

# ADDIS ABABA UNIVERSITY

## SCHOOL OF GRADUATE STUDIES



### EVALUATION OF ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS AGAINST CHOCOLATE SPOT DISEASE (*BOTRYTIS FABAE*) ON FABA BEAN



By

**Roman Mesfin**

June 2010

Addis Ababa

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June 2010

Addis Ababa

### Declaration

I, the undersigned, declared that this is my own original work, has not been presented for a degree to any other university and that all sources of materials used for the thesis have been duly acknowledge.

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Date: \_\_\_\_\_

The work has been done under my supervision

Advisor: Tesfaye Alemu, Ph.D

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## ABBREVIATIONS

cm: centimeter

g: gram

g/l: gram per liter

MIC: Minimum inhibitory concentration

mg: milligram

ml: milliliter

mm: millimeter

PDA: Potato dextrose agar

PG: Polygalacturonase

rpm: rotation per minute

RH: relative humidity

SAR: systemic acquired resistance

Spp.: species

TLC: Thin layer chromatography

## ABSTRACT

Aqueous, ethanol, methanol, chloroform and ethyl acetate extracts of seven plants were screened for antifungal activity against *Botrytis fabae* at different concentrations using poisoned food techniques. The phytopathogenic fungi were isolated from diseased faba bean plants. Four effective plant extracts were selected *in vivo* test on the infection of *B. fabae* on faba bean plants, were evaluated and measured in comparison with sterile distilled water which was used as control. The result revealed that aqueous extracts of immature and matured leaf extracts of *C. macrostchys* completely inhibited mycelial growth at 30mg/ml and 50mg/ml respectively. *C. aurea* extract also inhibited fungal growth at 50mg/ml concentration. Most of the selected plants were highly extractable with ethanol especially, the immature and mature leaf extracts of *S. marginatum* and immature leaf extracts of *S. incunum* significantly inhibited the growth of *B. fabae* at a concentration of 30mg/ml, followed by immature leaf extracts of *D. stramonium* and mature leaf extracts of *S. incunum* at 40mg/ml concentration. From the methanol extracts *D. stramonium* and *C. aurea* showed the highest inhibition. It was observed that the chloroform extracts of most selected plants do not show significant effect but, *C. macrostchys* at 40mg/ml inhibit 62%. The ethyl acetate extracts of all selected plants do not show effectiveness on the inhibition of the selected isolate. It was observed that ethanol extracts showed highly significant antifungal activity followed by aqueous and methanol extracts. The chloroform and ethyl acetate extracts were inferior on inhibition of the fungal isolate. Methanol extracts of *D. stramonium*, *C. aurea*, ethanol extracts of *S. marginatum* and aqueous extracts of *C. macrostchys* were selected from the *in vitro* evaluation and tested *in vivo* on faba bean plants and at 20% show significant inhibition. Mixture of plant extracts and bioagents *Trichoderma harzianum* show significant inhibition on the growth of *B. fabae* on faba bean *in vivo* test. The semi purified fraction of chloroform and ethyl acetate extracts of the selected plants did not exhibit effective antifungal activity, but the aqueous solubilized fraction of all plants show significant inhibition.

**Key words:** Antifungal compounds, Biocontrol, *Botrytis fabae*, MIC, Plant extracts, Solvent

## 1. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. Plants are the sources of natural pesticides that make excellent leads for new pesticide development (Bobbarala *et al.*, 2009). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Doughari *et al.*, 2008).

Plant extracts have played significant role in the inhibition of seed-borne pathogens and in the improvement of seed quality and field emergence of plant seeds. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Patel, 2007). Natural plant products are biodegradable, exhibit structural diversity and complexity and rarely contain halogenated atoms. These can act directly as pesticides or may provide structure lead for pesticidal discovery.

Several plant families like Acanthaceae, Amranthaceae, and Magnoliaceae are known for their antifungal properties (Ashraf and Javaid, 2007). Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoid, which have been found *in vitro* to have antimicrobial properties. Extracts of many plants are now known to exhibit antimicrobial activity (Dewanjee *et al.*, 2007, Rani *et al.*, 2008).

Resistance to fungicides is one of critical causes of poor disease control in agriculture, followed by their phytotoxicity, resistance and pollution (Suieiman and Emua, 2009). There is a need to develop alternative agents for the control of pathogenic fungal diseases in plants. Plant-derived natural substances are considered as non-phytotoxic compounds and potentially effective against plant pathogenic fungi. The natural plant products have an ability to inhibit soil borne plant pathogen and control disease development in the host plant. These plant extracts have potential as environmentally safe alternatives and as components in integrated pest management programs (Bowers and Locke, 2004).

Although plant based pesticides are cheap, locally available, non-toxic and easily biodegradable limited efforts have been made to screen plants that are suspected to possess antimicrobial properties for effect against pathogenic micro organisms. Higher plants may contain secondary compounds that could effectively control plant diseases, but which are yet to be exploited and used as pesticides. Although there is a growing interest in the use of medicinal plants to control plant diseases, only about 2,400 plant species among more than 250,000 higher plants have been screened for phytoactivity (Nduagu *et al.*, 2008).

In recent years, antifungal agents such as plant-based essential oils and extracts were focus of attention to control phytopathogens in agriculture. Historically, many plant oils and extracts have been reported to have antimicrobial properties (Joseph *et al.*, 2008). It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds. The resurgence of interest in natural control of plant pathogens and increasing consumer demand for effective, safe, natural products means that quantitative data on plant oils and extracts are required (Hadizadeh, *et al.*, 2009).

Some phenolic compounds showed antifungal activity against common pathogens of cucumber, such as *Botrytis cinerea*, *Pythium ultimum*, and *P. aphanidermatum*, which suggests that the plant extract would induce synthesis of antifungal compounds and contribute to disease resistance (Daayf *et al.*, 2000). Antimicrobial substances originated from plant have been extensively studied for their use as an agrochemical with highly selective activity against some plant pathogens or as lead molecules for their synthesis of new chemical fungicides (Hur *et al.*, 2000). Therefore, the current research findings have been initiated to study *in vitro* and *in vivo* evaluation of some botanical plants extracts against chocolate leaf spot (*Botrytis fabae*) of Faba bean.

## **2. LITERATURE REVIEW**

### **2.1. Problems of chemical control**

Today, there is a growing movement in many countries to reduce the amount of chemicals being released into the environment. Among the notable hazards of misuse and overuse of pesticides are the induction of resistance in plant pathogens, occupational hazards, environmental pollution, destruction of natural enemies (ecological hazards) and residues in food which are poisonous and/or carcinogenic, mutagenicity, genetic damage and teratogenicity, acute and chronic residual toxicities and severe and acute mammalian toxicities. Due to their residual nature these synthetic chemicals can adversely affect a number of biological systems. With regard to acute toxicity, fungicides are reported to be less hazardous compared to insecticides and herbicides, though the mercurial compounds could be exceptional in this instance, and they are gradually being replaced.

Some fungicides are known to have phytotoxic side effects. Specifically acting fungicides have been observed to cause pathogen resistance. One example of this is the complete failure of the benzimidazole fungicides (benomyl, thiabendazole, carbendazin, thiophanate methyl) due to the development of resistance in a number of pathogenic fungi. Presently, such alternatives seem to come from natural plant products which are known to be of low mammalian toxicity and are highly biodegradable. The use of such pesticides in pest control strategies could result in an increased reduction of the amount of synthetic pesticides with positive effects on the environment (Englelska and Radgivar, 1995).

Keeping in view the draw back of chemical management of plant disease the use of plant extracts in the management of plant disease is gaining importance (Makovitzki *et al.*, 2007, Okereke *et al.*, 2007, Joseph *et al.*, 2008). The use of natural products for the control of fungal disease in plant is considered as an interesting alternative to synthetic fungicide due to their proven nature specificity, biodegradability, low toxicity, minimum residual toxicity in the ecosystem and lower negative impacts on the environment (Ke-qiang and HCvan Bruggen, 2001, Ogba and Ovibo, 2008). There has been a renewed interest in botanical pesticides because of several distinct advantages: (i) Pesticidal plants are generally much safer than conventionally used synthetic pesticides. Pesticidal plants have been in nature as its component for millions of years without

any ill or adverse effect on the ecosystem. (ii) Plant-based pesticides will be renewal in nature and would be cheaper. (iii) Some plants have more than one chemical as an active principle responsible for their biological properties. These may be either for one particular biological effect or may have diverse biological effects. The chances of developing quick resistance to different chemicals are highly unlikely. A number of antifungal compounds of diverse skeletal patterns have been found in plants. However, only a few commercial products from plant are used in practical plant protection (Choi *et al.*, 2004).

## **2.2. Important Botanical plants**

### **2.2.1. Neem**

As a fungicide, neem oil is mainly used as a preventative and when disease is just starting to show. Neem oil is effective against rots, mildews, rusts, scab, leaf spot and blights. Neem leaf extract gave the highest inhibition for seed born pathogenic fungi. The growth of *Fusarium oxysporum f.sp.ciceri*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Sclerotinia sclerotium* inhibited by neem oil (Singh *et al.*, 1980). Neem leaf extracts inhibited mycelial growth of *Phytophthora*, (Ramos *et al.*, 2007). The mycelial growth of *Fusarium moniliforme*, *Botryodiplodia theobromae*, *Aspergillus niger* and *Aspergillus flavus* was inhibited (Nwachukwu and Umechuruba, 2001).

### **2.2.2. Eucalyptus**

The *Eucalyptus* extracts were effective in inhibiting mycelial growth of the phyto-pathogenic fungi. *Pythium* species *P. aphanidermatum*, *P. graminicola*, *P. ultimum* and *Rhizoctonia solani* are inhibited by *eucalyptus* extracts. Antifungal activity of the *eucalyptus* extracts against soil-borne fungal pathogens of the grasses could provide environmentally friendly alternative to chemical fungicides for managing the pathogens. The pathogenic fungal isolates of *Botrytis cinerea* and *Phomopsis* sp. were effectively inhibited by the extracts. Six fungal pathogens of *Bipolaris coicis*, *Botrytis cinerea*, *Fusarium moniliforme*, *Magnaporthe grisea*, *Phomopsis* sp. and *Rhizoctonia solani* were also severely affected by the extract of *E. unigera*. Moderate inhibitions of mycelial growth were also found in *Colletotrichum gloeosporioides*, *C. coccodes*, *Fusarium graminearum*, *F. oxysporum*, *F. solani* and *Phomopsis soje* (Hur *et al.*, 2000).

### 2.2.3. Garlic (*Allium sativum*)

Garlic has anti-feedant (insect stop feeding), bacterial, fungicidal, insecticidal, nematicidal and repellent properties. Among the natural fungicide substance, garlic (*Allium sativum*) extract has been found active in various trials in vitro and to a less degree in vivo. *Allium sativum* L. show highest inhibition on germination of *Phytophthora infestance*. The inhibition rate on germination of sporangia and zoospores increased with increasing concentration. Garlic extract from fresh bulbs had stronger inhibiting effect against the germination of sporangia and zoospores. However, there was no significance difference between the extract of immediately made and those which was kept for a week under 4<sup>0</sup>c (Ke-Qiang, 2001)

## 2.3. Major Groups of Antimicrobial Compounds from Plants

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant.

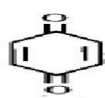
### 2.3.1. Simple phenols and phenolic acids

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenyl propane-derived compounds which are in the highest oxidation state. Caffeic acid is effective against viruses, bacteria, and fungi. Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. More highly oxidized phenols are more inhibitory. The mechanisms for phenolic toxicity to microorganisms include Substrate deprivation, Membrane disruption, and enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999)



### 2.3.2. Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are highly reactive. They provide source of stable free radicals, complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes and render substrates unavailable to the microorganism.



Quinone

### 2.3.3. Flavones and flavonoids

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). Flavonoids are hydroxylated phenolic substances. Since they are synthesized by plants in response to microbial infection, they have not been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes. Flavonoid compounds exhibit inhibitory effects against multiple viruses (Cowan, 1999).

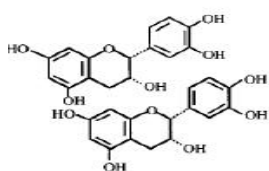


Flavones

### 2.3.4. Tannins

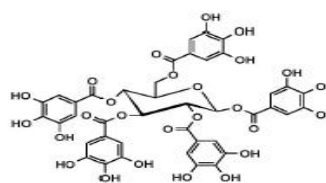
Tannins are found in almost every plant part. They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone

units. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, Complex with cell wall, membrane disruption, metal ion complexation, Substrate deprivation etc... Tannins can be toxic to filamentous fungi, yeasts, and bacteria.



Structure of tannin

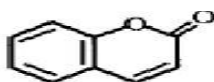
Condensed tannin



Hydrolyzable

### 2.3.5. Coumarins

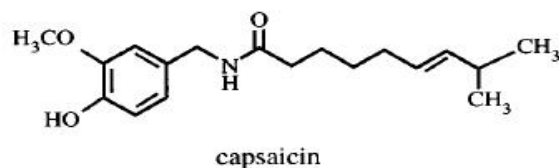
*Coumarins* are phenolic substances made of fused benzene and  $\alpha$ -pyrone rings. Phytoalexins, which are hydroxylated derivatives of coumarins, have antifungal activity.



Coumarins

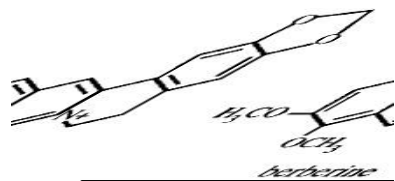
### 2.3.6. Terpenoids and Essential Oils

Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Essential oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, and they occur as diterpenes, triterpenes, and tetraterpenes, as well as hemiterpenes and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenes or terpenoids are active against bacteria, fungi, viruses, and protozoa (Cowan, 1999).



### 2.3.7. Alkaloids

Alkaloids rank among the most efficient and therapeutically significant plant substances. They are chemically very diverse group of organic nitrogen compounds. Generally they are extremely toxic though they do have a marked therapeutic effect in minute quantities. Pure, isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents all over the world for their antispasmodic and bactericidal effects (Ciocan and Bara, 2007). Alkaloids from medicinal species have been used in folk medicine as anti-infectious agents. Mode of action is intercalated into cell wall and DNA (Abad, 2007).

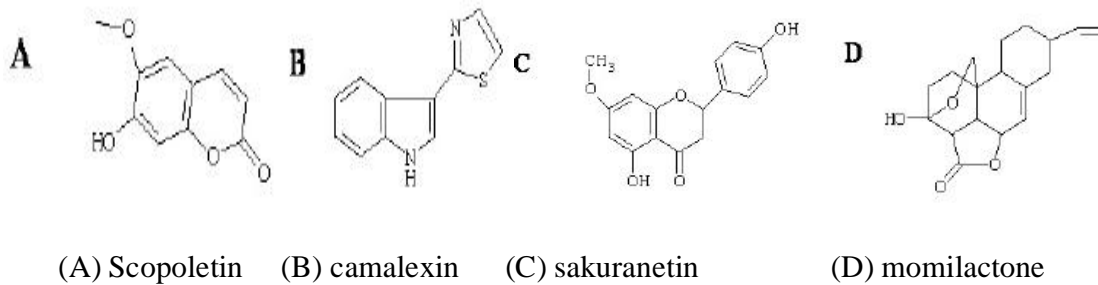


### 2.4. Phytoanticipins versus Phytoalexins

To arrest the spread of pathogens, plants possess an innate immunity that involves different layers of defence responses. Some of these defences are preformed and others are activated after recognition of pathogen elicitors, and include reinforcement of the cell wall, biosynthesis of lytic enzymes and production of secondary metabolites and pathogenesis related proteins (Lamothe *et al.*, 2009).

To protect themselves, plants accumulate an armoury of antimicrobial secondary metabolites. Some metabolites represent constitutive chemical barriers to microbial attack (phytoanticipins) and others inducible antimicrobials (phytoalexins). They are extensively studied as promising plant and human disease-controlling agents. The antimicrobial plant compounds that have received more attention in plant defence are the phytoalexins. These are antimicrobial compounds which require *de novo* expression of the enzymes involved in their biosynthetic pathways after elicitation. Production requires transcriptional and/or translational activity in the

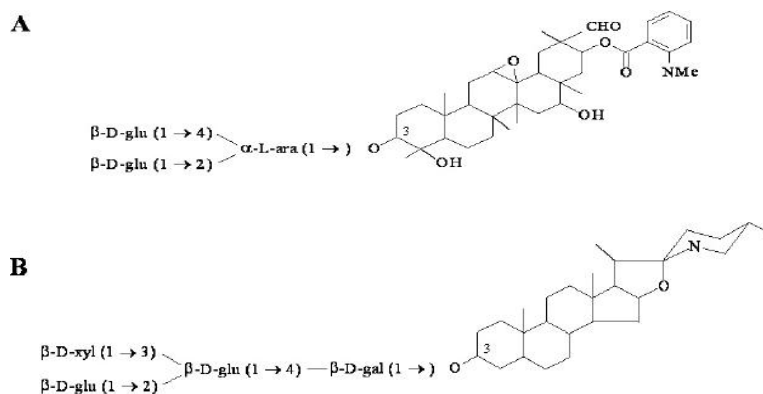
plant once the pathogen has been detected. The induced response mechanism also involves the trafficking and secretion of antimicrobial compounds to the infection site. The role of phytoalexins in defence mechanisms was intensively studied mean while to constitutive compounds has been paid less attention (Deepak *et al.*, 2007). Antimicrobial phytoalexin structures:



Phytoanticipin are low molecular weight antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents. Some phytoanticipins are found at the plant surface. Others are sequestered as preformed compounds in vacuoles or organelles and released through a hydrolyzing enzyme after pathogen challenge. Because the enzyme involved in the final liberation of the molecule is not formed *de novo* these compounds are not considered as phytoalexins (Lamothe *et al.*, 2009).

Saponins are glycosylated phytoanticipins that are found in a wide range of plant species. Because they have potent antimicrobial activities it is proposed that the natural role of these molecules in plants is to confer protection against potential pathogens. Avenacins are oat root saponins. The antifungal activity of avenacin is associated with its ability to form complexes with sterols present in fungal membrane leading to pore formation and loss of membrane integrity.

The major saponin in tomato is  $\alpha$ -tomatine. This is accumulated in healthy plants in its biologically active form. *Septoria lycopersici* produces tomatinase, an extracellular enzyme that hydrolyses  $\alpha$ -tomatine to  $\beta$ -tomatine, which is less toxic to the fungus. The degradation product of  $\alpha$ -tomatine is able to suppress the defense response. Therefore pathogen resistance cannot only be attributed to the disappearance of the antimicrobial compound, but also to the capacity of the degradation product of the phytoanticipins to suppress defense responses. Antimicrobial phytoanticipin structures (A) avenacin A-1 (B)  $\alpha$ -tomatine



#### 2.4.1. Biotechnological Applications of Phytoanticipins and Phytoalexins in Phytoprotection

Phytoanticipins and phytoalexins in the plant defence response are to develop biotechnological applications in crop protection. Phytoalexins provide a potentially interesting weapon to be used in agricultural techniques. The use of phytoalexin itself as a phytoprotectant presumes that the molecule is only toxic against pathogenic agents. Moreover, the synthesis or isolation of phytoalexins is very expensive compared to commercial fungicidal molecules. An alternative approach would be the production of plants that express a higher quantity of phytoalexins either by spraying with phytoalexin elicitors, by pre-immunization through a non pathogen inoculation, or by genetic transformation (Lamothe *et al.*, 2009).

#### 2.5. Important diseases of faba bean

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes and is now cultivated on large areas in many countries due to its high nutritive value in terms of energy and protein contents (24-30%). China leads in production, followed by Ethiopia, Egypt, Italy and Morocco. The crop occupies the largest area among the pulses in Ethiopia. Even though Ethiopia is the world's second largest producer of faba bean, its share is only 6.96% of world production and 40.5% of Africa.

These crops are an integral part of cool season agricultural systems throughout the country, providing an inexpensive source of protein, minerals (iron, zinc, calcium) and vitamins (B1, B2, C) in human diet and livestock rations and a source of biological nitrogen fixation in cereal rotation systems (Khalil and Erskine, 2001). Therefore, increasing the crop production is one of the most important targets of agricultural policy in several countries.

The production of faba bean is constrained by several yield limiting factors, of which diseases are the main factors. Faba bean is constrained mainly by the fungal diseases, chocolate spot (*Botrytis fabae*), rust (*Uromyces fabae*), alternaria (*Alternaria alternata*) and downy mildew (*Peronospora viciae*) which cause yield losses. Powdery mildew, fusarium, sclerotinia stem blight and rhizoctonia root rots were also prevalent diseases of faba bean (McKenzie and Morrall, 1975). *Alternaria* spp., *Cladosporium* spp., and *Coniella pulchella* Hohnel cause leaf spots. Significant problems were encountered recently with aphid transmitted viruses of which the most damaging are faba bean necrotic yellow virus and bean yellow mosaic virus (Khalil and Erskine, 2001). The parasitic weed, broomrape (*Orobanche crenata*), one of several species able to parasitise the roots of faba bean, causes heavy yield damage (Khalil and Erskine, 2001).

*B. cinerea* causing the grey mould of numerous vegetable crops is a weak pathogen on *V. faba*, producing limited, non-coalescent and non-sporulating lesions. Ascochyta blights are important foliar diseases of faba bean in many countries. The pathogens are seed borne in their respective hosts. The pycnidia can be seen with a naked eye. The fungus survives in the contaminated seeds which directly infect the developing seedling causing its emergence deficiency (Kaiser *et al.*, 1997). Chocolate spot of faba bean caused by *Botrytis fabae* Sard. is the most widespread and destructive disease in Ethiopia with yield reductions of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987, cited in Sahile *et al.*, 2009). In some countries, chocolate spot is caused by *Botrytis cinerea*, but in Ethiopia, only *B. fabae* is known to cause the disease (Sahile *et al.*, 2009).

### **2.5.1. The Genus *Botrytis***

Fungi of the genus *Botrytis* are important pathogens of many agronomically important crops, such as grapevine, tomato, bulb flowers, and ornamental crops. *Botrytis* diseases appear primarily as blossom blights and fruit rots but also as leaf spots and bulb rots in the field and in stored products. *Botrytis* species are necrotrophs, inducing host-cell death resulting in progressive decay of infected plant tissue. The pathogen produces abundantly sporulating gray mycelium on infected tissue. Macroconidia (mitotically produced spores) can be transported by wind over long distances.

*Botrytis* overwinters in the soil as mycelium in decaying plant debris and as sclerotia, melanized mycelial survival structures. Some species frequently produce a sexual teleomorphic stage in which ascospores are produced in an apothecium. When collected in nature, apothecia are found under cool weather conditions, arising from sclerotia, which have developed on decayed plant parts in moist soil. *Botrytis* and its sexual form *Botryotinia* Whetzel comprise 22 species are classified within the family *Sclerotiniaceae* Whetzel (Inoperculate Discomycetes). Delineation of species has traditionally been based on morphological characteristics, especially macroconidium ontogeny, and species have been named based on host association. Most species have a worldwide distribution or occur wherever their host crops are grown (Staats, 2005).

The genus *Botrytis* comprises one generalist, *B. cinerea*, infecting over 200 eudicot hosts, especially senescing or otherwise weakened or wounded plants. All other species are considered specialists with a narrow host range. They infect only one or a few closely related species within the same plant genus with the exception of *B. fabae*, which can infect species of the genera *Vicia*, *Lens*, *Pisum*, and *Phaseolus*, all belonging to *Fabaceae*. Specialized species occur on corolliferous monocotyledons and on members from the four eudicot families *Fabaceae*, *Ranunculaceae*, *Geraniaceae*, and *Paeoniaceae*. Narrow host range *Botrytis* species parasitize living leaves or bulbs of their hosts but may also occur as saprophytes. In many cases, host-specific *Botrytis* species are able to cause primary lesions on a non host, but these primary lesions fail to expand (Staats, 2005).

Several specialists are able to infect members of the same plant family or the same plant species. Three species of *Botryotinia* have been described occurring on the buttercup family *Ranunculaceae*; *B. calthae* occurs on *Caltha palustris* L., *B. ranunculi* on *Ranunculus* spp., and *B. ficariarum* on *Ficaria verna*. As many as seven *Botrytis* species occur on *Allium* spp., which is one of the largest genera of the petaloid monocotyledons. *Allium* includes several economically important species, such common onion, garlic, chives, and leek, all of which are infected by *B. byssoidea*, *B. aclada*, and *B. allii*. *B. byssoidea* and *B. aclada* are considered to be its parental species. Other *Botrytis* species infecting *Allium* spp. have a more restricted host range; they have become specialized on wild *Allium* spp. or infect only one or two economically important *Allium* crops (Staats, 2005).

### **2.5.2. The pathogen *Botrytis fabae* (Chocolate spot disease)**

*B. fabae* has been known to cause chocolate Spot on Faba bean (*Vicia faba*) and has also been associated with apple (*Malus pumila*) leaves. Sardina (1929) was the first to associate *Botrytis* with chocolate spot in Spain. He considered a new species, *B. fabae* to be the primary cause of chocolate spot, but *B. cinerea* could cause similar lesions. *B. fabae* is more pathogenic than *B. cinerea* in terms of the number of conidia formed and the rate of disease spread in vitro (McKenzie and Morrall, 1975).

#### **2.5.2.1. Symptoms of Chocolate spot disease**

Chocolate spot is first seen as reddish to chocolate brown, slightly flattened spots appearing on lower leaves. This is its non-aggressive phase, which is thought to have little effect. In wet conditions the disease will spread quickly and be termed aggressive. As lesions increase in number, generally on the upper leaf side, they may remain small, expand, or merge. Under favorable conditions of continuous high humidity, the disease become aggressive and lesions may coalesce causing blackening and partial defoliation. If the disease becomes severe at an early stage of growth before the fruit matures, the entire crop can be lost (Gourley and Delbridge, 1973, Matthews, 2003).

#### **2.5.2.2. Morphology and physical factors that affect growth of *Botrytis fabae***

The sclerotia, mycelium and conidia over winter remain in or on plant debris and in soil. *Botrytis* conidia are dispersed in rain and wind, and when a conidium lands on a seedling it then germinates and penetrates the host tissue. As mycelia progresses into host cells, the infected cells collapse and disintegrate. Environmental factors can have a profound effect on disease incidence. Humidity has a major effect on lesion expansion after infection; in dry weather, infections can remain dry for over a month. Humidity less than 66% cause very slow lesion expansion, and are generally directly related to the rate of lesion expansion. Sporulation at 10 and 20 °c occurred on field bean leaves when the relative humidity of the air was 92-100%. Soil pH has also shown a strong relationship with disease incidence. Acidic soil had significantly greater disease incidence than plants in basic soil. *B. fabae* infects only a few closely related plant species, including *Vicia sativica* and *V. narbonensis* (Buzi *et al.*, 2003). Severely affected plants lost over 50% of foliage and pod development was poor (Koike, 1998).

### 2.5.2.3. Biology and Epidemiology of *Botrytis fabae*

The fungus overwinters as sclerotia which sporulate and produce conidia in spring in wet conditions. The conidia contaminate all the plant organs and cause the chocolate spots. The fungus thrives in an optimum temperature of 15 -20 °C. High RH (>80%) is necessary for conidial sporulation on the spots. In dry weather the fungus remains dormant in the host tissues or grows slowly. In humid conditions there is rapid interance and extra cellular spreading of the disease\_(Gourley and Delbridge, 1973). In recent work examining the effects of the essential oil of *Ocimum basilicum* on fungal pathogens of broad bean were shown to possess powerful antifungal activity both *in vitro* and *in vivo* (Oxenham, 2005).



Chocolate spot in beans Source: (Diego, 2009)

As *B. fabae* spread and sporulate form lesions, *V. faba* tissues produce as a post-infection defence response against fungal pathogens, low-molecular-weight secondary metabolites, such as furanoacetylenic phytoalexins inhibits the fungal growth. The greater ability of *B. fabae* to colonize broad bean tissues seems to be related to its capacity to detoxify broad bean phytoalexins and to reduce their toxic effects. *B. fabae* is able to produce pectin degrading enzymes, such as polygalacturonase (PG), during development of chocolate spot. These enzymes were indicated as the principle cause of plant cell death during lesion development. The central role of pectin-degrading enzymes in *B. fabae* was host cell wall breakdown. Pectolytic enzymes have been suggested to be responsible for plant cell death (Buzi *et al.*, 2003).

#### **2.5.2.4. Disease Management**

##### **2.5.2.4.1. Cultural management**

The proportion of successful isolations of *B. fabae* from leaves in a bean crop decreased with increasing distance from debris from a previous bean crop. This highlights the importance of the source of inoculums and of rotating bean crops between locations at a distance from each other. Rotating crops, burning infected debris, removing volunteer bean seedlings and reducing non-crop hosts from the cropping vicinity reduce the inoculums level. Increasing spacing between plants reduce the sporulation that requires high relative humidity.

##### **2.5.2.4.2. Chemical management**

There are a range of fungicides available to control Botrytis, and selection of the most appropriate fungicide could depend on the level of disease pressure present. Fungicides containing mancozeb, chlorothalonil, carbendazim, or procymidone have activity against Botrytis. If disease pressure is high then carbendazim or procymidone are the preferred fungicides. It is worth remembering that these products are protectants and are most effective if applied before disease development. The performance of a chemical can be enhanced through various non chemical tactics to reduce inocula. Additionally, with in chemical control, numerous variables can influence the performance of a chemical. Some examples of variables affecting success of chemical-application technology include the following: time of applications, spray dilution, chemical rate per unit area and rainfall. Beginning spray programs prior to or at first sign of disease are best (Pernezny et al., 2008).

##### **2.5.2.4.3. Biological management**

The widespread use of chemical crop protection is becoming increasingly unsatisfactory due to public health and environmental concerns, and selection of more virulent isolates. Most sustainable and environmentally acceptable control may be achieved using biocontrol agents. Biocontrol systems of antagonistic microorganisms have involved various modes of actions by competition for nutrients and space and/or induction of host resistance mechanisms. Microbial

antagonists helps to establish potential biocontrol with no environmental and health problems (Mekbib *et al.*, 2006).

The antifungal activity of some compounds is due to their ability to affect function or the structure of the fungal cell. Such compounds include enzymes, antibiotics and proteins. Cell wall- degrading enzymes such as chitinases,  $\alpha$ -1, 3-glucanases, proteases and cellulases are involved in the antagonistic activity of biocontrol agents against phytopathogenic fungi. Fungal proteases may play a significant role in cell wall lysis. Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds in proteins. *B. fabae* have shown widespread resistance to conventional fungicides. Alternative control measures are being explored with biological control using various *Bacillus* isolates and Actinomycetes because of their greater capacity for antibiotic production (Jayalakshmi *et al.*, 2009)

Most commercial serine proteases are produced by microorganisms belonging to genus *Bacillus*, *Streptomyces*, and *Trichoderma*. The antifungal potential of proteases have evaluated from *T. flavus* and *T. harzianum* against *B. fabae* the causal agent of brown spot disease in faba bean and characterized the proteases and determine their effect *in vitro* against spore germination and mycelium growth rate, extracellular polygalacturonase and carboxymethyl cellulose activities of *B. fabae* ( Haggag *et al.*, 2006).

#### **2.5.2.4.4. Resistance varieties**

Recently the worldwide faba bean germplasm collected has been made available for evaluation and crossing. As a result some lines showing resistance to chocolate spot were obtained and used as genetic resources for resistance to chocolate spot disease. Using new varieties which produce more yield than local varieties, and have improved resistance to chocolate spot diseases (Khalil and Erskine, 2001).

#### **2.5.2.4.5. Integrated disease management**

Integrated disease control for faba bean studies conducted on farmers' fields under different environmental conditions showed that newly released varieties with resistance to chocolate spot responded less to fungicidal applications. These findings led to the development of improved disease control packages. The improved package for the integrated control of chocolate spot

disease uses the resistant faba bean sown, treated with the fungicide Diathane. The use of new, resistant varieties has reduced the use of chemicals drastically. The integrated control package for faba bean necrotic yellow virus which recommends a specific early maturing variety, early sowing rouging of infected plants and spraying with the aphicide Pirimor reduced the virus incidence (Khalil and Erskine, 2001).

## **2.6. Characteristics of botanical plants**

### **2.6.1. *Datura stramonium* L.**

The plant *Datura stramonium* *attafaris*, *astenagrt* (in Amharic) is known by a variety of names Jimson weed, Moon Flower, Thorn Apple, Stinkweed, Mad Apple. Jimson weed grows in most habitats, but thrives in high-nutrient soil. It is found throughout many parts of the world. The plant is an annual that reaches five to six feet in height. It has dark green leaves, large, 7 to 20 cm long irregular teeth, and white flowers. *D. stramonium* flowers from May to September, and the seeds, which contain the most alkaloid, appear in the fall (Stuart, 2005, Geeta and Gharaibeh 2007).

The flowers are one of the most distinctive characteristics of *D. stramonium* they are trumpet-shaped, white and 5-12.5 cm long. The fruit are walnut-sized, egg-shaped, and covered in prickles; they split into four chambers, each with a few kidney-shaped seeds. The flowers open and close at irregular intervals during the evening, earning the plant the nick name Moonflower. The seed pod has fewer spines but they are very stout. They're fairly well behaved, however if you allow the seed to drop from the pods onto the ground they will reseed themselves perhaps thicker than your prefer. They are excellent bloomers once they get started. All parts of the plant emit a foul odor when crushed (Apple, 2006).

Both seeds and leaves are used in medicine. Dried, ground leaves are mixed with butter and used as a fungicide to treat scalp infections. The vapor of boiled seed pods is inhaled to alleviate the pain of toothache. The black seeds are poisonous and a few seeds in a cup of tea or coffee are enough to produce instant death resulting from heart paralysis and in smaller quantity the powder from the seeds when mixed in local drinks such as areke, tej, and tella causes temporary insanity, a practice often used to make the drink more strong. All parts of the plant are considered poisonous (Amare Getahun, 1976). Fresh leaves are used for rubbing and dressing against 'fore

fore' (dandruff) and swellings (Fisseha Mesfin *et al.*, 2009). *D. stramonium* has alkaloid and flavonoid contents with antioxidant activity. Flavonoids are a group of polyphenolic compounds with known properties which include inhibition of hydrolytic oxidative enzymes and anti-inflammatory action (Kumar *et al.*, 2008).

The antiasthmatic and antispasmodic effects of the drug are mainly due to the presence of the alkaloids. Atropine is used to treat nerve gas poisoning, peptic ulcers, diarrhea and bronchial asthma. *D. stramonium* is documented to palliate the pain caused by muscular rheumatism, neuralgia, hemorrhoids, fistula, abscesses and similar inflammations. Careful consideration of the toxicity of the plant is required before its use (Hammouda *et al.*, 1997).



*Datura stramonium* Source: (Apple, 2006)

Goats will occasionally eat Jimsonweed, and subsequently die a slow and painful death. Tropane alkaloids are some of the few substances which cause true hallucinations which cannot be distinguished from reality (Wiebe *et al.*, 2008)

Leaf trichome density is considered a mechanism of defense in plants to prevent damage by herbivores. Trichome density is a component of plant resistance to herbivores in most populations of *D. stramonium*. Selection is expected to vary spatially and temporally in plant-animal interactions, and this constitutes the raw material of the co evolutionary process (Valverde *et al.*, 2001).

### 2.6.2. *Solanum marginatum* L.f

Common name White Margined Nightshade is in the family Solanaceae. The plant up to one meter in height and quite common in damp spots on roadsides. It is easily recognizable from the more common imbway (*Solanum incanum*) by its larger leaves that are white beneath and the large fruit so the flower is white. *S. marginatum* is a shrub native to Ethiopia. The ornamental oval, undulated leaves have a white margin, and are white underneath, pinnately lobed covered with fine white stellate pubescence which in age disappears from the upper surface except near the margin (Wright, 1905). The ripe yellow fruits are boiled and used as a soap substitute in clothes. The seed, roasted lightly, is used as medicine for weak heart and stomach complaints. The fruits are also used in leather tannery (Amare Getahun, 1976).

It is andromonoecious with long- styled hermaphrodite flowers and medium or short styled female sterile flowers which produce fertile pollen. It has potential economic importance because its fruit are source of solaaodine, asteroid alkaloid used in the commercial production of sex hormones (Dulberger *et al.*, 1981). Fruit and Leave of *s. marginatum* are Steroidal Sapogenin Yielding Plants (Singh and Kaushal, 2007).



*Solanum marginatum* Source: (Singh and Kaushal, 2007)

Effect attributed to Spirosolane glycoalkaloid bearing *Solanum* spp. led to investigate the potential antifidant/insecticidal activity. Crude plant extract obtained by extraction with ethanol of dried powdered fruits and leaves of *S. marginatum* showed asignificant inhibitory effect on larvae growth of *Tribolium castaneum* and *Manduca sexta* (Weissenberg, 1988). Areal part of *S. marginatum* which is prepared by infusion, decoction can take orally and have medicinal value for cough, administered as bath for general body joints pain and clean ailments (Manuel *et al.*, 2005).

### 2.6.3. *Solanum incanum* L.

Inboye (Amharic) Ingulla (Tigrigna) Apple of Sodam (English), vernacular names Thorn apple, grey bitter apple, Snake apple is in the Family Solanaceae. Shrub or herb widely spread, very common in overgrazed range areas or road- sides. It is considered an indicator for low-fertility soils. The globose fruits are bright orange in color and children are thus tempted to eat them. It is herb or shrub with spines on the stem, leaves, stalks and calyces, and with velvet hairs on the leaves. Flowers in clusters along the branches corolla pale to deep blue, purple, occasionally white. Fruit spherical, green, often striped or mottled with white, turning yellow to orange-brown when ripe. They are mostly toxic, fruits are used to treat gonorrhoea and in leather tannery by mixing with urine (Amare Getahun, 1976).



*Solanum incunum* Source: (Hyde and Wursten 2010)

*S. incanum* contains saponin steroids, in particular glycoalkaloids, which are found in all parts of the plant, but in highest concentrations in the fruit. Alkaloids such as solasodine are used commercially as precursors for the production of steroidal compounds for medicinal use, mainly as contraceptives. Flavonoids and chlorogenic acid, a phenolic derivative, have also been isolated. Solamargine has shown promise for treatment of liver, lung and breast cancer (Beaman-imbaya and Muhammed, 1976).

A methanol extract of the fruits showed broad-spectrum antifungal activities. It has a strong effect against the fungus that causes athlete's foot, *Trichophyton mentogrophytes*. The water extract of the fruit showed antibacterial activity against *Bacillus subtilis*, *Micrococcus flavus* and *Pseudomonas aeruginosa* and the methanol extract against *Staphylococcus aureus* and *Micrococcus flavus*. Chlorogenic acid has insect repellent properties and when ingested by insects decreased growth and development.

Throughout tropical Africa a sore throat, stomach-ache, headache, painful menstruation, liver pain and pain caused by onchocerciasis, pneumonia and rheumatism are treated with *S. incanum*. For these purposes, leaf, root and fruit decoctions are drunk, roots are chewed and sap swallowed, leaf paste, root infusions and pounded fruits are applied externally or rubbed into scarifications, leaf sap is used for washing painful areas, and ash of burnt plants is mixed with fat and applied externally. For relief of toothache a root infusion is used as mouth wash, fruit or root is rubbed on the gums or smoke of burning seeds is inhaled (Mirutse Giday *et al.*, 2009). Another widespread use of *S. incanum* is in the treatment of venereal diseases. Different plant parts are also widely used in the treatment of skin problems, including skin infections, ringworm, burns, sores, rashes, wounds, warts, ulcers and benign tumors. The fruits are poisonous and used as a traditional medicine for many ailments (Hyde and Wursten 2010).

In Uganda, Tanzania and South Africa extracts of leaves or flowers are used as ear drops to cure inflammations. In Senegal, Kenya, Uganda and Zimbabwe different plant parts are used to treat snakebites: a decoction of the roots is drunk, roots are chewed and sap is swallowed, and young chewed leaves or pulped fresh roots are applied to the bite wound. In Ethiopia fruit sap is mixed with butter and applied to cattle to control ticks and the boiled fruits are used as soap and in tanning leather. *S. incanum* is used against different ailments (stomach problems, snake bites, chest pain, tonicities, skin wounds of cattle (Mirutse Giday, 2001).

Methanol extract of fruits showed a very strong inhibition. The ethanol extract of ripe berries was tested for its growth inhibiting effect with breast cancer cells. The proliferative capacity of the cells was strongly suppressed in the presence of the extract. Solanaceous plants are biologically active because of two groups of their compounds, glycoalkaloids and steroid alkaloids. Glycoalkaloids interfere with membranes of the cells. They disrupt the integrity of cells. Leaves root and seeds of *S. incunum* boiled in butter taken orally used for stomachache disorder (Tilahun Teklehaymanot and Mirutse Giday, 2007).

Hygroscopic crystals of *S. incanum* displayed antibacterial activity against both gram-positive and gram-negative organisms. In addition, it showed anti-Candida properties, but its action against dermatophytes was the most prominent. The susceptibility of various organisms responsible for dermatomycosis to these solanum crystals was demonstrated in clinical trials on persons infected with *Microsporum* organisms resistant to the topical antifungal agents

frequently employed for such infections. *S. incanum* is effective against *Microsporium nanum*, *Microsporium gypseum*, *Microsporium canis*, *Microsporium equinum*, *Keratinomyces ajelloi*, *Microsporium distortum*, *Trichophyton tonsurans*, *Cryptococcus neoformans*, *Geotrichum candidum*, *Candida albicans* *Colletotrichum lindemuthianum* (Beaman-mbaya and Muhammed, 1976).

#### **2.6.4. *Calpurnia aurea***

*Calpurnia aurea* is in the family Fabaceae. It is a yellow-flowered small tree or shrub widely distributed in Africa. Chemical investigations of *C. aurea* have resulted in the isolation of a series of quinolizidine alkaloids. The leaves and twigs of Ethiopian *C. aurea* yielded 13-hydroxylupanine. The South African species yielded the well known alkaloids: hydroxylupanine, calpurnine, virgiline and its pyrrolylcarboxylic acid ester as found in Ethiopian sample. *C. aurea* is used for the treatment of amoebic dysentery and diarrhea in animals, killing head lice in humans and ticks in animals, syphilis, diarrhea, leishmaniasis, tapeworm, trachoma, *Tinea capitis*, wound, scabies, elephantiasis and different swellings.

In South Africa, *Calpurnia* leaves and powdered roots are used to destroy lice and to relieve itches. Unspecified parts are used to destroy maggots and the leaves are used to treat allergic rashes, particularly those caused by caterpillars. In East Africa, leaf sap is used to destroy maggots in wounds. In Nigeria, the seeds are used to treat abscesses. In Ethiopia it is used to treat stomach complaints, headache, eye diseases, amoebic dysentery, scabies (skin infection caused by ticks) and as an insecticide. *C. aurea* Leaf is used for treatment of wound, scabies, and different swellings (Gupta1 *et al.*, 2010).

The methanol extracts of the leaves and stem of *C. aurea* were screened for antibacterial properties. The level of these phenolic compounds in the methanol extracts of the leaves and stem of *C. aurea* were considerable. The stem extract higher levels of total phenol and flavonoids than the leaf extract. On the other hand, the leaf extract possessed higher levels of proanthocyanidins and total flavonols. The antibacterial activity of the methanol extracts of the leaves of the *C. aurea* is much higher than of the stem. Polyphenols are the major plant compounds with antioxidant activity. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching

singlet and triplet oxygen, or decomposing peroxides. The results suggest that phenolics are important components of this plant (Adedapo *et al.*, 2008).

Leaves or Fruit powder of *C. aurea* mixed with water or honey is taken orally have therapeutically used for diarrhea (Tilahun Teklehaymanot and Mirutse Giday, 2007). *C. aurea* in Ethiopia are used for treatment of skin disorders (Fisseha Mesfin *et al.*, 2009)



*Calpurnia aurea* source: (Adedapo *et al.*, 2008)

#### **2.6.5. *Clematis hirsute* and *Clematis simensis***

*Clematis* is a member of the Ranunculaceae family. It is a varied genus, made up of mostly woody, deciduous climbing plants, though a few are evergreen and a few herbaceous. Leaves are opposite on the stem and mostly compound with three to five leaflets. The leaf stalk twines like a tendril and is responsible for giving the plant support. It is commonly used for skin disorders. Leaf extracts of *C. hirsute* was indicated to have strong antifungal activity on certain species of fungi. But this medicinal plant species was reported to treat earache by traditional healers, which needs further study to confirm its antibacterial activity (HaileYineger and DelenasawYewhalaw, 2007).

*Clematis* species have been traditionally used for the treatment of inflammatory conditions by the Chinese and indigenous Australians. Both *C. hirsute* and *C. glycinoides* are used as a remedy for headache. The extracts of the young shoots of *C. vitalba* showed activity against pathogenic yeast and yeast-like microorganisms. Total extracts of *C. recta* and *C. hirsute* showed a fungicidal effect. The volatiles of *C. hirsute* and *C. simensis* under investigation are yellow liquids with a characteristic pungent odor. The oils were miscible with ether and ethanol and immiscible with water. Protoanemonin represented the major constituent in *C. hirsute* while eugenol was the major component in *C. simensis*. Butylated hydroxyl toluene was present in relatively high percentage in these species. 3-acetylanisole was present in minute amount in *C.*

*simensis*, Linalool, epoxy linalool; piperitone, thymol and carvacrol were present in low percentages in *C. hirsute*.

The antimicrobial activity observed for the volatile constituents of *C. hirsute* is probably due to the presence of several compounds reported to have antibacterial or antifungal activities. Both eugenol and piperitone have fungicidal activity against *C. albicans*. It was reported that thymol and eugenol exhibited antibacterial activities by bacterial membrane damaging mechanism. A broad spectrum of activity and synergistic effect was observed using combination of thymol and carvacrol. The high percentage of eugenol together with protoanemonin and acetyleugenol may attribute to the activity of *C. simensis* against *B. subtilis*, *S. aureus* and *C. albicans*.

The volatile constituents of *C. hirsute* and *C. simensis* showed a pronounced anti-inflammatory activity. Eugenol, present in high percentage in *C. simensis* is reported to have anti-inflammatory activity. The results of the anti-inflammatory testing suggested the use of *C. hirsute* and *C. simensis* under investigation for the treatment of rheumatic arthritis and other inflammatory conditions similar to the other *Clematis* species. However, toxicity studies are required to prove the safety of these plants (Areej *et. al*, 2008).

#### **2.6.6. *Croton macrostachyus***

Common names (Amharic): Bisana, (Tigrigna): *tambuk*, (English): broad-leaved croton. *C. macrostachyus* is in the family Euphorbiaceae common in secondary forests, on forest edges along rivers, around lakes, in moist or dry evergreen land forests, wooded grasslands and along roadsides. In Ethiopia it occurs in Dry, Moist and Wet Weynadega and Dega as well as in upper altitudes of Dry Kolla agro climatic zones in Tigray, Gondar, Gojam, Wollo, Bale Shewa, Illubabor, Kefa, Sidamo and Hararge regions. Apart from its medicinal uses, the tree is also used as firewood, timber, forage (young leaves) and as mulch (Gilbert, 1989, Azene Bekele *et al.*, 1993).

*C. macrostachyus* and *D. stramonium* in Ethiopia are used for treatment of skin disorders (Fisseha Mesfin *et al.*, 2009). Juice prepared from pounded and squeezed fresh leaves of *C. macrostachyus* was applied as a lotion to cure lesions of patients suffering from abiato (shererit) (Haile Yineger and Delenasaw Yewhalaw, 2007, Giday Yirga, 2010). The aqueous and diethyl ether extracts of the stem bark of *C. macrostachyus* possess anti-inflammatory properties

(Kamanyi, *et al.*, 2009). The species *C. macrostachyus* and *C. hirsute* were found to have the highest diversity of medicinal applications (Tilahun Teklehaymanot, 2009).

Boiled leaf decoction is drunk or ashes taken orally as treatment for cough; juice from fresh leaves is applied on wounds to hasten clotting. Root decoction is used as an anthelmintic for tapeworm, as a purgative, and for malaria and venereal diseases. Bark from the stems and roots is boiled in water and newly born babies are bathed in the mixture as a remedy or skin rash. The genus *croton* is particularly rich in secondary metabolites like alkaloids, terpenoids, and flavonoids. The most common class of compounds of *croton* is represented by diterpenoids. Several species of the genus are aromatic, indicating the presence of volatile oil constituents.

The bark-slash emits a peppery smell and the bark of young branches exudes a colourless sticky slime. The bark is an ingredient of an effective purgative and vermifuge in Ethiopia. An extract of leaves is used against itchy scalp in Ethiopia, where also a decoction of young leafy shoots is an ingredient of a prescription for jaundice, and for an eruptive disease resembling small-pox. In Sudan a vegetable salt is prepared from the leaves. The seed is recognized as poisonous in Ethiopia and is used as a fish-poison, and a preparation of the seed is instilled into the ear for ear-troubles. In Tigre the crushed leaves and seed admixed are drunk in water for tapeworm, the fruit is eaten and a root-decoction drunk for venereal disease, and the seed eaten to induce abortion. The plant in West Africa is used for stomach-ache, guinea-worm sores, pain-killers, ear treatments, vermifuges, cutaneous, subcutaneous parasitic infection venereal diseases, tumours, cancers, resins, Yellow fever, Typhoid and measles (Cyrus, 2008).

Methanol leaf extracts of *C. macrostachyus* was screened for larvicidal activity against late third instar larvae of *Anopheles arabiensis* Patton, a potent malaria vector in Ethiopia. The plant extracts demonstrated varying degrees of larvicidal activity against *Anopheles arabiensis* (Karunamoorthi and Ilango, 2010). Leaf powder of *C. macrostachyus* mixed with water is taken orally against Diarrhea, dysentery, stomach disorder and hepatitis. Rubbing and dressing with Latex from leaves against quaqucha (*Tinea versicolor*). *C. macrostachyus* was preferred among the medicinal plants that were reported by more informants as a remedy to diarrhea (Fisseha Mesfin *et al.*, 2009).

## 2.7. Combination of biocontrol microbial agents and plant extracts against plant pathogens

Combination of effective organisms and manipulation of microbial communities rather than using a single strain was suggested for more consistent and effective control (Van *et al.*, 2001). Chocolate spot, caused by *Botrytis fabae* is the most serious disease of beans and is capable of devastating in an unprotected crop. Controlling *B. fabae* by biocontrol agents seemed to be better than and preferable to the chemical control. Actinomycetes, and particularly Streptomyces, play a major role in antagonistic interaction for different plant pathogens because of their greater capacity for antibiotic production. In addition, *Trichoderma harzianum* was considered as a biocontrol agent for phytopathogenic fungi, mechanisms of this biocontrol are mycoparasitism competition and fungicidal action because of the capacity to produce antibiotics or hydrolytic enzymes.

A number of *Trichoderma harzianum* isolates produce a wide variety of fungal cell wall-degrading enzymes, such as pectinases, cellulases, chitinase, and proteases. Chitin and  $\beta$ -1,3-glucan are the main skeletal polysaccharides of fungal cell walls suggest that chitinase and  $\beta$ -1,3-glucanases act as key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action. Hence fungal cell wall-degrading enzymes of *Trichoderma* spp are of special importance in plant defense mechanisms. Plants are capable of producing an immune response after primary pathogen infection, which is known as systemic acquired resistance (SAR). The activation of SAR correlates with the expression of pathogenesis-related genes, including acidic and basic  $\beta$ -1, 3-glucanase and chitinase, which supposedly act against the cell walls of the pathogen. *Trichoderma* spp. can induce systemic resistance in plants that is phenotypically similar to SAR (Jayalakshmi *et al.*, 2009).

Species of *Trichoderma* are known to have proteins, enzymes and other metabolites that are directly involved in the interaction between *Trichoderma* and target fungi. Species of *Trichoderma* produced much more cellulolytic, amylolytic and chitinolytic activities than *B. fabae*. Only the species of *Trichoderma* secreted amylase and chitinase. Several reports have demonstrated positive relationship between the production of cellulase, chitinase and protease and the ability to control plant diseases, these enzymes are induced in *Trichoderma* during the

parasitic interaction and can inhibit the growth of several fungal plant pathogens by degrading cell walls (Saber *et al.*, 2009).

The contrasting effects of *B. fabae* and the biocontrol agents on the growth, productivity and yield of faba bean may be due to the pathogenicity of *B. fabae*, effect of leaf extracts, and the anti-Botrytis effect of both *Trichoderma* and Streptomyces. The pronounced recovery of the growth, productivity and yield of infected plants by adding *Trichoderma* with Streptomyces or mixture of extracts rather than adding individual treatments could be ascribed to the additive effects of both bioagents in minimizing chocolate spots caused by *B. fabae* (Mahmoud *et al.*, 2004).

### **3. OBJECTIVES OF THE STUDY**

#### **3.1. The general objective:**

To screen, evaluate, test and determine the antimicrobial activity of some plant extracts against chocolate spot (*Botrytis fabae*)

#### **3.2. The specific objectives:**

- ∅ To isolate and identify chocolate spot (*B. fabae*)
- ∅ To screen and determine the MIC of plant extracts against *B. fabae*

## 4. MATERIALS AND METHODS

### 4.1. Collection of botanical plant materials

The plant parts of *Croton machostachyus* (leaf), *Solanum incanum* (leaf), *Solanum marginatum* (leaf and seed), *Calpurnia aurea* (leaf), *Clematis simensis* (leaf), *Clematis hirsute* (leaf), and *Datura stramonium* (leaf and seed) used in this study were collected in December 2009. *C. macrostachyus* and *S. incanum* were collected from Nazerath and Debrezeit areas. *S. marginatum* and *C. simensis* were collected from North Shewa zone (Debrelibanos). *D. stramonium* was collected from Nazerath and Melkassa, *C. aurea* and *C. hirsute* were collected from Menagesha state forest, East Shewa.

The plants were selected for further investigation/findings based on the indigenous knowledge. Voucher specimens of the plants were deposited and identified at the National Herbarium of Department of Biology, AAU.

Table 1. Plants that were used for antifungal activities assay

Name	Family	Plant part used
<i>Croton machostachyus</i>	Euphorbiaceae	Leaf
<i>Solanum incanum</i>	Solanaceae	Leaf
<i>D. stramonium</i>	Solanaceae	Leaf and Seed
<i>S. marginatum</i>	Solanaceae	Leaf and Seed
<i>Calpurnia aurea</i>	Fabaceae	Leaf
<i>C. simensis</i>	Ranunculaceae	Leaf
<i>C. hirsute</i>	Ranunculaceae	Leaf

### 4.2. Preparation of plant extracts

The plant material was separated in to its selected parts (seed and leaf), fresh plant material were washed through under tap water followed by sterilized distilled water and cut in to smaller size of about 1-3cm long. The leaf and seeds were shade dried at room temperature for two weeks,

and then pounded using sterile mortar and pestle into fine powder and kept in refrigerator until use (Selvaraj and Narayanasamy, 1993, Singh *et al.*, 2007).

#### **4.2.1. Preparation of aqueous extract**

Crude plant leaf extract was obtained by separately infusing 50 g of each plant material in 250 ml distilled water to give 20% (w/v) in a 1000 ml conical flask, kept on shaker for 24hrs at 121 rpm (Naduagu, *et al.*, 2008). The infusion was filtered afterwards through double layer cheese cloth and with Whatman No 1 filter paper and filtrates were centrifuged for 15 min at 6000 rpm. Supernatant of the extract was preserved in air tight bottle until further use in refrigerator (Naduagu, *et al.*, 2008).

Fifty (50) gram of dried powdered seeds *D. stramonium* and *S. marginatum* were soaked in 250 ml of sterilized distilled water. Then mixture was kept on rotary shaker for 72 hrs and filtered through double layered muslin cloth and then with Whatman No 1 filter paper. Filtrates were centrifuged for 15 min at 6000 rpm and the supernatant was kept in air tight bottle in refrigerator for further studies (Rani, *et al.*, 2008).

#### **4.2.2. Preparation of Solvent extracts**

Fifty (50) gram of air dried powdered plant material was placed in 250 ml of methanol, ethanol, ethyl acetate and chloroform in conical flask each and kept in rotary shaker at 121 rpm for 24 hrs. After 24hrs they were filtered with double layer muslin cloth and then Whatman No 1 filter paper and concentrated under reduced pressure in rotary evaporator at 40 °c. The gummy residue was further dried in a water bath until the solvent was removed. After solvent evaporation, the remaining crude extracts were diluted with sterilized distilled water and kept in air tight bottle on refrigerator until use (Dewanjee *et al.*, 2007, Bhaskarwar *et al.*, 2008, Rajeendran and Ramakrishana, 2009).

#### **4.2.3. Solvent Solvent fractionation**

The crude methanol leaf extract (5g) of *D. stramonium*, *S. marginatum*, *C. macrostychus* and *C. aurea* was taken and suspended in 50 ml of distilled water. The solution was transferred into a 500ml separatory funnel and shaken after addition of 50ml chloroform three different times. The solvent layers were then allowed to separate. The chloroform layer was collected in a flask

separately. The water suspension residue was further partitioned with ethyl acetate to obtain ethyl acetate fraction. The chloroform fraction and ethyl acetate fraction was reduced to dryness on a rotary evaporator at 40°C and the aqueous residue was used for antifungal activity (Mahasneh and El-oglah, 1999, Okpuzor *et al.*, 2009 Benamar *et al.*, 2010).

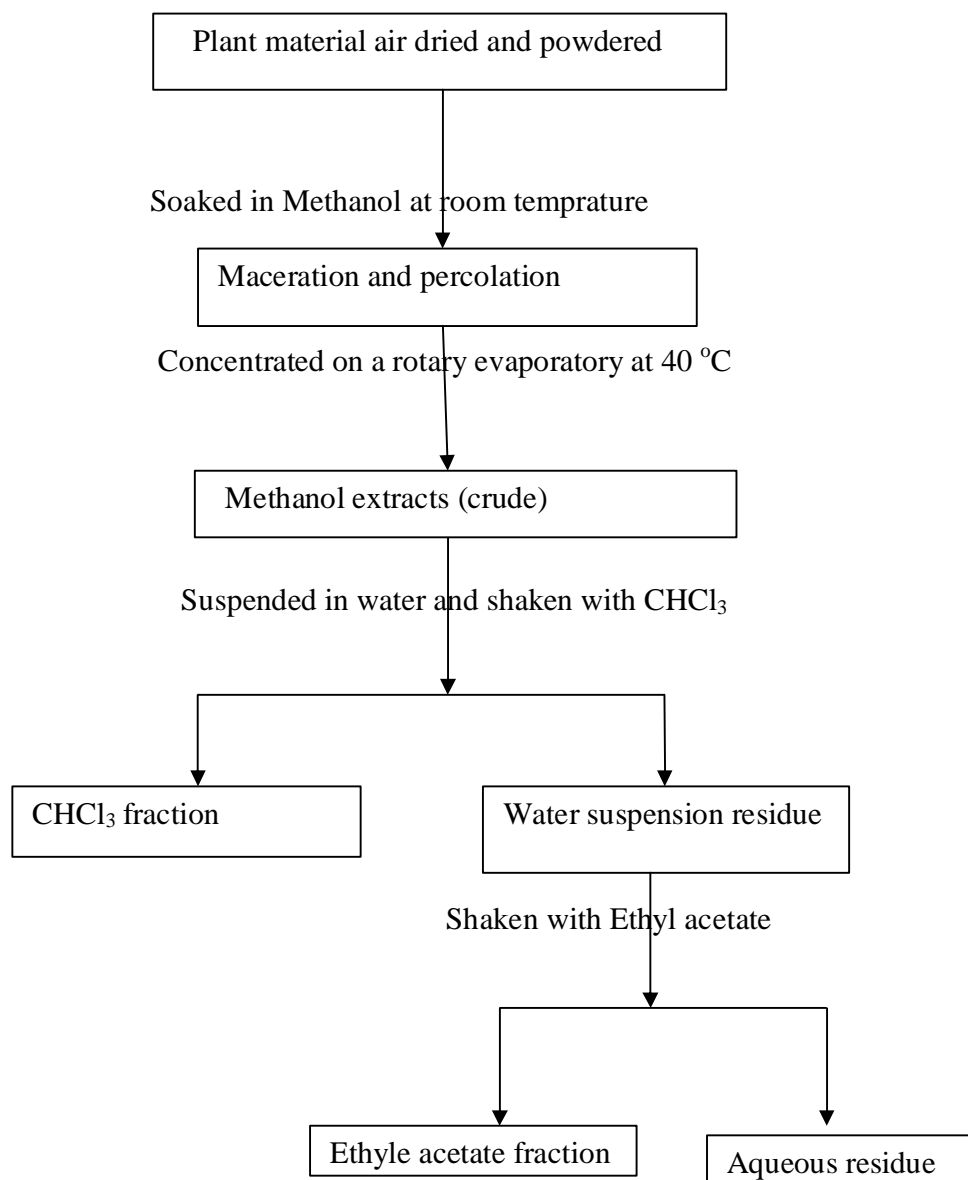


Fig.1.Flow chart for fraction of the crude extract for in vitro antifungal testing with some modifications (Mahasneh and El-oglah, 1999).

Stock solutions of standard fungicide Sancozeb 80%WP were prepared, using analytic balance 2g were weighed and dissolved in 100ml of sterile water and serially diluted to different concentrations. Neem oil was solubilized with 0.5% of Tween-20 and diluted with sterile water to make 20 % (Bobbarala *et al.*, 2009).

### **4.3. Isolation and identification of plant pathogens**

*B. fabae* was isolated from diseased plants of faba bean grown at a field. Faba bean leaves and seeds infected with chocolate spot were collected from farmer's field in September 2009. Sections of 3-5mm<sup>2</sup> were cut from the margin of the infected lesion and surface sterilized with 70% ethanol for 2 min and rinsed thrice with sterile water in three Petri plates. The sterile pieces were blotted dry using sterile filter paper and placed (3 pieces per plate) on potato dextrose agar (PDA) medium impregnated with streptomycin sulphate 0.1gm /L of the medium. The plates were incubated at 25°C for 7 days. The fungus was subcultured several times for purity. The fungal identification was confirmed by microscopic examination and comparison with reference slide cultures (Haggag, *et al.*, 2006, Okereke *et al.*, 2007).

### **4.4. In vitro evaluation of plant extracts**

#### **4.4.1. Aqueous extract**

The plant extracts were assayed for antifungal activity against the fungal isolate *B. fabae* obtained from diseased Faba bean plant. All tested plant extracts were subjected to antifungal assay by food poison technique. 20% aqueous plant extracts with different concentration were added to PDA impregnated with streptomycin sulphate (0.1g/L). 2g of standard fungicide was serially diluted at different concentration and added to PDA medium.

After complete solidification of the medium, 4mm disc of seven day old culture of the test organism were transferred on the hole cut at the center of the Petri plate using sterile cork borer. Plates were incubated at 25°C for seven days. The Petri dish containing media devoid of extract but with same amount of sterile distilled water served as control. All plates were with three replicates and were arranged in completely randomized block design. After full growth of the control plate's size of colony diameter measured in mm and percentage inhibition of mycelial growth was calculated using the formula (Mohana and Raveesha, 2007).

$$\text{Percentage inhibition} = \frac{C-T}{CX100}$$

Where C, average increase in mycelial growth in control plate and T, average increase in mycelial growth in treatment plate

#### **4.4.2. Solvent extracts**

Four solvent extracts using ethanol, methanol, ethyl acetate and chloroform obtained from leaf and seeds of the test plants (Table1) were subjected to antifungal assay. The gamy residue of all solvent extracts were dissolved in 2ml of 9% ethanol and 8 ml of sterile water after removal of the solvent diluted with sterile distilled water adjusted to 20% concentration. To the autoclaved PDA medium, different amount of the 20% extracts were added in to the medium. After complete solidification of the medium, 4mm disc of seven day old culture of *B. fabae* using sterile cork borer was inoculated in to PDA at the center of the Petri plate. Plates were incubated at 25°C for seven days. Plates with out plant extracts served as control. All plates were with three replicates. Size of colony diameter was measured after full growth of the control plates. Effective solvent extracts which was tested in inhibition of mycelial growth of *B. fabae in vitro* test were further subjected to *in vivo* test.

#### **4.5. Determination of minimum inhibitory concentration (MIC)**

The methanol, ethanol and water extracts of different plants that showed significant antimicrobial activity in the previous test were selected for determination of MIC. The gamy residues of all crude solvent extracts were dissolved with 2ml of 9% ethanol and 8ml of distilled water in test tubes to obtain 10ml stock solution. Different amount of the plant extracts and standard fungicide was separately transferred to PDA medium. After complete solidification, 4mm mycelial disc of *B. fabae* inoculated at the center. They were incubated at 25°C for seven days. The plate containing the least concentration of extract and standard fungicides showing no visible sign of growth was considered as MIC (Andreuos, 2001).

#### **4.6. *In vivo* experiment**

The effectiveness of the leaf extract of plants which were found to be effective against the pathogen *in vitro* was tested on Faba bean against the disease. Based on the previous results methanol leaf extracts of *D. stramonium*, *C. aurea*, ethanol leaf extracts of *S. marginatum* and water extracts of *C. macrostachyus* were selected for the *in vivo* test (Muthulakshmi and Seetharaman, 1993).

3.5kg soil mixture of sand, humus and clay (1:1:1) were autoclaved and placed on sterile 20cm diameter plastic pots. Five seeds of faba bean per pot were sown, grown in a glasshouse and water regularly. The dry leaf extract of *D. stramonium*, *C. aurea*, *S. marginatum* and *C. macrostachyus* were sprayed on faba bean plants raised in the glasshouse when the plants were 20 days old after bearing four leaves stage. They were sprayed with each leaf extract separately i) 2, 7 and 14 days prior to the artificial inoculation of the pathogen ii) two days after inoculation of the pathogen (Carabet, 2007). Mixtures of plant extracts and *T. harzianum*, plant extracts, *T. harzianum* alone were also sprayed to the leaf 2 days before inoculation of the pathogen *B. fabae* (Mahmoud, *et al.*, 2004).

All pots were covered with transparent plastic bag to get moisture. Control pots were treated with sterile water. Disease severity and disease reduction were assessed 10 days after inoculation. The percentages of disease severity determined in relation to the control pots. The effect of the treatments on the growth of Faba bean was determined by measuring size of lesion of leaves after ten days. The experiment had five replicates and repeated thrice and was arranged in completely randomized block design (Haggag, *et al.*, 2006). Percent inhibition is calculated by the formula:  $C - T / TX100$

Where C, Size of lesion of control leaves and T, size of lesion of treatment leaves

#### **4.7. Re isolation of the pathogen**

Pathogenicity was confirmed by inoculating isolates onto faba bean plants. The pathogen *B. fabae* was re isolated from the leaf lesion of the control plants in the *in vivo* experiment. Leaf lesions were cut in to pieces and surface sterilized with 70% ethanol for 2 min and rinsed thrice with sterile water in Petri plates. Pieces dried with sterile filter paper and plated on PDA

medium and incubated at 25°C for 7 days. The fungus was subcultured to purify, and identification was by Comparison with the previous isolate.

#### **4.8. Separation of compounds using thin layer chromatography (TLC)**

The plant extracts dissolved in methanol, ethanol and water a small amount of the extract were analyzed to separate the extract using thin layer chromatography. The extracts were spotted on the gel and placed in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. TLC tank containing the solvent system: Hexane: Acetic acid: ethanol in the ratio 2: 1: 3 and Hexane: Ethyl acetate in the ratio of 1: 1 (v/v). When the solvent reached the top of the plate, the plate was removed from the developing chamber, dried, and the separated components of the mixture were visualized. The separated components on the TLC plates were viewed using ultraviolet light at 254 nm wavelength (Babay *et al.*, 2004, Benamar *et al.*, 2010).

The extracts were evaluated for the presence of flavonoids, saponins, steroids and tannins as follows: One gram of MgSO<sub>4</sub> powder and two drops of concentrated HCl was added to 3ml of each plant extract. A red coloration indicates the presence of flavonoids. Two milliliters of the extracts in separate test tubes were vigorously shaken for two minutes. An observation of frothing in the extract indicates the presence of saponins. One milliliter of concentrated H<sub>2</sub>SO<sub>4</sub> was added to 1ml of each plant extracts. No red colouration indicates the presence of steroids. Two drops of 5% FeCl<sub>3</sub> were added to 1ml of each plant extracts. A dirty-green precipitate shows the presence of tannins (Hassan *et al.*, 2004, cited in Nduagu *et al.*, 2008).

#### **4.9. Data Analysis**

SPSS 13.0 Version statistical software package was used for statistical analysis of percentage inhibition in each case. This was applied to determine whether differences between treatments were significant when subjected to Tukey HSD at 0.05 subset.

## 5. RESULTS

Antifungal activities of matured, immature leaf and seed extracts of seven botanical plants were assayed and evaluated on the growth and development of the pathogen *B. fabae*. The effectiveness antifungal activity of aqueous and different solvent extracts of these plants were determined on the basis of evaluated percentage inhibition in colony diameter of the test fungus. The validity of experimental result of percent inhibition obtained at different concentration was checked by various statistical parameters as it has indicated in (Table 2, 3, 4, 5) The data revealed that significant reduction in growth of *B. fabae* was observed with extracts of seven botanical plants and the extracts have showed significance difference in their efficacy.

### 5.1. Antifungal activity of methanol crude plant extracts against *B. fabae*

The result of Table 2 revealed that highly significant 100% inhibition on the growth of *B. fabae* was observed in immature leaf extract of *D. stramonium* at a concentration of 40mg/ml followed by matured leaf extract of *D. stramonium*, *C. aurea* and immature leaf extract of *S. marginatum* 98.15%, 94.44 % and 90.19% respectively. Where as the methanol extracts of *S. incunum*, *C. simensis* and *C. hirsute* inhibit the growth of *B. fabae* with less extent at concentration of 40mg/ml 65.37%, 70% and 64.08% respectively.

Table 2. Percent of inhibition of methanol leaf and seed extracts at different concentration against *B. fabae*

Plant extracts	Concentration in mg/ml									
	2.5		5		10		20		40	
	G%	I%	G%	I%	G%	I%	G%	I%	G%	I%
<i>C. aurea m.</i>	38.83	56.86	27.17	69.81	21	76.67	16.83	81.3	5	94.44
<i>C. macrostchys m.</i>	48	46.67	40.17	55.37	33.33	62.97	22.17	75.37	19.17	78.7
<i>D. stramonium m.</i>	34.16	62.04	22.33	75.19	13	85.56	6.17	93.14	1.67	98.14
<i>S. incunum m.</i>	68.33	24.08	56.83	36.85	45.17	49.81	39.67	55.92	31.17	65.37
<i>S. marginatum m.</i>	40	55.56	31	65.56	26.66	70.37	21	76.67	14.67	83.7
<i>C. hirsute m.</i>	63.33	29.63	58.5	35	39.17	56.48	35.67	60.37	32.33	64.08
<i>C. simensis m.</i>	70.83	21.3	50.17	44.26	35.83	60.19	30.17	66.48	27	70
<i>C. macrostchys imm.</i>	35.67	60.36	27	70	24.33	72.97	21.17	76.48	14.67	83.7
<i>D. stramonium imm.</i>	28	68.89	14.83	83.52	12	86.67	9.33	89.63	0	100
<i>S. incunum imm.</i>	44	51.11	34.5	61.67	27.67	69.26	17.17	80.92	14	84.44
<i>S. marginatum imm.</i>	42	53.33	34	62.22	26.67	70.37	11.17	87.59	8.83	90.19
<i>D. stramonium seed</i>	31.17	65.38	20.83	76.86	15.83	82.41	11.83	86.86	9.67	89.26
<i>S. marginatum seed</i>	35	61.11	26.83	70.19	22.33	75.19	14	84.44	11.83	86.86
<i>Neem oil</i>	19.5	78.33	12	86.67	8.9	90.11	1.5	98.33	0	100

Key: G%, Percentage of growth, I%, Percentage of growth inhibition, m, mature and imm, immature

## 5.2. Antifungal activity of aqueous crude plant extracts against *B. fabae*

The result shown in Table 3 indicated that the highest percent of inhibition on the growth of *B. fabae* was obtained with immature leaf extracts of *C. macrostchys* and *D. stramonium* at 40mg/ml and the growth was totally inhibited. Matured leaf extracts of *C. macrostchys*, *D. stramonium* and *C. aurea* also show highest inhibition with the same concentration, (40mg/ml) 95.37%, 94.44%, and 93.88%, respectively. Immature leaf extracts of *S. marginatum* *S. incunum*, seed extracts of *D. stramonium* and *S. marginatum* at 40mg/ml concentration inhibit 90.93%,

84.44%, 89.07% and 89.07 % respectively. *C. hirsute* also showed antifungal property but with less extent.

Table 3. Percent of inhibition of aqueous leaf and seed extracts at different concentration against *B.fabae*

Plant extracts	Concentration in mg/ml									
	2.5		5		10		20		40	
	G%	I%	G%	I%	G%	I%	G%	I%	G%	I%
<i>C. aurea m.</i>	31.33	65.19	21.5	76.11	15.83	82.41	7.67	91.47	5.5	93.89
<i>C. macrostchyus m.</i>	29.83	66.86	18	80	10.5	88.33	6.83	92.41	4.17	95.37
<i>D. stramonium m.</i>	26.5	70.56	18.33	79.63	16.83	81.3	8	91.11	5	94.44
<i>S. incunum m.</i>	45.33	49.63	36.5	59.44	30	66.67	15.67	82.59	13	85.56
<i>S. marginatum m.</i>	41	54.44	30.83	65.74	25.3	71.88	18	80	10.67	88.14
<i>C. hirsute m.</i>	90	0	78.33	12.97	76.5	15	75	16.67	70	22.22
<i>C. simensis m.</i>	70.5	21.67	43.16	52.03	33	63.33	28.17	68.7	21.5	76.11
<i>C. macrostchyus imm.</i>	21.15	76.11	13.5	85	11.83	86.86	1.83	97.97	0	100
<i>D. stramonium imm.</i>	27.17	69.81	15.83	82.41	10.67	88.14	4.5	95	0	100
<i>S. incunum imm.</i>	37	58.89	26.83	70.19	21	76.67	14.33	84.08	14	84.44
<i>S. marginatum imm.</i>	37.33	58.52	25.66	71.48	19.67	78.14	13.17	85.37	8.17	90.92
<i>D. stramonium seed</i>	45.67	49.26	23	74.44	19.5	78.33	16.17	82.03	9.83	89.08
<i>S. marginatum seed</i>	45	50	22	75.56	17.33	80.74	13.5	85	9.83	89.08
<i>Neem oil</i>	19	78.89	14	84.44	11.08	87.68	1.7	98.11	0	100

Key: G%, Percentage of growth and I%, Percentage of growth inhibition m, mature and imm, immature

### 5.3. Antifungal activity of ethanol crude plant extracts against *B. fabae*

The effect of leaf and seed extracts of the test plants on the growth of *B. fabae* is indicated in (Table 4). The most effective extracts have obtained the highest percent of inhibition of the mycelial growth of *B. fabae* were matured leaf extracts of *S. incunum*, *S. marginatum*, immature leaf extracts of *S. incunum*, *S. marginatum* and *D. stramonium* at 40mg/ml completely inhibited the growth of *B. fabae*. Matured leaf extracts of *D. stramonium*, seed extracts of *S. marginatum*

and *D. stramonium* inhibit at 40mg/ml concentration 96.48%, 95.19% and 92.96% respectively. Matured leaf extracts of *C. aurea*, *C. hirsute* and *C. macrostchys* at 40mg/ml concentration inhibit 91.48%, 91.48%, and 89.63% respectively.

Table 4. Percent of inhibition of ethanol leaf and seed extracts at different concentration against *B.fabae*

Plant extracts	Concentration in mg/ml									
	2.5		5		10		20		40	
	G%	I%	G%	I%	G%	I%	G%	I%	G%	I%
<i>C. aurea m.</i>	54.83	39.08	42.5	52.78	25	72.22	16.83	81.3	7.67	91.48
<i>C. macrostchys m.</i>	66.33	20.3	50.83	43.52	23.17	74.26	16.17	82.03	9.33	89.63
<i>D. stramonium m.</i>	43.17	52.03	32.67	63.7	18.17	79.81	9.17	89.81	3.17	96.48
<i>S. incunum m.</i>	39.5	56.11	31	65.56	19.5	78.33	14.67	83.7	0	100
<i>S. marginatum m.</i>	35.17	60.92	17.83	80.19	10.33	88.52	3.17	96.48	0	100
<i>C. hirsute m.</i>	55.5	38.33	40	55.56	28	68.89	17.17	80.92	7.67	91.48
<i>C. simensis m.</i>	52.67	41.48	28.67	68.14	23.83	73.52	20	77.78	11.17	87.59
<i>C. macrostchys imm.</i>	55.33	38.52	39.5	56.11	32.5	63.89	20.66	77.03	12.67	85.92
<i>D. stramonium imm.</i>	33.5	62.78	18	80	15.33	82.97	11	87.78	0	100
<i>S. incunum imm.</i>	37.33	58.52	19.33	78.52	14.5	83.89	7.5	91.67	0	100
<i>S. marginatum imm.</i>	34.5	61.67	17.17	80.92	13.83	84.63	3	96.67	0	100
<i>D. stramonium seed</i>	35.17	60.92	21.5	76.11	16.67	81.49	14.17	84.26	6.33	92.97
<i>S. marginatum seed</i>	41.5	53.89	16.83	81.3	12.83	85.74	8.33	90.74	4.33	95.19
<i>Neem oil</i>	20	77.78	13	85.56	10	88.89	1.67	98.14	0	100

Key: G %, Percentage of growth and I%, Percentage of growth inhibition m, mature and imm, immature

#### 5.4. Antifungal activity of chloroform crude plant extracts against *B. fabae*

The effect of chloroform extracts of matured, immature leaf and seed of the seven medicinal plants was tested against *B. fabae* and the result is indicated in Table 5. Most of the plant extracts and plant products did not inhibit the growth of *B. fabae* even at higher concentration. The immature and matured leaf extracts of *C. macrostchys* inhibited the growth of *B. fabae* at

40mg/ml 61.85% and 65.93% respectively. The immature leaf extracts of *S. incunum*, matured leaf extracts of *D. stramonium* seed extract of *D. stramonium* and immature leaf extract of *D. stramonium* inhibited at 40mg/ml 41.83%, 38.52%, 37.41%, and 34.81% respectively. Matured leaf extracts of *S. incunum*, *S. marginatum* and *C. simensis* inhibited the growth of *B. fabae* with less extent at 40mg/ml 26.67%, 28.15% and 27.04% respectively.

Table 5. Percent of inhibition of chloroform leaf and seed extracts at different concentration against *B.fabae*

Plant extracts	Concentration in mg/ml									
	2.5		5		10		20		40	
	G%	I%	G%	I%	G%	I%	G%	I%	G%	I%
<i>C. aurea m.</i>	89	1.11	81	10	75	16.67	64.33	28.52	60.33	32.97
<i>C. macrostchys m.</i>	81	10	62.67	30.37	45.33	49.63	41.66	53.71	34.33	61.86
<i>D. stramonium m.</i>	89.33	0.74	85	5.56	78	13.33	59	34.44	55.33	38.52
<i>S. incunum m.</i>	90	0	90	0	89.33	0.74	71.67	20.37	66	26.67
<i>S. marginatum m.</i>	89.33	0.74	87	3.33	82.33	8.52	71.33	20.74	64.67	28.14
<i>C. hirsute m.</i>	89	1.11	86	4.44	84.67	5.92	62.67	30.37	61	32.22
<i>C. simensis m.</i>	89	1.11	87.33	2.97	86.67	3.7	77	14.44	65.67	27.03
<i>C.macrostchys imm.</i>	82.67	8.14	59.33	34.08	41	54.44	33.33	62.97	30.33	66.3
<i>D.stramonium imm.</i>	89	1.11	82	8.88	74	17.78	68.67	23.71	58.67	34.81
<i>S.incunum imm.</i>	89	1.11	88	2.22	85.67	4.81	64	28.89	52.67	41.83
<i>S.marginatum imm.</i>	88.67	1.48	85	5.56	78.33	12.97	68	24.44	62.33	30.74
<i>D. stramonium seed</i>	88.33	1.86	85	5.56	75.33	16.3	63.33	29.63	56.33	37.41
<i>S. marginatum seed</i>	89.33	0.74	84	6.67	81.33	9.63	67	25.56	60.33	32.97
<i>Neem oil</i>	20	77.78	13	85.56	6	93.33	4.4	95.11	0	100

Key: G %, Percentage of growth and I%, Percentage of growth inhibition m, mature and imm, immature

## 5.5. Determination of minimum inhibitory concentration (MIC)

*B. fabae* was tested in this study to see the *in vitro* antifungal activity of matured, immature leaf extracts and seed extracts of seven botanical plants the standard fungicide Sancozeb and Neem oil which were used as control.

### 5.5.1. MIC for the crude aqueous extracts

The crude water extracts of matured leaf of *D. stramonium*, *C. macrostchys*, *S. incunum*, *S. marginatum*, *C. simensis*, *C. aurea*, *C.hirsute*, immature leaf extracts of *C. macrostchys* *D. stramonium*, *S. incunum*, *S. marginatum*, seed extracts of *D. stramonium* and *S. marginatum* were solubilized by sterilized distilled water and then were tested against the test pathogen (*B. fabae*). The result of the *in vitro* susceptibility of the tested pathogen to the crude aqueous extract was indicated in (Fig.2). Immature leaf extracts of *C. macrostchys* and *D. stramonium* show better antifungal activity with MIC value of (30mg/ml). Crude matured leaf extracts of *C. aurea*, *C. macrostchys*, *D. stramonium* and immature leaf extracts of *S. marginatum* exhibited equal MIC (50mg/ml) and *C. simensis* showed inhibition with MIC value of (80mg/ml) against *B. fabae*.

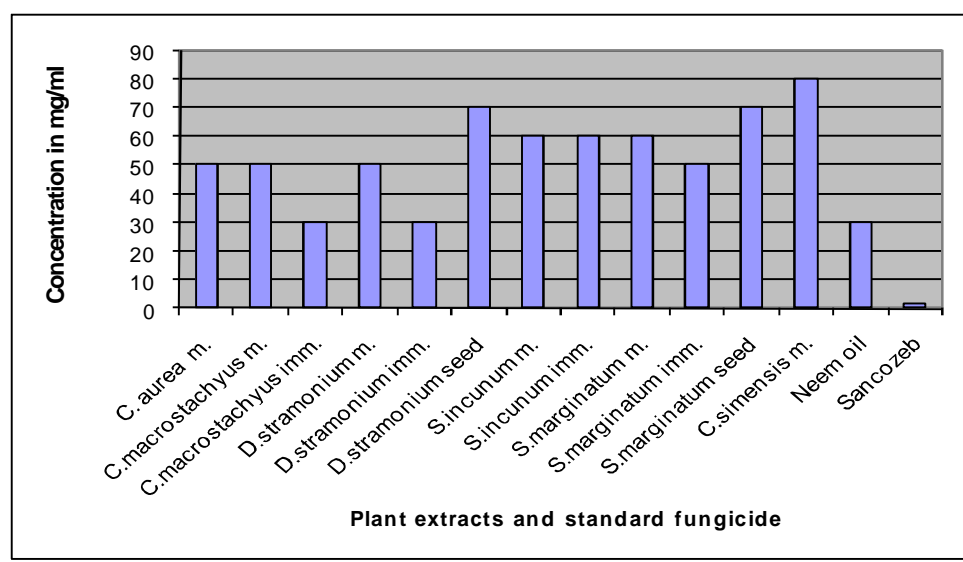


Fig.2. MIC for the crude aqueous extracts of plants and standard fungicide

imm = immature and m = mature

### 5.5.2. MIC for the crude ethanol extracts

The crude ethanol extracts of mature, immature leaves and seeds of seven medicinal plants were solubilized by 2 ml of 9% ethanol and 8 ml of sterile distilled water and then were tested against *B. fabae* have indicated significance difference in inhibition (Fig 3). The matured leaf extracts of *S. marginatum*, immature leaf extract of *S. incunum* and *S. marginatum* inhibited and totally prevented the growth of *B. fabae* at a concentration of 30mg/ml. Immature leaf extracts of *D. stramonium* and matured leaf extracts of *S. incunum* at 40mg/ml, matured leaf extracts of *D. stramonium* and seed extracts of *S. marginatum* at 50mg/ml prevented the mycelial growth of *B. fabae*. The seed extracts of *D. stramonium*, leaf extracts of *C. aurea*, and *C. hirsute* at a concentration of 60mg/ml, mature leaf extracts of *C. macrostchys* and *C. simensis* at 70mg/ml inhibited the growth of test fungi.

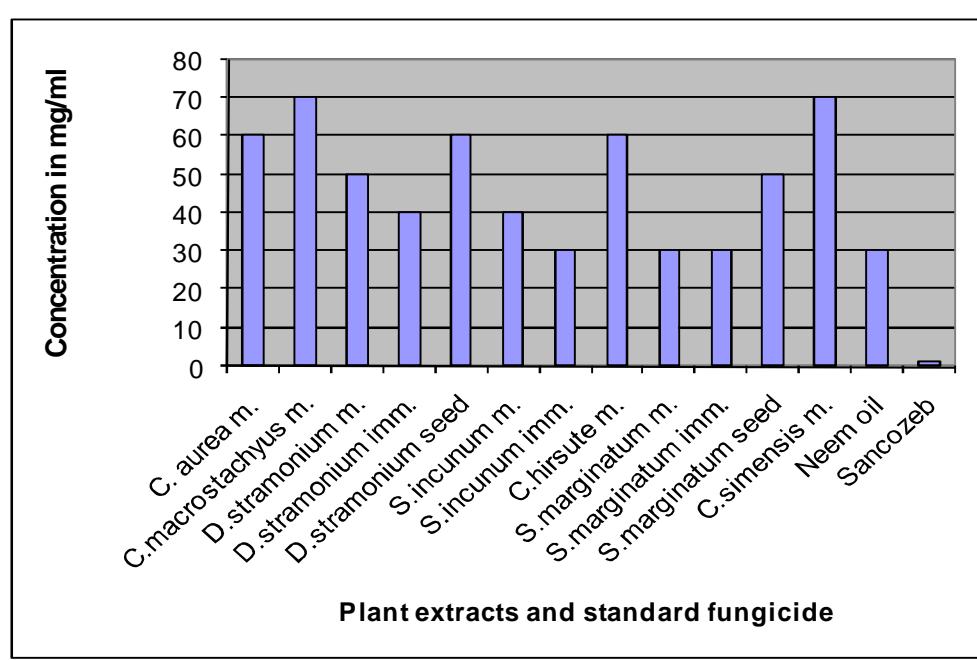


Fig.3. MIC for the crude ethanol plant extracts and standard fungicide

imm = immature and m = mature

### 5.5.3. MIC for the crude methanol extracts

The result (Fig 4) has revealed that highly significant MIC on the growth of *B. fabae* was the immature leaf extracts of *D. stramonium* at 40mg/ml concentration followed by matured leaf extracts of *D. stramonium*, *C. aurea* and, immature leaf extracts of *S. marginatum* with MIC at 50mg/ml. The Seed extracts of *D. stramonium*, *S. marginatum* and immature leaf extracts *S. incunum* inhibited growth of *B. Fabae* at 70mg/ml. Immature and mature leaf extracts of *C. macrostchys*, mature leaf extracts of *S. incunum*, *C. simensis* and *C. hirsute* inhibited the mycelial growth of *B. fabae* at 80mg/ml.

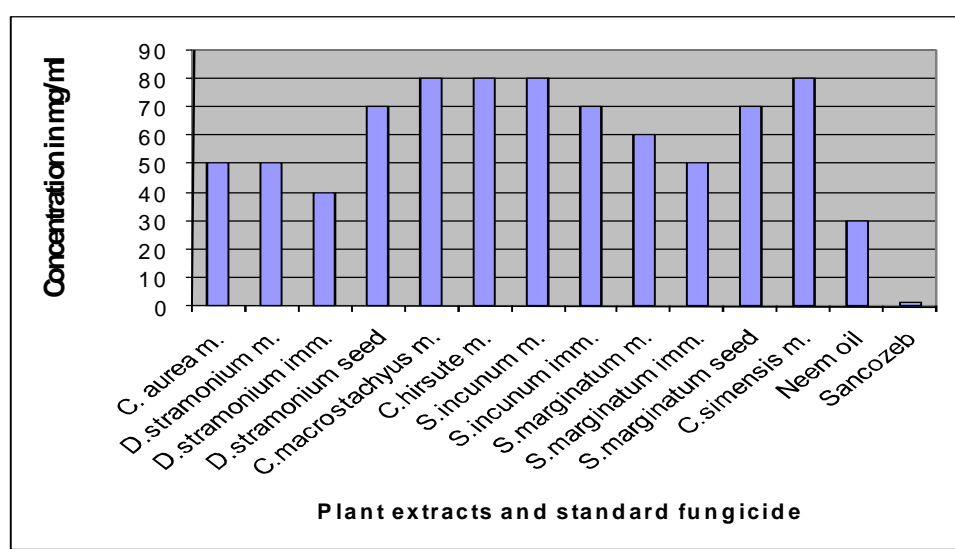


Fig.4. MIC for the crude methanol plant extracts standard fungicide

imm = immature and m = mature

### 5.6. The semi purified fractions of selected plant extracts

The semi purified fraction of plant extracts were tested at different concentration and the aqueous solubilized fraction of all extracts revealed better antifungal activity against *B. fabae*. However, chloroform and ethyl acetate fractions did not show inhibitory effect.

### 5.7. Evaluation of different plant extracts on Faba bean

The treatment with extracts of leaf material of *D. stramonium*, *S. marginatum*, *C. macrostchys* and *C. aurea* inhibited development of lesions caused by *B. fabae*. The activity of the extracts

against *B. fabae* was tested as pre inflectional treatment two days before inoculation, on faba bean plants with different concentration indicated different percent of inhibition of symptom development by various extracts (Table 6). The 20% extracts of *D. stramonium* reduced the lesion development by 94.77%, at lower concentration; 10% or 5% the extract exerted efficacy between 81.48% and 69% respectively. In the case of *S. marginatum* extract applied as 20% and 10% the efficacy was up to 95.37 and 81.2% respectively. The extract of *C. macrostchys* and *C. aurea* at 20% reduce the attach intensity by 92.59 and 95.09% respectively.

Table 6. Effectiveness of plant extracts at different days of application before *B. fabae* inoculation on faba baen plants

Treatment	2		7		14	
	Mean ± SE	% Inh.	Mean ± SE	% Inh.	Mean ± SE	% Inh.
Control	21.6 ± 1.03	0	19.4±2.23	0	20.6±1.54	0
<i>D.stramonium</i> 5%	6.66 ± 0.45	69	7.13±0.33	63.25	10.4±0.51	49.51
<i>D.stramonium</i> 10%	4.0 ± 0.24	81.48	4.39±0.50	77.37	6.86±0.58	65.39
<i>D.stramonium</i> 20%	1.14 ± 0.17	94.77	1.4±0.29	92.78	1.68±0.42	91.94
<i>C. aurea</i> 5%	10.6 ± 0.19	50.92	10.93±0.41	43.7	10.53±0.31	48.88
<i>C. aurea</i> 10%	5.6 ± 0.19	74.07	5.66±0.28	70.82	7.13±0.23	65.39
<i>C. aurea</i> 20%	1.06 ± 0.29	95.09	1.48±0.34	92.47	2.12±0.23	89.66
<i>S. marginatum</i> 5%	7.0 ± 0.43	67.59	7.73±0.61	60.15	9.93±0.65	51.8
<i>S. marginatum</i> 10%	4.06 ± 0.19	81.20	5.53±0.33	71.50	6.33±0.51	69.27
<i>S.marginatum</i> 20%	1.0 ± 0.24	95.37	1.66±0.30	91.44	1.8±0.34	91.13
<i>C.macrostchys</i> 5%	10.0 ± 0.37	53.7	10.31±0.62	46.75	10.6±0.43	48.54
<i>C.macrostchys</i> 10%	5.66 ± 0.24	73.8	6.06±0.51	68.76	7.13±0.50	65.39
<i>C.macrostchys</i> 20%	1.6 ± 0.19	92.59	1.8±0.17	90.72	2.12±0.23	89.66

SE=Standard error of mean; Inh.: inhibition

The pre inflectional treatment seven days before inoculation with extracts of *D. stramonium*, *S. marginatum*, *C. macrostchys* and *C. aurea* on the tested plants showed different percent of

inhibition by various plant extracts. Leaf extracts of *D. stramonium* show better inhibition at all concentrations. The extract of *D. stramonium* 20%, 10% and 5% applied seven days before inoculation reduced the attach intensity by 92.78%, 77.37% and 63.25% respectively. Leaf extracts of *S. marginatum*, *C. aurea* and *C. macrostchys* at 20% inhibited growth of *B. fabae* by 91.44%, 92.47% and 90.72% respectively. Even at lower concentration reduced the attach intensity by 60.15%, 43.7 % and 46.75% respectively.

The activity of extracts against *B. fabae* was tested as pre infectional treatment 14 days before inoculation of faba baen plant with different concentration. Regarding the persistency of extracts, studies revealed along duration of efficacy of the extracts based on *D. stramonium*, *S. marginatum*, *C. macrostchys* and *C. aurea* (Table 6). At 20% concentration all the applied extracts have showed inhibition on the development of *B. fabae* on the tested plants.

Mixture of plant extracts and biocontrol *T. harzianum* were applied on Faba bean plants 2 days before inoculation. The result (Table 7) indicated that mixture of *T.harzianum*, *D.stramonium* and *C. aurea* showed significant inhibition on the lesion development of *B. fabea*.

Table 7. Effectiveness of mixtures of plant extracts and biocontrol applied two days before inoculation of *B. fabea* on faba baen plants

Treatment	Mean± SE	% Inh.
Control	17±0.7 <sup>c</sup>	0
<i>T. harzianum</i>	1±0.26 <sup>b</sup>	94.11
<i>D.stramonium</i> and <i>C. aurea</i>	0.33±0.15 <sup>ab</sup>	98.06
<i>S.marginatum</i> and <i>C.macrostchys</i>	0.6±0.16 <sup>ab</sup>	96.47
<i>T.harzianum</i> , <i>D.stramonium</i> and <i>C. aurea</i>	00±00 <sup>a</sup>	100

SE=Standard error of mean; Inh.: Inhibition;

The value means of five replicates ± standard error. The value followed by different alphabets differs significantly when subjected to Tukey HSD at 0.05 subset.9.

## 6. DISCUSSION

In the present study, seven medicinal plants were screened for their antimicrobial activity against *B. fabae*. Four plants with highest antifungal activity were tested *in vivo* as a botanical control agent against *B. fabae* on faba bean. The screening revealed that most of the botanical plants were effective for inhibition of mycelia growth as demonstrated by poisoned food technique at different concentration.

The antifungal activity of matured and immature aqueous leaf extracts of *C. macrostchys* showed significant antifungal activity on the growth of *B. fabae*. The immature aqueous leaf extracts of *C. macrostchys* showed better inhibition than the matured leaf extract, and the active compound were soluble in water. The *in vivo* evaluation for antifungal activity of aqueous extracts of *C. macrostchys* at different concentration revealed that 20% of the extract show significant activity on the faba bean plant.

The antifungal assay of the three solvent extracts of *C. macrostchys* revealed that the ethanol extract showed the highest antifungal activity and followed by methanol extract, suggesting that the active compound is better extracted with ethanol than other solvents. Chloroform extracts of *C. macrostchys* showed inhibition at 40mg/ml were 60.86% which is significantly different from the other tested plants, indicating that the active compound of *C. macrostchys* was also soluble in chloroform. Therefore, aqueous extracts of *C. macrostchys* was used to test the efficacy controlling mycoflora of faba bean plant.

The antimicrobial activity of aqueous and methanol stem bark extracts of *C. macrostchys* against anti-inflammatory property has been demonstrated by (Kamanyi, *et al.*, 2008). Cyrus, (2008) evaluated the plant *C. macrostchys* for ear treatments, cutaneous, subcutaneous parasitic infection, venereal diseases, Yellow fever, Typhoid and measles. Karunamoorthi and Ilango (2010) demonstrated that the methanol extract of *C. macrostchys* have effective larvicidal activity against *Anopheles arabiensis*. Fisseha Mesfin *et al.* (2009) also reported that *C. macrostchys* was preferred among the medicinal plants that were reported by more informants as a remedy to diarrhea. Aqueous leaf extracts of *D. stramonium* significantly suppressed the radial growth of *B. fabae*. There was a gradual decrease in fungal colony diameter with the increase in extract concentration. Immature leaf extracts of *D. stramonium* showed the highest

antifungal activity with MIC of 30mg/ml. Aqueous seed extracts of *D. stramonium* also have antifungal activity at 70mg/ml. The antifungal activity of the three solvent extracts of *D. stramonium* revealed that methanol extract showed the highest antifungal activity followed by ethanol extract, suggesting that the active compound were better extracted with methanol, ethanol than chloroform.

The chloroform extract did not show inhibitory effect even at higher concentration. Aqueous, methanol and ethanol extracts of *D. stramonium* showed the highest antifungal activity in the *in vitro* test. The methanol extract selected to test *in vivo* on faba bean plants showed inhibition against *B. fabae*. The effectiveness of water extracts of *D. stramonium* on the spore germination of *Phaeoisariopsis personata* and *Puccinia arachidis* Rust disease of Ground Nut was reported by Ganapathy and Narayanasamy (1993). Arjunan *et al.* (1993) also demonstrated that the efficacy of *D. stramonium* on the incidence of sterility mosaic and mite population in Pigeon pea.

The antimicrobial activities of *D. stramonium* against human pathogenic microorganism were evaluated earlier. Hammouda *et al.* (1997) evaluated that the Anticholinergic, antiasthmatic and antispasmodic effects of *D. stramonium* were mainly due to the presence of the alkaloids, hyoscyamine and scopolamine. Fisseha Mesfin *et al.* (2009) reported that fresh leaves of *D. stramonium* used for rubbing and dressing against 'fore fore' (dandruff). The presence of alkaloids and flavonoids in *D. stramonium* was demonstrated by Kumar *et al.* (2008). The ethanol extracts of *S. marginatum* showed maximum inhibition in the target fungal colony of the selected isolate *B. fabae*.

Bioassay (antifungal) directed to ward to the evaluation of relative phytotoxicity of various extracts of aerial part of the plant is found to be in the following order: ethanol > aqueous > methanol > chloroform indicating that bioactive compounds of *S. marginatum* are highly extracted with ethanol and little with chloroform. Based on the *in vitro* result the ethanol leaf extract of *S. marginatum* tested on the *in vivo* test of Faba bean plants against *B. fabae* show significant effect. Manuel *et al.* (2005) evaluated that areal part of *S. marginatum* which is prepared by infusion and decoction has medicinal value for cough. Weissenberg (1988) also demonstrated that crude ethanol extract of dried powdered fruits and leaves of *S. marginatum* showed inhibitory effect on larvae growth of *Tribolium castaneum* and *Manduca sexta*.

The *in vitro* evaluation of antifungal activity of aqueous extracts of *S. incunum* at different concentration revealed that 70mg/ml concentration completely inhibited the tested pathogen *B. fabae*. The antifungal activity assay of the three solvent extracts of *S. incunum* revealed that ethanol extracts showed highest antifungal activity, suggesting that the bioactive compound is better extracted with ethanol than methanol and chloroform. Active compounds of *S. incunum* are soluble with ethanol and slightly soluble with water. The antimicrobial activity of *S. incunum* against gonorrhoea has been evaluated by Amare Getahun (1976). Mirutse Giday *et al.* (2009) evaluated that methanol extract of the fruits showed broad-spectrum antifungal activities. It has a strong effect against the fungus that causes athlete's foot, *Trichophyton mentogrophytes*.

The antifungal bioassay of different solvent leaf extracts of *C. aurea* against chocolate spot disease of Faba baen confirmed the presence of significant inhibitory effect which extracted methanol and aqueous extract of the plant. This indicated that the active compound was soluble in methanol and water and slightly soluble in ethanol. The chloroform extract was inferior against the fungus suggesting that *C. aurea* active compounds are not soluble or slightly soluble. The methanol extract of *C. aurea* show significant inhibition *in vivo* on the faba baen plant against *B. fabae*. Adedapo *et al.* (2008) evaluated that polyphenols are major plant compounds with antioxidant activity, methanol extracts of leaves and stems of *C. aurea* have antibacterial activity.

The antifungal activity of ethanol leaf extracts of *C. hirsute* against chocolate spot disease show significant inhibition, where as the methanol extract requires higher concentration. Chloroform and water extracts of *C. hirsute* show insignificant activity even at higher concentration, suggesting that the bioactive compound against the pathogen is less extractable, while more soluble in ethanol. This result corresponds to the result reported by other investigators Areej *et al.* (2008) evaluated that the oils of *C. hirsute* and *C. simensis* were miscible with ethanol and immiscible with water. The antifungal bioassay of different solvent leaf extract of *C. simensis* against *B. fabae* confirms the presence of significant effect of ethanol extract of the plant. The active compound of *C. simensis* is slightly soluble in methanol and water. The antimicrobial activity of *C. hirsute* and *C. simensis* was reported earlier. Volatile constituents showed a pronounced anti-inflammatory and antifungal activity (Areej *et al.*, 2008).

The aqueous immature leaf extracts of *C. macrostchyus* and *D. stramonium* significantly suppressed the growth of *B. fabae* which was significantly different from other aqueous extracts included in this study. Aqueous extracts of *C. hirsute* were poor in performance comparing to the other extracts. Following the immature leaf extracts of *C. macrostchyus* and *D. stramonium* matured leaf extracts of *C. macrostchyus* showed better inhibition which was significantly different from the matured leaf extracts of *D. stramonium* and *C. aurea*. Extracts of different concentration of all aqueous extracts exhibited gradual increase in antifungal activity with the increase in extract concentration. Water could be the medium to extract antifungal compound for most of the selected plants in this study especially for *C. macrostchyus*, *D. stramonium* and *C. aurea* (Fig.2).

Both species of *Solanum* were best extracted by ethanol following by *D. stramonium* and seed extract of *S. marginatum*. Immature leaf extracts of *S. marginatum*, *S. incunum* and mature leaf extract of *S. marginatum* showed antifungal activity against *B. fabae* with out any significant difference between them. Most of the selected plants were extractable with ethanol.

As shown in Table 2 methanol extracts of *D. stramonium* displayed antifungal activity which is significantly different from the other plant extracts. Especially the immature leaf extract exhibited highest antifungal activity followed by the matured leaf extract. *C. aurea* also show inhibition for the tested organism significantly different from the immature leaf extract of *S. marginatum*. Both *Clematis* spp. and *S. incunum* were less extracted with methanol.

The result of this study showed that all the seven tested plants have varied antifungal activity against the tested organism. Among the seven plants *D. stramonium*, *S. marginatum*, *C. macrostchyus*, *C. aurea* and *S. incunum* was found most effective against the selected strain in aqueous extracts and most solvents. Significant reduction in growth of *B. fabae* was observed with extracts of *D. stramonium*, *S. marginatum*, *C. macrostchyus* and *C. aurea* in the pot experiment. In most screened botanical plants in this study the immature leaf extracts have more inhibitory effect than the matured one. The difference in efficacy of different extracts could be attributed to the presence of the active principles that are extracted by different solvents which may be influenced by several factors such as maturity of the plant, method of extraction and type of extracting solvent. It is evident from the result that most the plant extracts showed inhibition on the mycelial growth of the isolated fungus.

Among the plant extracts *D. stramonium*, *S. marginatum*, *C. macrostchyus* and *C. aurea* proved to be effective in greenhouse test applied as extract from dry leaf material inhibited *B.fabae* growth. The effectiveness declined when lower extract concentrations were applied. Exploitation of preparations based on natural substances, which can limit plant pathogens development comes into higher and higher prominence, especially restricting traditional chemical preparation application. It results from comparable efficiency of bio-preparations to fungicide pesticides.

Combining certain microorganisms with broad-spectrum activity showed promise for increased consistency of suppression of chocolate spot caused by *B. fabae*. In experiments directed at comparing application of individual versus combined plant extract and microbial treatments, the combination of *T. harzianum*, *D. stramonium* and *C. aurea* provided significant inhibition. All tested different concentrations of the *extract* provided significant inhibition and Tukey HSD results shows statistically significant difference between treated and control groups. Mahmoud *et al.* (2004) evaluated that the additive effects of bioagents and extracts in minimizing chocolate spots caused by *B. fabae*.

These botanical plants responsible for antifungal activity could be possibly the presence of active compounds. To indicate the presence of active compounds TLC was developed and different compounds were visualized with UV 254nm (Appendix 6). *D. stramonium*, *S. marginatum*, *C. macrostchyus*, *C. aurea* and *S. incunum* formed strong foam with water during this study indicating that they contain saponins. Addition of concentrated H<sub>2</sub>SO<sub>4</sub> to extracts no red coloration was seen in extracts of *D. stramonium*, *S. marginatum* and *S. incunum* indicates the presence of Steroids. Weissenberg (1988) evaluated the presence of Steroidal glycoalkaloids in *solanum* spp. Addition of drops of 5% FeCl<sub>3</sub> to extracts resulted in dirty green precipitate in extracts of *C. macrostchyus*, *C. aurea*, *S. incunum* and *S. marginatum* indicates the presence of Tannins. Extracts of *C. macrostchyus* and *C. aurea* have showed red coloration after the addition of MgSO<sub>4</sub> and concentrated HCl indicates the presence of Flavonoids. This result was also reported by (Adedapo *et al.*, 2008).

## 7. CONCLUSION AND RECOMMENDATION

### 7.1. CONCLUSION

- ∅ Ethanol extracts of all screened and tested plants revealed relatively more effective against the pathogen than other solvents extracts.
- ∅ Active compounds of *D. stramonium*, *S. marginatum* and *C. aurae* are extractable with water and most extracted solvents.
- ∅ The results of this investigation revealed that ethanol, methanol and aqueous extracts of all the seven plants possess diverse antifungal activity against the test fungus *B. fabae*.
- ∅ The differentiating activities against the selected isolate of these seven extracts encourage developing broad spectrum antifungal in the future.
- ∅ The potential benefits of using mixture of fungal isolate and plant extracts to suppress diseases under the pot conditions.
- ∅ *In vivo* application of *T. harizianium* against *B. fabae* reduced the disease incidence of the pathogen. However, more efforts to search good antagonistic fungal strains are still needed.

### 7.2. RECOMMENDATION

- ✓ All the tested plant contains antifungal agents and need further purification for better efficacy.
- ✓ A continuous effort to search good botanical extracts and antagonistic microorganisms is primarily needed.
- ✓ More investigations are needed to investigate this regard for isolation and characterization of antifungal compounds and recommendation in field applications.
- ✓ The result of the present study can be further exploited for formulating integrated disease management schedule of chocolate spot disease.

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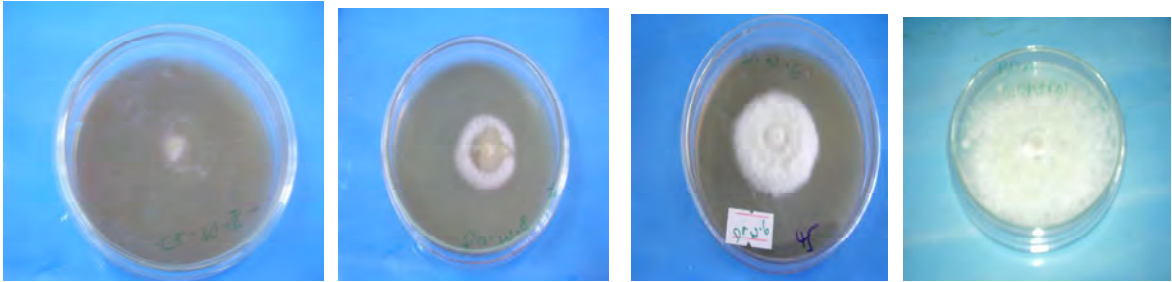
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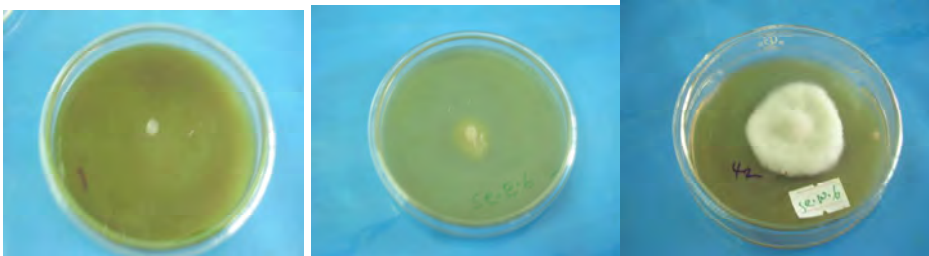
## 9. APPENDICES

### Appendix 1. Antifungal activity of aqueous plant extracts against *B. fabae*



Aqueous extracts of *D. stramonium*

control

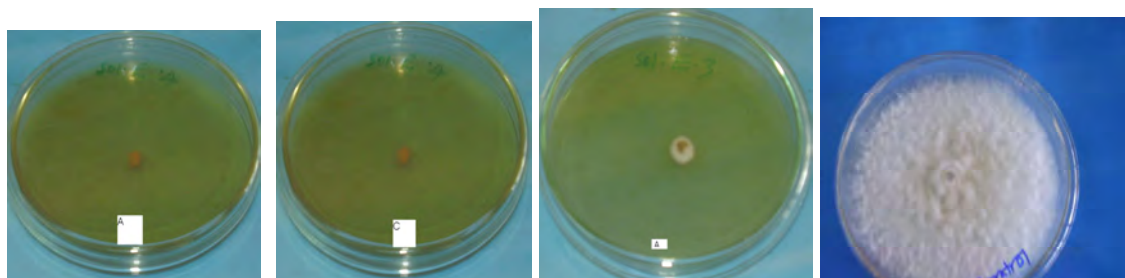


Aqueous extracts of *C. aurea*



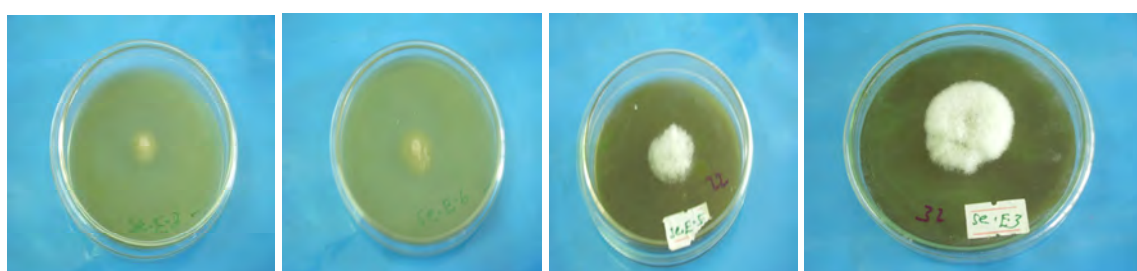
Aqueous extracts of *C. macrostchyus*

Appendix 2. Antifungal activity of ethanol plant extracts against *B. fabae*



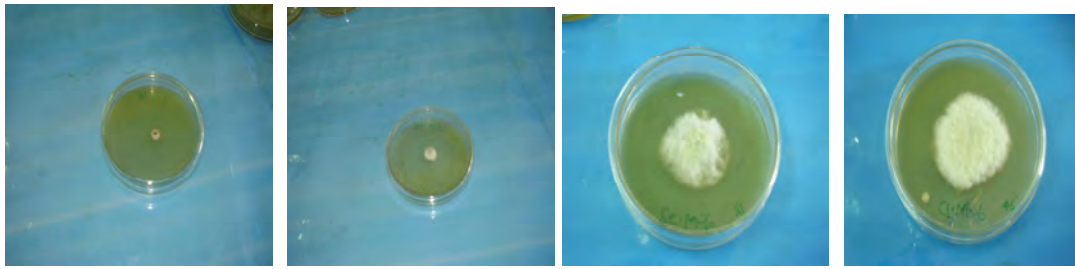
Ethanol extracts of *S.marginatum*

control

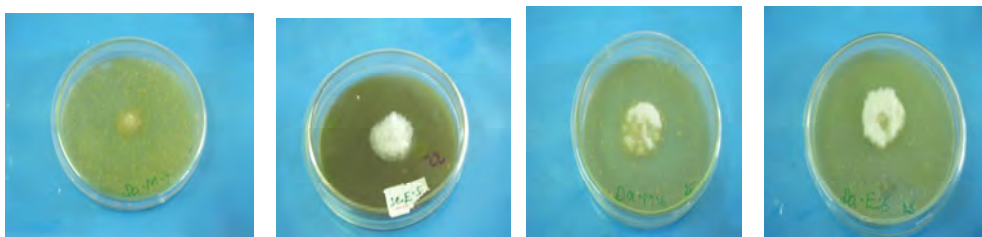


Ethanol extracts of *C. aurea*

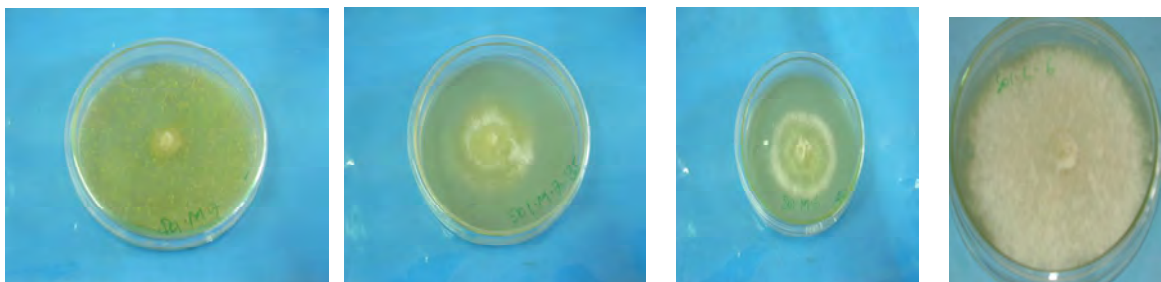
Appendix 3. Antifungal activity of methanol plant extracts against *B. fabae*



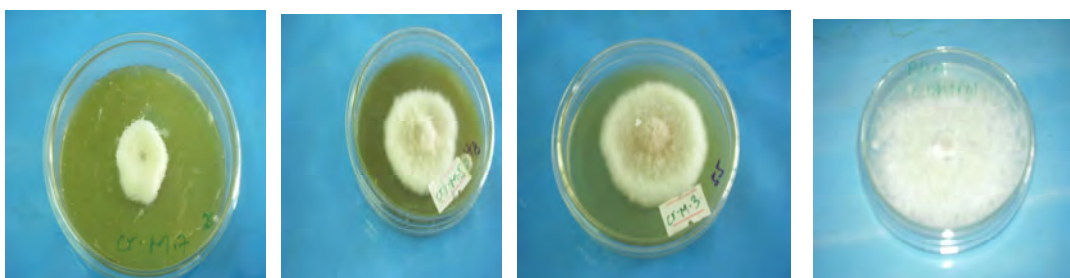
Methanol extracts of *C. aurea*



Methanol extracts of *D. stramonium*



Methanol extracts of *S. incunum*



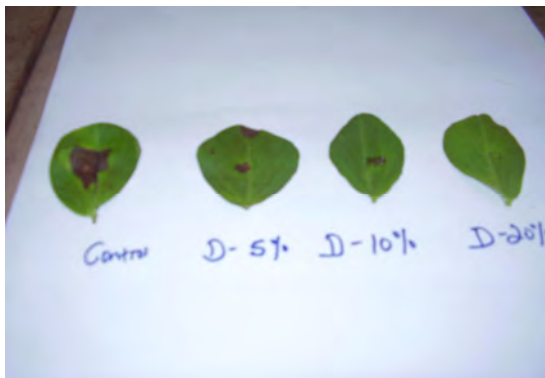
Methanol extracts of *C. macrostchys*

control

Appendix 4. Pot experiments for evaluation of antifungal activity plant extracts against *B. fabae*



Appendix 5. Effectiveness of plant extracts, inoculation after 14<sup>th</sup> days after treatment



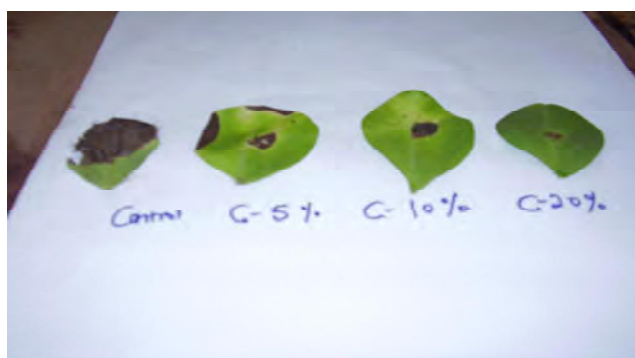
*D. stramonium* extract treated leaves



*S. marginatum* extract treated leaves



*C. aurea* extract treated leaves

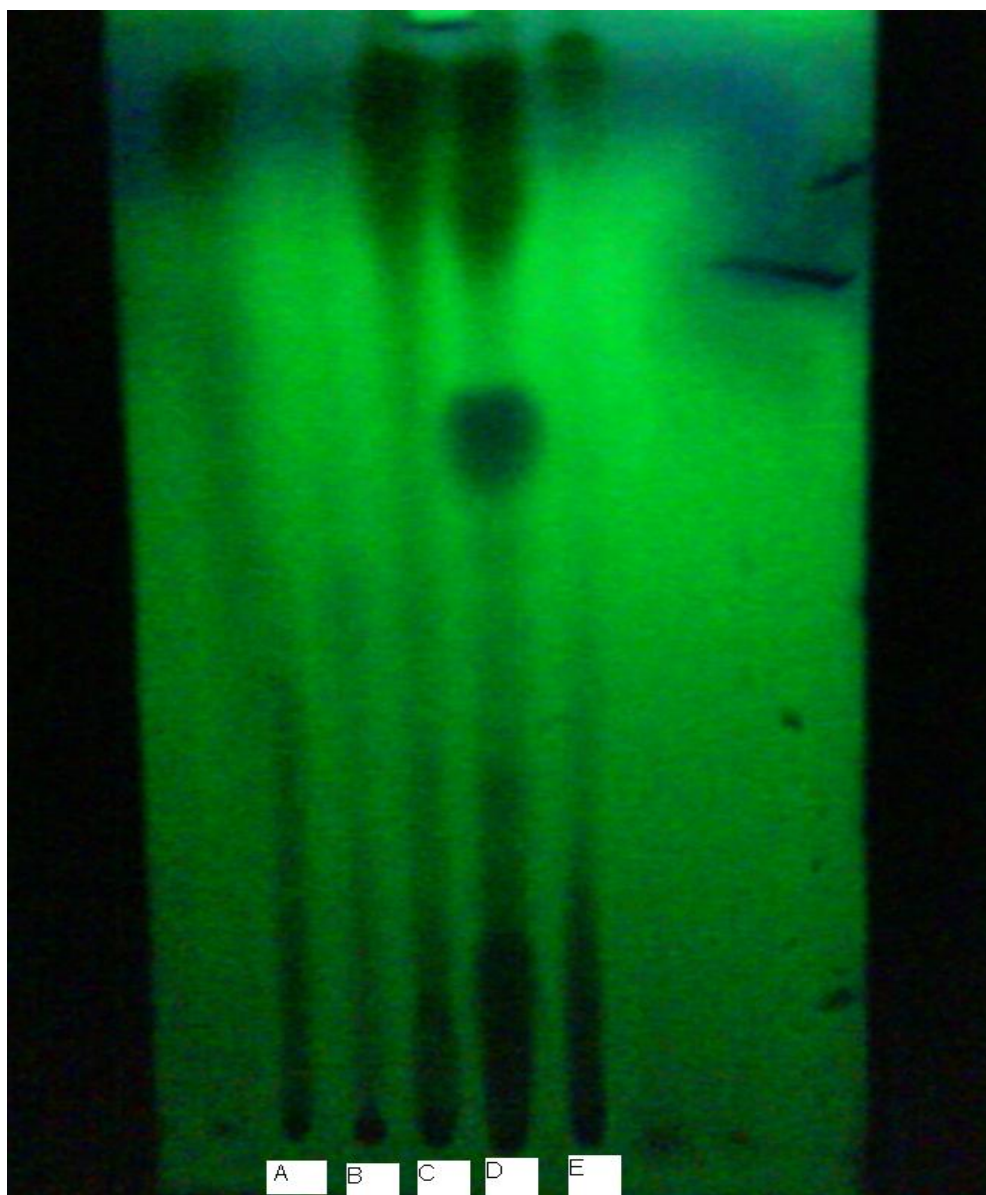


*C. macrostchyus* extract treated leaves



Plant extracts and biocontrol mixture treated leaves

Appendix 6. TLC developed for the plant extracts to indicate the presence of active compounds



A - Methanol extract of *D. stramonium*, B - Aqueous extracts of *C. macrostchys*, C- Ethanol extracts of *S. marginatum*, D- Methanol extracts of *C. aurea* and E- Ethanol extracts of *C. simensis*

