EVALUATION OF ANTIFUNGAL ACTIVITY OF SOME INVASIVE ALIEN SPECIES PLANT EXTRACTS AGAINST COFFEE BERRY DISEASE (*Colletotrichum kahawae* Bridge and Waller) IN ETHIOPIA

M.Sc Thesis

By

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Jimma, Ethiopia

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Suse Merga Jirata

A Thesis

Submitted to School of Graduate Studies, Jimma University, College of Agriculture and Veterinary Medicine, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant Pathology

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APPROVAL SHEET

DEDICATION

To my beloved brother the late Teferra Merga and my father Merga Jirata, who had a great dream for my successes in education, but passed away at early age and few months before, without seeing any of those long journeys.

]

DECLARATION/ STATEMENT OF THE AUTHOR

I, the under signed, declare that this thesis is my original work and has not been presented for any institute or anywhere for the awards of any academic degree or diploma. All the sources of materials used for this thesis have been duly acknowledged. This thesis submitted in partial fulfillment of the requirement for an M.Sc degree at Jimma University College of Agriculture and Veterinary Medicine and is deposited at the University Library to be made available to borrowers under rule of the Jimma University library.

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LIST OF ACRONYMS ANDABRIVATIONS

CBD	Coffee Berry Disease
CRD	Completely Randomized Design
CSA	Central Statistics Authority
DI	Disease Index
FAO	Food and Agricultural Organization
GDP	Gross Domestic Product
ha	Hectare
hr	Hour
IAR	Institute of Agricultural Research
ICO	International Coffee Organization
IAS	Invasive Alien Species
JARC	Jimma Agricultural Research Center
JUCAVM	Jimma University College of Agriculture and Veterinary Medicine
M.a.s.l	Meter above sea level
MIC	Minimum Inhibition Concentration
PDA	Potato Dextrose Agar
Psi	Pressure per Square Inch
RCBD	Randomized Complete Block Design
SAS	Statistical analysis system
Spp.	Species
USA	United States of America
USD	United States Dollar
µm	Micrometer
µl	Micro litter
rpm	Revolution Per Million

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ABSTRACT

Coffee is one of the most important trade commodities in the world next to petroleum. Arabica coffee (Coffea arabica L.) is the world most important commercial coffee species. Coffee berry disease (CBD) caused by Colletotrichum kahawae is the major threat to Arabica coffee production in Ethiopia and the world. The use of fungicides against the disease has shown to induce negative effect on environment and result in the appearance of fungicide resistant pathogen biotype. It is also expensive for farmers in countries like Ethiopia. Thus it is important to look for alternative management strategies of the disease. This study aimed at evaluating the antifungal activities of aquous, ethanol and acetone extracts of five different invasive alien species in vitro against C. kahawae mycelia growth. Then we tested two of the best performed invasive plant extracts on detached green berries and seedlings in vivo against disease development applying the extracts at 3 different times of application (at the time of inoculation, 48 hrs before inoculation and 48hrs after inoculation) of the pathogen, in completely randomized design with three replications. The extracts were from Parthenium hysterophorus, Lantana camara, Prosopis juliflora, Eichhornia crassipes and Mimosa diplotricha. All the crude extracts showed in vitro antifungal activity at 20 %(w/v). The study indicated that the inhibitory effect of the extracts depends on the type of plant species used, solvent type and time of application of the extracts. Generally, L.camara and P.juliflora ethanol and acetone extracts significantly reduced radial growth of the pathogen compared to the control. L.camara reduced radial growth of the pathogen with 76.23 and 84.3%, and P.juliflora with 62.63 and 66.96% in 70% ethanol and acetone respectively. Furthermore, L.camara ethanol and acetone extracts reduced disease severity on detached green berries to 20 and 29.1% when applied before inoculation of the pathogen. Likewise, ethanol and acetone extract of L. camara applied before innoculation highly inhibited the effect of the pathogen,and shows highly significant difference as compared to the untreated control in vivo. The study indicated the possible use of extracts of L.camara as an alternative means of CBD management but further study at field conditions should be carried out to verify the result.

Key words*:* Biological Control, Coffee Disease, Coffee, Coffee Disease Control, Phytoproducts, Phytochemicals

1. INTRODUCTION

Coffee is a non-alcoholic and stimulant beverage crop, which belongs to the family *Rubiaceae* and the genus *Coffea*. It is not only one of the highly preferred international beverages, but also one of the most important trade commodities in the world next to petroleum (Labouisse *et al.,* 2008). On average, its trade generates about US $9.7 billion annually (Vega, 2008). In Ethiopia, the total area covered by coffee is about 1104300km^2, with a total production of 6008 (000bags) in crop year 2011 and provides a livelihood for 84.73 million population of coffee farming families (ICO database, 2011). Arabica coffee accounts for about 70% of the world coffee production and known for the preparation of high quality beverage (Anthony *et al*., 2002). Ethiopia is believed to be the country of origin of Arabica coffee and Arabica coffee is the only species grown in Ethiopia (Paulos and Demel, 2000).

Coffee is the single most important cash crop that has been contributing a lion's share to the Ethiopian economy (Arega *et al,* 2008). It is the major source of foreign currency for Ethiopia and contributes more than 35% of the total export earnings (FAO/WFP, 2008), over 5% of the GDP, 12% of the agricultural output, and 10% of the government revenues (CSA, 2009). Thus, it is a cornerstone in the export economy of the country. Despite the significant role that coffee plays in the economy of the country, the crop suffers from many production constraints.

Limiting factors of coffee production include three major fungal diseases, coffee leaf rust (or orange rust), Coffee berry disease (CBD) and coffee wilt disease or *tracheomycosis* caused by *Hemileia vastatrix* Berkeley and Broome, *Colletotrichum kahawae* Bridge and Waller and *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) respectively. Anteshia bag is also a major insect pest of coffee in Ethiopia (Martin *et al.*, 2002*)*. Coffee berry disease (CBD) is the top major disease of coffee in Ethiopia. It was first observed in Ethiopia in 1971 (Mulinge, 1973; Arega *et al.*, 2008). Coffee berry disease (CBD) is the major threat to Arabica coffee production since its outbreak (Arega *et al.*, 2008). It is an anthracnose of green coffee berry, caused by the infection of a fungal

pathogen *Colletrichum kahawae*. Tefestewold (1995) pointed out the concomitant occurrence of CBD in southwest and southeast of Ethiopia in the early 1970s. Furthermore, the disease spread to Shewa and Gamugofa in 1977 and Hararghe in 1978 (Van der Graaff, 1981). Eventually, it was also disseminated to other coffee growing areas.

CBD causes significant yield losses. The average national yield losses were about 28% between 1974 and 1978 as reported by Van der Graaff (1981) and Merdasa (1985). The average yield losses ranged from 51% to 81% from Wondogent, Gera and Jimma experimental plots (Merdasa, 1985). Similarly, in Hararghe, the losses were estimated to be as high as 100% (Tefestewold, 1995). Eshetu and Girma, (1992) estimated coffee yield loses exceeds 40% at Gera. The severity of CBD and the losses caused are often under estimated annually since young coffee berries drop off from the tree at an early stage of the disease (Tefestewold, 1995). By and large, CBD causes 25% national average crop losses to total harvestable coffee yield every year in Ethiopia (Eshetu *et al*, 2000). At present, CBD has rapidly spread to all coffee growing areas of Ethiopia and still significant crop loss is recorded (up to 100% on susceptible land races) although the magnitudes vary from place to place and from time to time (Arega *et al*, 2008).

The most important method of protecting the plant against fungal attack was the use of synthetic fungicides such as Daconil and Dealan (Critchtt and Merdasa, 1984). However, later these products including Dyrene and Octave were banned for a number of side effects (Eshetu *et al*, 2000). Thus, use of fungicides against CBD has been shown to induce negative effects on beneficial microorganisms that can antagonize the CBD pathogen (Masaba, 1991). The high cost of pesticides, the appearance of fungicide resistant pathogen biotype and other social and health related problems of the conventional agriculture on the environment have increased interest in sustainable agriculture and biodiversity conservation (Vander Vossen, 1985). As a result, there is an increase in the need to search for safe, efficacious and environmentally friendly bio-fungicides (Anonymous, 1987; Hostettman *et al.,* 2000).

Plants produce antifungal agents by secondary metabolism to protect themselves from fungal attack, and therefore many plant species possess substantial antifungal activity. Angeh (2006) developed a procedure that yielded excellent activity against plant fungal pathogens from *Melianthus comosus* in laboratory and in a preliminary field trial. Thus, the use of plant extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides based on the availability of natural products (Angeh, 2006).

Many workers have reported antimicrobial activities of plant extract (phytoproducts) and gaining due attention because of their proven attributes such as specificity, biodegradability and low toxicity (Adityachaudhur, 1991). In Ethiopia, information on antimicrobial activity of invasive plant species extracts against fungal phytopathogens is very scanty. However, the use of common invasive plant species on fungal phytopathogens have a great potential in suppressing various plant pathogenic fungi (Satya *et al*., 2005; Mdee, 2009) and that may serve as better biological alternative in substituting the employment of chemical fungicides. The use of Invasive alien species as a natural bio-fungicide has arena of advantages. For instance, IAS may be such successful competitors due to resistance towards different pathogens. These plants therefore contain active principles to resist fungal attach and has excellent antifungal activity (Enright, 2000).

In an approach toward the development of eco-friendly antifungal compounds, crude methanolic extracts of *Agapanthus africanus* leaf extracts were screened *in vitro* against eight economically important plant pathogenic fungi. Result shows that, mycelial growth was inhibited significantly in five test organisms, (100% or >97%) namely *Botrytis cinerea*, *Sclerotium rolfsii*, *Botryosphaeria dothidea*, *Rhizoctonia solani* and *Mycosphaerella pinodes* and showed high degree of control against *Fusarium oxysporum*(77%), *Pythium ultimum*(64%) and *Alternaria alternate* (60-80%). Subsequently, these crude extracts were tested *in vivo* in the green house against *Mycosphaerella pinodes*, the cause of black spot or *Ascochyta blight* in peas. Treatment of detached pea leaves with crude extracts both before and after inoculation with *M. pinodes* spore, resulted in significant differences among extracts in suppressing lesion development, treatment of leaves with extracts 30 min before

inoculation was visible lesion development than the post inoculation treatment, but at different MICs (Tegenye *et al*., 2008).

Aqueous, methanol and ethanol extracts of 46 plants belonging to 32 different families of the plant kingdom were screened for antifungal activity against eight important species of *Fusarium* viz., *Fusarium aquiseti*, *F. oxysporum*, *F. moniliforme, F. semitectum, F. graminiearum, F. proliferatum, F. solani* and *F. lateritium* isolated from maize, paddy and sorghum seeds. Among the 46 plants screened aqueous extracts of *Acacia* nilotica, *Achras zapota, Datura stramonium, Emblica officinalis, Eucalyptus globules, Lawsonia inemis, Mimusops elengi, Peltophorum pterocarpum, Polyalthia longifolia, Prosopis Juliflora Pucina granatum* and *Sygigium cumini* have recorded significant antifungal activities against one or the other Fusarium species tested. Among the different solvent extracts tested against *F. proliferatum*, methanol and ethanol extracts of *A. nilotica*, *M. elengi* and *P. Juliflora* r3secorded highly significant antifungal activity (Satish *et al*., 2009).

Effect of plant extracts of three different species; *Ocimum gratissimum, Acalypha wilkesiana* and *Acalypha macrostachya* were assessed for inhibitory effect on the radial growth of *Cercospora purpurea* of *Persea americana* (Avocado). Results showed that the ethanolic extract showed a total inhibition of the pathogen, while for the aqueous extract, inhibition was over 60%. The use of unsterilized water for extraction also showed appreciable inhibition of over 60% on the radial growth of the fungus (Ogbo and Oyibo, 2008).

Generally, the cost of cultivating the plant has prompted the investigation of ready available material such as weeds. Furthermore, if IASs are used as raw materials for plant-derived fungicides then there are large quantities of materials ready available for use. Additionally collection may protect indigenous plants and minimizes the impacts of IAS (management by utilization) on crop production, biodiversity and the environment; and at the same time may create economic uses and jobs based on these unwanted species.

General objectives

- To investigate the antifungal activity of some invasive alien species (IAS) plant extracts against coffee berry disease (CBD pathogen (*C. kahawae*).

Specific objectives

- To evaluate the antifungal activity of IAS plant extracts and solvent type on the radial growth of *C. kahawae*.
- To determine the minimum inhibition concentration (MIC) for the most effective IAS plant extracts against the radial growth of the fungus *C. kahawae in vitro* test.
- To evaluate the effect of IAS plant extracts, solvent type and application time on the development of CBD on detached green coffee berries and seedlings.

2. LITERATURE REVIEW

2.1Historical Background of Coffee

Coffee belongs to the genus *Coffea*, in the *Rubiaceae* family. It is a non alcoholic stimulant beverage crop plant. It is not exactly known when and how coffee was initially used as a stimulatory beverage in its home–land, the African Continents (Sylvain, 1955), and distributed to other part of the world (Berthaud and Charries, 1988). The stimulatory effect of roasted coffee beans were known to the native of Africa when the Arabs brought coffee (*Coffea arabica* L.) beans from Ethiopia to Yemen probably through Arabian and Persian invader during the 13th century and established the first plantation. The consumption of coffee as a stimulatory beverage was expanded through the establishment of coffee houses in Turkey (1554), Venice (1615), France (1664), Vienna (1650), London (1652), and the rest of the world (Waller *et al*., 2007).

Coffee was taken from Ethiopia to Yemen perhaps as early as AD 575 (Wellman 1961). Following its introduction to Yemen and the great popularity of the beverage, the cultivation of coffee was extended to various Asian and Latin American countries. The Dutch were the first Europeans to take seed from Yemen and then to Java during the 1690s (Wrigley, 1988). From Java it was spread to Ceylon (now Sri-Lanika) in 1700. The British smuggled seven coffee seeds from Yemen and established them in India. The Dutch took one or a few plants from Java to botanical garden in Amsterdam (Bayetta, 2001), which becomes the necessary relay station for introduction to various Latin American countries.

2.2 Economic Significance and Production Status of Coffee

The increase in the use of coffee as one of the best stimulant beverages, has favored the expansion of coffee cultivation and commerce. There are about 103 species of genus *Coffea*, all exclusively restricted to the tropical forests of Africa, Madagascar and islands of the Indian Ocean (Mascarene Islands). Of all the species, at present, only two economic species of coffee namely *Coffea arabica* L. (Arabica coffee) and *Coffea canephora Piere* ex

Froehne (Robusta coffee) have commercial value in the world coffee industry. *Coffea arabica* is the only species occurring in Ethiopia and is geographically isolated from the rest of the *Coffea* species. It is naturally restricted to two isolated mountain forests on the western and eastern sides of the Great Rift Valley in southern Ethiopia. It is the most popular and widely cultivated coffee species in the world, the more important economic species because of its superior quality, dominating 70% of total coffee production. The remaining proportions come from Robusta coffee, which originates from the equatorial lowland forest of west and central Africa (Girma *et al*., 2008).

From a commercial point of view, it is one of the most important agricultural products in the world in economic terms, being second only to petroleum among the global commodities and many countries depend heavily on the revenue from coffee exports. It generates 10 to12 billion US dollars annually to the producing countries and provides job opportunities for some 20 million people who grow, process, distribute and market the product (Wrigley, 1988). The world production of coffee has increased steadily from about four million Mg in the mid 1960's to about 6.5 million in the 1990's.

Brazil is the world's leading producer and exporter of coffee and the second largest consumer (Bayetta, 2001). The second producer is Vietnam followed by Indonesia and Colombia according to ICO database (2012). Ethiopia is the world's fifth largest producer of coffee, and Africa's top producer (ICO database 2012). Ethiopia is usually among the top ten countries and produces coffee annually including forest, garden and plantation coffee. 75% of the coffee is produced as garden coffee by small farmers, 25% is collected in forest and semi forest coffee systems, and merely 5% is plantation coffee (EIAR, 2008). Coffee is by far Ethiopia's most important export crop (one-third is exported to Germany) and, with 41%, contributes decisively to the country's foreign currency income. Furthermore, the livelihood of same 15 million people directly or indirectly depends on coffee (Behailu *et al.,* 2008).

2.3. Coffee Diseases in Ethiopia

In Ethiopia, production and productivity of coffee is significantly reduced by numerous diseases. Limiting factors of coffee production include three major fungal diseases, coffee leaf rust (or orange rust), coffee berry disease (CBD) and coffee wilt disease or tracheomycosis caused by *Hemileia vastatrix* Berkeley and Broome and *Colletotrichum kahawae* Bridge and Waller and *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) respectively (Martin *et al.*, 2002).

Coffee wilt disease or tracheomycosis caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) is becoming important in some regions of Central and West Africa, not only on Robusta but also on Arabica. It is a vascular disease causing yellowing and wilting of the trees. *Fusarium stilboides* Wollenw (telemorph: *Gibberella stilboides*), the causal agent of the coffee bark disease, is also present in some African countries, particularly in Ethiopia, Kenya, Malawi and Tanzania. Its characteristic symptom is a scaling of the bark leading to stem cankers and a progressive dying back of the whole tree (Martin *et al.*, 2002).

Other coffee leaf rust diseases (powdery, yellow rust or grey rust), caused by the fungus *Hemileia coffeicola* Maubl and Rog., have not been considered so important economically as leaf rust. The symptoms of the disease are characterised by a dusty or powdery coating of yellow uredosori covering the underside of the coffee leaves, in contrast to *H. vastatrix* that forms distinct blotches or pustules (Adejumo, 2005). Bean discoloration (*Pseudomonas syringae*), leaf blight (*Ascochyta tarda*), root-rot (*Armillaria mellea*), brown-eyespot (*Cercospora coffeicola*) and damping off diseases of seedlings (*Rhizoctonia* spp., and *Pythium* spp.), Fruit-rot (*Fusarium* spp.), and thread-blight (*Corticium kolleorega*) were recorded associated with coffee (Merdassa Ejetta, 1985; Eshetu *et al*. 2000). Coffee berry disease (CBD) is the top major disease of coffee in Ethiopia, which attack mainly the green berries of coffee. CBD was first observed in Ethiopia in 1971 (Mulinge, 1973). Since then it spread and was found in all coffee growing areas were favorable environmental conditions.

2.3.1 Coffee Berry Disease (CBD)

2.3.1.1 Origin and spread

Coffee berry disease (CBD) is an anthracnose of the green and ripening berries caused by the fugues *Colletorichun kahawae* sp nov. It was first detected in 1922 in the Sotik area, south of Mountain Elgon in western Kenya, on newly established plantations of imported Arabica coffee with a narrow genetic base. CBD is believed to have originated from Coffea eugenioids, a wild diploid coffee found in high altitude forests in Western Kenya and Eastern Uganda. The pathogen is thought to be a harmless parasite of *C. eugeniodes*, surviving as a micro-epiphyte on the bark of this coffee species (Muller, 1984).

By 1951, the disease was reported from all the main coffee growing areas of western districts and central provinces of Kenya. The pathogen then spread to Zaire in 1993 (Vander Vossen, 1985) and was subsequently reported in Cameroon in 1958 (Muller, 1984), in Uganda in 1959 on the slopes of Mt Elgon at an altitude of 1890 m, and in Tanzania in 1964. Arega et al., (2008) pointed out the concomitant spread of CBD to Southwest and Southeast of Ethiopia in the early 1970s. Furthermore, the disease extended to Shewa and Gamugofa and Harraghe areas of the country in the year 1971 and 1978, respectively (Hindorf and Arega 2006). In 1971 it appeared simultaneously in a number of pocket areas in the main coffee producing regions of Sidamo (Wondo Gent), Illubabor (Gore) and in 1972 in the Kaffa region (Wushi-Wush, near Bonga). In 1987, the disease spread to almost all the coffee growing regions (Bayetta, 2001).

The dispersal agents of the CBD pathogen vary, depending on the distance of movement. The conidia (spores) of *C. kahawae* are primarily water-borne. The Conidia are hard and firmly attached to the diseased tissue when dry, but when wetted, the conidial masses swell up, become slimy and individual spores are quickly dispersed in water. Indeed, dispersion within the tree is by water running along branches and dripping through the tree canopy. Dispersal between the trees could be by wind-driven rain flash and by vectors such as pickers or insects. Other dispersal agents such as insects, birds or humans can carry out

dispersal from one plantation to another nearby plantation during handling and picking (Masaba *et al.,* 1984). Long range dispersal such as location-to-location and from country to country seems to take place solely through the movement of infected planting material, seeds or seedlings by humans.

2.3.1.2 Importance of the disease

CBD is the major factor threatening Arabica coffee production in Ethiopia, Kenya, Tanzania and other African countries. It causes significant yield losses. The average national yield losses were about 28% between 1974 and 1978 (Arega, 2006). Similarly in Hararghe, the losses were estimated to be as high as 100% (Eshetu *et al*., 2000). The severity of CBD and the losses caused are often under estimated annually since young coffee berries drop off the tree at an early stage of the disease. Largely, CBD causes 30% national average crop losses to total harvestable coffee yield every year in Ethiopia (Eshetu *et al*., 2000). At present, CBD has rapidly spread to all coffee growing areas of Ethiopia and still inflicting significant crop loss (up to 100% on susceptible land races) although the magnitudes vary from place to place and from time to time (Arega, 2006). In Kenya, average crop loss due to CBD was estimated at not less than 30% and reaching 50 to 80% in years of no severe fungicide spray programmers. CBD is also severe in Tanzania (31 to 68%), Uganda (35 to 50% and Cameroon (up to 80%) losses.

The importance of CBD can also be judged from the cost incurred to control the disease using chemicals. In Ethiopia, it was estimated that on a national bases, spraying of all coffee farms costs about US $30 million. In Kenya, the cost of chemical control is estimated to be 30 to 35% of total production costs (Masaba *et al*.., 1984). Even after spraying a certain level of crop loss is unavoidable due to the difficulties of efficient application and that no one chemical is 100% efficient in controlling the disease.

2.3.2 The Genus *Colletotrichum* species

Taxonomy of the genus *Collototrichum*, the genus *Colletotrichum* is classified into *eumycota* to the major sub division of deuteron *mycoxina*, class *coelomyoetes* (Sutton, 1980 and Farr *et al.,* 1989), order *Melanconiales* (Agrios, 2005) and family *melanconiaceae* (Ielingwoth *et et.al.,* 1991). The taxonomy of the species of the genus *Colletotrichum* is frequently revised and is still in a state of confusion (Sutton, 1980). Representative of the genus *Colletotrichum* are ubiquitous and often polygamous causing a variety of disease symptoms commonly known as anthracnose on fruits, leaves and stems, die – back on branches, root rot, leaf spot, blossom rot, fruit and seedling branches of a wide range of crops. The genus has also been recorded worldwide both as pre- harvest and post harvest causes of crop loss (Sutton, 1980).

2.3.2.1 Morphology and Biology of the Genus *Colletotrichum* species

The genus *Colletotrichum* is described based on morphological and biological structures like mycelium immersed, branched, septate, hyaline, pale brown or dark brown conidiomata a circular, subculture, epidermal sub epidermal (Sutton, 1980).

2.3.2.2 Pathogenic variability among CBD isolates

The results of three independent studies evidenced no host specialization (physiologic races) in the CBD pathogen population in Ethiopia Tefesetewold, (1995).sampled in keffa, Sidamo and Hararghe on three CBD resistant cultivar (741 ,744 and 74110 and land race from Sidamo (kurme) and found significant variations in aggressiveness among the isolates. Similarly, twelve *Colletorichum kahawae* isolates sampled from four mountain rain forest coffee areas in Harenna, Bonga Sheka, and Yayu and inoculated with seedlings of three widely grown CBD resistance cultivars and a susceptible check the isolate were pathogenic and varied in aggressiveness (Arega, 2006). These results emphasize that horizontal resistant in the host populations practically advantageous to deploy resistant coffee varieties in CBD management.

2.3.3 The *Colletorichum* Pathogen on Coffee

There are different strains of *Colletorichum* occurring on the bark of coffee shoots as harmless saprophytes. In Kenya, Gibbs (1969) identified four groups as *Collectotrichum* strains from coffee, there saprophytic (ccm, cca, and ccp) and a pathogenic strain causing CBD, based on the morphology of their colonies on malt extract agar. He defined the pathogenic strain as slow growing with profuse grayish-black aerial mycelium and conidia that born directly on hyphae. Waller *et al.*, (1993) further defined the different strains of *Colletroichum* and recognized that the ccp strain was *colletorichum acutatum*; ccm and cca were morphs of *collectorichum gloeosporiodes* and referred to the pathogenic strain that cause CBD as *Collectrotrichum coffeanum*. The review by Van der Graaff (1981), however, indicated that taxonomically, this could not be correct, as this name had originally been given to an isolate from Brazil that is not pathogenic to green coffee berries.

Waller *et al.* (1993) studied the biochemical and pathogenic characteristics of a wide range of *Colletotrichum* isolates including the CBD pathogen. Based on this study they introduced a new species name of *Colletorichum kahawae* sp nov. The slow-growing greenish-grey cottony mycelium of fresh isolates has been recognized as a distinctive and consistent characteristic of CBD that enables it to be separated from other non-pathogenic isolates of *Colletroichum* obtained from coffee. Additionally, the early work of Gibbs (1996) and others was confirmed, showing that the conidia are born directly on the branches of the hyphae and not to on acervuli (flat disc shaped fruiting structures present in masses in the necrotic parts of the lesions) (Waller *et al.*, 1993). Reports of Wrighley (1988) and other authors, however, have implied that the conidia are produced in acervuli.

2.3.4 Disease cycles

Muller (1984) and other workers also reviewed the disease cycle of CBD. The CBD pathogen overwinters as conidia on the yellowish browning bark of the coffee tree, on mummified berries of the previous year and on leaf flecks. The conidia are water-borne and distributed by water splashes. They require the presence of liquid water or 100% relative

humidity and an optimum temperature of about 22^0C for germination. Conidia germinate into germ-tubes (short hyphae) which in turn produce apresoria. The apresoria adhere to the plant cuticles and produce infection pegs, which penetrate the cuticle to cause infection. At the optimum temperature of about 22^0C and suitable humidity, the whole process of successful penetration takes about five hours. Under optimum conditions, the time between infection and lesion development (incubation period) takes two to three or two to four weeks. The lesion is brownish black and on its surface acervuli develop in which conidia are produced under conditions of high air humidity (Van der Graaff, 1981).

According to Waller *et al.* (1993), the conidia are born directly on hyphae with no acervuli conidia. When humidity is high, conidia are formed massively and a pink layer of conidia covers the lesion. If condition are adverse (no rain and no favorable humidity and temperature), growth is arrested and cork cambium is formed that seals off the lesion. Such lesions turn brown and are called ‘scab’ lesions. Heavily diseased berries are often shed, but some remain throughout the year on the tree if not interfered with by coffee pickers, becoming potential of inoculums for the next season’s crop. The development of CBD infection varies with the stages of berry development (Muller, 1984). The pinhead stage (up to eight weeks after flowering) is resistant and berries showing new lesions are few. Susceptibility increases substantially during the expanding stage (9 to 16 weeks after flowering) and endosperm stage (17 to 24 weeks). Fully expanded berries (25 to 32 weeks) are much less susceptible. At ripening (33 to 35 weeks), the susceptibility again increases, but the lesion only covers the purple and therefore damage is not important. Since the pathogen does not penetrate the beans. However, it might create a problem during wet processing as the muscilageous part of the bean cover sticks to the beans. CBD as this stage is called brown blight of coffee.

2.3.5 Disease Symptoms

CBD attacks all stages of the crop, from the flower, including flower buds, to ripe cherries, but is characterized by an anthracnose of green berries (Bayetta, 2001). Lesions may also occur on young berry stalks, causing them to shed before lesions are visible on the berry

itself. The disease also affects ripening berries causing a 'brown blight' phase. This phase is characterized by typical dark, sunken lesions that envelop the red berry (Bayetta, 2001). The coffee berry disease may also infect flowers under very wet conditions, and causes brown lesions on petals. This disease does not kill trees, but crop losses can be more than 80%. Spore dispersal within the tree is by rain splash. Disease spread from tree to tree and from farm to farm is by coffee pickers, birds or infected seedlings (Bayetta, 2001).

2.3.6 Epidemiology

The occurrence and intensity of CBD varies from place to place and from season to season, depending largely on the presence or absence of favorable climatic conditions (Bayetta, 2001). The disease is initiated mainly from diseased berries and infected plant, and appears every year again on previously infected coffee trees (Gassert, 1979; Tefestewold, 1995). Conidia are the asexual spores and major inoculums that can be dispersed easily by rain splash and winds over short inoculums that can be dispersed easily by rain splash and winds over short distance such as dispersal within tree canopy and from tree to tree. For dispersal of CBD inoculums, tree tops are extremely important as a source of inoculums while longer distance dispersal of CBD inoculums has apparently been by passive vectors that may carry viable spores (Tefestewold, 1995).

2.3.6. 1 Conditions that favour development of the Pathogen

CBD severity is highly dependent upon climatic factors, particularly temperature, rainfall, and humidity. Rain is necessary for the dispersal of conidia of *Colletotrichum* species. Optimum temperatures for conidium germination and mycelium growth are 20 to 22°C for *C. kahawae* on coffee. Appressorium formation occurs at the same temperatures and at relative humidity approaching 100%. Climatic conditions conducive to *C. kahawae* typically occur at high elevations (>1,600 m); disease incidence is minimal below 1,000 m *(*Griffiths and Waller, 1971).

Agricultural practices may also influence disease severity. Currently, CBD is managed through host resistance, cultural practices, and chemical control. However, these management practices could be used more efficiently as part of an IPM strategy that can be applied by producers of Arabica coffee. Such an approach requires prior knowledge of the pathosystem (host/parasite/environment) interactions that govern disease development.

2.3.7 Managements of Coffee berry disease

2.3.7.1 Resistant Variety

For several years it has been observed that some variability for resistance exist among coffee cultivars. Blue mountain, certain lines of local Bronze and kabura Kiseya were considered to have some degrees of resistance. Jackson hybrid Bourbon Mayaguez mibirzi 49 and local Bronze 8 & and 12 provide fair resistance. Rume sudan and Hibrdo de Timor. Timor were indicated as having high levels of resistant, and k7 has shown some field resistance to CBD, differences, in disease severity between coffee trees were also detected in Ethiopia after the appearance of CBD (Tefestewold, 1995).

2.3.7.2 Chemical control

However, fungicides such as Daconil and Dealan which were considered as promising chemicals against CBD. However, later these products including Dyrene and Octave were banned for a number of side effects (Eshetu *et al*., 2000). The use of fungicides against CBD has shown to induce negative effects on beneficial microorganisms that can antagonize the CBD pathogen (Masaba, 1991). The high cost of pesticides, the appearance of fungicide resistant pathogen biotype and other social and health related problems of the conventional agriculture on the environment have increased interest in sustainable agriculture and biodiversity conservation. These problems make it essential to look for alternative strategies that can ensure competitive coffee production.

2.3.7.3 Cultural and biological control

The use of fungicides against CBD had shown to induce negative effects on microorganisms antagonistic to the CBD pathogen and subsequent loss of a natural biological control mechanism. This effect was studied in more detail by Masaba (1991), who found that there was a large difference between the micro-flora on sprayed and unsprayed coffee. A number of organisms (filamentous fungi, yeast and bacteria) that showed significant antagonisms toward the CBD pathogen were present on unsprayed coffee and elements of the micro bacteria were shown to have a natural biocontrol effect on the disease. Treatments on green coffee berries with *Epicocum nigrum* archived good control of CBD when compared to leaves treated with copper based fungicides. These indicate promising use of antagonistic micro-organisms for CBD management.

Extract from coffee fruits leaves stems and roots contains allopathic substance which includes purine alkaloids like the bromine, paraxanthine, theophylline and phenolic compound like caffeic acid, p-coumaric acid and vanellic acid. Caffeine of the bromine and tleophlline has variable inhibitory effects on growth and aflatoxin production of *Aspergillus* and *Penicillium*, *Cladosporium* spp. and *Colletotrichum gloeosporioides*. However, the effects of coffee extracts on *Colletotrichum coffeanum* and their influence in the identification and control are not yet completely investigated (Tefestewold, 1995).

2.4 Invasive Alien Species

Alien species are non-native or exotic organisms that occur outside their adaptive ranges and dispersal ranges (Raghubanshi *et al*., 2005). According to CBD (2001), IAS are species introduced deliberately or unintentionally from outside their natural habitat, where they have the ability to establish themselves, invade, out-compete natives and take over the new environment. These invasive species are widely distributed in all kinds of ecosystems throughout the world, and include all categories of living organisms. Nevertheless, plants, mammals and insects comprise the most common types of invasive alien species in

terrestrial environments (Raghubanshi *et al*., 2005). Invasive species have now affected every ecosystem types on the planet and considered as the second greatest global threat to biodiversity, after habitat destruction (Raghubanshi *et al*., 2005). Moreover, climate change such as global warming triggers suppression of native biodiversity by invasive alien species. The invasive species impact on native species directly competing for resources such as food and breeding sites as well as indirectly by altering habitat and modifying hydrology, nutrient cycling and other ecosystem processes. These impacts dramatically change the ecosystem both positively and negatively (Raghubanshi *et al*., 2005).

Threats to biodiversity and habitat destructions are interacting phenomena. Habitat destruction can make areas more vulnerable to invasive species, and species invasions can result in the destruction of habitats (Raghubanshi *et al*., 2005). Apart from their threat to biodiversity and ecosystem services, invasive species have significant social, ecological and economical impacts. They reduce agricultural yields, irrigated crop lands, grazing areas, water availabilities, and contribute to spread of vector born diseases. According to Shackleton *et al*. (2006), IASs can be integrated into the livelihoods of the local people in a number of ways (Raghubanshi *et al*., 2005).

According to Raghubanshi *et al*. (2005), IASs have unique characteristics over the native ones. They do not need special environmental requirement for seed germination, have rapid seedling growth and produce seeds for longer period of time as long as environmental condition permit, they are also highly tolerant to climatic and edaphic variations and have an ability to compete and drive off other species from their habitat. Moreover, they can reproduce sexually and asexually. Forest invasive species can negatively affect forest ecosystem or damage specific forest products. *P. juliflora*, like any invasive species, is invasive only under conditions that are favorable to their spread (Geezing *et al*., 2004). The recent global assessment and global data sets showed that there are 1348 serious agricultural weed species and 1041 wide spread agricultural weedy species (Daehler, 1998). IASs have affected 30% of threatened birds, 11% of threatened amphibians, 8% of threatened mammals and 15% of threatened plants (Baillie, 2004). In 2004, IUCN identified 81 IAS in

South Africa, 49 in Mauritius, 44 in Swaziland, 37 in Algeria and Madagascar, 37 in Kenya, 28 in Egypt, 26 in Ghana and Zimbabwe, and 22 in Ethiopia (Raghubanshi *et al*., 2005).

2.4.1 Status, distribution and economic importance of IAS in Ethiopia

IASs are of great concern to Ethiopia, posing particular problems on biodiversity of the country, agricultural lands, range lands, national parks, water ways, lakes, rivers, power dams, roadsides and urban green spaces with great economic and ecological consequences. In Ethiopia, there are about 22 IAS (McGinley, 2007). Foremost among these are parthenium weed (*Parthenium hysterophorus*), prosopis/ Mesquites (*Prosopis juliflora),* water hyacinth (*Eichhornia crassipes*), cactus (*Euphorbia stricta*) and lantana weed (*Lantana camara)*. They have been identified by the Environmental Policy and the National Biodiversity Strategy and Action Plan as a major threat to biodiversity of the country and economic well being of its people. However, little attempt has been made in terms of research and management of IAS. Their high seed production capacity and spread, adaptation to wide climatic and soil conditions, spread by animal movement and their association with pastoralists way of life and overgrazing are challenges to their management in Ethiopia (Taye, 2007). Control of *Parthenium* by farmers resulted in some of them developing skin allergies, itching, fever, and asthma. The social cost of *Parthenium* in Ethiopia was measured by Disability Adjusted Life Years and its equivalence in terms of monetary value was estimated at 2,535,887 to 4,365,057 USD. *Prosopis* form impenetrable thicket that prohibits free movement of people and animals and its thorns damage eyes and hooves of animals. More resources have to be invested to tackle the IAS problem as the estimated loss is disproportionate to the cost of investment on IAS research and development activities (Taye *et al.,* 2011).

2.4.2. Review on use of Invasive Alien Species plant extracts as Plant Disease control

Abera et al., 2010 evaluated the antifungal potential of aqueous and ethanol extracts of eight different plant species invitro and *in vivo* against *Colletotrichum kahawae* in completely randomized design with three replications. The extracts were from *Hagenia abyssinica, Allium sativum, Phytolacca dodcandera*, *Croton macrostachyus*, *Maesa lanceolata, Eucalyptus globules, Eucalyptus citriodera* and *Lippia adoensis*. Subsequently, two most effective plant extracts were tested *in vivo* against the disease on detached green coffee berries and seedling applying the extracts at 3 different times of application (at the time of inoculation and 48 h before and after inoculation) on the pathogen. The study indicated that the inhibitory effect of the extracts depended on the type of plant species used, method of extraction and time of application of the extracts. Generally, *A. sativum* and *C. macrostachyus* aqueous and ethanol extracts were the most effective plants that significantly reduced radial growth of the pathogen compared to the control. *A. sativum* reduced radial growth of the pathogen in ethanol and aqueous extracts by 83, 100%, respectively, and *C. macrostchyus* by 68 and 88%, respectively. Furthermore, *A. sativum* extracts consistently reduced disease severity on detached green berries and seedling in greenhouse at all times of application. Nevertheless, the efficacy of *C. macrostachyus* on detached green berries and seedlings was inconsistent and variable based on method of extraction and time of application of the extracts. The study indicated the possible use of extracts of *A. sativum* as an alternative means of CBD (coffee berry disease) management.

Johnny et al., 2010 evaluated the antifungal activities of leaf extracts from fifteen selected plants; *Alpinia galangal* L., *Alstonia spatulata* Blume., *Annona muricata* L., *Blechnum orientale* L., *Blumea balsamifera* L., *Centella asiatica* L., *Dicranopteris linearis*, *Dillenia suffruticosa*, *Litsea garciae* Vidal., *Melastoma malabathricum* L., *Momordica charantia* L., *Nephrolepis biserrata* (Sw.)., *Pangium edule* Reinw., *Piper betle* L., and *Polygonum minus* Huds., on the growth and sporulation of *Colletotrichum gloeosporioides* isolated from mango. The result showed as *A. galanga* extracts were the most effective and exhibited highest antifungal activities against *C. gloeosporioides.* Methanol crude extract reduced radial growth of *C. gloeosporioides* by 66.39%, followed by chloroform crude extract

63.26%, and 61.56% for acetone crude extracts. The exact concentrations that have definite potential to fully restrict the growth of *C. gloeosporioides* (MICs) for *A. galanga* is 15.00 mg/mL in methanol, 17.50 mg/mL in chloroform, and 17.50 mg/mL in acetone. The sporulation assay also revealed that *A. galanga* leaves crude extracts showed highest inhibition of spore germination of *C. gloeosporioides* overall at concentration of 10 mg/mL; with 68.89% inhibition by methanol extracts, 64.13% by chloroform extracts, and 62.86% by acetone extracts.

Ogbo and Oyibo (2008) tested effects of three plant extracts (*Ocimum gratissimum*, *Acalypha wilkesiana* and *Acalypha macrostachya*) for inhibitory effect on the radial growth of on post harvest pathogen of *Persea americana (Cercospora purpurea)*. Ethanol extracts of the three plants caused total inhibition of *C. purpurea*. Crude extract of sterilized and un sterilized water significantly reduced the radial growth of the fungus. However, the fungistatic effect of *O. gratissimum* was highest and was followed by *A. macrostachy* and then *A. wilkesiana*.

3. MATERIALS AND METHODS

3.1 Description of the Study Area

The research was conducted at Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) Plant Pathology laboratory and greenhouse during 2011/2012. The study area is situated at about 356 km southwest of Addis Ababa located at about $7^{0}33^{'}$ N latitude and $36^{0}57$’ E longitude at an altitude of 1710 m a. s. l. The mean maximum and minimum temperatures are 26.8^{0}C and 11.4^{0}C, respectively and the mean maximum and minimum relative humidity are 91.4% and 39.92%, respectively (BPEDORS, 2000). The mean annual rainfall of the area is 1500 mm (Melaku, 2008).

3.2 Plant Materials Used for the Study

Five invasive alien species namely *Parthenium hysterophorus* /Congress weed/, *Lantana camara* /Lantana weed/, *Prosopis juliflora* /Mesquite/, *Eichhornia crassipes* /Water hyacinth/ and *Mimosa diplotricha* were used. Leaves of the plant materials were collected individually in summer, from their natural habitats. Samples of *Parthenium hysterophorus*, *Lantana camara* and *Mimosa diplotricha* were collected from around Jimma whereas *Prosopis juliflora* and *Eichhornia crassipes* were collected from around East Shewa (Awash; Welenchiti woreda) and Koka, respectively. The samples of plant species were identified and each species were deposited at herbarium, Biology Department, Jimma University.

3.3 Culture Media and Culture Conditions

Potato dextrose agar (PDA) was prepared by dissolving commercially formulated dehydrated (powdered) PDA. The PDA powder were mixed with sterilized water in a flask at the rate of 39 gm/l (company recommendation) and heated until melting. The media were autoclaved at 121°C at atmospheric pressure of 15 psi for 15 min. The liquid media were maintained under aseptic condition and allowed to cool to about 50°C. Streptomycin sulphate powder were added to the nutrient media at the rate of l gm/1 to avoid bacterial contamination and the

media then was poured into sterilized Petri-dishes at the rate of about 20 ml per Petri dish and allowed to solidify.

3.4 Sample Preparation

3.4.1 Preparation of fungal pathogen, culturing and sub-culturing

Culture of *Colletotrichum kahawae* were kindly obtained from Jimma Agricultural Research Center (JARC) and then further sub-cultured on fresh media to obtain pure CBD cultures. Blocks of fungal colonies were cut out with a sterile surgical blade from the leading edge of the actively growing portion and transferred to fresh agar media and incubated in a cooled incubator as before.

CBD culture can be stored in two ways: maintenance of pure culture in sterile distilled water and maintenance of the pathogen on susceptible host plant berries. Storage of CBD culture in sterile distilled water has long been familiar and is the most commonly used technique at JARC. To store the isolated pure CBD culture, screw-capped culture tubes were filled with half (10 ml) distilled water, loosely closed and autoclaved to sterilize the water. After the tube cultures have cooled down overnight in a refrigerator, small blocks of fungal colonies were cut from the actively growing margins on agar plates of the pure culture and transferred to the culture tubes under aseptic conditions. The culture tubes were then tightly closed with screw caps and sealed with Para-film. Altogether, 20 culture tubes prepared in this way were stored in a refrigerator at 4°C.

According to the procedure of Van der Graaff, (1981) and Bayetta (2001), berries having the same size and developmental stage were collected from the same line of coffee selection 370 CBD susceptible varieties at JARC in July 2012, and surface sterilized with 5% Sodium hypochlorite for two minutes and rinsed 3 times for 3 minutes with distilled water. Then after, the berries were placed on clean plastic Petri dish covered with sterile water saturated sponge to obtain 100% humidity. Conidial suspensions were prepared by washing PDA medium in distilled water and filtering through a double layer of cheese cloth. Inoculums

suspension at the recommended concentration (2 x 10^6 conidia/ml of water) was inoculated on 24 berries of the first batch (Bl). A month after the first inoculation, the CBD pathogen was isolated from B1 berries and was inoculated on 20 berries for second batch (B2).

3.4.2 Plant materials preparation and extraction

The collected plant samples of each species was washed under tap water, surface sterilized with 5% sodium hypochlorite solution for two minutes followed by thorough repeated rinsing with sterile water. The plant samples were then air dried at room temperature, ground in a mill and kept in dark bottles for the subsequent activities.

Plant samples from each species were individually extracted using maceration techniques following standard procedures (Amadioha, 2002), with some modification. Aqueous extracts were prepared by dissolving each crushed plant samples in sterilized distilled water in (w/v) of 100 g/500 ml and were shacked by orbital shaker at 130 rpm for one and half hour and allowed to stand for 48 hr, and then filtered by cheese cloth followed by filter paper Whatman No. 1.

Accordingly, 100 g of each dried powder plants were dissolved in 500 ml of 70% ethanol and acetone separately and were shacken by orbital shaker at 130 rpm for one and half hour and allowed to stand for 48 hr, and then filtered by cheese cloth followed by filter paper Whatman No. 1. The organic solvent was evaporated under vacuum oven at 40^0C under room temperature in a pre- weighed labeled glass dish. Then, the remaining extracts were weighted and diluted by adding appropriate quantity of sterilized water to make 20% extract. The stock extracts were then transferred to labeled sterile screw capped bottle and stored at 4^0C until further use.

3.5. Antifungal Assays

3.5.1 *In vitro* anti fungal assay

In this experiment, the antifungal activity of some invasive alien species (IAS) extracts and solvent type on the radial growth of *Colletotrichum kahawae* were determined. The effect of the plant extracts on the radial growth of *C. kawahae* was determined by using the method described by Amadioha, (2002). Accordingly, one milliliter of the respective extract was separately spread on the surface of the pre-solidified PDA contained in the Petri dishes. Petri dishes without extracts were used as control. Five-millimeter fungal block were cut out with a sterile cork borer from the 7 to 10 day old culture at the actively growing portion on PDA and placed in the center of 9 cm diameter Petri dish with three replications for each treatment combinations. The plates were then incubated at room temperature at 25^0C for 21 days. Then, the radial growth of the fungus for each treatment was measured as average of three diameters taken at right angle for each colony with ruler on the 21 days of inoculation. The percentages of inhibition of the concentrations of plant extracts on radial growth of the fungus were calculated as the formula described by Amadioha, (2002) and Ogbo and Oyibo (2008):

$$\text{Inhibition} \quad \% = 100 - \left[\frac{gowth\ of\ fungus\ in\ extract}{growth\ of\ fungus\ in\ control} x100 \right]$$

3.5.2 Determination of minimum inhibitory concentration (MIC)

A serial micro dilution assay methods (Eloff, 1998c) were used to determine the minimum inhibitory concentration (MIC) values for plant extracts. To apply it to measuring antifungal activities, a slight modification were made to suit fungal growth conditions (Masoko *et al*., 2005). Accordingly, the plant extract with the most antifungal activity (from the first experiment) were tested against radial growth of *C. kahawae* at different concentrations (50 mg/ml, 100 mg/ml 150 mg/ml and 200 mg/ml) on mycelia growth to determine minimum inhibition concentration *(MIC)* by dilution methods of the extract (v/v).

3.5.3 *In Vivo* antifungal test

3.5.3.1 Detached green coffee berries

In this experiment, the effect of plant extracts with the most inhibition percentage under *in vitro* test, solvent type and application time on the development of coffee berry disease was determined on detached green coffee berries. Accordingly, detached green coffee berries were inoculated following standard methods (Van der Graaff, 1981). Berries having the same size and developmental stage were selected and harvested from the same line of coffee selection 370. Then, the berries were surface sterilized with 5% Sodium hypochlorite for two minutes and rinsed three times for three minutes with sterile water. Thereafter, the berries were placed on clean plastic Petri dish covered with sterile water saturated sponge, to obtain 100% humidity. Twenty four berries were used in each replication. For inoculation of the berries, aqueous conidia suspension of 2×10^6 conidial/ml was used. The concentration of conidia in the suspension was determined using a haemocytometer (Van der Graaff, 1981).

Twenty five µl conidia suspension were placed at the center of the berries. Berries were treated in different time of application with the most inhabitant plant extract in the *in vitro* test. The selected IAS plant extracts were sprayed on berries by using an air-pressurized hand sprayer at 48 hr before spore inoculation, at the time of spore inoculation, and 48 hr after spore inoculation. The control was not treated. The interaction between plant extracts and inoculated pathogen were determined on 21 day after inoculation. The disease assessments on green coffee berries were done using 0 to 5 score scale measurements (Table 1). Disease index on berries was calculated using the following equation (Van der Graaff, 1981).

$$DI= 100 (b_1+2b_2+3b_3+4b_4+5b_5)/5(b_0+b_1+b_2+b_3+b_4+b_5)$$

Where: b0= number of berries in class 0, b_1= number of berries in class 1, b_2= number of berries in class 2, b_3= number of berries in class 3, b_4= number of berries in class 4, and b_5= number of berries in class 5 respectively.

Table 1. Classification for CBD assessment on detached green berries

Class	Symptom	Code for DI
0	No symptom	b_0
1	Minute brown spot lesion less than 1 mm in diameter	b_1
2	Black lesion of 1-5 mm in diameter	b_2
3	Black lesion of 6-10 mm in diameter	b_3
4	Black lesion of 11-20 mm in diameter	b_4
5	Black lesion of greater than 20 mm in diameter	b_5

Source; Van der Graaff, (1981)

3.5.3.2 *In vivo* seedling test

The plant extracts with the most inhibition percentage under *in vitro* test were further tested in green house according to Tegene, (2007) by some modification, on susceptible coffee seedling to evaluate their ability to control coffee berry disease caused by *C. kahawae* two days after, before and at the same time of inoculation. Plant extracts were sprayed on the seedlings by using an air-pressurized hand sprayer.

Coffee seedlings were raised in green house from freshly harvested seeds of coffee cultivar 370. To obtain seedlings, ripe cherries were picked from Jimma Agriculture Reseach Center and dried under shade after removing the pulp by hand. Seeds of coffee cultivar 370 were prepared by removing the parchment and soaked in sterile distilled water and kept for 48 hours. There after, seeds were sown (10 seeds/pot) in heat sterilized and moistened forest, compost and sandy soil at 2:1;1 ratio in disinfected plastic pot arranged on benches and covered with mulch in greenhouse. Six weeks after sowing, the emerging seedlings were kept at 20-25 ^{o}C. Two days before inoculating the hypocotyls, seedlings were sprayed with sterile distilled water and covered with plastic sheet for 48 hours to obtain 100% relative humidity.

Mycelia colonies of each isolate were carefully removed with a sterile scalpel from the PDA medium while washing with sterile distilled water to harvest conidia from 10 days old cultures. The suspension of each isolate was stirred with magnetic stirrer for 10-15 minutes and filtered through double layers of cheese clothes. After repeating the procedure again the spore concentration of each suspension was adjusted to 2 x 10^6 conidia/ml.

Plastic pots containing eight seedlings/pot of coffee cultivar 370 were inoculated with the fungal spore suspension by stem brushing procedure with fine camel hairbrush (Van der Graaff 1981) with different time of application (2 days before, at the same time and after inoculation) of the crude extracts of 70% ethanol and acetone respectively at the rate of 200 mg/ml using an air pressurized hand sprayer. The control was not treated. Complete Randomize Block design was employed to see the interactions of IAS crude extracts and the pathogen.

For *C. kahawae* severity (leaf area with symptoms) was assessed. Severity was rated using the symptom classifications (0-4 scale, Table 2) according to Van der Graaff, (1981). The severity grades were converted to disease severity index as follows;

$$DI= 100 (b_1+2b_2+3b_3+4b_4)/4(b_0+b_1+b_2+b_3+b_4)$$

Where: b0= number of seedlings in class 0, b_1= number of seedlings in class 1, b_2= number of seedlings in class 2, b_3= number of seedlings in class 3, and b_4= number of seedlings in class 4.

Severity assessments were examined. The area under disease progress curve (AUDPC) for coffee seedlings was calculated from severity assessments according to Garrett and Mundt, (2000).

$$AUDPC = \sum_{i=1}^{n-1} [0.5(x_i + x_{i+1})][t_{i+1} - t_i]$$

where x_i is the percentage of disease severity at ith assessment, t_i is the time of the ith assessment in days from the first assessment date and n is the total number of days disease was assessed. Disease incidence was assessed on three weaks after innoculation.

Table 2. Assessment key for rating CBD infection level in coffee seedlings using 0-4 scale.

Grade	Symptom	Code for DI
0	No symptom	b_0
1	Minute brown spot lesions 0.5mm in diameter.	b_1
2	One brown lesion more than 0.5mm in diameter	b_2
3	Wide brown lesions with numerous black dots, but the top remains alive.	b_3
4	Black lesions girdling the stem. Top killed.	b_4

Source; Van der Graaff, (1981)

3.6.Data analysis

Data were subjected to analysis of variance using SAS version 9.2 (SAS institute Inc, 2008). Single and interaction effect of factors were determined using the GLM procedure of SAS. Whenever significant interactions were observed between factors, the level of one factor was compared at each level of other factors. Mean values among treatments were compared by the Tukey's test at α = 0.05% level of significance.

4. RESULTS

4.1 *In Vitro* Antifungal Activity of Invasive Alien Species Plant extracts against *C. kahawae*

Crude extracts of all selected invasive alien species plant extracts inhibited the mycelia growth of C. *Kahawae* in *in vitro* test. The *in vitro* results indicated significant differences ($p<0.001$) among IAS plant extracts tested and solvent types used (Table 3). All the crude extracts showed *in vitro* antifungal activity at 20 % (w/v).

Among the five IAS plant extracts screened, 70% acetone extract of *L camara* inhibited the mycelia growth of target pathogen at concentration of 200 mg/ml (84.13%) followed by 70% ethanol extract *of L camara* (76.23%) and 70% acetone extract of *P. juliflora (*66.96%) (Plate 1). The aqueous extract of *M. diplotricha* and 70% ethanol extract of *P. hysterophorus* showed less inhibition of target pathogen with mean percentage inhibition of 15.76 (Table 3).

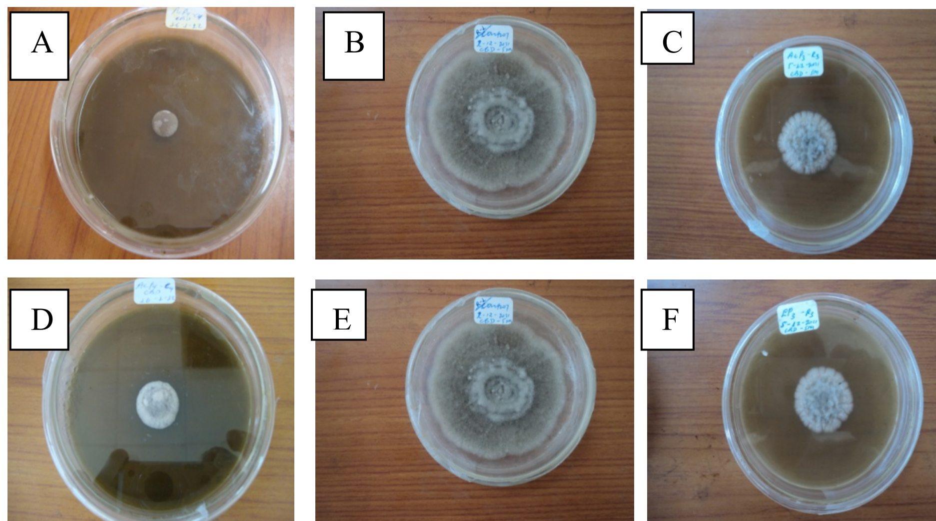

Plate 1. *In vitro* antifungal activity of the most effective Invasive alien plant extracts against C. kahawae:
A) *L.camara* acetone extract against *C. kahawae* pathogen, B) Control plate, C) *P. juliflora* acetone extracts against *C.kahawae* pathogen D) *L.camara* 70% ethanol extract against *C.kahawae* pathogen, E) Control plate, F) *P. juliflora* 70% ethanol extract against *C. kahawae* pathogen.

All extracts of *E. crassipes*, *P.hysterophorus* and *M.diplodia* 20 % (w/v) reduce mycelia growth of target pathogen when compared to untreated plates (the control) (Table 3).

Table 3. Effect of invasive alien plant extracts and solvent type on radial mycelia growth of *C. kahawae*

Solvent type	Invasive alien plant spp.	Radial growth(mm)#	Inhibition %
Aqueous extract	*Eichhornia crassipes*	57.00^{d}	35.43
	Parthenium hysterophorus	61.33^{d}	30.56
	Prosopis juliflora	60.66^{d}	31.26
	Lantana camara	59.33^{d}	32.80
	Mimosa diplotricha	74.33^{b}	15.76
70% Ethanol extract	*Eichhornia crassipes*	59.33^{d}	32.76
	Parthenium hysterophorus	74.33^{b}	15.76
	Prosopis juliflora	33.00^{e}	62.63
	Lantana camara	21.00^{fg}	76.23
	Mimosa diplotricha	59.66^{d}	32.40
70% Acetone extract	*Eichhornia crassipes*	72.33^{bc}	18.03
	Parthenium hysterophorus	59.66^{d}	32.50
	Prosopis juliflora	29.16^{ef}	66.96
	Lantana camara	14.00^{g}	84.13
	Mimosa diplotricha	62.33^{cd}	29.46^{d}
	Control	88.33^{a}	0.00
CV = 5.29%			

#Means with the same letter are not significantly different (Tukey α=0.05).

The percent inhibition of 70% ethanol and acetone extracts of two IAS plants namely *P. juliflora* and *L.camara* were more than 50% inhibition against test fungi. The other three plants were less significant in their antifungal activities on the mycelia growth of coffee berry disease causal agent *C. kahawae* when compared to the above two IAS plants of ethanol and acetone extracts (Table 3).

Ethanol and acetone extracts of P. *juliflora* and *L.camara* antifungal activity were tested against *C. kahawae* at different concentrations to determine minimum inhibitory concentration (MIC). The results showed that both crude extracts of *P. juliflora* and *L.camara* at 50,100,150 and 200 mg/ml concentration significantly reduced the mycelia growth of the target pathogen, *C.kahawae* as compared to the untreated plate (control). 70% ethanol

extracts of *P. juliflora* at 50 mg/ml concentration reduce the radial mycelia growth of the target pathogen to 56 mm (Table 4). Acetone extract of *L. camara* at 200 mg/ml showed 14 mm less radial mycelia growth with high inhibition on *in vitro* test under laboratory condition (Table 3). The minimum inhibition concentration of 70% ethanol and acetone crude extracts of *L.camara* and *P.juliflora* were 50 mg/ml.

Table 4. Effect of plant extracts, solvent type and rate of plant extracts on the minimum inhibition concentration of radial mycelia growth 21 days after inoculation

Plant species	Solvent type	Concentration(mm)	Radial growth(mm)#
Prosopis juliflora	Ethanol	5	56.00^{b}
		10	44.67^{c}
		15	$37.33d^{e}$
		20	33.00^{ef}
	Acetone	5	40.50^{cd}
		10	34.33^{def}
		15	31.33^{efg}
		20	29.17^{fg}
Lantana camara	Ethanol	5	$35.33d^{ef}$
		10	30.50^{fg}
		15	26.00^{gh}
		20	21.00^{h}
	Acetone	5	37.33^{de}
		10	29.0^{fg}
		15	25.17^{gh}
		20	14.00^{i}
		Control	82.33^{a}
CV = 4.80 %			

Means with the same letter are not significantly different (Tukey α=0.05).

4.2 *In vivo* antifungal activity of the most suppressive plant extracts against C. *kahawae*

4.2.1 Detached Coffee berries

Green detached coffee berries were treated with crude extracts from *P. juliflora* and *L. camara* with 70% ethanol and acetone extracts (Plate 2). Both IAS plant extracts were applied before, at the same time and after inoculation of *C. kahawae* spores. The result showed significant difference (P< 0.0001) among the extracts in suppressing lesion development compared to time of application (Table 5).

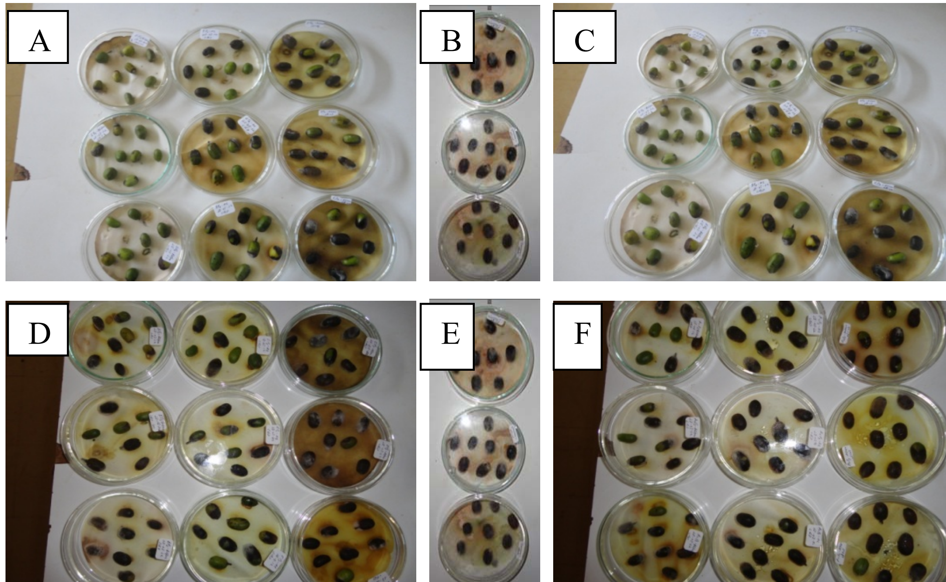

Plate 2. Detached green berries treated with extracts of *L. camara* and *P.juliflora* extracted by ethanol and *acetone* at different time of application 21 days after inoculation
A=*L. camara* extracted by ethanol at different time of application, B= control plate, C=*P.juliflora* extracted by ethanol at different time of application, D= *L.camara* extracted by acetone at different time of application, E= control plate, F=*P.juliflora* extracted by acetone at different time of application.

Green berries treated by ethanol and acetone extracts of *L. camara* 48 hr before inoculation was visibly (Plate 2) significantly suppressing lesion development to 20% and 29.1% reducing disease severity, respectively, than the other inoculation treatments (Table.5).

Berries treated by *P.juliflora* 70% ethanol extract 48 hr after, 70% acetone extract at the same time and after *C. kahawae* spore inoculation and *L. camara* acetone extract 48 hr after inoculation reduce disease lesion, the disease severity percentage were 92.5 , 92.5, 93.3 and 85.8% showed high severity percentage with no significance difference with the control (Table 5) .

Table 5. Effect of IAS plant, solvent type and time of application on detached coffee berries; test severity % on 21 day after inoculation

Plant Species	Solvent Type	Time of Application#			Control
		Before	At the same time	After	
Prosopis juliflora	Ethanol	64.1^{cd}	47.5^{e}	92.5^{a}	100.0^{a}
	Acetone	75.0^{bc}	92.5^{a}	93.3^{a}	100.0^{a}
Lantana camara	Ethanol	20.0^{g}	43.3^{ef}	75.8^{bc}	100.0^{a}
	Acetone	29.1^{fg}	57.5^{de}	85.8^{ab}	100.0^{a}

Application of different IAS plant extract at different time Before = Detached coffee berries treated by IAS plant extract before 48 hr *C. kahawae* strain inoculation, At the same time = Detached coffee berries treated by IAS plant extract at the same time of spore inoculation. After = Detached coffee berries treated by IAS plant after 48 hr spore inoculation spore inoculation.

#Means with the same letter are not significantly different (Tukey α=0.05).

Detached coffee berries treated by *L. camara* 70% ethanol and acetone extracts 48 hr before spore inoculation reduced disease incidence significantly to 37.5 and 41.66% when compared to untreated berries (Fig.1). Coffee berries treated by *L.camara* 70% ethanol extract at the same time with the pathogen spore inoculation reduced disease incidence significantly to 50% when compared to untreated berries. Coffee berries treated by 70% ethanol and acetone extracts of *P. juliflora* and acetone extract of *L. camara* 48 hr after inoculation by the pathogen spore gave, disease incidence 100%, 100% and 91.66% respectively, and have no significant difference with the control.

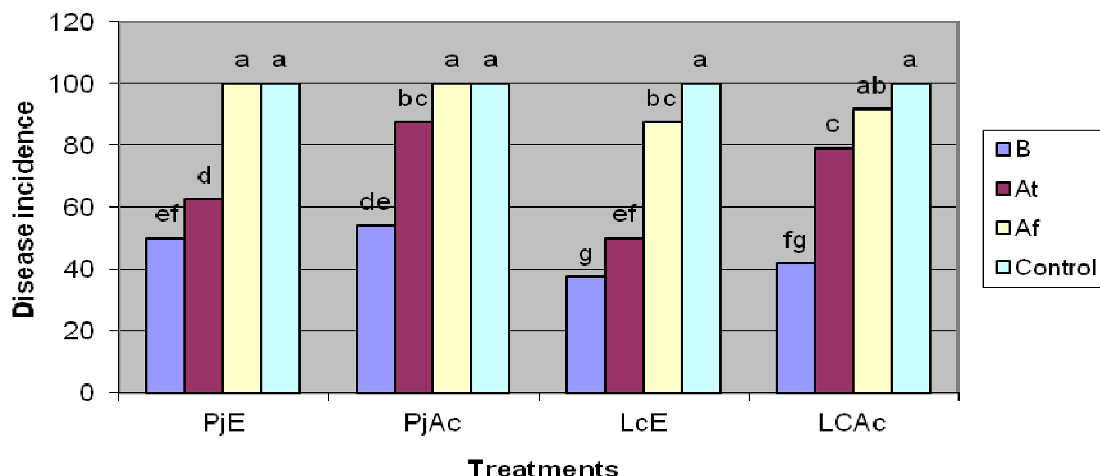

Fig. 1 The effectiveness of *L. camara* and *P. juliflora* extracts on detached coffee berry disease incidence 21th day after pathogen inculcation with different time of inoculation.

PjE= *P. juliflora* ethanol extract, PjAc= *P. juliflora* acetone extract, LcE= *L. camara* ethanol extract. LcAc= *L. camara* acetone extract, B= extract before *C. kahawae* spore inculcation, At= extract applied at the same time *with C. kahawae* spore inculcation, Af= extract applied at the same time *with C. kahawae* spore inculcation, Control= Untreated seedlings inoculated only by *Colletotrichum kahawae* spore.

4.2.2 Coffee seedling test

Coffee seedlings were treated with 70% ethanol and acetone crude extracts of *P .juliflora* and *L. camara* at 20%(w/v) concentration 48hr before, at the same time and 48hr after inoculation with *C. kahawae* spore, showed significant difference among extracts in suppressing lesion development compared to untreated (Appendix V). Coffee seedlings treated with ethanol extract of *L. camara* two day before inoculation showed highly significant difference (P=<0.0001) in suppressing lesion development than the post inoculation treatment. It reduces the severity to 0. Acetone extract of *L.camara* also reduces the severity to 0.83%. Seedlings treated with 70% ethanol and acetone crude extracts of *L. camara* applied at the same time with the *C. kahawae* spore inoculation reduced severity to 7.50% and 6.67% respectively. 70% ethanol and acetone crude extracts of *P. juliflora* applied before inoculation of *C. kahawae* spore, the severity was 17.70%, 6.66% respectively (Table 6).

Table 6. Effect of IAS plant, solvent type and time of application on coffee seedlings; test severity % on 21 days after inoculation

Plant Species	Solvent Type	Time of Application#			Control
		Before	At the same time	After	
Prosopis juliflora	Ethanol	17.50^{de}	28.33^{c}	38.33^{b}	83.00^{a}
	Acetone	6.66^{fg}	15.00^{ef}	24.17^{cd}	83.00^{a}
Lantana camara	Ethanol	0.00^{g}	7.50^{fg}	10.83^{ef}	83.00^{a}
	Acetone	0.83^{g}	6.67^{fg}	25.83^{cd}	83.00^{a}

Application of different IAS plant extract at different time Before = Coffee seedlings treated by IAS plant extract before 48 hr *C. kahawae* strain inoculation, At the same time = Coffee seedlings treated by IAS plant extract at the same time of spore inoculation. After = Coffee seedlings treated by IAS plant after 48 hr spore inoculation spore inoculation.

#Means with the same letter are not significantly different (Tukey α=0.05).

4.2.2.1 Effect of different plant extracts and solvent type on disease progress

The IAS plant extracts tested on detached coffee berries for the control of coffee berry disease causing pathogen were evaluated under green house conditions and a significant interaction between the IAS plant used and solvent type at different time of inoculation was observed in terms of AUDPC (Fig.2). Higher AUDPC value was observed in the control treatment compared to ethanol and acetone extracts of *P.juliflora* and *L.camara* applied before, at the same time and after inoculation of the pathogen.

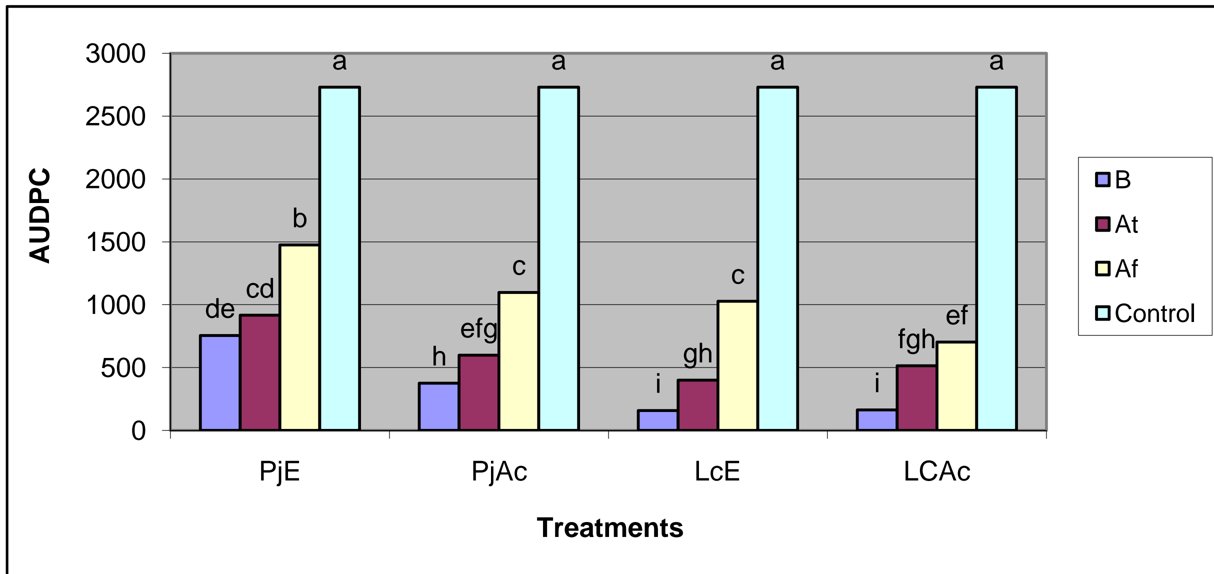

Fig.2 Area under disease progress curves (AUDPC) for coffee seedlings treated by ethanol and acetone extracts of *P. juliflora* and *L.camara.*
PjE= *P. juliflora* ethanol extract, PjAc= *P. juliflora* acetone extract, LcE= *L. camara* ethanol extract. LcAc= *L. camara* acetone extract, B= extract before *C. kahawae* spore inculcation, At= extract applied at the same time *with C. kahawae* spore inculcation, Af= extract applied at the same time *with C. kahawae* spore inculcation, Control= Untreated seedlings inoculated only by *Colletotrichum kahawae* spore.

Ethanol and acetone extract of *L. camara* applied before innoculation highly inhibited the effect of the pathogen,and showed highly significant difference (P<0.0001) compared to the untreated control (Table 6)

Coffee seedlings treated with *L. camara* 70% ethanol extract 2 days before pathogen spore inoculation reduced disease incidence significantly to 0% and extract applied at the same time with the pathogen also reduces disease incidence significantly to 4.16% when compared to untreated (Fig. 3).

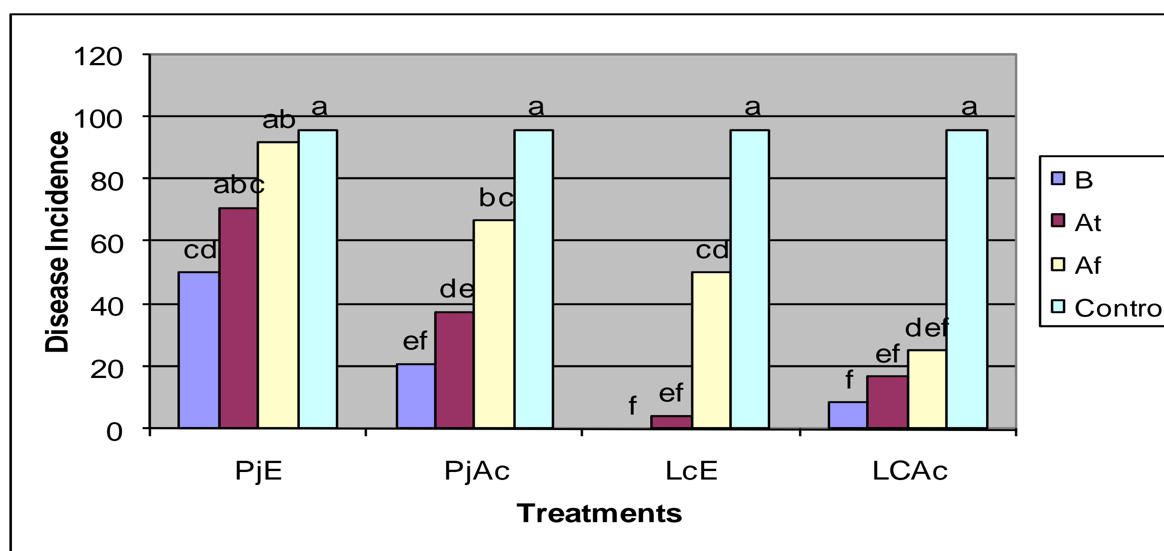

Fig. 3 The effectiveness of *L. camara* and *P. juliflora* extracts on coffee seedling incidence 21th days after pathogen inoculation with different time of inoculation. PjE= *P. juliflora* ethanol extract, PjAc= *P. juliflora* acetone extract, LcE= *L. camara* ethanol extract. LcAc= *L. camara* acetone extract, B= extract before *C. kahawae* spore inoculation, At= extract applied at the same time *with C. kahawae* spore inculcation, Af= extract applied at the same time *with C. kahawae* spore inoculation, Control= Untreated seedlings inoculated only by *C. kahawae* spore.

Coffee seedlings treated with *P. juliflora* 70% acetone extract 2 days before pathogen spore inoculation reduced disease incidence significantly to 8.33% when compared to untreated. Coffee seedlings treated by *P. juliflora* 70% ethanol extract at the same time with inoculation and 2 days after inoculation by the pathogen spore, disease incidence 70.83% and 91.66% respectively have no significance different compared to the control and would be more effective when applied as preventive CBD management rather than as curative disease management method.

5. DISCUSSION

Plants are important sources of potentially useful structures for the development of new chemotherapeutic agents. Plants and their secondary metabolites have shown great potential as antifungal source. The first step towards this goal is the *in vitro* antimicrobial activity (Bikash and Bijaya, 2011).

In this study, the *in vitro* and *in vivo* interaction between solvent type (aqueous 70% ethanol and acetone) and IAS with different time of applications were studied under laboratory and greenhouse conditions. The interaction effects with different solvent types and IAS used significantly varied in terms of radial growth inhibition and disease development during inoculation test.

Extracts of five plants with three different solvent types showed different effects on the pathogen development; all extracts inhibited mycelia growth of the target pathogen when applied 200 mg/ml extract. The fungi toxic effects of ethanol and acetone plant extracts of different plant species indicate the importance of many plant species as possible natural source of fungicidal material. Many workers have reported antifungal activities of different plant species and stressed the importance of plants as possible source of natural fungicides (Tewari and Dath, 1984; Lakshmana, 1990; Tewari, 1995; Philp and Sharm, 1997; Ogbebor and Adekunle, 2005).

In the *in vitro* test, antimicrobial potential of crude extracts tested from different IAS plants, acetone extract of *Lantana camara* shows significant different. It inhibited *Colletotrichum kahawae* radial mycelia growth with 84.13% as compared to the others. The variation (the broad spectrum fungicidal potential) among the plants may be attributed to the different chemical nature of the compounds present in the plant and their part (Afshorypuor *et al*, 1995).

Based on the *in vitro* results, the *in vivo* antifungal activity of the crude extracts of *Lantana camara* and *Prosopis juliflora* ethanol and acetone extracts were tested against *Colletotrihum*

kahawae on detached green berries and seedling inoculation test with different time of application. The crude extracts suppressed lesion development on detached green coffee berries and seedling test to variable degrees. Depending on time of inoculation, the ethanol extracts of *Lantana camara* with 200 mg/ml concentration were most effective both when applied before and at the same time of inoculation on detached berry with *C. kahawae* spore inoculated.

In vivo experiments resulted on the potential of plant extracts to control fungal infections of coffee berry and seedling under controlled conditions. Furthermore, according to Roger and Tiyoli (1996), control measure based on the reduction of initial inoculums at the early stage, before spore infection establishes, is the most desirable method of coffee berry disease control. *Colletotrichum kahawae* can develop within days under favorable moisture and temperature conditions and currently available fungicides must be applied before the pathogen invades host tissue to ensure successful control. Interestingly this study confirmed that 70% ethanol and acetone extract of *L. camara* has the potential to be applied as preventive measure against infection of coffee berry disease caused by *Colletotrichum kahawae* spores at concentration 200 mg/ml.

The application of crude extracts of *Lantana camara* and *Prosopis juliflora* ethanol and acetone extract seems to be a promising convencent, effective and economical alternative for the majority of Ethiopian subsistence farmers who cannot afford synthetic chemicals. Additionally, the risk associated with synthetic chemicals as well as consumer resistance towards its application in agriculture must be accepted as important issues when considering in depth studies on the application potential of natural products in agriculture. The phytochemicals are known to have antimicrobial activity. The curative properties of plants are perhaps due to the presence of various secondary metabolites which are the non nutritive plant compounds. These molecules possess interesting biological activities which attracted several researchers to their elucidation to provide knowledge that will lead to the advancement medicine. The results obtained in the present investigation showed the presence of phytochemicals which take part in defense mechanism of the plants. This suggests a phytochemical basis shown from previous studies to be correlated with invasive potential in

exotic plants (Mitchel and power, 2003, Carpenter and Cappuccino. 2005, Cappuccino and Carpenter. 2005).

This is the first report about antifungal activities of *L. camara* ethanol and acetone extract in Ethiopia against coffee berry disease the causal agent of *Colletotrichum kahawae.* These facts indicated that the chosen strategy to screen against pathogen was reasonable and practical. In a similar study, antimicrobial activity of crude methanolic and acetone extracts of *Lantana camara* was determined against eight test fungal strains. The fungitoxic spectrum of the test plant's leaf and stem extracts indicated maximum percentage growth inhibition at 1000 μgml^{-1} concentration against *Alternaria alternata*. Thus phytochemicals from *L, camara* have a broad antimicrobial spectrum and might be a novel source of antimicrobial drugs

Antagonistic characteristics and phytochemical screening of invasive Alien species has been tested in Nepal Himalaya by hot and cold process of methanolic and water extracts of the alien species (*Ageratina adenophora*, *Eichhornia crassipes*, *Lantana camara*, *Mikania micrantha* and *Parthenium hysterophorus*) against nine phytopathogenic fungi at different concentrations(50,100, 150, 200 mg/ml). The extracts were found to be more effective against the fungal pathogens that may be due to the presence of different bioactive compounds in the plants and their action towards the pathogens. The antifungal activity of the methanolic and aqueous extracts varied. The fungal pathogens were found to be more sensitive towards the extracts tested. *L. camara* extract was most effective towards the fungal pathogens inhibiting all the tested ones. Of the nine tested fungi, *F. eridiforme*, *F. moniliforme*, *F. proliferatum* and *Sclerotium rolfsii* were inhibited by one more concentration of each plant extracts. Among the fungi, *Candida albicans* was most resistant being inhibited by the extract of *L. camara* only. Thus, the study improves knowledge on the scope and use of the invasive Alien plants to control different pests (Bikash and Bijaya, 2011). This shows the broad spectrum of *L.camara* extracts in controlling many fungal phytopathogens.

6. SUMMARY AND CONCLUSIONS

6.1. Summary

Coffee is the single most important cash crop that has been contributing a lion's share to the Ethiopian economy. Coffee berry disease (CBD) is the top major disease of coffee in Ethiopia. It is the major threat to Arabica coffee production since its outbreak in Ethiopia in 1971.

The antifungal activities of crude extracts from aqueous, ethanol and acetone extracts of five invasive alien plant species namely *Parthenium hysterophorus*, *Lantana camara*, *Prosopis juliflora*, *Eichhornia crassipes* and *Mimosa diplotricha* were evaluated in vitro against *C. kahawae* mycelia growth. Crude extracts of all selected invasive alien plants inhibited the mycelia growth of C. *Kahawae in vitro* test. All the crude extracts showed *in vitro* antifungal activity at 20 % (w/v). The *in vitro* results indicated significant differences ($p<0.001$) among plant species tested and solvent types used.

Among the five plants screened *Lantana camara* and *Prosopis juliflora* ethanol and acetone extracts significantly reduced *C. kahawae* mycelia growth compared to the control. *L.camara* reduced radial mycelia growth of the pathogen with 76.23and 84.3%, and *P.juliflora* with 62.63 and 66.96% in 70% ethanol and acetone respectively under in vitro test.

The minimum inhibition concentration of 70% ethanol and acetone crude extracts of *L.camara* and *P.juliflora* were tested against *C. kahawae* at 50,100,150 and 200 mg/ml to determine minimum inhibitory concentration (MIC). The results showed that the MIC for both crude extracts of *P. juliflora* and *L.camara* were 50 mg/ml.

Furthermore, two of the best invasive alien species plant extracts under *in vitro* (*Lantana camara* and *Prosopis juliflora*) were tested on detached green coffee berries and seedlings *in vivo* against disease development at three different times of application (at the time of

inoculation, 48 hrs before inoculation and 48hrs after inoculation) of the pathogen, using completely randomized design with three replications. *Lantana camara* ethanol and acetone extracts reduced disease severity on detached green berries to 20% and 29.1% when applied before inoculation of the pathogen, and on seedling it highly inhibited the effect of *C.kahawae* and shows highly significant difference as compared to the untreated control. It reduces the severity to 0. Acetone extract of *L.camara* also reduces the severity to 0.83%.

6.2. Conclusions

The greatest severity and incidence reduction of coffee berry disease was observed on detached coffee berries and seedling treated with ethanol and acetone extracts of *L. camara* applied two days before inoculation with *C. kahawae* suggesting that the extracts would be more effective when applied preventively as opposed to curatively. The study indicated that the inhibitory effect of the extracts depends on the type of IAS, solvent type and time of application of the extracts. The study indicated the possible use of ethanol extracts of *L.camara* as an alternative means of CBD management.

6.3. Recommendations

The use of ethanol extracts of IAS plants in agricultural practices has advantage technology in preparation of the crude extracts easily to transfer to the farmer and thus promote sustained coffee production. Further studies are required to determine the minimum concentration required for maximum disease control under filed conditions, the frequency and mode of application of the plant extracts, investigation in the active ingredients of the extracts and mode of action are also necessary.

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8. APPENDICES

Appendix I Variance analysis of some invasive alien species extracts and solvent type used on radial mycelia growth of *C. kahawae in vitro* test

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Plant	4	6811.91667	1702.97917	158.36	<.0001
Method	3	15915.84583	5305.28194	493.35	<.0001
Rep	2	3.03333	1.51667	0.14	0.8689
plant*method	12	6776.21667	564.68472	52.51	<.0001
Error	38	408.63333	10.75351		
Corrected Total	59	29915.64583			

Appendix II Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type used minimum inhibition concentration on radial mycelia growth *C. kahawae in vitro* test

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	1161.60	1161.60000	275.41	<.0001
method	1	277.35	277.35000	65.76	<.0001
concentration	4	25648.68333	6412.17083	1520.28	<.0001
rep	2	25.22500	12.61250	2.99	0.0622
plant*method	1	120.41667	120.41667	28.55	<.0001
plant*conc	4	332.81667	83.20417	19.73	<.0001
plant*method*conc	8	282.73333	35.34167	8.38	<.0001
Error	38	160.27500	4.21776		
Corrected Total	59	28009.10000			

Appendix III Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type used and time of application on detached green coffee berries; test severity percentage

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	4408.33333	4408.33333	187.33	<.0001
method	1	1518.75000	1518.75000	64.54	<.0001
time	3	21067.70833	7022.56944	298.41	<.0001
rep	2	44.01042	22.00521	0.94	0.4037
plant*method	1	102.08333	102.08333	4.34	0.0459
plant*time	3	3255.20833	1085.06944	46.11	<.0001
plant*method*time	6	2170.83333	361.80556	15.37	<.0001
Error	30	705.98958	23.53299		
Corrected Total	47	33272.91667			

Appendix IV. Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type and time of application on detached green coffee berries; test incidence percentage

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	833.33333	833.33333	64.00	<.0001
method	1	833.33333	833.33333	64.00	<.0001
time	3	22408.85417	7469.61806	573.67	<.0001
rep	2	26.04167	13.02083	1.00	0.3798
plant*method	1	13.02083	13.02083	1.00	0.3253
plant*time	3	286.45833	95.48611	7.33	0.0008
plant*method*time	6	1445.31250	240.88542	18.50	<.0001
Error	30	390.62500	13.02083		
Corrected Total	47	26236.97917			

Appendix V. Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type used and time of application on coffee seedlings; test severity Percentage

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	1150.52083	1150.52083	129.81	<.0001
method	1	102.08333	102.08333	11.52	0.0020
time	3	43519.43750	14506.47917	1636.77	<.0001
rep	2	4.94792	2.47396	0.28	0.7584
plant*method	1	533.33333	533.33333	60.18	<.0001
plant*time	3	396.35417	132.11806	14.91	<.0001
plant*method*time	6	447.91667	74.65278	8.42	<.0001
Error	30	265.88542	8.86285		
Corrected Total	47	46420.47917			

Appendix VI. Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type used and time of application on coffee seedlings; test AUDPC

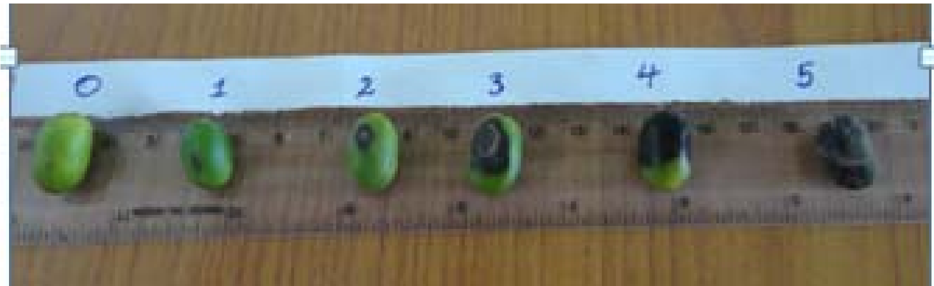
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	952477.01	952477.01	222.45	<.0001
method	1	307132.00	307132.00	71.73	<.0001
time	3	40905131.82	13635043.94	3184.48	<.0001
rep	2	25754.03	12877.02	3.01	0.0645
plant*method	1	142209.81	142209.81	33.21	<.0001
plant*time	3	343298.37	114432.79	26.73	<.0001
plant*method*time	6	309784.56	51630.76	12.06	<.0001
Error	30	128451.49	4281.72		
Corrected Total	47	43114239.8			

Appendix VII. Variance analysis of *P. juliflora* and *L. camara* extracts, solvent type used and time of application on coffee seedlings; test incidence percentage

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
plant	1	10208.33333	10208.33333	142.98	<.0001
method	1	1575.52083	1575.52083	22.07	<.0001
time	3	40638.02083	13546.00694	189.73	<.0001
rep	2	253.90625	126.95313	1.78	0.1863
plant*method	1	1302.08333	1302.08333	18.24	0.0002
plant*time	3	3671.87500	1223.95833	17.14	<.0001
plant*method*time	6	2278.64583	379.77431	5.32	0.0008
Error	30	2141.92708	71.39757		
Corrected Total	47	62070.31250			

Appendix VIII. Crude Extracts of invasive alien species plant extracts

Appendix IX. Detached green berries by (1-5) scale disease measurement



Appendix X. Greenhouse seedling arrangements

Appendix XI. The effectiveness of *L.camara* extracts on coffee seedling inoculation 21st day after pathogen inculcation

control control treated with *L.camara* before and at resp.