENT AND COLLABORATION WITH UNIVERSITY OF BOTSWANA UNDER THE SUPERVISION OF DR. D.T. TAKUWA (ANALYTICAL CHEMISTRY)

This letter serves to introduce to you Phumelela Peace Mwelasi, R135600Z who is a student in the department of Horticulture at the Midlands State University. To fulfill the requirements for MSc Crop Protection, the student is expected to conduct and submit a research dissertation.

We call upon your organization to consider being involved in the research and practical development of the above student. Due to the nature and depth of their scientific research, our students may require the assistance from institutions such as your own. We would much appreciate whatever support and assistance you can offer this student.

We are also glad that through the communications with this student, Dr. D.T. Takuwa under your department has shown interest in the proposed research by our student and upon discussion and assessment of the student's needs we would like to accept Dr. D.T. Takuwa as an additional co-supervisor . This student has currently two supervisors, namely, Ms. R.M. Mudyiwa and Dr. E. Ngadze in the speciality of plant pathology.

We also acknowledge and note that such collaboration would follow all academic procedures and any publications that might be an output through such efforts. Morever, we thank you for the extension of future collaboration with our department, faculty staff and/or students.

S. Muzemu (Mr)
(Acting Chairperson - Department of Horticulture)

Appendix IX: Letter of Co-supervision at the Department of Biological Sciences (UB

MIDLANDS STATE UNIVERSITY

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FACULTY OF NATURAL RESOURCES MANAGEMENT AND AGRICULTURE
Department of Horticulture

18 August 2015

Professor M. Setshogo (H.O.D Department of Biological Sciences)
C/O Professor K.B. Khare (DOBS-Mycology)
University of Bostwana
4775 Notwane Road
Gaborone, Botswana
Tel: +267 355 4465

MIDLANDS STATE UNIVERSITY
NATURAL RESOURCES MANAGEMENT
HORTICULTURE
18 AUG 2015
PRIVATE BAG 9055,GWERU
ZIMBABWE TEL/FAX: 054 - 260233

Dear Sir/Madam

RE: LETTER OF INTRODUCTION OF MSc. PROTECTION STUDENT AND COLLABORATION WITH UNIVERSITY OF BOTSWANA UNDER THE SUPERVISION OF PROFESSOR K.B. KHARE (DOBS-MYCOLOGY)

This letter serves to introduce to you Mr Phumelela Peace Mwelasi, R135600Z who is a student at the Midlands State University, Zimbabwe. To fulfil requirements of MSc. Crop Protection, which is under the Department of Horticulture in the Faculty of Natural Resources Management and Agriculture, the student is expected to submit an original research based thesis.

We call upon your organization to consider being involved in the research and practical development of the above student. Due to the nature and depth of their scientific research, our students may require the assistance from institutions such as yours. We could much appreciate whatever support and assistance you can offer this student.

We are also glad that through the communications with this student, Dr. D.T. Takuwa (Department of Chemistry) who agreed to be one of the co-supervisors particularly on the chemistry requirements of his research. Upon discussion and assessment of the student's needs, we would like to also accept Professor K.B. Khare as an additional co-supervisor particularly looking at the mycology-biological component of the student's research. This student has other two supervisors from Zimbabwe, namely, Ms. R.M. Mudyiwa and Dr. E. Ngadze in the specialty of plant pathology.

We also acknowledge and note that such collaboration would follow all academic procedures and any publications that might be an output through such efforts. Moreover, we thank you for the extension of future collaboration with our department, faculty staff and/or students.

S. Muzemu (Mr)
(Acting Chairperson - Department of Horticulture)