EFFICACY OF *Leonotis nepetifolia* L. AND *Ocimum gratissimum* L. EXTRACTS IN CONTROLLING ASCOCHYTA BLIGHT (*Phoma exigua)* OF FRENCH BEAN (*Phaseolus vulgaris* L.)

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been previously presented in this University or any other institution for the award of any degree.

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RECOMMENDATION

This thesis has been submitted with our approval as supervisors according to Egerton University regulations.

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DEDICATION

This thesis is dedicated to my mother F. Ochola and late father, Daniel Ochola. Special dedication to Trevor Ivan and Liam Austin.

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ABSTRACT

French bean (*Phaseolus vulgaris* L.) is an important export vegetable crop that faces serious production challenge due to Ascochyta blight (*Phoma exigua)*. The objectives of this study were to determine the; (i) antifungal activities of methanol extract of *Leonotis nepetifolia* and *Ocimum gratissimum* against *Phoma exigua in vitro*, (ii) fungicidal effects of extracts of the two test plants on the incidence and severity of Ascochyta blight, and yield of French bean. Methanol extracts of composite leaves and tender stems of each test plant were diluted to obtain final concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.31, and 0.16 mg ml^{-1} (w/v) for use in antifungal tests. The pathogen was isolated from infected French bean pods and used for inoculation in greenhouse and field experiments. Laboratory bioassays were conducted using Kirby-Bauer disc diffusion method in a completely randomised design in triplicate. The diameters of zones of inhibition and the minimum inhibitory concentrations of the methanol extracts against the pathogen were determined. The *in vivo* experiments were conducted at Egerton University and Kenya Agricultural and Livestock Research Organization – Njoro fields. The experiments were laid in a 2 × 6 factorial arrangement of randomized complete block design with three replicates. Methanol extracts of *O. gratissimum* and *L. nepetifolia* at 20.0, 10.0, 5.0, 2.5 and 1.25 mg ml^{-1} were sprayed per plot at 14 days after emergence (DAE). The *in vivo* data were collected on disease incidence and severity, yield; and percent disease index (PDI) and disease severity index (DSI) determined per plot. The data were subjected to analysis of variance using Statistical Analysis System software and treatment means separated using Tukey's HSD test. Correlation analysis between the botanical rates, disease incidence and severity, and yield per plot was conducted to determine the effect of the test plants on yield of French bean. The extracts showed antifungal activity at all the concentrations tested with minimum inhibitory concentration (MIC) of 1.25 mg ml^{-1} for both *L. nepetifolia* and *O. gratissimum*. There were concentration-dependent antifungal activities of extracts of the test plants with average inhibition zones (at 40.0 mg ml^{-1}). *Ocimum gratissimum* and *L. nepetifolia* at 5.0 mg ml^{-1} resulted in 4.2 and 12.5% reduction of disease incidence, respectively. *Ocimum gratissimum* at 10.0 mg ml^{-1} and *L. nepetifolia* at 20 mg ml^{-1} caused 13.5 and 13.1% reduction in Ascochyta blight severity in the field. Use of methanol extracts of the two test plants resulted in a significant increase (between 42.6 – 91.4 and 49.8 – 95.7 % for *Ocimum gratissimum* and *Leonotis nepetifolia*, respectively) in yield of French bean per plot. These findings provide insight on the potential of the two plant extracts to control Ascochyta blight and to promote low risks, cheap bio-pesticides for the smallholder farmers.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CIAT	International Center for Tropical Agriculture
CRD	Completely Randomized Design
DAE	Days after emergence
DAI	Days after incubation
DMSO	Dimethyl sulphoxide
DSI	Disease Severity Index
EU	European Union
EurepGAP	European Good Agricultural Practices
GlobalGAP	Global Good Agricultural Practices
HAI	Hours after inoculation
HBI	Hour before inoculation
HCDA	Horticultural Crops Development Authority
IPM	Integrated Pest Management
KALRO	Kenya Agricultural and Livestock Research Organization
LN	*Leonotis nepetifolia*
MIC	Minimum Inhibitory Concentration
NaOCL	Sodium hypochlorite
OG	*Ocimum gratissimum*
PDA	Potato Dextrose Agar
PDI	Percent Disease Index
RCBD	Randomized Complete Block Design
SAS	Statistical Analysis System

CHAPTER ONE
INTRODUCTION

1.1 Background information

French bean (*Phaseolus vulgaris* L.) is an important export horticultural crop in Kenya. The crop is mainly grown in medium to high altitude areas of Central, Eastern, Rift Valley, Nyanza and Western Provinces in Kenya (Monda *et al.*, 2003). The French bean sub-sector benefits more than one million people in Kenya with the majority being women and youths (Lenne *et al.*, 2005). Smallholder farmers account for about 80% of the total French bean production, most of whom own less than 0.25 hectares of land under production (HCDA, 2010).

In the recent years, there has been a decline in French bean production due to problems caused by insect/mite pests, diseases (Monda *et al.*, 2003) and nematodes. French bean is prone to attack by multiple diseases caused by viruses, bacteria and fungi. For a long time now, the diseases that have been considered to be economically important in major French bean producing regions in East Africa have mainly been fungal diseases such as rusts (*Uromyces appendiculatus*), angular leaf spot (*Phaeoisariopsis griseola*), anthracnose (*Colletotrichum lindemuthianum)* among others (Lemessa *et al.*, 2005). However, Ascochyta blight caused by *Phoma exigua* var. *exigua* is emerging as one of the problematic diseases in French bean production. The pathogen can stay on the seed for over two years and can also spread from old plant tissues and diseased plants (French, 2006). The fungus can spread systemically throughout the plant and is also seed borne (Schwartz, 1989). The pathogen thrives under cool, humid temperature conditions (Hanson *et al.*, 1993). The earliest symptoms of the disease appear as black concentric lesions on the leaves that later spread to other parts of the plant, such as the stem and pods. When infected seeds are used for sowing and infection occurs early in growth cycle of susceptible cultivars, and under favourable climatic conditions for the survival of the pathogen, yield losses can be up to 100%. This is especially so when the pathogen attacks the stem of the plant resulting in girdling of the stem and eventual breaking and drying of the plant. Under favourable conditions when disease severity is high, the plant loses leaves and pods (Bardas *et al.*, 2008) resulting in a decrease in final yield. Symptoms of the disease on the pods may be confused with that of anthracnose. Transmission of the pathogen depends on the initial source of the inoculum; if the source is infected seeds especially farmers' own recycled seeds, the crop emerges when it has already been attacked by the pathogen.

However, most of the time the source of infection is the over-seasoning bean debris which is mechanically dispersed by water or rain droplets onto healthy susceptible bean cultivars.

Several measures have been suggested in controlling bean pathogens and managing the diseases. Seed treatments, use of certified seed and foliar sprays with various synthetic fungicides have been the norm in attempts to manage Ascochyta blight in beans (Singh and Reddy, 1990). Due to variability in the races of the pathogen, and continuous emergence of new races, cultivar resistance soon breaks down after deployment (Akem *et al.,* 2004). Thus alternative approaches to managing the pathogen have to be sought. Use of synthetic fungicides has been fronted as the first line among farmers in controlling fungal pathogens in plants including Ascochyta blight (Akem *et al.,* 2004). However, sustained control of diseases using synthetic and oil-derived pesticides has produced perverse effects such as mammalian toxicity and ecological hazards (Pinto *et al.,* 2010). In addition, extensive application and lack of adequate control in synthetic fungicide application has resulted in adverse effects in the ecosystem and human health. Most of the synthetic antimicrobials are not easily biodegradable into simpler forms and results in chemical residue in the food chain for longer duration causing toxicities and other adverse effects to different mammalian systems (Moosavy *et al.,* 2008). Moreover, synthetic chemicals contaminate the environment, results in pathogen resistance and are usually expensive to the local smallholder farmers. These risks have induced the search for alternative environmentally friendly and low-risk disease control methods (Okigbo and Ogbonnaya, 2006).

Secondary plant metabolites or bioactive compounds from plants with fungicidal properties are an option in the management of pathogens (Curtis *et al.*, 2004). Previous studies of several species from the Labiatae family have confirmed the fungicidal and insecticidal potency of the plants (Belmain *et al.,* 2001; Nashwa and Abo-Elyousr, 2012; Sohani *et al.,* 2012). The potential and action of botanical extracts from several plants in the Labiatae family to control plant pathogen has already been demonstrated (Ayanwuyi *et al.,* 2009; Pinto *et al.,* 2010). These plant metabolites or bioactive compounds are biodegradable to nontoxic products, have low impact on human health and can be incorporated to integrated pest and disease management programs (Bouda *et al.,* 2001). This shows the possibility of new control agents to be developed from the plants (Lee *et al.,* 2001; Key *et al.,* 2003). The chemical analysis and antimicrobial activities of *Ocimum gratissimum* and *Leonotis nepetifolia* has been widely studied *in vitro* (Ayanwuyi *et al.,* 2009; Pushpan *et al.,* 2012; Sobolewska *et al.,* 2012).

Ocimum gratissimum is an aromatic, perennial herb, with much branched erect stem and oppositely arranged leaves (Orwa *et al.*, 2009). It has antifungal potential on French beans against *Colletotrichum lindemuthianum* apart from its ability to suppress conidial growth on the host (Pinto *et al.*, 2010) due to the presence of eugenol that has antimicrobial, insecticidal and antihelminthic properties (Sadiq *et al.*, 2012). *Leonotis nepetifolia*, commonly known as Klip Dagga or Lion's Ear, is a frost hardy perennial herbaceous plant in the Lamiaceae family. Studies have shown that *Ocimum* sp. and *Leonotis* sp. have antimicrobial activity under *in vitro* conditions (Ayanwuyi *et al.*, 2009; Pinto *et al.*, 2010). Hence this study was conducted to determine the antifungal activity of these plants against Ascochyta blight of French bean.

1.2 Statement of the problem

There has been a decline in French bean production in Kenya due to the adverse effects of pests and diseases. Ascochyta blight has contributed to a significant reduction in French bean productivity. The disease attacks all parts of the crop, but of significant importance is its effect on the pods that result in deterioration in quality and quantity of French bean produce. This has resulted in low quality and yield especially in smallholder farmers' fields. Attempts to control the disease using synthetic fungicides has not only failed to yield appreciable results but has also resulted in negative impacts on the environment and human health. Moreover, the synthetic fungicides are expensive thus most smallholder farmers sometimes find it difficult to apply the recommended rates and end up using lower rates thus resulting in development of resistance by plant pathogens. This forces producers to either increase the dosage or frequency of application, a move that comes with increase in the cost of production. The unregulated use of the synthetic fungicides has also resulted in the rejection of tonnes of French bean exported to the European markets due to maximum residue limits (MRL). This is due to non-compliance with EurepGAP and GlobalGAP requirements concerning food safety, environmental protection, occupational health and safety and animal welfare. This therefore necessitates search for an alternative cheap approach that is environmentally friendly and acceptable by consumer to control the pathogen and the disease.

1.3 Objectives

1.3.1 Broad objective

To improve French bean production through application of *Leonotis nepetifolia* and *Ocimum gratissimum* extracts to control Ascochyta blight.

1.3.2 Specific objectives

To determine the;

1. Antifungal effects of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. extracts against *Phoma exigua in vitro*.
2. Effects of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. extracts on the incidence and severity of Ascochyta blight, and yield of French beans.

1.4 Hypotheses

1. *Leonotis nepetifolia* and *O. gratissimum* extracts have no antifungal effects against *Phoma exigua* var. *exigua in vitro.*
2. *Leonotis nepetifolia* and *O. gratissimum* extracts have no effect on incidence and severity of Ascochyta blight and yield of French bean.

1.5 Justification

French bean is an important export vegetable crop in Kenya, Tanzania, Uganda, Zambia, Zimbabwe and many countries in north of Africa. The crop accounts for 60% of all export vegetables and 21% of horticultural exports, thus significantly contributing to national revenue in Kenya. It is grown as a cash crop by both large and smallholder farmers, thus creating employment opportunities for the rural communities especially to the women and youth. It is estimated that more than one million people benefit from the sub-sector in Kenya. Emergence of Ascochyta blight as an important disease in farmers' fields has resulted in yield reduction especially in smallholder. Smallholder farmers bear the impact of the disease as they are not able to control the disease due to financial constraints of buying the synthetic fungicides.

These problems have necessitated research for alternative control measures for the pathogen or an integrated disease management (IPM) system for the disease. Various indigenous plant extracts have been tested and demonstrated to have the bio-control potential and are user and environmental friendly. Development of *L. nepetifolia* and *O. gratissimum* plant based bio-pesticide would therefore be a valuable additional tool for use in an IPM system

designed to control Ascochyta blight in French beans. Several publications have reported the use of *O. gratissimum* L. in controlling fungal diseases. However, the bio-control potential of *L. nepetifolia* has not been fully exploited, although indigenous knowledge about its use as a fungicide in its crude form is known among the smallholder farmers. The active ingredients in most botanical extracts have been reported to be volatile and non-toxic to mammals hence solving the problem of chemical residue limit set by the European markets. Therefore, there was need for further studies on the potential and refinement of the chemical extracts for the control of Ascochyta blight and increased productivity for the benefit of the smallholder farmers in Kenya. The use of botanical extracts will result in substantial reduction in the cost of production for the smallholder resource poor farmers and improve on their livelihood. Commercialization of the technology will result in economic development especially when it is adopted by majority of French bean farmers who are mainly smallholders.

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CHAPTER TWO

LITERATURE REVIEW

2.1 French bean production and economic importance

French bean (*Phaseolus vulgaris* L.) is an annual vegetative crop in the legume family with both dwarf and climbing growth characteristics (Arunga *et al.*, 2010). The stalked flower head is in the form of a short raceme that eventually results in the production of pods that vary in colour depending on the cultivar. The production of high value horticultural crops has been identified as a key pathway out of poverty and a prospect for national development (HCDA, 2010). French bean is an important export vegetable crop in Kenya, Tanzania, Uganda, Zambia, Zimbabwe and North Africa (Iruria *et al.*, 2002). It accounts for 60% of all export vegetables and 21% of horticultural exports in Kenya (HCDA, 2010), thus contributing 35 – 40% of foreign export exchange annually. The crop is grown as a cash crop by both large and smallholder farmers (CIAT, 2006) and thus creating employment opportunities for the rural communities especially to the women and youth. Despite the total area under production of French bean declining from 4798 Ha in 2011 to 4,128 Ha in 2012, the yields and value increased by 12 and 5% with the total production of 44,000 MT valued at Ksh 1.7 Billion in 2012 (HCDA, 2012). The leading counties producing French bean are Murang'a, Kirinyaga and Meru accounting for 43, 25 and 7% of the total production, respectively. French bean is primarily grown for export with a small quantity consumed in the domestic market. The farm gate prices for the product have stagnated over the years on an average of KSh. 40 per Kilogram and below (HCDA, 2012).

Global French bean production has been on the decline due to problems caused by insect/mite pests, diseases and excessive use of pesticides (HCDA, 2012). French bean is susceptible to several diseases that are caused by bacterial, viral and fungal pathogens. Ascochyta blight has emerged as one of the most important fungal diseases in French bean production. The pathogen attack the plant at different growth stages with yield losses of up to 100% having been recorded when infected seeds are used for sowing and the climatic conditions are favourable for the pathogen survival (Allen *et al.*, 1996).

In Kenya, French bean is mainly grown in medium to high altitude areas of Central, Eastern, Rift Valley, Nyanza and Western Provinces (Ndegwa *et al.*, 2010) for export. The horticultural industry in Kenya is credited as the fastest growing (≥20% p.a.) sub-sector in the last six years. However, it faces serious challenges due to zero tolerance to quarantine pests,

diseases and EurepGAP certification requirements for all exports to EU supermarkets (HCDA, 2010). Since the crop is grown mainly by smallholder (80%) and medium-scale farmers (Minot and Ngigi, 2003), who own less than 0.25 Ha, the burden of the stringent regulation set by the European market fall squarely on them. Imposition of ban on French bean export due to non-compliance with the export market regulations would have a negative impact on an estimated more than one million people who benefit from the sub-sector (Lemessa *et al.*, 2011) with the majority being women and youths.

2.2 Constraints to French bean production

French bean growers experience many production constraints such as marketing, fluctuation in prices, rejection of beans by middlemen, high cost of seed for production and insect and disease problems (Ndegwa *et al.*, 2010). Major pests that hinder French bean production are whiteflies (*Bemicia tabaci*), aphids (*Aphis fabae)*, Red spider mites (*Tetranychus* spp.), bean stem maggot (*Ophiomyia* spp.), thrips (*Frankliniella* spp.), African bollworm (*Helicoverpa armigera*) among other common field pests (Ndegwa *et al.*, 2010). The pest situation is worse in most production areas as the farmers grow French bean during the short rain season, when there is high incidence of crop pests and thus high severity of damage. For quite a long time now, the most prevalent diseases in major French bean producing regions in the country have mainly been fungal diseases with rusts (*Uromyces appendiculatus*), angular leaf spot (*Phaeoisariopsis griseola*) and anthracnose (*Colletotrichum lindemuthianum*) being the most important and widely distributed diseases in East Africa (Wahome *et al.*, 2011). However, in the recent past, Ascochyta blight (*Phoma exigua*) has emerged as a disease that requires serious attention with regard to control and management. Continuous cultivation of local varieties and cultivars of French bean has resulted in increased incidence of diseases in the field as the varieties perform poorly in relation to diseases resistance (Arunga *et al.*, 2010). Moreover, French bean seeds are mostly imported and expensive for small-scale farmers (Van Rheenen, 2001). This has forced most small-scale growers to recycle their own seeds, a practice that has led to deterioration in quality of French bean, thus fetching lower prices due to differences in quality and low yield (Minot and Ngigi, 2003).

2.3 Ascochyta blight distribution

Ascochyta blight is a destructive disease that is caused by a fungal pathogen *Phoma exigua*. The pathogen is distributed worldwide with devastating effects on Faba bean in Europe, North America, South America, Asia and Australia (Chalkley, 2014). Bretag *et al.* (2006)

observed that in 436 seed lots tested in Australia, 94.8% of isolates were *Mycosphaerella pinodes*, 4.2% *Phoma medicaginis* and 1.0% *Ascochyta pisi*. Skolko *et al.* (1954) reported that 85% of Canadian Ascochyta blight-infected seed lots were infected by *A. pisi*. In France, *M. pinodes* is the dominant pea pathogen, but *A. pisi* can be found in southern France (Le May *et al.*, 2005). In Africa, the distribution of the pathogen has been recorded in all bean production areas with cool, wet and humid conditions in Burundi, Rwanda, Tanzania, Uganda, Zambia and Kenya. In eastern Africa, areas where Ascochyta blight is a constraint to bean production include highlands of south-west Uganda, the slopes of mount Elgon, the highlands of Kenya, Tanzania, Rwanda and Burundi (Sengooba and Male-Kayiwa, 1990). The impact of the diseases varies between crops, countries, seasons and cropping systems, and yield loss data collected under well-defined conditions is scarce (Tivoli and Banniza, 2007). However, Ascochyta blights are considered major diseases in many areas where legumes are grown. Ascochyta blight severity is high particularly if the pathogen attacks bean seedling (Plate 1.0) under favourable conditions (Allen *et al.*, 1996).

Plate 1.0: French bean plant attacked by Ascochyta blight at seedling stage.
Source: Authors own photograph.

2.4 Biology and development of Ascochyta blight

All pathogens responsible for Ascochyta blights belong to the genus *Phoma* (formerly *Ascochyta*), with the different species *pisi* and *pinodes* (on pea), *rabiei* (on chickpea), *lentis* (on

lentil), *fabae* (on faba bean), *viciae* (on vicia) (Tivoli and Banniza, 2007) and *exigua* on beans. *Phoma exigua* has been isolated from more than 200 host genera around the world (Marcinkowska *et al.*, 2005). The pathogen is known as a wound-pathogen in different species of plants and sometimes causes severe dumping off when infected seeds are used for sowing. It deploys a polyphagous life cycle with ability to survive in plant debris for several seasons (MacLeod and Sweetingham, 1999). Sources of infection in a new crop are mainly from infected seeds, air-borne ascopores spread from infected plants or residue from the previous crop (Frate *et al.*, 2007). Ascospores produced in the perithecia on plant debris after the short rains are transferred a few kilometres from the infected to the new host plant if they are dispersed by air currents, although most land a few meters from the source (Tivoli and Banniza, 2007). The conidia are dispersed from pycnidia by rain or water splash from overwintering debris or infected plants to the nearby plants (Chalkley, 2014).

The pathogen is associated with the formation of pseudothecia at the end of the cropping season that allows the survival of the pathogen in the plant debris (Tivoli and Banniza, 2007). The pseudothecia release ascospores after rainfall and thus they are dispersed over long distances by wind. This explains the importance of plant debris as a source of primary inoculum for Ascochyta blight development in the subsequent seasons. The pathogen attack pods, cotyledons, developing hypocotyl, leaves and other aerial parts of bean plant (Moore *et al.*, 2013). This results in dark grey to black concentric rings on the leaves which later coalesce and with time, the centres of the diseased tissues fall out. Stem attack is characterised by sunken lesions that girdle the stem resulting in breakage and eventual death of the parts above the diseased area. If pods are present at the time of the attack or the pathogen is spread to the developing/developed pods, they become infected and lesions with concentric rings appear as the disease progresses (Frate *et al.*, 2007). Lesions produced on the surface of the pods become very deep with dark brown centres containing numerous pycnidia and/or conidia that are pale pink to yellow in damp conditions (Chalkley, 2014).

Seedlings established from contaminated seeds often have dark brown to black sunken lesions on the stems and cotyledons that senesce prematurely, with subsequent stunted growth of the plants. The disease intensity is weather dependent and thus, high yield loss is common in cool, wet and high relative humidity areas (Tivoli and Banniza, 2007). Ascochyta blight is polycyclic and secondary cycles are generally due to a succession of pycnospores released from plant tissue to tissue or from plant to plant (Tivoli and Banniza, 2007). When the spore lands

on susceptible host plant, it germinates, enter the plant and continue spreading through the plant tissues resulting in leaf blight and stem and pod lesions (Frate *et al.*, 2007). Leaves or leaf axils tend to be the first plant tissues that are infected, followed by stems, pods and seeds.

2.5 Control strategies of Ascochyta blight

Integrated disease management has been recommended in controlling Ascochyta blight. This involves cultural practices, use of clean seeds, certified seeds, and strategic use of foliar fungicides (Moore *et al.*, 2013). Cultural control of the pathogen can be achieved through crop rotation for at least two years with non-host crops, use of uncontaminated certified seeds, intercropping, field sanitation and destruction of infected crop residues (Khan *et al.*, 2013). Since the fungus is disseminated in the presence of water, fields should not be entered for cultivation or pesticide applications when the plants are wet thus avoiding unnecessary movement in infested fields which minimizes spread of the disease. This helps in maintaining hygiene on and off the farm (Moore *et al.*, 2013). Monitoring blight development at regular intervals can help recognize disease symptoms at an initial stage which is essential in blight control.

2.5.1 Use of tolerant or resistant varieties

The most economical, environmentally sustainable and recommended method of controlling bean diseases is through host plant resistance (Wahome *et al.*, 2011). Development of French bean cultivars with improved resistance to biotic stresses has been the goal in bean breeding (Sharma *et al.*, 2007). French bean varieties that are resistant or tolerant to Ascochyta are available from seed companies. Current cultivars only possess low resistance to the pathogen which can breakdown easily (Gan *et al.*, 2006). This is due to the contribution of the sexual stage (teleomorph) of the pathogen to emergence of new races or pathotypes (Barve *et al.*, 2003; Bretag *et al.*, 2006; Gan *et al.*, 2006). The existence of many physiological races of *Phoma exigua* and the high variability of the pathogen means that host resistance alone may not be sufficient. This is because varieties that are resistant to one race may be susceptible to another or when favourable conditions (extended cool and wet periods) for diseases development prevail, the resistance often break down (Duc *et al.*, 2014). Moreover, the resistance of plants decreases with plant age especially when the particular cultivar has partial resistance thus resistance alone may not provide adequate disease control in the field (Chongo and Gossen, 2001; Chongo *et al.*, 2003). This has forced many farmers to opt for use of foliar fungicides. Integrated use of genetic resistance and effective program of foliar fungicides

application has been found effective in controlling Ascochyta blight (Gossen *et al.*, 2011). However, due to several negative impacts associated with use of these synthetic fungicides, an alternative approach is needed in managing this disease.

2.5.2 Chemical control

Seed borne inocula as well as infected or contaminated seeds are the major routes of introduction of Ascochyta blight to a healthy crop (Akem *et al.*, 1999; Gan *et al.*, 2006). Seed treatments and foliar sprays with various synthetic fungicides have been suggested for controlling the pathogen (Gossen *et al.*, 2011). Use of broad spectrum fungicides in seed treatment can limit fungal pathogens that may be present on the seed or in the soil. However, seed dressings will only protect emerging seedling from seed borne Ascochyta blight. This means that seed dressing alone is not effective in protecting the emerging seedling from rain-splashed Ascochyta blight inoculum (Markell *et al.*, 2008). This therefore calls for further spraying with synthetic fungicides after emergence of the crop. However, use of foliar fungicides may not be cost effective if the disease incidence and severity is low in the field.

Frequent use of synthetic chemicals to control the pathogen, without adequate control, has led to several problems. Synthetic chemicals have been associated with development of pathogen resistance to the chemicals, environmental pollution, human health risks, expensive to the smallholders (Pinto *et al.*, 2010) and reduced acceptance of bean exports in the European and other international markets (HCDA, 2010). In order to mitigate several problems associated with synthetic pesticides, numerous research works have been dedicated towards prospecting for alternative biopesticide products with low toxicities (Ntonifor *et al.*, 2011). This has necessitated the search for alternative low-risk control methods (Okigbo *et al.*, 2008), with the use of botanical extracts being widely researched on and adopted in most places.

2.5.3 Use of botanical extracts

The search for alternative biocontrol agents that are environmentally friendly and readily available has been extended to the wild plants that have been found to have antimicrobial activities. Previous studies using extracts of *Miconia argyrophylla* and *Origanum vulgare* showed that the plants are effective in reducing the severity of bean pathogens (Pinto *et al.*, 2010). Studies on the inhibitory potential of botanical extracts against phytopathogens

showed that leaf extracts of test plants had highest concentration of active ingredients, with a few exceptions (Pinto *et al*., 2010).

Indigenous plants with secondary plant metabolites or bioactive compounds with fungicidal properties are an option in the management of pathogens. Use of botanical extracts and essential oils in controlling a wide range of pathogens has been documented (Quintal *et al*., 2011; Mangang and Chhetry, 2012; Sen and Batra, 2012). Plants in the Lamiaceae family have been used for centuries as folk medicine with plants in the genus *Leonotis* and *Ocimum* being recognized for their therapeutic potentials (Imran *et al*., 2012; Usharani *et al*., 2013). Previous studies of several species from the Labiatae family have confirmed the antifungal and insecticidal potency of the plants (Pinto *et al*., 2010; Othira *et al*., 2011). These plant bioactive compounds act against a limited number of pathogen species, are biodegradable to non-toxic products, have low negative impact on human health, and can be incorporated into integrated pest and disease management programs (Ukeh *et al*., 2010). Mixtures of plant extracts from Neem, *Ocimum* and *Eucalyptus* with other plants have also been shown to exhibit insecticidal activity against pod borers on cowpeas (Oparaeke *et al.,* 2005). Although much of the literature on natural plant products in the agricultural field concerns insect control, plant extracts and plant essential oils are effective antimicrobials against foliar pathogens (Bowers and Locke, 2000). The effectiveness of *Azadirachta indica* and *Xylopia aethiopica* in controlling cowpea anthracnose both *in vitro* and *in vivo* has already been confirmed (Amadioha and Obi, 1998).

Inadequate research has been conducted on the efficacy of botanical extracts against other bean pathogens under field conditions (Amadioha, 2003) and thus efficacy data on suppressive effects of plant extracts and essential oils under field conditions are lacking. The stage of plant growth and part of the plant used for extraction of the essential oils should be carefully chosen to obtain high chemical content (Pinto *et al*., 2010). This is because distribution of secondary metabolites and the essential oils tend to be higher on the leaves and tender stems of the plants (Shai *et al*., 2009). However, geographic origin, climatic and ecological factors have little influence on the chemical composition of the secondary metabolites and essential oils of indigenous plants (Amvam *et al*., 1998). The effectiveness of indigenous plant extracts is determined by the concentration and the exposure of the organism to the active ingredients in the biopesticidal plant (Othira *et al*., 2011). Alcohol extract of *Piper nigrum* has been reported to have antifungal activities against bean anthracnose under field conditions if the extract are sprayed before symptoms development (Amadioha, 2003). This is

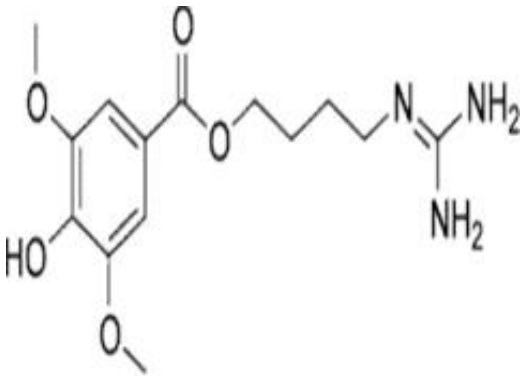
an indication that the stage and the time when the plant extracts are applied to control pathogens may be crucial in controlling plant diseases.

Leonotis nepetifolia (Plate 2.0 a), commonly known as Klip Dagga or Lion's Ear, is a frost hardy perennial herbaceous plant in the Lamiaceae (mint) family. This plant has a clump forming growth form, with a mature plant attaining a height of 2.4 m and a spread of 1 m. The plant has strongly angled stems and produces leaves that are smooth, triangular in shape, opposite pairs and with serrated margins (Udaya Prakash *et al.*, 2013). The inflorescence is verticillate and flowers are borne in rounded, spiny clusters that encircle the stem. As the stems elongate, new flower clusters continue to develop above the older ones. The tubular flowers that peak out of the spiny heads are orange, velvety and long. It produces orange yellow coroneted verticillate inflorescence with distinct stem, leaves and even inflorescent odour (Imran *et al.*, 2012). This characteristic has been explored and found to be associated with the repellence potential of the plant against most crop insect pests. The plant grows in patches along roadside or barren unused agriculture waste land during rainy season.

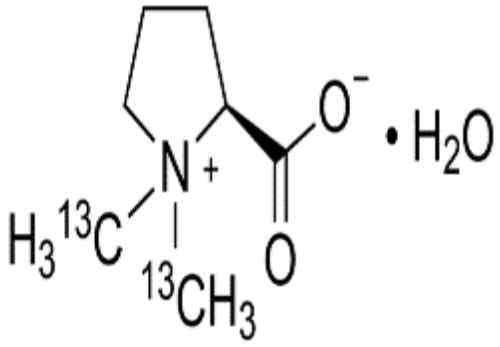
(a) (b)

Plate 2.0: *Leonotis nepetifolia* (a) and *Ocimum gratissimum* (b) growing by the road sides

Preliminary qualitative and quantitative phytochemical analysis of *L. nepetifolia* revealed that it contains significant levels of alkaloids, phenolics, flavonoids, tannins, steroids, glycosides and saponins (Imran *et al.*, 2012; Usharani *et al.*, 2013). Alkaloids are produced by plants as a defence mechanism against environmental stresses such as animals or pathogen attack. Alkaloids obtained from *L. nepetifolia* are rich in leonurine and stachydrene that are known for antimicrobial activities (Pingale *et al.*, 2013).



Leonurine



Stachydrene

The type of solvent used in the extraction of secondary metabolites from the plant determines which metabolites are present within the plant parts as different solvents have different potential to extract the constituent secondary components (Imran *et al.*, 2012). For instance, alkaloids, phenolic, flavonoids and glycosides are reported to be present in chloroform extract; tannins, steroids and saponins found in petroleum ether extract; phenols, flavonoids and glycosides are present in methanolic extract; alkaloids, phenolic, flavonoids and saponins in aqueous extract while acetone extract showed presence of steroids (Imran *et al.*, 2012). Chloroform, ethyl acetate, and methanol have been reported to be suitable for the extraction of secondary metabolites in plants (Usharani *et al.*, 2013). However, amongst these solvent systems, the ethanolic extract has been reported to show better activity followed by acetone and ethyl acetate.

Extraction of phytochemical constituents of *L. nepetifolia* from different parts of the plant using different solvents has been found to show difference in the antibacterial potency, antioxidant, larvicidal and pesticidal properties (Imran *et al.*, 2012). These studies show that plant extracts from organic solvents provide more antimicrobial activity than aqueous extracts because of the high solubility of the secondary metabolites in the former solvent. This results in differential toxicity or the antimicrobial activity of the extracts. Ethyl acetate extracts of *L. nepetifolia* obtained from leaves, stem and roots showed antibacterial potency at lower

concentrations compared with the other solvents like acetone, chloroform and ethanol (Udaya Prakash *et al.*, 2013). These findings show that there is need to test different solvent systems to know the biological efficacy of plant and different plant parts. Medicinal properties of *L. nepetifolia* have been demonstrated in treating human infections caused by diverse pathogens (Dhawan *et al.*, 2013), where the plant exhibited various biological activities such as antifungal and antibacterial. Little research, however, has been done on the antimicrobial activity of the plant extracts against plant pathogens.

Essential oils of *Ocimum basilicum, O. americanum,* and *O. gratissimum* are among other *Ocimum* species that have been reported in various studies to have insecticidal, antifungal and antibacterial activity on plant pests and diseases (Amvam *et al.*, 1998; Apinya *et al.*, 2009). *Ocimum sanctum, O. gratissium, O. canum, O. basilicum, O. kilimandscharicum, O. ammericanum, O. camphora* and *O. micranthum* grow in different parts of the world and are known to have medicinal properties (Prakash and Gupta, 2005). The *Ocimum* species have been reported to yield oil of diverse nature, commonly known as basilic oils that are made up of mainly eugenol and methyl eugenol among other minor components (Janine *et al.*, 2005; Matasyoh *et al.*, 2007). The presence of phytochemical bases in *O. gratissimum* accounts for its usefulness as a medicinal plant with many subspecies *(*for example *O. viride* Linn, *O. suave* Linn, *O. basilicum* Linn and *O. canum* Sims) being reported for their numerous medical uses (Mshana *et al.*, 2000). The aerial parts (leaves, flowers and stem) of these plants contain essential oils with good percentage of eugenol. The leaves of *O. sanctum* L. are chief source of essential oils followed by the inflorescence and stem, with the flowers containing more essential oils than leaves in *O. basilicum* while the roots and fruits are almost completely devoid of any essential oil (Prakash and Gupta, 2005).

Ocimum gratissimum (Plate 2.0 b) is an aromatic, perennial herb, with much branched erect stem and oppositely arranged leaves. The plant is a variable polymorphic species often with subspecies, varieties mainly based on difference in chemical content, the morphology of the fruiting calyx and the degree of hairiness (Sulistiarini, 1999). Chemical analysis of extracts from the plant has indicated that *Ocimum* species contain Eugenol (l-hydroxy-2-methoxy-4-allylbenzene), a phenolic compound that has been found to be largely responsible for the therapeutic potentials of the plant (Akinmoladun *et al.*, 2007). It has been demonstrated that eugenol isolated from *O. gratissimum* presents antimicrobial, insecticidal, antihelminthic and fungistatic properties (Janine *et al.*, 2005). Previous studies have confirmed the antimicrobial

and antifungal activities of *O. gratissimum* on common bean pathogens. The crude extracts from the aerial parts of the plant has been demonstrated *in vitro* to be active against fungal pathogens that attack common beans (Pinto *et al.*, 2010). Moreover, essential oils of *O. gratissimum* leaves have fungistatic properties on *Aureobasidium pullulans, Microsporum gypseum, Trichophyton rubrum, Candida albicans* and *Cryptococcus neoformans* (Amvam *et al.*, 1998). However, evaluation of antimicrobial activity of the extracts under field conditions has not been determined. Development of *L. nepetifolia* and *O. gratissimum* plant based bio-pesticide would be a valuable alternative tool for use in an IPM system designed to control Ascochyta blight of French bean.

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CHAPTER THREE

ANTIFUNGAL ACTIVITY OF METHANOL EXTRACTS OF *Leonotis nepetifolia* AND *Ocimum gratissimum* AGAINST ASCOCHYTA BLIGHT (*Phoma exigua*) OF FRENCH BEAN

ABSTRACT

The potential of crude plant extracts as antimicrobial agents has been demonstrated both in animal and plant health. The antimicrobial activities in these plants are due to phytochemicals produced by the plants as defence mechanism against biotic stresses. A study was conducted to determine antifungal activity of methanol extracts of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. against Ascochyta blight pathogen (*Phoma exigua)* of French bean *in vitro*. Composite leaf and stem samples were shade-dried, ground to fine powder using electronic hammer mill and extracted using methanol. Two-fold serial dilutions were performed to obtain final concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.31, and 0.16 mg ml^{-1} (w/v) used in the laboratory bioassays against the pathogen. The bioassays were performed according to Kirby-Bauer disc diffusion method. The experiment was laid out in a completely randomised design (CRD) replicated three times. The extracts showed antifungal activity at all the concentrations tested with minimum inhibitory concentration (MIC) of 1.25 mg ml^{-1} for both *L. nepetifolia* and *O. gratissimum*. There was plant extract concentration-dependent increase in the diameter of zones of inhibition. The average diameter of inhibition zones for the two plant extracts at 40.0 mg ml^{-1} were comparable to those from the synthetic fungicide (Carbendazim). These results showed that the two test plant extracts possess antifungal activity against the pathogen and can be used as natural alternatives to the synthetic fungicides in the management of Ascochyta blight of French bean.

Key words: Ascochyta blight, *Leonotis nepetifolia, Ocimum gratissimum, Phoma exigua*

INTRODUCTION

French bean (*Phaseolus vulgaris* L.) is an important export horticultural crop accounting for 60 and 21% of all vegetable and horticultural exports in Kenya (HCDA, 2010), respectively, thus contributing to 35-40% of foreign export exchange earnings annually. The crop is grown for commercial purposes by both large and smallholder farmers, thereby creating employment opportunities for the rural communities especially to women and youth. In the recent years, there has been a decline in French bean production due to problems caused by biotic stresses (Fernandez *et al.*, 2000). The major insect pests that affect French bean production in Kenya are bean stem maggot (*Ophiomyia* spp.), thrips (*Frankliniella* spp.), bean aphids (*Aphis fabae*), Red spider mites (*Tetranychus* spp.), African bollworm (*Helicoverpa armigera*) and white flies (*Bemicia tabaci*) (Nderitu *et al.*, 2007). Leguminous crops are frequently attacked by fungal diseases such as grey mould or chocolate spot, Ascochyta blight, anthracnose, powdery and downy mildews and rusts. The relative importance of these diseases and their effect on yield vary across geographical locations. Ascochyta blight caused by *Phoma exigua* is an emerging economically important disease that has resulted in decline in French bean production in the recent past. Ascochyta blight affects large areas in many countries where pulses are cultivated and cause considerable qualitative and quantitative losses (Ghazanfar *et al.*, 2011).

For long time, research has been directed at other diseases considered to be important in major French bean producing regions. However, with the increase in incidence of Ascochyta blight in French bean production areas, there is need to refocus the research agenda. The pathogen can stay on the seed for over two years and thus can be spread from overwintering debris and infected seeds (Maurin, 1993). Seed dressing and foliar sprays with various synthetic fungicides have been suggested for control of the pathogen (Gossen *et al.*, 2011). The synthetic fungicides are known to cause more harm to the environment than benefits and therefore there is need for an alternative approach to controlling the disease.

Secondary plant metabolites or bioactive compounds from plants with antifungal properties are an option in the management of pathogens (Adjou *et al.*, 2012). Previous studies of several plant species from the Labiatae family have demonstrated their antifungal activities against plant pathogens (Dikbas *et al.*, 2008). This shows the possibility of new control agents to be developed from them. Amongst some of the widely studied and promising botanical control agents are *Ocimum gratissimum* and *Leonotis nepetifolia* (Apinya *et al.*, 2009; Ngoci

et al., 2013). Studies have shown that *Ocimum* sp. and *Leonotis* sp. have antimicrobial properties *in vitro* (Apinya *et al*., 2009). A study was therefore conducted to determine the antifungal activities of methanol extracts of *O. gratissimum* and *L. nepetifolia* against *Phoma exigua*, *in vitro*. The findings of this study were expected to provide the basis for integrating *O. gratissimum* and *L. nepetifoia* extracts in integrated pest management (IPM) of Ascochyta blight by smallholder French bean farmers.

MATERIALS AND METHODS

3.2 Collection of test plants and preparation of extracts

The plant materials used in this study consisted of composite fresh plant samples (leaves, succulent stems and fruits) of *O. gratissimum* and *L. nepetifolia*. These plants are natural weed species and were abundantly found in undisturbed and/or in cultivated fields and along road reserves. The plants were collected from fallow fields at Egerton University and its environs, and identified by a taxonomist in the Department of Biological Sciences, Egerton University. Voucher specimens of test plants were deposited in the Biotechnology Laboratory, Egerton University.

The fresh composite plant samples were shade-dried for three weeks to complete dryness and ground to fine powder using electronic hammer mill. The resulting powders were weighed, placed in air-tight containers and stored in cool place until extraction. Sequential extractions were performed on 250 g of each plant powder by soaking in 2 litres of 100% methanol and left to stand for 24 hours (h). The mixture was then filtered using Whatman filter paper No. 2. The filtrate was concentrated and methanol recovered using a Büchi Rotavapor (Model R-200, Switzerland). The concentrated plant extracts were transferred into 90 mm Petri dishes and placed at room temperature to complete dryness. Stock solutions of the extracts were prepared by dissolving 1000 mg of the plant extract in 25 ml of Dimethyl sulphoxide (DMSO) to yield a final concentration of 40 mg ml^{-1}. Two-fold serial dilutions were prepared to obtain the final concentrations of 0.16, 0.31, 0.63, 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 mg ml^{-1} (w/v) that were used to determine the antifungal activities of the two plant extracts.

3.3 Isolation and culturing of the pathogen

Thirty nine grams (39 g) of prepared potato dextrose agar (PDA) was autoclaved at 121 °C for 15 minutes and allowed to cool to about 45 °C. Streptomycin sulphate ($0.1gl^{-1}$) was added to the media to suppress bacterial growth. About 10 ml of the PDA was aseptically

poured into 90 mm Petri dishes with pre-drawn diametrical lines on the bottom and allowed to solidify. Infected French bean pods were obtained from farmers' fields and used as source of initial inoculum of *Phoma exigua*. Small angular cuttings were made from the pods and surface sterilized using 0.5% sodium hypochlorite (NaOCl) for about two minutes. The sterilized pod tissues were then rinsed three times in sterile distilled water, blot-dried in sterile blotting paper and placed on PDA media. The plates were sealed with laboratory parafilm and incubated in dark conditions at 21 °C for 7 days. Further sub-culturing and single spore isolation were conducted to obtain pure cultures of the pathogen that was used for the bioassays. To ascertain the identity and virulence of the isolated pathogen, pathogenicity tests were conducted following Koch's postulates. This was done by inoculating healthy French bean plants with 1.0 $\times 10^6$ spore/ml under greenhouse conditions, and the experiment repeated two times.

3.4 Antifungal bioassays

The antifungal bioassays of the extracts were done by adopting Kirby-Bauer disc diffusion method (Kirby *et al*., 1966) with modifications. About 10 ml of the PDA, prepared according to the procedure described in section 3.3, was aseptically poured into 90 mm Petri dishes with pre-drawn diametrical lines on the bottom and allowed to solidify. The fungal spores were obtained by flooding 7-day old cultures in petri dishes with sterile distilled water. The petri dishes were then gently scrubbed using a sterile spatula, and the spores harvested using cotton gauze. Haemocytometer was used to quantify the spores to a final concentration of 1.0 x 10^6 spore/ml. Spore suspension (1 ml) of Ascochyta blight pathogen, prepared as above, was dispensed at the centre of the Petri dish and evenly spread all over the surface of the media using a sterile glass rod.

Sterile 4 mm filter paper discs were impregnated with 10 µl of 40 mg ml^{-1} extract of *O. gratissimum* and *L. nepetifolia*. This was repeated with the other rates (0.16, 0.31, 0.63, 1.25, 2.5, 5.0, 10.0, and 20.0 mg ml^{-1}) to obtain enough discs for the three replicates. The discs were left undisturbed in the laminar flow hood for 3 h to allow for partial drying. The treated discs were then aseptically placed on the surface of the media inoculated with the pathogen. Dimethyl sulphoxide (DMSO) and Carbendazim were used as negative and positive controls, respectively. The plates were sealed with laboratory parafilm and incubated in dark conditions at 21 °C. The treatments were replicated three times and arranged in a completely randomized design (CRD) and the experiment repeated three times. Antifungal activity of the extracts was determined by measuring the zones of inhibition (mm) 7 days after incubation (DAI). The

extracts were considered to be active against the pathogen whenever a zone of inhibition equivalent to or greater than 10.0 mm was recorded. The lowest concentration of the botanical extracts that inhibited growth of the fungus was considered to be the minimum inhibitory concentration (MIC) for the individual plant extract.

3.5 Statistical analysis

The data collected on inhibition zones induced by the plant extracts were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, 2001) software. Treatment means were separated using Tukey's HSD test whenever ANOVA showed significant treatment effects.

RESULTS AND DISCUSSION

The antifungal activity of methanol extracts of *O. gratissimum* and *L. nepetifolia* are presented in Table 1.0, Fig.1.0 and Plate 3.0 below. The extracts of the two plants exhibited antifungal activity through inhibition of mycelia growth of *P. exigua in vitro*. There was significant ($P \leq 0.05$) concentration-dependent antifungal activity of the extracts demonstrated by increased diameters of zones of inhibition (Table 1.0). An increase in the concentration of the extracts resulted in a progressive increase in diameter of the inhibition zone (Fig. 1.0). Although the antifungal activity of the positive control (Carbendazim) was significantly ($P \leq 0.05$) higher compared to the highest rates of the two extracts, the rates resulted in excellent inhibition of the pathogen *in vitro*. Minimum inhibitory concentration (MIC) of 1.25 mg ml^{-1} was recorded for both test plants.

Table 1.0: Zones of inhibition (mm) of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on *Phoma exigua*

	Inhibition zones (mm) of the plant extracts	
Concentration (mg/ml)	***Ocimum gratissimum*††**	***Leonotis nepetifolia*††**
DMSO (0.00)	0.00 ± 0.00	0.00 ± 0.00
Carbendazim	18.67 ± 0.26	18.67 ± 0.26
0.16	2.83 ± 0.86	3.88 ± 0.84
0.31	7.83 ± 0.37	8.42 ± 0.29
0.63	9.92 ± 0.23	9.88 ± 0.21
1.25	10.50 ± 0.19	10.33 ± 0.19
2.50	11.33 ± 0.36	10.67 ± 0.28
5.00	11.83 ± 0.47	11.58 ± 0.26
10.00	12.00 ± 0.39	11.92 ± 0.26
20.00	13.25 ± 0.41	12.25 ± 0.35
40.00	14.08 ± 0.26	14.13 ± 0.20

††Figures shown are the Means ± SE of three replicates. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Generally, there was no significant ($P \leq 0.05$) species-dependent antifungal activity of the two plant extracts at all the concentrations tested (Fig. 1). The two botanicals showed the potential to be effective in controlling *P. exigua in vitro*.

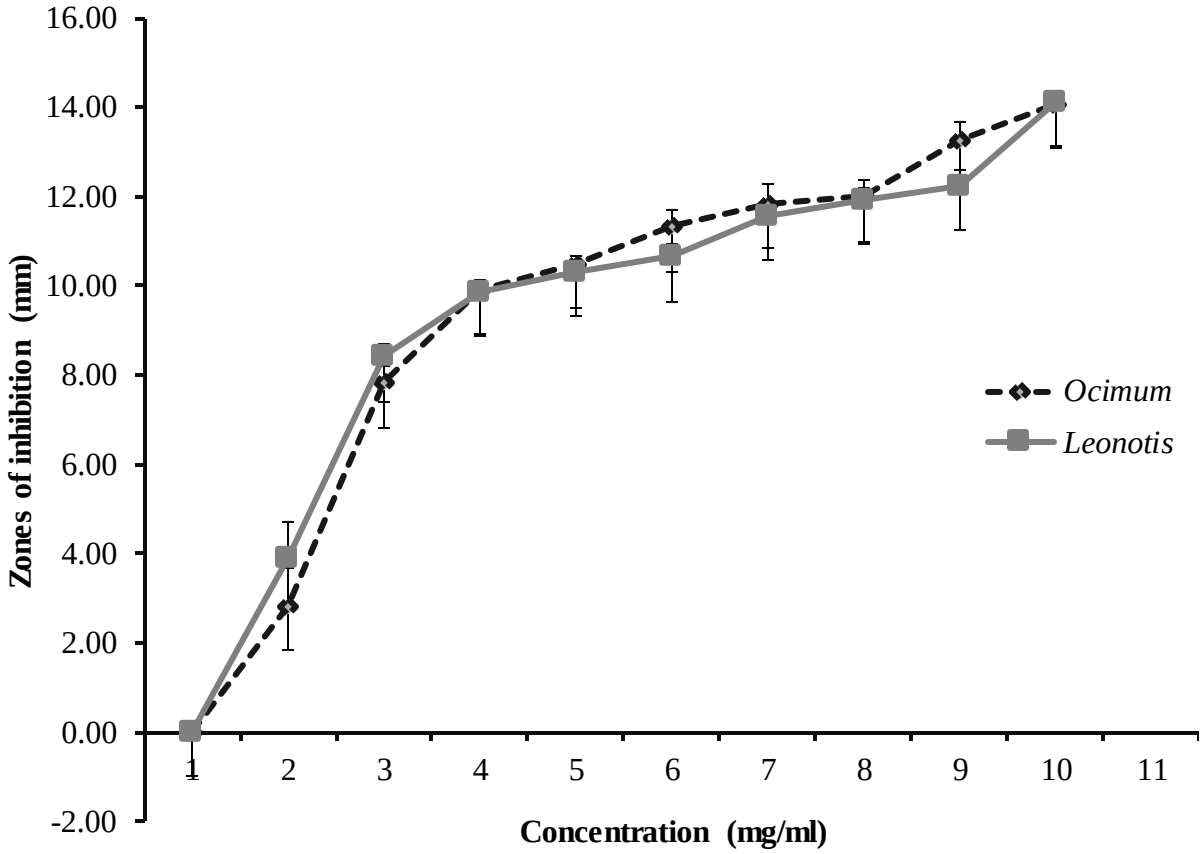
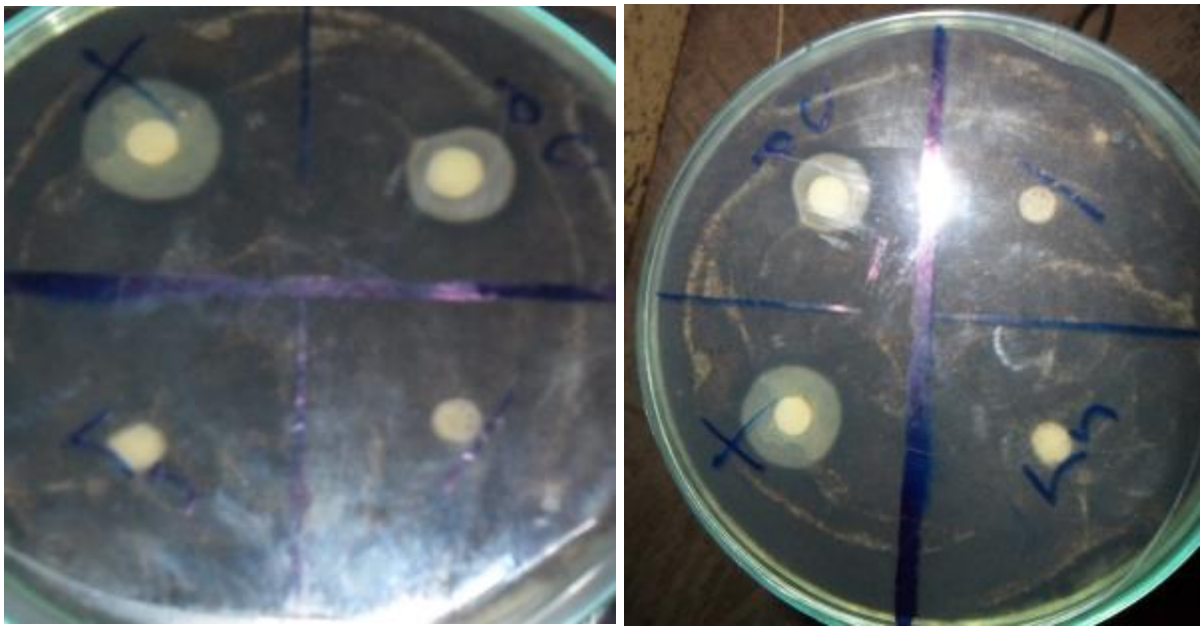


Figure 1.0: Antifungal activity of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* against *Phoma exigua in vitro*

The antibacterial and antifungal activity of plant extracts has been demonstrated in previous studies (Bajpai and Kang, 2012; Obeidat *et al.*, 2012; Ngoci *et al.*, 2013). The observed bioactivity may be attributed to the presence of phytochemicals or secondary metabolites that have been reported to be active against most phytopathogens. Phytochemical analysis of the two test plants has shown that *O. gratissimum* contains alkaloids, resins, tannin, phenolics, glycosides, saponin, and steroidal terpenes while *L. nepetifolia* contains alkaloid, phenolic, flavonoids, tannins, steroids, glycosides and saponins at different proportions (Koche *et al.*, 2012). These compounds are known to be produced by the plants as a natural defensive mechanism against pests and diseases and thus explain the bioactivity of the two plants. Therapeutic studies have confirmed the effectiveness of *Leonotis* spp. against both Gram

negative and Gram positive bacteria (Ngoci *et al.*, 2013). *Leonotis nepetifolia* has been demonstrated to have antimicrobial activity against human diseases caused by bacterial and fungal pathogens.



(a) (b)

Plate 3.0: Inhibition zones induced by (a) *Ocimum gratissimum* and (b) *Leonotis nepetifolia* extracts at 5.0 and 10.0 mg ml^{-1} respectively

The potential antifungal activity of the plant extracts against phytopathogens has been demonstrated in this study. The diameters of inhibition zones obtained across all the concentrations of *L. nepetifolia* tested conforms to those reported in previous studies. Ngoci *et al.* (2013) reported that *L. nepetifolia* resulted in zones of inhibition greater than 20 mm for both Gram positive and Gram negative bacteria. This slight variation could be attributed to difference in susceptibility of the test pathogen to the active ingredients in the plant. The activity of *O. gratissimum* against phytopathogens has also been reported in previous studies (Nakamura *et al.*, 1999). The findings of this study on the activity of *O. gratissimum* extract are in conformity with those obtained in the previous studies by other researchers (Koche *et al.*, 2012).

Antifungal activities of crude extracts of these two plants were dose-dependent as an increase in concentration resulted in increased inhibition of the pathogen. These results are similar to those obtained by Koche *et al.* (2012), who reported an increase in the activity of *O.*

gratissimum with increase in the concentration of the extract against *Escherichia coli* and *Listeria monocytogenes*. The increase in activity could partly be attributed to higher rates of diffusion due to increase in concentration of the active ingredients per unit volume of the solvent. This study has demonstrated the potential of *L. nepetifolia* and *O. gratissimum* to be used as natural alternatives to the synthetic fungicides in managing Ascochyta blight of French bean.

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CHAPTER FOUR

EFFECT OF METHANOL EXTRACTS OF *Leonotis nepetifolia* L. AND *Ocimum gratissimum* L. ON THE INCIDENCE AND SEVERITY OF ASCOCHYTA BLIGHT (*Phoma exigua)* AND ON YIELD OF FRENCH BEAN

ABSTRACT

The antifungal activities of indigenous plant extracts against several phytopathogens have been demonstrated *in vitro*. However, the antifungal activities of the extracts have not been tested under field conditions to ascertain their efficacy. The objective of this study was to determine the efficacy of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. extracts on the incidence and severity of Ascochyta blight on French beans *in vivo*. Four certified French bean seeds were sown per plastic pot (20 cm in diameter) filled with sterilized field soil under greenhouse conditions. The pots were divided into two sets with 12 treatments and the experiment arranged in a randomized complete block design with three replicates. Plants in the first set were inoculated with a spore suspension (1.0×10^6 spore/ml) of *Phoma exigua* 48 h before treatment application; while the second set were inoculated 48 h after treatment application. The experiment was laid in a 2 × 6 factorial arrangement of RCBD with three replicates. Methanol extracts of *O. gratissimum* and *L. nepetifolia* at 20.0, 10.0, 5.0, 2.5 and 1.25 mg ml^{-1} were applied per plot at 14 days after emergence (DAE). Data were collected on disease incidence and severity, percent disease index (PDI) and disease severity index (DSI) and crop yield per plot. Analysis of variance (ANOVA) was conducted and means separated using Tukey's HSD test whenever ANOVA showed significant treatment differences. Correlation ($P \leq 0.05$) analysis between the botanical rates, disease incidence, disease severity and yield per plot was conducted. *Ocimum gratissimum* and *L. nepetifolia* at 5.0 mg ml^{-1} resulted in reduction of disease incidence by 4.2 and 12.5% respectively. *Ocimum gratissimum* extract at 10.0 mg ml^{-1} and *L. nepetifolia* extract at 20.0 mg ml^{-1} reduced Ascochyta blight severity to 13.5 and 13.1%, respectively, in the field. There was a positive correlation between disease incidence and severity, while yield per plot was negatively correlated to disease incidence and severity. Results showed that methanol extracts of the two plants were active against Ascochyta blight of French bean under field conditions and thus can be used as natural alternatives to synthetic fungicides in managing the disease.

Key words: Antifungal activity, Ascochyta blight, *Leonotis nepetifolia, Ocimum gratissimum, Phoma exigua, in vivo*

INTRODUCTION

French bean (*Phaseolus vulgaris* L.) is an important export horticultural legume crop that is facing serious production constraint from biotic stresses. Pests (white flies, bean flies, aphids) and diseases such as rust (*Uromyces appendiculatus*), bacterial blight (*Xanthomonas campestris* pv. *phaseoli*), and anthracnose (*Colletotrichum lindemuthianum*) have resulted in significant decline in production of French bean. Most smallholder farmers in Kenya grow French bean during the short rain seasons that are characterized by moderate humidity and most of the time relatively high temperature conditions that are conducive for the growth and thriving of most fungal phytopathogens. This results in fast disease development, high incidence and severity (Granke and Hausbeck, 2010; Lopez *et al.*, 2012).

Ascochyta blight (*Phoma exigua*) is a fungal disease that attacks several plants in the Fabaceae family. The pathogen has a wide host range and has been reported to be pathogenic to chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), field pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik), vetch (*Vicia* spp.), common bean (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* L.) among other crops (Kaiser, 1991). The importance of the pathogen on French bean is increasing due to its adverse effects on pods, the main harvestable component of the plant. The pathogen is spread by overwintering conidia in infected plant debris and seeds through dispersal by rain or water splash (Davidson *et al.*, 2011). Complete plant death is common when Ascochyta blight attacks seedlings under favourable climatic conditions (Bretag *et al.*, 2006). Multiple sprays of foliar fungicides has been recommended in controlling Ascochyta blight under field conditions (Demirci *et al.*, 2003). However, there is limited economic gain of foliar fungicide application since the fungicides may result in yield increase or no increase at all. Moreover, although the fungicides application may result in reduction in disease severity, the resulting yields may not offset the cost of the foliar fungicide application (Dermici *et al*, 2004). The unregulated use of synthetic fungicides on export commodities has also resulted in rejection of the produce in numerous occasion in the international markets due to maximum chemical limit. The foliar fungicides are also known to cause several environmental hazards when frequently used in the field. These concerns have resulted in search for alternative antimicrobial agents that are environmentally safe and cheap for controlling phytopathogens.

Researchers have sought to explore secondary metabolites and other plant tissue constituents as alternative routes to ameliorate the challenges associated with synthetic

fungicides (Adamu *et al*., 2005). Indigenous plant extracts have been shown to be active against plant pathogens under field conditions (Amadioha, 2003). The plant extracts contain active ingredients that if harnessed, can be used as natural substitutes to the foliar synthetic fungicides used in plant protection. *Ocimum gratissimum* and *Leonotis nepetifolia* are plants in the Lamiaceae family that have been widely studied for their antimicrobial activities against animal and plant pathogens (Alo *et al*., 2012; Rajalakshmi *et al*., 2013). The efficacy of these plants have been demonstrated only under *in vitro* conditions and their possible use for controlling pathogens need further testing under field conditions. Although these plants are effective in controlling the test plant pathogens *in vitro* under these conditions, their adoption as safer alternative to synthetic fungicides has been limited because their effectiveness in the field is still undocumented. Hence a study was conducted to determine the effect of O. *gratissimum* and *L. nepetifolia* extracts on the incidence and severity of Ascochyta blight of French bean *in vivo*.

MATERIALS AND METHODS

4.2 Experimental Sites

a) Greenhouse experiment

The experiment was conducted in the glasshouse in the Department of Biological Sciences, Egerton University, Njoro campus.

b) Field Experiments

Sites description

The experiments were conducted at Egerton University and Kenya Agricultural and Livestock Research Organization (KALRO) – Njoro, Kenya during the period July – December, 2014

i). Egerton University site

The site lies at latitude 0° 23´ S, longitude 35° 35´ E, and at an altitude of 2238 m above sea level. The annual mean precipitation is 1000 mm, and the annual mean temperature is 15.9 °C. The site is situated in the agro-ecological zone III and has mollic andosols loam soils (Jaetzold and Schmidt, 1983). The site has relatively high altitude as well as high annual rainfall which are suitable for French bean production and also Ascochyta blight development and spread.

ii). KALRO – Njoro site

The site lies at latitude 0° 20´ S, longitude 35° 56´ E and 2166 m above sea level. It is located in the lower highlands (LH_3) with an average annual rainfall of 931 mm and annual mean temperature of 14.9° C. The soils are vitric mollic andosols that are well drained, deep to very deep, dark reddish brown, consisting of heavy textured friable silty clay humic top soils (Jaetzold and Schmidt, 1983).

4.3 Evaluation of the botanicals under greenhouse and field conditions

4.3.1 Greenhouse experiment

Two experiments were conducted to determine the efficacy and mode of action of the two plant extracts, when applied before and after inoculation with the pathogen, to control Ascochyta blight under greenhouse conditions. Teresa French bean variety was used in the experiment as it is the most commonly grown French bean variety by farmers, and is also susceptible to Ascochyta blight infection. Four certified seeds were sown in plastic pots (20 cm in diameter) filled with sterilized field soil. The pots were watered twice (in the morning and evening) daily. The pots were divided into two sets with 12 treatments per replicate and replicated three times. The plants were maintained in the greenhouse until 14 days later when they were ready for inoculation. The rates that were considered active against the pathogen in *in vitro* bioassays were used for *in vivo* studies.

a) Experiment 1:

The plants were inoculated with spore suspension (1.0×10^6 spore/ml) of *P. exigua* using an atomiser until runoff and covered with humid polythene bags for 48 h to enhance disease development. The methanol extracts of *L. nepetifolia* and *O. gratissimum* at five rates (20.0, 10.0, 5.0, 2.5 and 1.25 mg ml^{-1}), were sprayed per pot using a hand sprayer 2 days after inoculation (DAI). Carbendazim and distilled water were used as positive and negative control, respectively.

b) Experiment 2:

The methanol extracts of *L. nepetifolia* and *O. gratissimum* at five rates (20.0, 10.0, 5.0, 25 and 1.25mgml^{-1}), were applied per pot and the plants allowed to stand for 24 h to complete dryness. The plants were inoculated with spore suspension (1.0×10^6 spore/ml) using an atomiser and covered with humid polythene bags for 48 h to enhance disease development. Carbendazim and distilled water were used as positive and negative control respectively.

Disease incidence was assessed by counting the total number of plants that showed disease symptoms 14 days after inoculation. The percentage disease incidence was determined using Equation 2.0 below. Disease severity per plot in both experiments was visually assessed on a scale of 0 – 9 (where 0 = no infection, 1 = l%, 2 = 5%, 3 = l0%, 4 = 20%, 5 = 40%, 6 = 60%, 7 = 80%, 8 = 90%, and 9 = complete (100 % of leaf area affected by the pathogen) (Warketin *et al.*, 1996). The disease severity were converted to disease severity index using the formula;

$$\text{DSI}\,(\%) = \frac{\text{ISR}}{\text{TNPP} \times \text{HSR}} \times 100 \qquad \text{(Equation 1.0)}$$

Where; DSI = Percent disease severity index, ISR = Individual severity rating, TNPP = Total number of plants per plot, HSR = Highest severity rating.

Disease incidence per plot were converted to percentage disease incidence (PDI) according to Amadioha (2003) as in Equation 2.0 below.

$$\text{PDI}(\%) = \frac{\text{NDP}}{\text{TNPP}} \times 100 \qquad \text{(Equation 2.0)}$$

Where; PDI = Percent disease index per plot, NDP = number of diseased plants, TNPP = Total number of plants per plot.

4.3.2 Field experiment

The field was ploughed and harrowed to attain a fine soil tilth. The experiment was laid out in a 2 × 6 factorial arrangement of randomized complete block design (RCBD) with six treatments replicated four times. Each plot measured 2.5 m in length and 2.0 m wide. The seeds were sown at a spacing of 0.4 m × 0.3 m (0.4 m between the rows and 0.3 m between the plants) resulting in six rows of French bean per plot. Two seeds of Teresa French bean variety were sown per hole at an appropriate depth. Insect pests were controlled by spraying the plots when the pest population had reached economic threshold using Actara (Thiamethoxam). Other cultural practices such as weeding were carried out regularly when there was need.

The five rates (20.0, 10.0, 5.0, 2.5 and 1.25 $mgml^{-1}$) of methanol extracts of *L. nepetifolia* and *O. gratissimum* were applied to the plots at 14 DAE. Carbendazim and distilled

water were used as positive and negative control, respectively. The plots were allowed to air-dry for 24 h before the plants were inoculated with spore suspension (1.0×10^6 spore/ml) of the pathogen, and covered with humid polythene bags overnight to enhance disease development. Data on incidence and severity of Ascochyta blight were collected from four middle rows in each plot.

Disease severity was assessed at 14, 21 and 28 DAI based on a visual scale of 0 – 9 (where 0 = no infection, 1 = l%, 2 = 5%, 3 = l0%, 4 = 20%, 5 = 40%, 6 = 60%, 7 = 80%, 8 = 90%, and 9 = complete (100% of the leaf area affected by the pathogen) (Warketin *et al.*, 1996). The disease severity were converted to disease severity index using the formula above (Equation 1.0). The treatments were independently randomized for each experimental site but the same experimental procedure was used for both sites.

4.4 Effects of the botanical extracts on yield of French bean

The study was conducted to evaluate the effect of using the crude extracts on yield of French bean. Effectiveness of the botanicals in managing Ascochyta blight was evaluated by comparing the yields of French bean in plots where the botanicals were applied to the control plots. Harvesting was done when the crop had attained horticultural maturity at interval of 4 – 5 days between the harvests. Freshly harvested pods were weighed per plot and total yield (kg per plot) obtained at the end of the season. The total yield per plot were extrapolated to obtain the final yield in $KgHa^{-1}$.

4.5 Statistical analysis

The data collected on DSI, PDI and French bean yield were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, 2001) software and means separated using Tukey's HSD test whenever ANOVA showed significantly different treatment effects. Correlation ($P \leq 0.05$) analysis between the treatment concentrations, disease incidence, severity and yield per plot was conducted.

RESULTS AND DISCUSSION

(a) Greenhouse experiment

The effect of *O. gratissimum* and *L. nepetifolia* extracts on incidence and severity of Ascochyta blight (*Phoma exigua*) on French bean under greenhouse conditions are presented in Tables 2.0 and 3.0. There was a significant ($P \leq 0.05$) difference in disease incidence when the extracts of *O. gratissimum* and *L. nepetifolia* were applied 48 h before and after inoculation with Ascochyta blight (*P. exigua*) pathogen in the greenhouse (Table 2.0). Disease incidence was high when the extracts were applied 48 HAI compared to when the extracts were applied 48 HBI.

Table 2.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on incidence of Ascochyta blight of French bean under greenhouse conditions at Egerton University (July – December, 2014).

	Percentage (%) disease incidence (PDI) ††			
	48 HBI		48 HAI	
Rate (w/v)/ Plant species	OG	LN	OG	LN
Water (0.0)	79.2 ± 11.0	79.2 ± 11.0	100.0 ± 0.0	100.0 ± 0.0
Carbendazim	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.25	33.3 ± 4.2	62.5 ± 7.2	100.0 ± 0.0	100.0 ± 0.0
2.5	29.2 ± 11.0	45.8 ± 4.2	87.5 ± 12.5	87.5 ± 12.5
5.0	4.2 ± 4.2	12.5 ± 7.2	50.0 ± 0	87.5 ± 12.5
10.0	16.7 ± 4.2	16.7 ± 8.3	50.0 ± 20.4	62.5 ± 12.5
20.0	29.2 ± 4.2	8.33 ± 4.2	75.0 ± 14.4	12.5 ± 12.5

†† The values are the Means ± SE of three replicates in two separate experiments. HBI = Hours before inoculation, HAI = Hours after inoculation, OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Extracts of *O. gratissimum* at a rate of 5.0 mg ml^{-1} and *L. nepetifolia* at a rate of 20 mg ml^{-1} resulted in low disease incidences of 4.2 and 8.3%, respectively, when applied 48 HBI

(Plate 4.0 a). There was no significant ($P \leq 0.05$) difference among the other rates tested, although they were still better than the negative control. Application of the two plant extracts 48 HAI resulted in insignificant ($P \leq 0.05$) disease incidence responses compared to the negative control (Plate 4.0 b), except for *L. nepetifolia* at 20 mg ml^{-1}. The rate of pathogen spread within the pots was fast and was not significantly different from the negative control pot. This was an indication that the extracts were effective against the pathogen more as protectant agents than eradicants. They thus can be used to effectively protect the plant against the pathogen when conditions are favourable for the disease development. Carbendazim completely controlled disease development in the experiment.

(a) (b)

Plate 4.0: French bean plants (a) sprayed with 20 mg ml^{-1} *Leonotis nepetifolia* and (b) in negative control in the greenhouse

Table 3.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on severity of Ascochyta blight on French bean under greenhouse conditions at Egerton University (July – December, 2014)

	(DSI) Disease severity index (%) ††			
	48 HBI		48 HAI	
Rate (w/v)/ Plant species	OG	LN	OG	LN
Water (0.0)	50.5 ± 6.1	50.5 ± 6.1	81.9 ± 5.3	81.9 ± 5.3
Carbendazim	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.25	10.7 ± 0.5	25.9 ± 1.7	52.8 ± 5.8	68.1 ± 2.7
2.5	10.7 ± 2.3	18.1 ± 1.4	41.7 ± 3.6	50.0 ± 3.9
5.0	1.4 ± 1.3	3.7 ± 1.9	13.9 ± 1.6	33.3 ± 5.1
10.0	4.6 ± 1.2	5.1 ± 2.6	6.9 ± 2.7	27.8 ± 2.3
20.0	6.0 ± 1.2	1.9 ± 1.2	13.9 ± 3.6	8.3 ± 5.3

†† The values are the Means ± SE of three replicates in two separate experiments. HBI = Hours before inoculation, HAI = Hours after inoculation, OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

There was a significant ($P \leq 0.05$) difference in disease severity when the plant extracts were applied 48 h before and after inoculation (Table. 3.0). Disease was more severe when the treatments were applied 48 HAI compared to application 48 HBI. High disease incidence and severity in experiment where extracts were applied 48 HAI with pathogen led to high disease development and even complete death of the plants before flowering (Plate 5.0 a). More than 90% of the plants in the experiment where extracts of the two test plants were applied 48 HAI showed disease symptoms 14 DAI. Those that had not been attacked within the first 14 days, developed symptoms later and their disease severity increased to high levels with time. Generally, disease development and disease progress was faster in pots where the extracts were applied 48 HAI compared with 48 HBI.

(a) (b)

Plate 5.0: French bean plants (a) attacked by Ascochyta blight (b) healthy French bean plant in the greenhouse

(b) Field experiments

There was a significant ($P \leq 0.05$) difference in the incidence and severity of Ascochyta blight in the two sites where the experiments were conducted. There was low disease incidence and severity for the time periods (14, 21 and 28 days) when disease progress was being monitored (Tables 6.0 and 7.0). The disease was very severe in the negative control plot where distilled water was applied. Complete death of plants was recorded in some plots where neither plant extract nor Carbendazim was applied. Distilled water provided moisture to French bean leaves and other tender parts of the plant thus providing conducive environment for conidia germination, disease development and spread to other parts of the plant. Disease incidence in the field was considerably lower in the plots where the plant extracts had been applied compared to the negative control plot. Extracts of *O. gratissimum* and *L. nepetifolia* at 10.0 mg ml^{-1} resulted in disease incidences of 35.9% and below (Table 4.0) at Egerton University experimental site. These results were lower compared to disease incidence in negative control plots with over 80% disease incidence.

Table 4.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on incidence of Ascochyta blight on French bean under field conditions at Egerton University (July – December, 2014)

Rate (w/v)/ Plant species	Percentage (%) disease incidence (PDI)			
	OG	% DR	LN	% DR
Water (0.0)	80.4 ± 5.1	0.0	80.4 ± 5.1	0.0
Carbendazim	2.8 ± 1.4	77.6	2.8 ± 1.4	77.6
1.25	54.4 ± 2.2	26.0	68.1 ± 3.1	12.3
2.5	45.5 ± 3.3	34.9	57.4 ± 1.4	23.0
5.0	39.3 ± 1.1	41.1	51.9 ± 0.4	28.5
10.0	35.7 ± 1.9	44.7	35.9 ± 2.8	44.5
20.0	37.5 ± 1.4	42.9	35.9 ± 3.5	44.5

†† The values are the Means ± SE of three replicates in two separate experiments. OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*, DR (%) = Disease Reduction. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

The other lower rates of the two test plants also resulted in a significant ($P \leq 0.05$) reduction in disease incidence to levels lower than the negative control. However, the synthetic fungicide (Carbendazim) was still superior to all the concentrations of the two test plants, with a very low disease incidence of 2.8%. The overall results on disease incidence and severity were relatively similar to those observed in KALRO – Njoro experimental site (Table 5.0 and Figures 2.0 & 3.0).

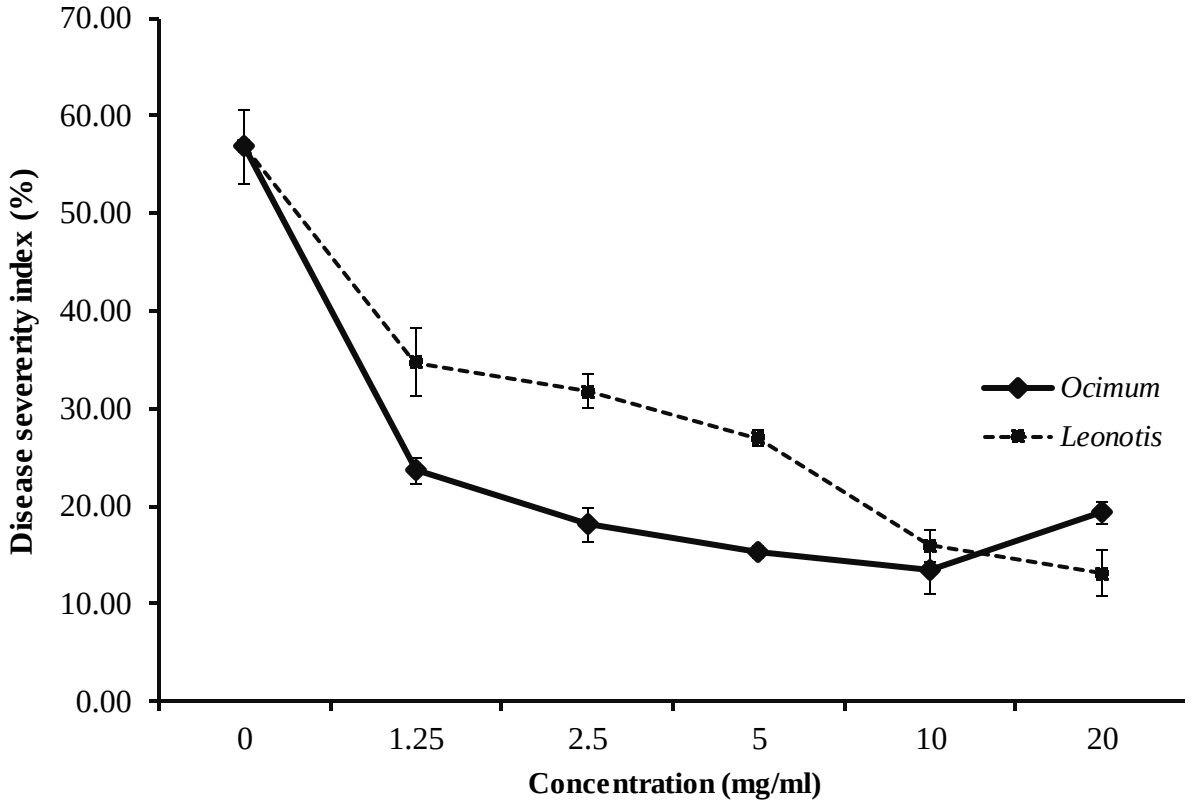

Figure 2.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on severity of Ascochyta blight on French bean under field conditions at Egerton University (July – Deccember, 2014)

Ocimum gratissimum, at 10.0 mg ml^{-1}, showed the highest antifungal activity as it resulted in reduction of disease severity to 13.5% (Fig. 2.0). The lower rates of *O. gratissimum* also showed substantial reduction in disease severity to below 23.6%. *Leonotis nepetifolia* showed high antifungal activity at the highest concentration of 20 $mgml^{-1}$, resulting in reduction of disease severity to 13.1% (Fig. 2.0). There was no significant ($P \leq 0.05$) difference between the activities of *O. gratissimum* at 10 mg ml^{-1} and that of *L. nepetifolia* at 20 mg ml^{-1} (Fig. 2.0). There was a steady decline in Ascochyta blight severity across the sites with an increase in the concentration of the extracts (Fig. 2.0 and 3.0), except for *O. gratissimum* at 20 mg ml^{-1} and 10 mg ml^{-1} at Egerton University and KALRO – Njoro, respectively. Generally, extract of the two plant showed a dose-dependent activity against in controlling the severity of Ascochyta bight in the field.

Table 5.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on Ascochyta blight incidence on French bean under field conditions at KALRO - Njoro (July – December, 2014)

	Percentage (%) disease incidence (PDI)			
Rate (w/v)/ Plant species	OG	% DR	LN	% DR
Water (0.0)	78.3 ± 4.1	0.0	78.3 ± 4.1	78.3
Carbendazim	3.2 ± 2.1	75.1	3.2 ± 2.1	75.1
1.25	52.9 ± 2.6	25.4	53.9 ± 1.9	24.4
2.5	43.6 ± 5.6	34.7	56.8 ± 2.1	21.5
5.0	37.7 ± 2.6	40.6	70.2 ± 1.6	8.1
10.0	41.2 ± 3.4	37.1	49.7 ± 8.6	28.6
20.0	43.6 ± 2.8	34.7	43.6 ± 2.8	34.7

†† The values are the Means ± SE of three replicates in two separate experiments. OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*, DR (%) = Disease Reduction. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Disease incidence was lower in the plots where extracts of *L. nepetifolia* at 20.0 mg ml^{-1} and *O. gratissimum* at 5.0 mg ml^{-1} were applied. This resulted in disease incidences of 43.6 and 37.7% for *O. gratissimum* and *L. nepetifolia,* respectively (Table 5.0). These were almost two times lower than in the negative control plots with over 78% incidence. However, there was no significant ($P \leq 0.05$) difference in the effect of the other botanical extract rates tested against Ascochyta blight incidence in the two experimental sites.

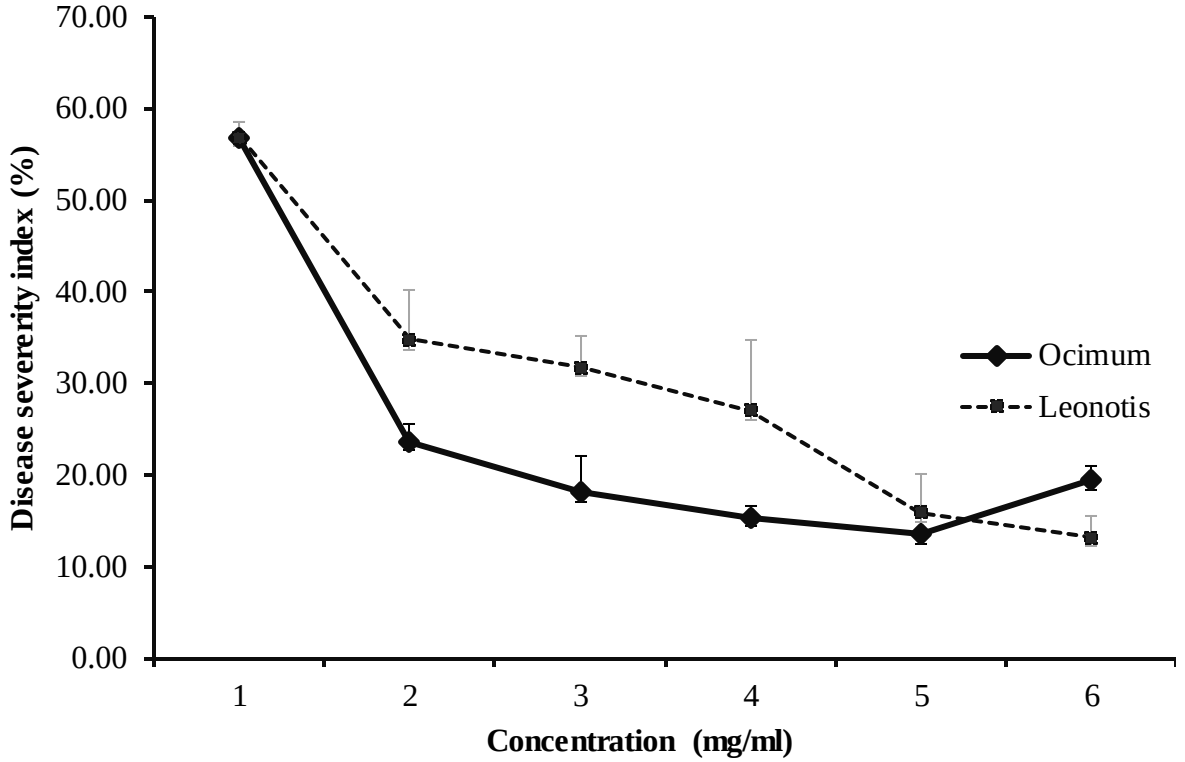

Figure 3.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on severity of Ascochyta blight on French bean at KALRO – Njoro in July – December, 2014

Application of the plant extracts resulted in lower disease severity in the field at KALRO – Njoro despite of the high disease incidences in the plots. Extracts of *O. gratissimum* resulted in considerably lower severity with no significant ($P \leq 0.05$) difference in the activity at all the test rates of *O. gratissimum* (Fig. 3.0). Extracts of *L. nepetifolia* were not very effective at reducing disease spread at lower concentrations and thus the higher severity at lower concentrations. However, *L. nepetifolia* extract was effective ($P \leq 0.05$) at higher concentrations of 10.0 and 20.0 mg ml^{-1} resulting in disease severity of less than 21.1% (Fig. 3.0). Ascochyta blight was so severe in the negative control plot (Plate 6.0) at 21 days after inoculation relative to the plots where the two test plant extracts had been applied.

(a) (b)

Plate 6.0: Healthy French bean (a) sprayed with 20 mg ml^{-1} *Leonotis nepetifolia* (b) Ascochyta blight attacked plant in negative control plot in the field

There was a significant ($P \leq 0.05$) difference in the rate of disease development with time between the two experimental sites (Table 6.0 and 7.0). Disease spread and severity was high in Egerton University site compared to KALRO - Njoro during the time this study was conducted. The field at Egerton University had high inoculum load compared to KALRO - Njoro since the neighbouring plots had common bean trials that had been ongoing for quite some time. Moreover, overseasoning bean debris also served as a source of inoculum in addition to the artificially inoculated pathogen load. On the contrary, the experiment plot at KALRO – Njoro was at an isolated area where only cereals (wheat and barley) had been planted prior to the experiment. The inoculum load was, therefore, low with limited possibility of external pathogen transfer to the field apart from the artificially inoculated load.

Table 6.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on Ascochyta blight progress at KALRO – Njoro field (July – December, 2014)

	Disease severity index (DSI) (%) ††					
	Ocimum gratissimum			*Leonotis nepetifolia*		
Rate/DAI	14	21	28	14	21	28
Water (0.0)	60.2 ± 1.7	63.2 ± 0.6	76.9 ± 1.9	60.2 ± 1.7	63.2 ± 0.6	76.9 ± 1.9
Carbendazim	0.5 ± 0.3	1.2 ± 0.3	12.8 ± 1.6	0.5 ± 0.3	1.2 ± 0.3	12.8 ± 1.6
1.25	23.5 ± 2.0	28.9 ± 1.3	48.3 ± 4.3	30.2 ± 5.4	34.9 ± 5.0	52.4 ± 0.8
2.5	22.3 ± 3.9	19.8 ± 4.4	35.7 ± 4.0	32.5 ± 3.4	34.6 ± 1.9	51.7 ± 2.6
5.0	16.9 ± 1.3	19.2 ± 1.9	36.5 ± 4.9	30.3 ± 7.7	40.6 ± 5.7	61.6 ± 2.5
10.0	19.9 ± 3.3	17.0 ± 1.0	38.7 ± 1.7	21.1 ± 4.1	22.4 ± 2.8	37.6 ± 3.1
20.0	23.2 ± 1.7	23.6 ± 1.5	40.5 ± 1.9	17.9 ± 2.5	19.4 ± 2.9	37.5 ± 3.3

†† The values are the Means ± SE of three replicates. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Table 7.0: Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on Ascochyta blight progress at Egerton University field (July – December, 2014)

	Disease severity index (DSI) (%) ††					
	Ocimum gratissimum			*Leonotis nepetifolia*		
Rate/DAI	14	21	28	14	21	28
Water (0.0)	56.8 ± 3.8	77.2 ± 2.4	87.3 ± 3.0	56.8 ± 3.8	77.2 ± 2.4	87.3 ± 3.0
Carbendazim	0.4 ± 0.3	13.1 ± 0.9	30.1 ± 1.8	0.4 ± 0.3	13.1 ± 0.9	30.1 ± 1.8
1.25	23.6 ± 1.3	43.9 ± 2.0	65.4 ± 3.1	34.7 ± 3.5	55.6 ± 2.6	70.8 ± 0.4
2.5	18.1 ± 1.7	39.1 ± 2.0	58.2 ± 6.3	31.8 ± 1.8	46.8 ± 1.9	61.1 ± 5.6
5.0	15.4 ± 0.7	36.8 ± 2.8	53.9 ± 6.2	26.9 ± 0.7	46.3 ± 3.0	63.2 ± 6.5
10.0	13.5 ± 2.4	30.5 ± 1.8	51.1 ± 0.6	15.9 ± 1.6	31.8 ± 0.2	53.8 ± 5.1
20.0	19.3 ± 1.6	33.9 ± 3.1	53.4 ± 3.2	13.1 ± 2.3	31.6 ± 3.9	48.4 ± 2.8

†† The values are the Means ± SE of three replicates. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Application of the two plant extracts resulted in a significant ($P \leq 0.05$) reduction in the incidence and severity of Ascochyta blight in the two experimental fields (Tables 6.0 and 7.0). High disease incidence in the field was highly positively correlated to disease severity and the two parameters resulted to reduction in average amount of fresh French bean yield per plot (Table 8.0). There was a significant ($P \leq 0.05$) positive correlation between disease incidence and severity in the two sites. Higher disease incidence resulted in a corresponding high severity within the plot, resulting in lower yields within the affected plots in both fields (Tables 9.0 and 10.0).

Table 8.0: Correlation analysis for site, botanical extracts, percent disease incidence and severity index and yield of French bean (July – December, 2014)

	Site	Botanical Extract	% Disease Index	Disease severity index	Yield
Site	1.00	0.00	0.07	0.09	- 0.40*
Botanical Extract		1.00	- 0.18*	- 0.18*	0.09*
% Disease Index			1.00	0.89***	- 0.40**
Disease severity index				1.00	- 0.42**
Yield					1.00

*, ** represent differences at 0.05 and 0.01 level of significance, respectively

There were significant ($P \leq 0.05$) differences in yields of French bean between the two experimental sites (Tables 9.0 and 10.0). Higher yields were recorded at the Egerton University field compared to KALRO – Njoro. The two test plants were equally effective in controlling the incidence and severity of Ascochyta blight in the field. Consequnetly, the rates were highly effective in controlling incidence and severity of Ascochyta blight in the field resulted in corresponding higher yields of French bean within the plots. This was evident across the two sites where the studies were conducted.

Table 9.0: Yield of French bean ($KgHa^{-1}$) from KALRO – Njoro field (July – December, 2014)

	Yield of French bean ($KgHa^{-1}$)	
Rate (w/v)/ Plant species	OG	LN
Water (0.0)	2036.4 ± 213.3	2036.4 ± 213.3
Carbendazim	3326.9 ± 309.3	3326.9 ± 309.3
1.25	2573.9 ± 543.8	2449.3 ± 256.2
2.5	2903.3 ± 374.5	2438.7 ± 209.9
5.0	2804.8 ± 404.9	3051.3 ± 515.3
10.0	2592.4 ± 600.2	2710.9 ± 198.1
20.0	2511.8 ± 473.4	2850.3 ± 541.6

†† The values are the Means ± SE of three replicates in two separate experiments. OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

Generally French bean yields at Egerton University field were higher compared to yield from KALRO – Njoro. This could partly be attributed to high soil fertility in the Egerton University field since common bean had been grown in the field prior to the study. This resulted in luxurious growth of the crop and thus high yields, and relatively high Ascochyta blight incidence.

Table 10.0: Yield of French bean ($KgHa^{-1}$) from Egerton University field (July – December, 2014)

Rate (w/v)/ Plant species	Yield of French bean ($KgHa^{-1}$)	
	OG	LN
Water (0.0)	1996.4 ± 400.7	1996.4 ± 400.7
Carbendazim	3953.8 ± 469.2	3953.8 ± 469.2
1.25	2703.0 ± 355.6	4337.1 ± 527.3
2.5	2806.7 ± 572.4	3324.8 ± 574.2
5.0	3498.1 ± 1030.3	3142.5 ± 352.6
10.0	3820.5 ± 192.2	3912.5 ± 97.9
20.0	3672.1 ± 485.9	3972.0 ± 614.9

†† The values are the Means ± SE of three replicates in two separate experiments. OG = *Ocimum gratissimum*, LN = *Leonotis nepetifolia*. Figures within a column whose SE values do not overlap are statistically different at ($P \leq 0.05$)

The efficacy of different rates of methanol extracts of *O. gratissimum* and *L. nepetifolia* on incidence and severity of Ascochyta blight of French bean were studied *in vivo*. The time of the application of the extracts resulted in variation in the activity of the two test plants. Disease incidence and severity were effectively reduced when the plant extracts were applied 48 hours before inoculation with the pathogen. This indicates that the extracts of the two test plants act as protectants rather than eradicants in controlling Ascochyta blight. These findings and conclusions conform to those reported by Amadioha and Obi (1998) on the activity of *Piper betle*, *Ocimum sanctum* and *Citrus limon*. Methanol extract of the two plants were active at higher concentration than the reported MIC values when the treatment were applied before pathogen inoculations. Pinto *et al.* (2010) reported antifungal activity of *O. gratissimum* at concentrations lower than the ones reported in this study. The variability in findings could be attributed to the chemical characteristic of the solvent used, the method used in the extraction and the diversity in the chemical composition of the natural product (Pinelo *et al.*, 2004).

Moreover, different pathogens may respond differently to the same active components from the same plant (Moharam *et al*., 2012).

The extent of disease severity in each plot was proportionate to the corresponding time when the extract was applied and the PDI in each plot. Disease incidence and development in the field was faster compared to the greenhouse study with time after treatment application and inoculation with *P. exigua* suspension. The fast spread and high disease incidence in the field can be attributed to favourable conditions compared to those in greenhouse. Moreover, pathogen dispersal in the field is more enhanced due to splashing effect of rain droplets and dispersal by wind aerosol spray. The lower diseased leaves also increased the source of inoculum in the field as they dropped and the pathogen was washed and splashed by water (Davidson *et al*., 2011). By extension, disease severity was high in the field compared to the greenhouse. The difference can be attributed to controlled water/wind dispersal and water application that results in reduced pathogen spread in the greenhouse. This explains the high severity recorded in the plots where ineffective concentrations of the extract were applied and on experiments where extracts were applied after inoculation.

The effect of the two plant extracts in the field was concentration dependent. Lower concentrations of *O. gratissimum* were relatively more effective than higher concentrations in controlling the incidence and severity of Ascochyta blight. This can possibly be due to reduced solubility of the extract at high concentration resulting in reduced efficiency in application using manual sprayer equipment (Sultana *et al*., 2009). This therefore translates to low quantities of the active ingredients per unit volume of the suspending solvent (distilled water). Higher concentration of *L. nepetifolia* resulted in low disease incidence and severity in the field. The findings of this study are in line with previous studies that have confirmed that the antifungal activity of plant extracts is concentration – dependent (Moharam *et al*., 2012).

Disease severity in the field intensified with time from the first treatment application and the subsequent intervals when disease progress was monitored. This could be due to fast degradation of the volatile active secondary metabolites in the plant extracts. The fast degradation of plant secondary metabolites to environmentally harmless products has been one of the widely held beneficial attribute of indigenous plant extracts (Singh *et al*., 2012). However, this property of plant-derived antifungal agents reduces their effectiveness in

controlling phytopathogens in the field thus necessitating several repeat applications for the period the plant is in the field. Extracts of *L. nepetifolia* and *O. gratissimum* reduced the incidence and severity of Ascochyta blight by inhibiting conidial germination and mycelial growth on French bean. These findings are in conformity to the finding of Dellavalle *et al.* (2011) on the antifungal activity of medicinal plant extracts against *Alternaria* spp.

There was a positive correlation ($P \leq 0.05$) between yield reduction and the high disease incidence and severity in the respective plots. Bretag *et al.* (1995) reported a close correlation between disease severity and reductions in yield of fields attacked by Ascochyta blight. The disease resulted in a significant reduction in active photosynthetic leaf area and thus a decrease in the photosynthetic efficiency of the remaining patches of green leaf area (Garry *et al.*, 1998). In field pea, Bretag *et al.* (2000) found that disease severity varied considerably between years and fields in the same region and they attributed this to differences in climatic conditions and in the availability of inoculum. In chickpea, Nene (1981) quoted yield losses ranging from 10 – 20 to 50 – 70 % depending upon the country and the year. Similar ranges of yield losses have been published for Ascochyta blight diseases on other crops (Bretag, *et al.*, 1995). Very few publications list yield loss assessments as an explicit objective, and consequently there is lack of yield loss data collected under well-characterised conditions.

The characteristic Ascochyta blight symptom of die-back and girdling of the stem results in reduced efficiency of the plant to transport the photosynthates from the source to the sinks. This could have contributed to low yields in plots with high disease due to interference with photosynthesis and the translocation of carbohydrates and nitrogenous compounds from the source (leaf) to the sinks (pods) (Garry *et al.*, 1996). Yield reduction could have also been contributed by interplay of other factors in the field such as high incidence of pests during the period of study. This study demonstrates the effectiveness of methanol extracts of *L. nepetifolia* and *O. gratissimum* in managing the incidence and severity of Ascochyta blight on French bean. By extension, the extracts resulted in improved yields per plot.

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

1. Methanol extracts of *L. nepetifolia* and *O. gratissimum* possess dose–dependent antifungal activity against *Phoma exigua* both *in vitro* and *in vivo*.
2. The extracts of the two test plants were effective in reducing the incidence and severity of Ascochyta blight and increasing the yield of French bean when applied before inoculation under greenhouse and field conditions.

5.2 RECOMMENDATIONS

1. Extracts of *L. nepetifolia* and *O. gratissimum* can be used as natural alternatives to the synthetic fungicides in managing Ascochyta blight of French bean. The two plants used in this study should therefore, be promoted for adoption in controlling Ascochyta blight and other fungal diseases of French bean.

2. The extraction method used in this study may not be practical for farmers and thus may be a hindrance to adoption of the botanicals for disease control. Therefore, other extraction methods need to be tested to identify one that gives optimal antifungal activity from the extracts, and that can be adopted by the farmers. Moreover, the scope of organic solvents used in the extraction should be widened if the extracts are to be suspended in water for the final use, methanol extracts of the test plants have low solubility in water.

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6.0 APPENDICES

Appendix 1. Mean squares for percentage disease incidence (PDI) and disease severity index (DSI) for treatment application 48 h before and after inoculation under greenhouse conditions

		48hrs before		48hrs after	
Source of variation	**df**	**PDI**	**DSI**	**PDI**	**DSI**
Replicate	2	169.3	22.9	1458.3*	164.6*
Treatment	11	1743.6**	621.1**	3939.4**	2092.2**
Error	22	126.7	15.3	397.7	45.8
CV		40.0	33.8	29.9	20.9

*, ** Significant at ($P \leq 0.05$) and ($P \leq 0.01$) respectively.

Appendix 2. Mean squares for percentage disease incidence (PDI) at 14, 21 and 28 days after treatment application and inoculation at Egerton University and KALRO – Njoro, 2014

Source of variation	**df**	**PDI 1**	**PDI 2**	**PDI 3**
Site	1	148.05*	8.16	808.21**
Replicate	2	107.23	86.73	11.00
Botanical	1	1268.77**	1332.83**	912.21**
Rate	4	640.30**	910.51**	694.14**
Site*Rate	4	167.83**	85.12	28.89
Botanical*Rate	4	236.29**	236.07**	249.36*
Error	43	35.58	34.30	73.62
CV		12.49	11.02	12.85

*, ** Significance at ($P \leq 0.05$) and ($P \leq 0.01$) respectively. PDI 1, PDI 2 and PDI 3 represent percent disease index at 14, 21 and 28 days after inoculation

Appendix 3. Mean squares for disease severity index (DSI) at 14, 21 and 28 days after treatment application and inoculation at Egerton University and KALRO – Njoro, 2014

Source of variation	**df**	**DSI 1**	**DSI 2**	**DSI 3**
Site	1	96.47	2767.74**	2895.51**
Replicate	2	62.54	51.13	54.44
Botanical	1	519.67**	764.48**	475.40**
Rate	4	253.95**	494.85**	434.20**
Site*Rate	4	28.11	27.27	32.02
Botanical*Rate	4	180.00**	158.15**	198.76**
Error	43	24.93	25.49	47.74
CV		12.49	15.38	13.55

*, ** Significance at ($P \leq 0.05$) and ($P \leq 0.01$) respectively. DSI 1, DSI 2 and DSI 3 represent percent disease severity index at 14, 21 and 28 days after inoculation

Appendix 4. Authors own Publications

a) Refereed Journals

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Antifungal Activity of Methanol Extracts of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. against Ascochyta Blight (*Phoma exigua*) on French Bean

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ABSTRACT

The potential of crude plant extracts as antimicrobial agents has been demonstrated both in animal and plant health. The objective of this study was to evaluate the fungicidal efficacy of methanol extracts of *Leonotis nepetifolia* L. and *Ocimum gratissimum* L. against Ascochyta blight (*Phoma exigua*) pathogen *in vitro*. Composite leaves and tender stems were shade-dried, ground to fine powder using electronic hammer mill and extracted using methanol. Two-fold serial dilution was performed to obtain final concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.31 and 0.16 mg mL^{-1} (w/v) used in the laboratory bioassays against the pathogen. The experiment was performed according to Kirby-Bauer disc diffusion method in a Completely Randomised Design (CRD) in triplicate. The extracts showed antifungal activity at all the concentrations tested with Minimum Inhibitory Concentration (MIC) of 1.25 mg mL^{-1} for both *L. nepetifolia* and *O. gratissimum*. There was plant extract concentration-dependent increase in the diameter of zones of inhibition. The average diameter of zones of inhibition for the two plant extracts at 40.0 mg mL^{-1} were comparable to the synthetic fungicide (Carbendazim) used as positive control. These results showed that the two plant extracts possess antifungal activity against the pathogen and can be used as natural alternatives to the synthetic fungicides in the management of Ascochyta blight on French bean.

Key words: Ascochyta blight, *Leonotis nepetifolia*, *Ocimum gratissimum*, *Phoma exigua*

b) Conference papers

1. S. O. Ochola, J. O. Ogendo, I. N. Wagara, J. O. Ogweno, J. G. Nyaanga and K. O. Ogayo. Effect of methanol extracts of *Leonotis nepetifolia* and *Ocimum gratissimum* on the incidence and severity of Ascochyta blight on French bean. 9TH Egerton University International Conference, 25TH – 27TH March, 2015, held at Egerton University, Kenya
2. S. O. Ochola, J. O. Ogendo, I. N. Wagara, J. O. Ogweno, J. G. Nyaanga and K. O. Ogayo. In vitro evaluation of *Leonotis nepetifolia* and *Ocimum gratissimum* extracts against *Colletotrichum lindemuthianum*. The 4TH RUFORUM Biennial Conference in Maputo – Mozambique, 21ST – 26TH July, 2014

3. S. O. Ochola, J. O. Ogendo, I. N. Wagara, J. O. Ogweno, J. G. Nyaanga and K. O. Ogayo. Indigenous practices for French bean production and management of spider mites and anthracnose among smallholder farmers in Nakuru County, Kenya. 8TH Egerton University International Conference, 26TH – 28TH March, 2014 held at Egerton University, Kenya.