CHAPTER I

1. INTRODUCTION

1.1 General Introduction

Nepal, being one of the agricultural country, most of the people are involved in agriculture. Vegetables are main source of farmers to uplift their economical status. Garlic is one of the important vegetable crop which has been used throughout recorded history for both culinary and medicinal purposes.

Garlic (*Allium sativum* Linn.) is the edible bulb from a plant in the liliaceae family. The most important part of this plant for medical purpose is compound bulb while aerial green parts are used as vegetable. It has been used as spice for thousands of years as well. The medicinal value of garlic is high, having antiseptic, antibacterial, antifungal, antiviral and antitumor properties.

Garlic was found growing wild in Central Asia and Mediterranean and is now grown all over the world. It is distributed through out Nepal to about 3000m (Manandhar 2002).

Garlic is perennial herb with aromatic leaves. Leaves are flat, narrow, acuminate, entire; scape slender, spathes long beaked; flowers in head, white; root fibrous. The bulbous structures consists of a number of small segments or ‘cloves’ enclosed within whitish or pinkish scaly sheath.

Garlic is grown globally, but China is by far the largest producer of garlic with approximately 10.5 billion kilogram (23 billion pounds) annually, accounting for over 77% of world output. According to statistical data, in the year of 2006/2007, the total production of garlic is 30308mt in the area of 4806ha and the productivity is 6.31mt/ha in Nepal.

Garlic is known for its nutritional value. It has moderate amounts of carbohydrate protein, fat, fiber and good amounts of calcium, phosphorous and potassium.

In Nepal, Garlic is infected by many diseases. Although it acts as antifungal activity, it has been found that various pathogens are susceptible to the garlic. Some of the diseases that lead to destruction of plant crop are as follows.

Table 1: Diseases of Garlic

S.N	Diseases	Pathogen
1.	White rot	*Scleroticum cepivorum*
2.	Basal rot	*Fusarium culmorum*
3.	Blue mold rot	*Penicillium spp.*
4.	Leaf blight	*Botrytis squamosa*
5.	Neck rot	*Botrytis porri*
6.	Leaf blight	*Stemphylium vesicarium*

Among these diseases, leaf blight of garlic is one of the destructive disease that is caused by the pathogen *Stemphylium vesicarium* (Wallr.) Simmon, which lies in class of Deuteromycetes. The fungus infects basically leaves of garlic showing symptoms of lesions of ellipsoidal to oval, brown to dark brown, sometimes purple, zonate, often becoming larger, light brown to tan and eventually kill the older leaves.

The pathogen when cultured in PDA medium, the colonies appear gray to grayish brown, hairy or velvety, reaching about 6cm in diameter after 7 days at 25^0 C. Mycelium immerged or partly superficial; hyphae septate, branched, smooth walled, hyaline Conidia solitary; broadly oval when juvenile, often with a swollen basal cell, usually oblong to broad-ellipsoid at maturity. Olivaceous brown to medium golden-brown, rounded at the apex with no pointed ends, verrucose; 25-45 × 15-25 mm on host, 25-50 × 12-30 mm on agar media, with 1-5 transverse and 1-2 complete or nearly incomplete series of longitudinal irregular septa, commonly constricted at the 2-3 of the major transverse septa, but 1 median transverse septum in juvenile conidia.

The medicinal properties such as antifungal, antiviral, anticancer and others are present in medicinal plants (Parajuli *et al.*, 1998). The active components or secondary metabolites like alkaloids, saponins, essential oils, tannins etc. are the main reasons for the medicinal properties (Kruger, 1992). Extracts of eight medicinal plants

which consists of highly medicinal values were used for the assessment of fungi toxicity. They are *Phyllanthus emblica* L., *Syzygium aromaticum* L.,*Cinnamomum camphora* Linn. Presl, *Solanum xanthocarpum* Schrad and Wendl, *Cuscuta reflexa* Roxb., *Allium cepa* L., *Equisetum diffusum* D.Don, *Cinnamomom zeylanicum* B. The extracts of these plants contain active chemical components that aid to control disease of various plants inhibiting the mycelial growth of pathogen.

Biological control is defined as 'any condition under which or practice where by survival or activity of the pathogen is reduced through the agency of any other living organisms except man himself with the result that there is reduction in incidence of disease caused by pathogen (Garrett,1965). It is a nature friendly ecological approach to overcome the problems caused by standard chemical methods of plant protection.

Baker and Cook (1974) have pointed out that the aim of biological control is the reduction of disease by (a) reduction of inoculum of the pathogen through disease survival between crops, decrease production or release of viable propagules or decreased spread by mycelial growth, (b) reduction of infection of the host by the pathogen and (c) reduction of severity of attack by the pathogen.

Trichoderma species are important biological control agents used in plant disease management. They are fungi that are present in nearly all agricultural soils and in their environments. The antifungal abilities of these beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then. They are imperfect fungi, with their teleomorph perfect stage belonging to Hypocreales of Ascomycetes. They are capable of secreting hydrolytic enzymes and cause mycoparasitism on fungal pathogens of plants. Besides, they produce antibiotics and toxins that inhibit the fungal plant pathogens. These fungi grow tropically towards hyphae of other fungi, coil about them in a lectin-mediated reaction and degrade cell walls of the target fungi by secretion of different lytic enzymes.

1.2 OBJECTIVES

- To isolate the pathogen from infected leaf of garlic and test its pathogenecity.
- To test the fungitoxicity of the extracts of 8 medicinal plants at different concentration against *Stemphylium vesicarium* (Wallr.) Simmons.
- To determine the minimum inhibitory concentration (MIC) of plant extracts used.
- To observe the efficacy of *Trichoderma* spp. to control leaf blight of garlic.

1.3 JUSTIFICATION OF THE STUDY

Garlic is considered as nature's 'wonder drug' which has powerful effects for a tremendous variety of common illnesses. Now, medicinal research indicates garlic may prevent and even reverse high blood pressure, heart disease, and cholesterol level and cancer. In fact, recently garlic has been used reasonably successfully in AIDS patients to treat cryptosporidium in China. It has also been used by at least one AIDS patient to treat toxoplasmosis, another protozoal disease (John S. James, 2007).

Above medicinal purposes shows that garlic is one of the most important and valuable vegetable that can flourish the income of farmers if it is grown precisely. However, farmers are always suffered from loss of productivity of this crop due to diseases in garlic and one of the most destructive disease is leaf blight, caused by *Stemphylium vesicarium* (Wallr.) Simmons. The history of severe outbreak of this disease in Thimi area proves that it may lead to a devastating epidemic of this disease.

The control of this disease through the use of different fungicides is common method that causes harmful effects in human health and environment. A report from Research council, Board of Agriculture, 1987 about pesticides residue on food suggests that fungicides have more of a carcinogenic risk than insecticides and herbicides together. These risks of fungicides can be overcome by the use of the plant extracts. The extracts of plants are less toxic and more systemic into the fungicides (Fawcett *et al*., 1970). Hence the plant extracts can be more effective to control the disease regarding with human health and environment.

1.4 LIMITATION OF THE STUDY

Due to time and economic factors, the study suffered from following limitations:

- The entire work was carried out in limited time for one year only.
- Only single pathogen was selected from host garlic for experiment.
- Eight selected aromatic plant species were subjected to assess fungitoxicity.
- TLC and GC were not carried out.
- The antifungal activity of extracts of medicinal plants was confined within the laboratory only.
- Field application was not conducted.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Test fungus

2.1.1 Isolation and pathogenicity of *Stemphylium vesicarium* (Wallr.) Simmons

Boiteux *et al.* (2007) identified the causal agent of a foliar disease of garlic as *Stemphylium vesicarium* (Wallr.) Simmons. Disease symptoms were reproduced on garlic leaves 8 to 10 days after inoculation in a greenhouse (temperature range 22-26 C). Onion and tomato, two previously reported hosts of *S. testcarnom* were also artificially infected with the isolate from garlic. This is believed to be the first report of *S. testcarnom* causing a severe leaf blight of garlic in the New World area.

Basallote-Ureba *et al.* (1999) identified a new leaf spot disease, characterized by white and purple lesions followed by extensive necrosis. Isolation and pathogenicity tests with fungal isolates taken from these spots indicated that *Stemphylium vesicarium* was the causal agent. Pseudothecia of the teleomorph stage, *Pleospora* sp., were found on leaf debris from affected plants. Inoculation of garlic and onion plants with residues carrying mature pseudothecia, or with ascospore suspensions obtained from the pseudothecia, resulted in the development of white and purple leaf spots. Wetness periods longer than 24 hr were required for symptom development under controlled conditions. Isolates of *S. vesicarium* from garlic and asparagus caused disease in all three hosts.

Chang *et al.* (2000) found a leaf spot disease occurred on *Allium tuberosum* Roth. (Chinese chive) in Korea. All the isolates of *Stemphylium* sp. from the lesions of the diseased plant parts were identified as *S. vesicarium* (Wallr.) Simmons, based on the morphological characteristics of conidia and conidiophores. Pathogenicity of the fungus was proved by artificial inoculation on Chinese chive plants. This is the first record of leaf blight on Chinese chive caused by *S. vesicarium* in Korea.

2.2 Antifungal activity of medicinal plant extract

2.2.1 Test plants

2.2.1.1 *Phyllanthus emblica* L.

Family: Euphorbiaceae

Local name: Amala

Distribution in Nepal: It is found throughout upto above 1600m.

Description: Deciduous tree about 15m high, leaves compound, leaflet 1-1.5cm long, 0.2-0.3cm wide. Flowers small, yellowish, densely clustered on the braches. The male flowers numerous, female flowers few, both sexes on the same branchlets.

Chemical constituents: Rich source of vitamin C and tannin; seed contains fixed oil phosphotide. (Manandhar, 2002)

2.2.1.2 *Syzygium aromaticum* L.

Family: Myrtaceae

Local name: Lwang

Distribution in Nepal: It is native of Indonesia but not in Nepal.

Description: A conical tropical evergreen myrtaceous tree reaching in height up to 14m. The bark is grey. The leaves are shiny dark green, elliptical in shape and very fragrant. Small crimson flowers grow in triple clusters at the ends of branches. The fruit is a purple drup about 2.5cm long.

Chemical constituents: The components of clove oil are eugenol, eugenol acetate, iso-eugenol and caryophyllene. (Manandhar, 2002)

2.2.1.3 *Cinnamomum camphora* Linn. Presl

Family: Lauraceae

Local name: Kapur

Distribution in Nepal: It is commonly found in subtropical zone (1500m)

Description: A large, robust tree occurring up to altitude of 1500m bark pale brown leaves opposite, large 20-30cm long elliptic-oblong, glabrous, glacucous beneath, coriaceous, nerves 3, not impresses above, flower pale yellow, pubescent in large panicles, fruits sub-globose, seated on slightly enlarged perianth.

Chemical constituents: The mature leaves and twigs yield an essential oil, camphor. The oil contains 1,8-cincol. Presence of limonene, camphene and p-cymene is also

reported. Although all the parts of plants contain oil, its yield is highest in leaves. The kernels yield a crystalline fat. (Dutta, 2007).

2.2.1.4 *Solanum xanthocarpum* Schrad and Wendl

Family: Solanaceae

Local name: Kantakari

Distribution in Nepal: Tropical and subtropical zones.

Description: A very spiny diffuse herb up to 1, 2 m tall commonly found through out Nepal. Leaves ovate or elliptic, sinuate or sub-pinnatifid, spines 1cm long, straight; flowers blue in lateral cymes; berries globose 1.2-2cm in diameters; glabrous, yellow or whitish and green blotched; seed glabrous.

Chemical constituents: Solancarpine, a gluco-alkoloid is found in the fruits. On hydrolysis it gives a crystalline compound and a sugar, Solacarpindin and Carpesterol are found in the fruit. (Dutta, 2007).

2.2.1.5 *Cuscuta reflexa* Roxb.

Family: Concovulaceae

Local name: Akashbeli

Distribution in Nepal: Up to 2400m altitude.

Description: The well known leafless greenish- white or yellow flowering parasite, very common in the scrub forests along the outer foot of the Siwaliks and in similar localities. The leafless character is carried even to the embryo which is destitute of cotyledons. The seed germinates on the ground but the plant does seem to drive much nourishment from it, its growth being sustained for the little while it is obliged to shift for itself, by the fleshy albumen in which the germ-plant is emended in the seed.

Chemical constituents: Amarbelin, Bergenin,Cuscutalin, Kaempferol,Luteolin, Mangiferin, Mannitol. (Datta, 2007)

2.2.1.6 *Allium cepa* L.

Family: Alliaceae

Local name: Pyaj

Distribution in Nepal: Distribute throughout Nepal to about 3000m, cultivated.

Description: Bulbous herb. Leaves all basal, hollow, linear, opposite, surfaces alike. Flowers many, white in dense umbels.
Chemical constituents: Onion contains an acrid, volatile oil, uncrystallizable sugar, gum, albumen, woody fiber, acetic and phosphoric acid, phosphate, citrate of calcium and water and sulphur compound, C6H12S2.

2.2.1.7 *Equisetum diffusum* D.Don

Family: Equisetaceae
Local name: Aankhle jhar
Distribution in Nepal: Distribution throughout Nepal to about 3300m, common in moist places.
Description: Spores bearing herb, Stems erect, tufted, very rough, branches densely whorled, short, arising from the base of the sheath of scale like leaves.
Chemical constituents: Aerial portion contains Megastigmane diglucosides. (Manandhar ,2002)

2.2.1.8 *Cinnamomom zeylanicum*

Family: Lauraceae
Local name: Dalchini
Distribution in Nepal: It is growing naturally between 900-1200m altitudes.
Description: Medium sized tree 7.5m in height and 1.35 in girth; bark is dark brown, leaves opposite or sometimes alternate, elliptic to oblong- lanccolate, glabrous, 3-nerved at base, pink when young; flowers pale yellow, pubescent, fruits black, ovoid, on the thickened peduncle and enlarged base of the perienth.
Chemical constituents: Essential oil with cinnamaldehyde and eugenol. (Dutta, 2007)

The loss of productivity occurs yearly due to plant disease. It becomes need to increase the cultivation and productivity by the proper management of plant diseases and pests. In order to overcome this problem, higher concentration of chemicals or pesticides was used but this increased the risk of high level toxic residues in the products which were not beneficial for human beings and environment (Cakir *et al.*, 2004). Thus there has been a growing interest on the research of the possible use of the plant extracts in the disease control which are less damaging to the human health

and environment (Costa *et al*., 2000; Duru *et al*., 2003). The extracts of plants also exhibited marked effect on germination of fungal spores as well (Dubey *et al*., 1991).

Asthana *et al.* (1982) studied the volatile activity of 20 plant species against *Helminthosporium oryzae*. The extracts of *Ageratum conyzoids, Cymbopogon martini* var. *motia, Eupatorium capifollium* and *Ocimum adscendens* exhibited absolute toxicity. The volatile antifungal constituents isolated as essential oils inhibited the mycelial growth completely at their minimum inhibitory concentrations of 250µl/1, 1000µl/1 and 200µl/1 respectively. The oils were non-toxic to paddy and checked the appearance of disease symptoms during symptoms during in-vivo trials.

Tripathi *et al*. (1983) studied the distribution of fungitoxicity at five different growth stages of *Iberis amara* against *Helminthosporium oryzae* by spore germination, poisoned food and modifier paper technique.

Upadhya *et al*. (1987) tested leaf extracts of 30 plant species against *Aspergillus flavus*. Only the leaf extracts of *Anisomeles ovata* showed absolute toxicity. Its essential oil inhibited the growth of mycelium of *Alternaria alternate, A. solani and A tenuis* by 0.2% concentration.

Miah *et al*. (1990) tested the efficacies of 16 plant extracts against the pathogens of five major rice disease namely sheath blight, sheath rot, blast, bakane and leaf scald. Out of those tested species, *Sapium indicum, Tagestes erecta, polyalthia longifolia, Leucaena leucocephala* exihibited more than 50% antifungal activity.

Yadav and Saini (1990) hydrodistilled six percent yield of essential oil of *Eupatorium triplinerve*. The essential oil showed antifungal and antibacterial activity.

Mishra and Tiwari (1990) screened ethanol extracts of leaves of *Claotropsis procera, Azadirachta indica* and *Datuara stramonium* to evatuate their toxicity at 1000ppm against *Pyricularia oryzae, Rhizoctonia solani, Fusarium moniliformae* and *Aspergillus niger*. Plants tested showed toxic principles against one or other tested pathogens.

Khan and Tripathi (1994) screened the extracts of different parts of 122 higher plants for their volatile antifungal activity against *Aspergillus niger* and *Curvularia ovoidea*. The flower bud extract of *Syzygium aromaticum* exhibited absolute toxicity against both tested fungi inhibiting mycelial growth completely.

Qusem and Abu-Blan (1996) studied the aqueous extracts of 64 weed species on growth and development of *Alternaria solani, Helminthosporium sativum* and *Rhizoctonia solani*. Plant pathogenic fungi were studied *in-vitro*. Extracts varied in the strength and persistence of their antifungal effects against three fungal species.

Adhikari (1998) investigated *Allium sativum, Artimesia vulgaris, Hydrocotyl asiatica* and *Melia azadirachta* for their antifungal activity against some filamentous and non filamentous fungi and bacteria.

Fiori *et al*. (2000) studied antifungal activity of leaf extracts and essential oils of some medicinal plants against *Didymella bryoniae*. They reported that the crude extracts of *Eucalyptus* and *Ageratum* species showed inhibitory effect on mycelia growth and germination of spores where as essential oil of *Cymbopogon citrates, Ageretum conizoids* and *Eucalyptus citriodora* provided 100% inhibition of the mycelia growth and germination of spores of *Didymella bryoniae.*

Kuwar and Tripathi (2002) extracted thirty two essential oils from higher plants and tested against *Aspergillus flavus, A. niger, Alternaria alternata* etc. The oil showed 100% inhibition in mycelial growth of *Alternaria alternate* at 600ppm oil concentration.

Parajuli *et al*. (2005) tested the fungitoxicity of E. oils of *Xanthoxyllum armatum, Nardostachs grandiflora, Juniperus recorva, Artimisia dubia, A.gmelinii* and *Thymus linearis* against *Alternaria brassicicola,* the causal agent of leaf spot disease if cabbage by poisoned food technique. The fungitoxicity was found to be variable with different oils.

Manandhar (2005) investigated antifungal activity of essential oils from six plants against *Bipolaris sorokiniana*. She reported appreciable inhibitory effects towards the

growth of colony of fungi. Among six plants essential oil of *Thymus linearis* was found to be the most effective in its fungitoxic properties.

Stoilova *et al*. (2007) has studied the antioxidant activity and the total phenols of ginger extract (*Zingiber officinale*) were studied. The total phenols of the alcohol extract were found to be 870.1 mg/g dry extract. The ginger extract inhibited the hydroxyl radicals 79.6% at 37^0C and 74.8% at 80^0C.

2.3 Antifungal activity of *Trichoderma* spp.

Abramsky *et al*. (1994) applied *Trichoderma harziamum* to cucumber and pepper seedlings as a peat-bran preparation incorporated into the propagative mixture in a commercial production nursery. On marketing day (after 18 and 30 days for cucumber and pepper, respectively),

Trichoderma-treated seedlings were much more developed and vigorous and had higher chlorophyll contents. Cucumber seedlings were then transplanted to a commercial greenhouse and analyzed over two successive growth cycles following soil fumigation with methyl bromide (500 kg/ha). Results revealed the *Trichoderma*-treated plants to be more resistant to damping-off disease.

Coskuntuna & Ozer (2007) tested *Trichoderma harzianum* to determine its effect on the mycelial growth of *Fusarium oxysporum* f. sp. *cepae* (FOC) in dual culture and its control of basal rot disease in pot- and field-grown onion sets. *T. harzianum* inhibited mycelial growth of the pathogen *in vitro*. Seed treatment with *T. harzianum* decreased disease incidence comparable to the imidazole fungicide, prochloraz in both pot and field experiments. It also enhanced bulb diameter of sets, especially in the pot experiment.

Iudem and Merih (2004) studied Interactions between *Trichoderma harzianum* strains and some soilborne plant pathogens (*Gaeumannomyces graminisvar. tritici, Fusarium culmorum* and *F. moniliforme*) on PDA medium. All *T. harzianum* strains tested produced ametabolite that inhibited growth of plant pathogenic fungi on PDA medium. When grown in liquid cultures containing laminarin,chitin or fungal cell

walls as sole carbon sources, 2 strains of *T. harzianum* produced 1,3-□-glucanase and chitinase in the medium.Higher levels of these enzymes were induced by *T. harzianum* T15.

Campbell *et al.* (1986) tested ten isolates of *Trichoderma harzianum* were tested for their ability to control lettuce seedling damping-off caused by introduced *Rhizoctonia solani. T. harzianum* isolates TRC 9 and 28 both reduced damping-off. Dual culture experiments were used to select isolates for the study of antibiotic production and mycoparasitism. *T. harzianum* isolate TRC 12 produced volatile and non-volatile antibiotics, whilst TRC 33 produced only non-volatile antibiotics. *T. harzianum* isolates 018-2/Y and TRC 9, 15 and 28 mycoparasitized *R. solani* by coiling around and lysing the host hyphae. It appeared that mycoparasitism was more important than antibiosis in the biological control of damping-off.

CHAPTER-3

3. MATERIALS AND METHODS

3.1 Materials

Different plant samples (used for testing antifungal properties), test fungus (*Stemphylium vesicarium* (Wallr.) Simmon), equipments, glasswares and chemicals were used in the experiments. All such materials are listed in Appendix I.

3.2 Methods

Portion of the research was carried out in the laboratory of NARC, Khumaltar and laboratory of Horticulture, Kirtipur and rest works in the laboratory of Central Department of Botany, Plant Pathology Unit, Kirtipur.

3.2.1 Collection of Diseased plants

During research period, the diseased leaf samples of garlic were collected from the fields of Thimi, Bhaktapur.

3.2.2 Isolation of Test fungus by Single spore Method:

The small part of infected leaves was firstly surface sterilized with Sodium hypochlorite (2%) solution and inoculated on a pre-sterilized petri plates containing Water Agar (WA) media. The spore suspension was made by putting some spores in test tube containing 2ml of distilled water. The spore suspension was again transformed on Water Agar (WA) media and was incubated at $20^0 \pm 3^0$C for 24 hours. After 24 hours, the single germinating spore on water agar (WA) media was transferred on a pre-sterilized petri plate containing Potato Dextrose Agar (PDA) media and was incubated in an inverted position at $20^0 \pm 3^0$C for one week. After one week, the mycelium growth of test fungus was observed.

3.2.3 PDA media Preparation

For preparing 1000ml of PDA media, 200gm of peeled potatoes were cut into small pieces and were boiled in 500ml of distilled water. The cooked potato pieces were filtered by muslin cloth on conical flask and the volume was maintained to 1000ml by

the addition of required distilled water. 20gm of agar and 20gm of dextrose were added to it and gently shaken to get thoroughly mixed. The flask was plugged and autoclaved for 15 minutes at 121^0C in 15lbs pressure.

3.2.4 WA media Preparation

For preparing 1000ml of WA media, 16gm of agar were added to 1000ml of distilled water and gently shaken to get thoroughly mixed. The flask was plugged and autoclaved for 15 minutes at 121^0C in 15lbs pressure.

3.2.5 Identification of Test fungus

The test fungus, *Stemphylium vesicarium* (Wallr.) Simmons was identified under the guidance of Professor and teachers of Central Department of Botany and NARC and with the help of the standard literature (Yu and Cho, 2001).

3.2.6 Pathogenicity test

For the test, 6 pots and potting mixture, sand and soil in the ratio of 5:2 were autoclaved at 121^0C in 15lbs pressure for 15 minutes. Equal amount of potting mixture was kept in each pot. The seeds of garlic were firstly surface sterilized with Sodium hypochlorite (2%) solution and were planted in each pot. Each pot was watered with sterilized distilled water at 2 days intervals. Regular watering was done for proper growth of plants in each pot. The inoculation of pathogen was carried out by leaf inoculation. The healthy leaf was first wiped with ethanol and then scratched with the help of sterilized sand paper. The one week old culture of pathogen was inoculated into the scratched portion. The triplicate was maintained for control and infected plants in each test. The symptoms appeared in the inoculated leaf were compared with the symptoms firstly noted.

3.2.7 Reisolation

The pathogen was reisolated from the inoculated plants in PDA media. Its characteristics were found to be similar with previous one. This proved that the pathogen in both the cases was *Stemphylium vesicarium* (Wallr.) Simmons which causes leaf blight disease of garlic.

3.2.8 Maintenance of Pure culture

The pure culture of test fungus was preserved by sub-culturing in sterilized petri plates and slants containing PDA media.

3.2.9 Control by using Different Plant extracts

For control of the test fungus, extracts of different medicinal plants were used. 8 different test plants were collected from different places of Kathmandu valley which are as follows.

Table 2: Medicinal plants used to test fungitoxicity

S.N.	Botanical name of plants	Parts used	Place of collection	Local name
1.	*Equisetum diffusum*	Whole plant	Kirtipur	Aankhle jhar
2.	*Phyllanthus emblica*	Leaves	CDB Garden	Amala
3.	*Syzygium aromaticum*	Fruits(cloves)	Kathmandu	Lwang
4.	*Solanum xanthocarpum*	Fruit	Kirtipur	Kanthakari
5.	*Cuscuta reflexa*	Stem	Kirtipur	Akashbeli
6.	*Allium cepa*	Bulb	Bhaktapur	Pyaj
7.	*Cinnamomum camphora*	Leaves	Kirtipur	Kapur
8.	*Cinnamomum zeylanicum*	Bark	Bhaktapur	Dalchini

3.2.10 Preparation of Plant extract

Five gram of required parts of each plant was firstly surface sterilized with the help of 2-3% sodium hypochlorite for 2-5 minutes and was thoroughly washed with sterile distilled water (three changes).The plant parts were grinded finely in sterilized mortar and pistil adding 5ml of sterile distilled water. The grinded part was filtered using sterile muslin cloth (autoclaved), and the obtained solution was centrifuged at 3000 rpm for 15 minutes, and the upper clear solution was separated. The volume of obtained filtrate of each plant after configuration was adjusted to 10ml by addition of required amount of distilled water. This final volume of extract was used as a "stock solution" i.e.; 100% concentration. Five different concentrations viz. 20%, 40%, 60%, 80% and 100% of each plant extracts were made in separate sterilized test tubes.

3.2.11 Preparation of One week Old culture

Inoculum disc from one week old culture is required for testing the antifungal activity of plant extracts. For preparation of one week old culture, the fungus from pure culture was transferred in sterilized petri plate containing PDA media and was placed in incubator at $25^0C \pm 3^0C$. After seven days the inoculum disc was taken from the culture plate for further experiment.

3.2.12 Antifungal Assay

The antifungal assay was assessed by using poisoned food technique (Grover & Moore, 1962). 0.5 ml volume of stock solution of each extract was aseptically poured into pre-sterilized petri plate followed by the addition of 9.5 ml of melted PDA. The petri plates were swirled gently to allow thorough mixing of the contents. This PDA media with extract solution served as treatment set. In the control set, no extract was used. The temperature of molten PDA was constantly monitored with the help of 75% ethanol sterilized thermometer. After solidification of the media, one inoculum disc of 5mm in diameter (prepared with the help of sterilized cork borer) of the test fungus was aseptically inoculated upside down at the centre of each petri plate and incubated at temperature $25^0C \pm 2^oC$ for 7 to 10 days. The number of concentrations in each experimental set including treatment and control sets were six (n=6). For each control set and treatment set, 3 replicates were maintained for each test. Average diameter of the fungal colonies was measured on the 7^{th} or 10^{th} day of incubation and the percentage of mycelial growth inhibition was calculated separately.

3.2.12 Calculation of Mycelial Growth inhibition:

The antifungal effect of the different concentrations of different plant extract was calculated in terms of percentage of mycelial growth inhibition (Rao & Shrivastava, 1994)

$$\%\ \text{inhibition of mycelial growth} = \frac{gc - gt}{gc} \times 100$$

Where,

gc= growth of mycelial colony after incubation period in control set.

gt = growth of mycelial colony after incubation period in treatment set.

3.2.14 Determination of Minimum Inhibitory Concentration (MIC) of Plant extract

MIC has been expressed as the minimum dose of the plant extract or essential oil required for complete (100%) inhibition of mycelial growth of the test fungus (Rao & Srivastava, 1994).

The MIC of different plant extract were tested against the test fungus by poisoned food technique employing different doses of extract till the minimum dose of the extract achieved for complete inhibition of mycelial growth of the test fungus.

3.2.15 Biological Control

Biological control was done using *Trichoderma* spp. For the test, three small well of equal distance, each of 5mm diameter were bored in PDA contained in petri plate using cork borer. In each well, conidial stock solution of the biological control agent i.e, *Trichoderma* spp. was poured with the help of dropper until the well were full. Then 5mm inoculum disc was prepared by boring the pure culture of the test fungus with a cork borer and the disc was put at the center of the media upside down. This PDA media with conidial solution of *Trichoderma* spp. was served as treatment set. In the control set, the media was inoculated only with the test fungus but was not treated with the biological control agent. For each control set and treatment set,3 replicates were maintained and all petri plates were incubated in an inverted position at $20^0 \pm 3^0$C for one week.

3.2.16 Conidial Measurement

For the conidial measurement, ocular micrometer was placed inside the eyepiece of a compound microscope and was calibrated by superimposing the graduations of stage micrometer. The number of division of ocular micrometer coinciding with the number of divisions in stage micrometer was noted down and the calibration factor was calculated by the help of the formula below.

$$\text{One ocular division} = \frac{\text{No. of div. on stage micrometer} \times 10\,\mu\text{m}}{\text{No. of div. on ocular micrometer}}$$

Now the stage micrometer was replaced by a fine slide of the test fungus (*S. vesicarium*) mounted in Cotton blue and Lacto-phenol. Size of the conidia was measured by determining the number of division of ocular micrometer. Measurement of different conidia was carried out for 10 times to find out the particular range of the size of conidia. The size of conidia was found as (23-44x14-24) µm.

The table for the measurement of length and breadth of conidia is given in appendix – IV.

3.2.17 Statistical Data Analysis

The correlation analysis was done by using SPSS window program (Version 11.5) to calculate the correlation coefficients of concentration of extracts of medicinal plants and mycelial growth of test fungus (Table: 5).

3.2.18 Photographs

The photographs of infected plant in field, all the test plants, conidia of test fungus and petri plates showing inhibition of mycelial growth at different concentration of different plant extracts were taken.

CHAPTER- 4

4. RESULTS

4.1 Isolation of test pathogen

The test fungus *Stemphylium vesicarium* (Wallr.) Simmons was isolated from the infected leaf of garlic which was collected from Thimi area. The study was conducted from January 2008 to November 2008. The severe attack was occurred by this pathogen on garlic plant during mid Jan and Feb.

The colony of pure culture of test pathogen isolated in PDA media was gray to grayish brown in color and velvety nature. The diameter of colony reached about 60.30mm after 7 days at 23^0C. Conidia solitary, olivaceous brown colour, 2-3 septate, rounded at the apex with no pointed ends. These characteristics were similar with description given by Yu and Cho (2001).

The size of conidia was found 23-44 x 14x 24 µm which is comparatively slightly smaller than size of conidia described by Yu and Cho (2001). The size of conidia of *S. vesicarium* is 25-45 x 15-25 µm (Yu and Cho, 2001).

Table 3 : Measurement of diameter of colony of test fungus after 7 days

S.No.	Diameter of colonies (µm)	Mean diameter(µm)
1.	62 µm	60.30 µm
2.	59 µm	
3.	60 µm	

4.2 Pathogenecity test

The pathogenicity test was carried out by transferring the inoculum of one week old culture of test fungus to healthy leaf of garlic. The symptoms appeared in the inoculated leaf were similar to the symptoms firstly noted from the infected leaf. The first symptoms were appeared in inocutated leaf after 10 days. The small lesions started to increase its size and infected whole leaf within 25 days. Thus, the fungus was found to be pathogenic and able to cause disease.

The pathogen was reisolated from inoculated plants in PDA media. Its characteristics were found to be similar with previous one. This proved that the pathogen in both cases was *Stemphylum vesicarium* (Wallr.) Simmons which causes the leaf blight disease of garlic.

4.3 Antifungal activity of different plant extracts against *Stemphylium vesicarium* (Wallr.) Simmons

Eight medicinal plant extracts were tested for their antifungal activity. The colony size of the test fungus at different concentrations of plant extracts were measured on the 7th day of incubation and the inhibitory effects of the test plants were calculated. The measurement of the diameter of the control set and the treatment sets was done by using the ruler and finally the inhibition percentages were calculated. For each concentration, the diameters of triplicates were measured and the mean is calculated. The inhibition percentages were finally calculated in reference to the colony diameter in control set. The number of concentrations in each experimental set is six (i.e. n=6). All the plant extracts showed their different potential of fungitoxicity. Following are the graphic diagrams for results of the experiment.

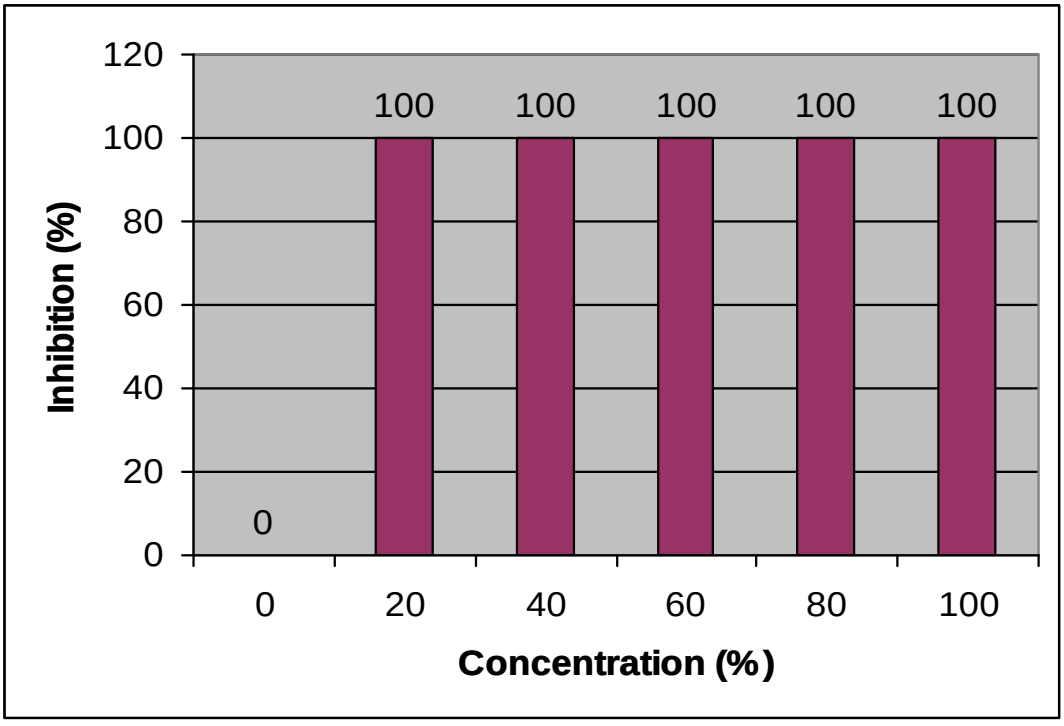

Figure 1: Antifungal activity of plant extract of *Cuscuta reflexa* against *Stemphylium vesicarium*(Wallr.) Simmons.

The extract of *Cuscuta reflexa* was found to be most effective one in controlling the test fungus.

The percentage of mycelia growth inhibition by its extract was found to be 100% even at low concentration of 20%.

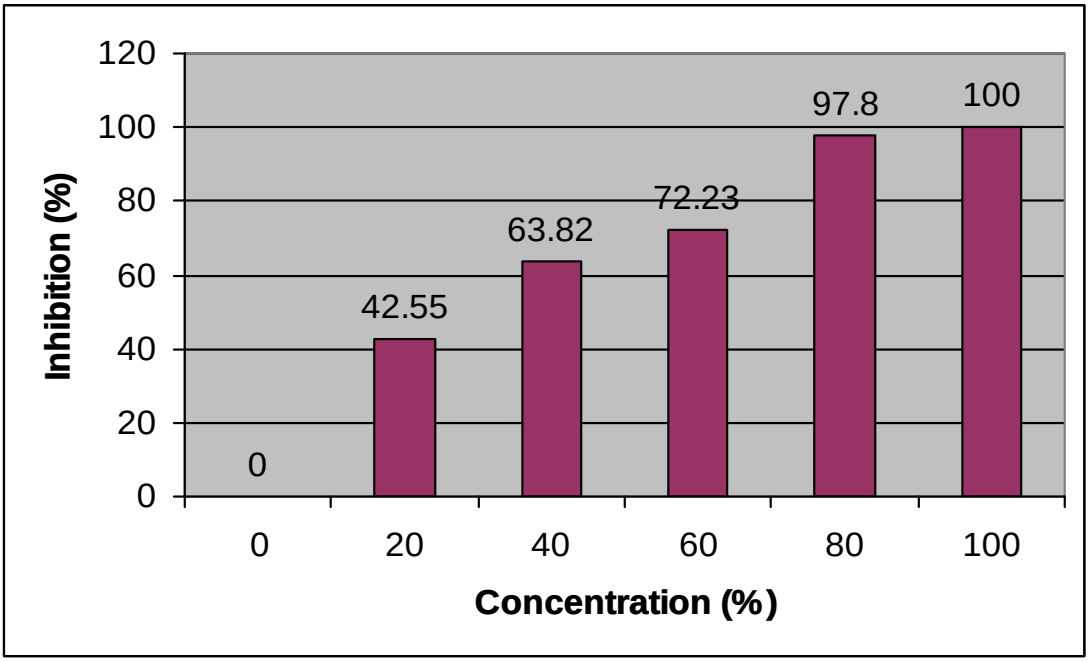

Figure 2: Antifungal activity of plant extract of *Syzygium aromaticum* against *Stemphylium vesicarium*(Wallr.) Simmons.

The above diagram shows the significant increase of inhibition of mycelia growth with increase of concentration.

The percentages of mycelial growth inhibition by the extract of *Syzygium aromaticum* were found to be 42.55%, 63.82%, 72.23%, 97.80% and 100% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

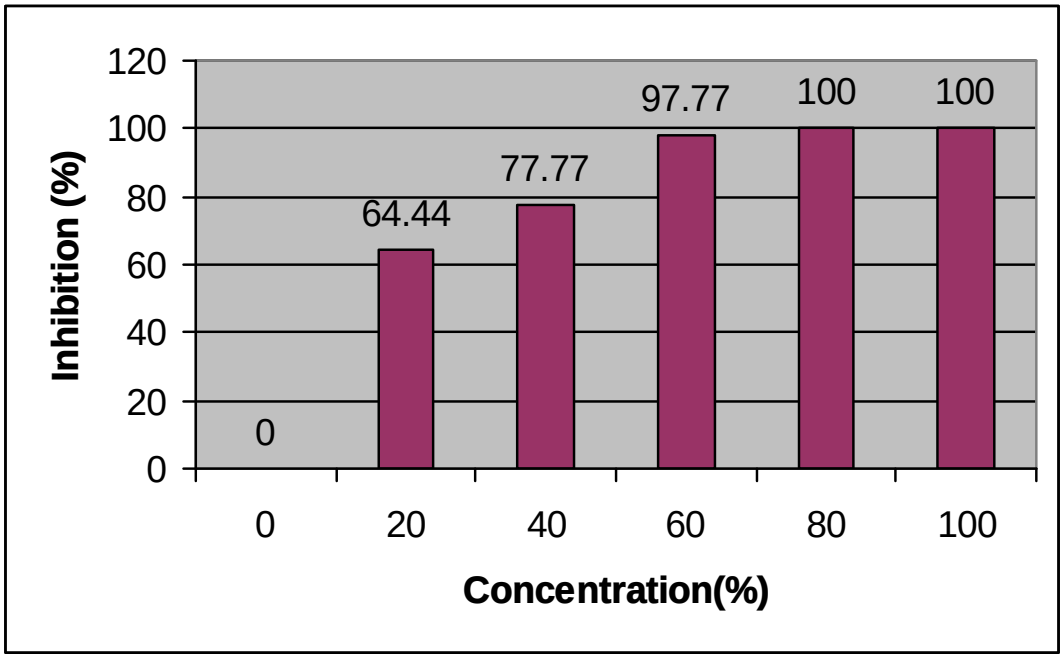

Figure 3: Antifungal activity of plant extract of *Allium cepa* against *Stemphylium vesicarium*(Wallr.) Simmons

The extract of Allium cepa also effective to inhibit mycelial growth of test fungus. The extract controlled the mycelial growth by 100% at the concentration of 80%.

The percentage of mycelial growth inhibition by the extract of *Allium cepa* were found to be 64.44%, 77.77%, 97.77%, 100% and 100% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

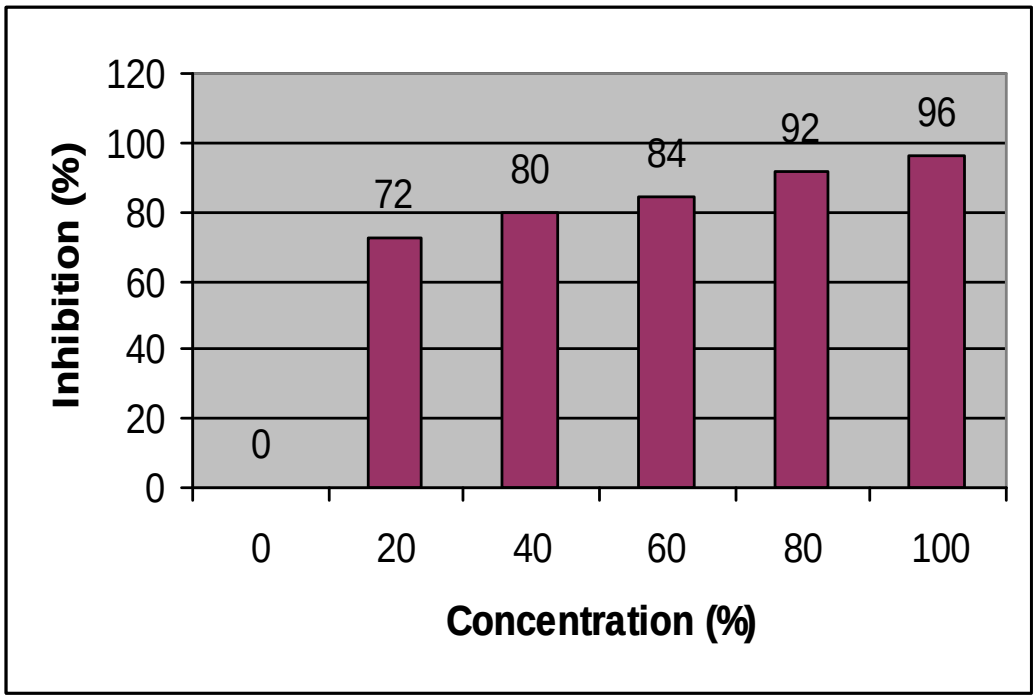

Figure 4: Antifungal activity of plant extract of *Cinnamomum zeylanicum* against *Stemphylium vesicarium*(Wallr.) Simmons.

The percentage of mycelial growth inhibition by the extract of *Cinnamomom zeylanium* were found to be 72.0%,80.0%, 84.0%, 92.0% and 96.0% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

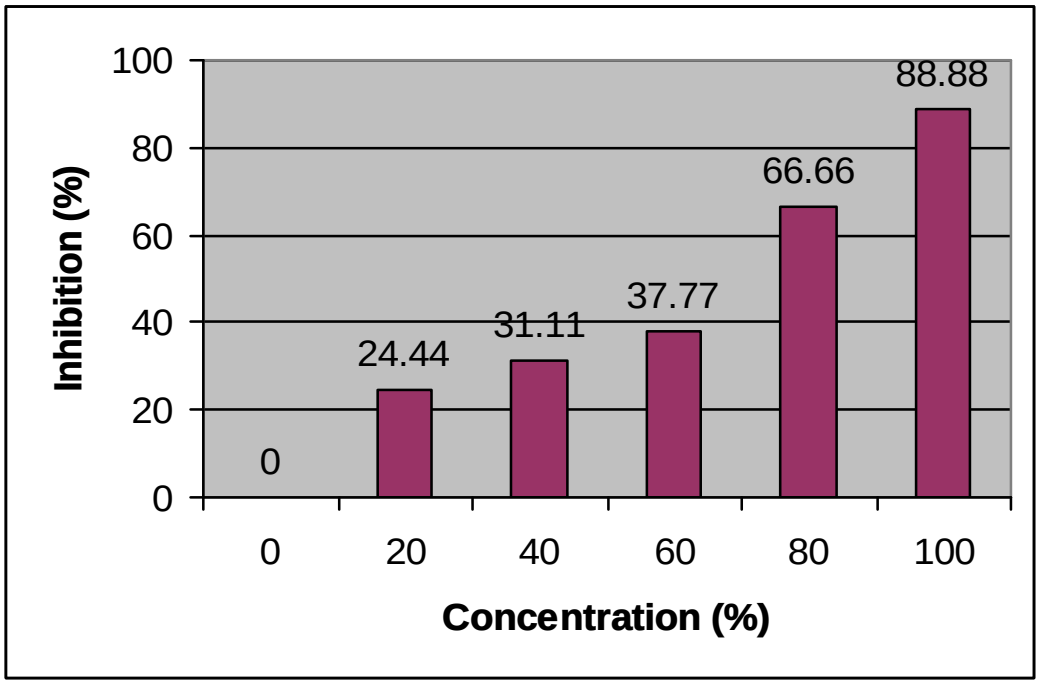

Figure 5: Antifungal activity of plant extract of *Solanum xanthocarpum* against *Stemphylium vesicarium*(Wallr.) Simmons.

The percentage of mycelial growth inhibition by the extract of *Solanum xanthocarpum* were found to be 24.44%, 31.11%, 37.77%, 66.66% and 88.86% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

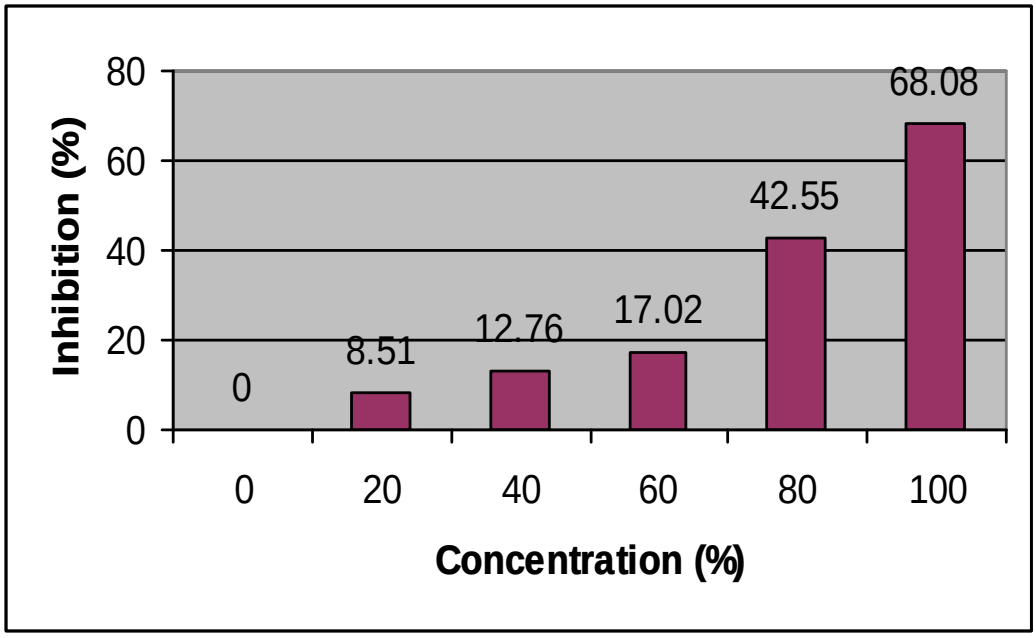

Figure 6: Antifungal activity of plant extract of *Phyllanthus emblica* against *Stemphylium vesicarium*(Wallr.) Simmons

The percentage of mycelial growth inhibition by the extract of *Phyllanthus emblica* were found to be 8.51%, 12.76%, 17.02%, 42.55% and 68.08% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

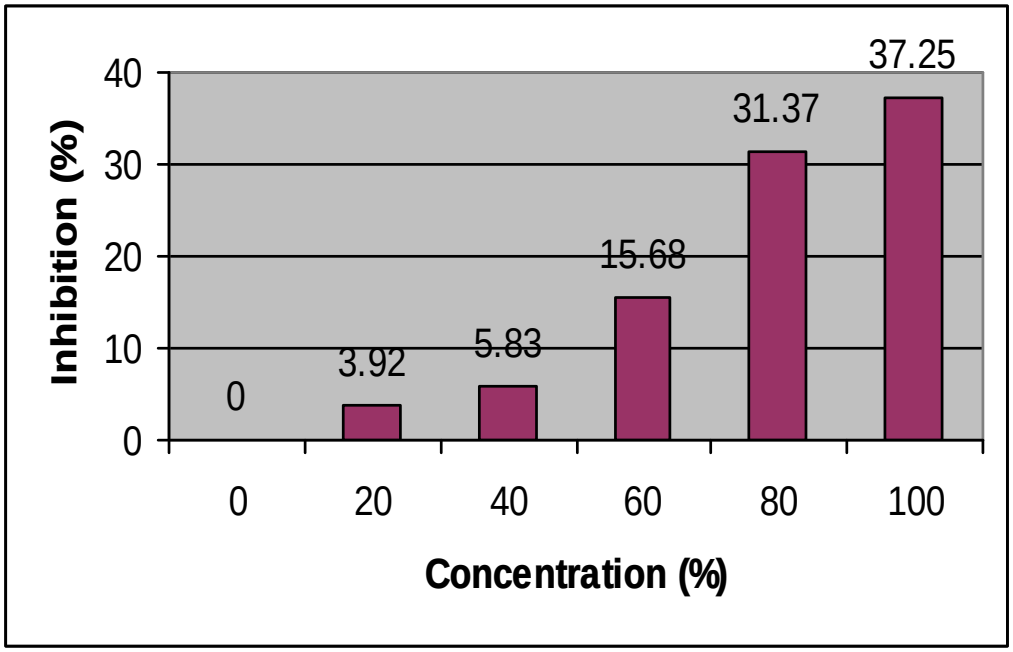

Figure 7: Antifungal activity of plant extract of *Cinnamomum camphora* against *Stemphylium vesicarium*(Wallr.) Simmons.

The percentage of mycelial growth inhibition by the extract of *Cinnamomum camphora* were found to be 3.92%, 8.83%, 15.68%, 31.37% and 37.25% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

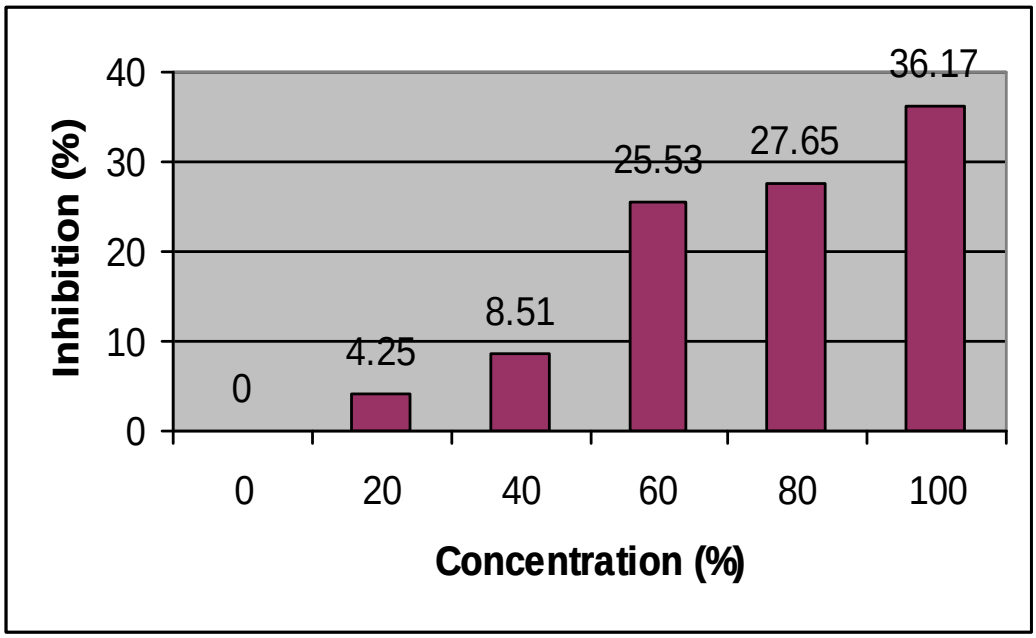

Figure 8: Antifungal activity of plant extract of *Equisetum diffusum* against *Stemphylium vesicarium*(Wallr.) Simmons.

The percentage of mycelial growth inhibition by the extract of *Equisetum diffusum* were found to be 4.25%, 8.51%, 25.53%, 27.65% and 36.17% at 20%, 40%, 60%, 80% and 100% concentrations respectively.

4.4 Minimum inhibitory concentration (MIC) of Plant extracts

MIC of plant extract can be expressed as the minimum concentration of the extract required for complete (100%) inhibition of mycelial growth of the test fungus (Rao & Srivastava, 1994).

MIC of plant extracts were obtained by poisoned food technique employing different concentrations of extract till the minimum concentration.

Table 4: Minimum inhibitory concentration (MIC) of Plant Extract

S.No.	Plant Extract	MIC (%)
1	*Cuscuta reflexa*	20
2	*Allium cepa*	80
3	*Syzygium aromaticum*	100

The above Table 4. shows that the extract of *Cuscuta reflexa* was found to have most lowest value of MIC. The minimum concentration of the extract of this plant required for complete inhibition of mycelial growth of test fungus was found to be 20%.

Similarly, the MIC of *Allium cepa* and *Syzygium aromaticum* was found to be 80% and 100% respectively.

4.5 Efficacy of *Trichoderma* spp. to control the test fungus

Isolates of *Trichoderma* spp. tested against *Stemphylium vesicarium* (Wallr.) Simmons by agar interaction tests and measuring the toxicity of culture filtrates. For this, 3 control sets and 3 treatment sets were incubated for 7 days at $20^0C \pm 3^0C$. The isolates of *Trichoderma* spp. are able to overgrow and completely inhibit the growth of test fungus. In control sets, there was only presence of inoculum of test fungus. The measurement of mycelial growth was found to be 67µm in control set while in treatment sets, the fast growth of *Trichoderma* spp totally checked the mycelial growth of test fungus showing its high efficacy to conrol the test fungus.

4.6 Statistical data analysis:

The statistical data analysis was done to find out the correlation between the extract of medicinal plants and the mycelial growth of test fungus.

Table 5: Correlation between different concentration of plant extracts and mycelial growth

S.No.	Test plants	Correlation Coefficient(r)	P- value
1.	*Syzygium aromaticum*	-0.95**	P < 0.001
2.	*Allium cepa*	-0.89**	P < 0.001
3.	*Cinnamomum zeylanicum*	-0.86**	P < 0.001
4.	*Solanum xanthocarpum*	-0.93**	P < 0.001
5.	*Phyllanthus emblica*	-0.91**	P < 0.001
6.	*Cinnamomum camphor*	-0.91**	P < 0.001
7.	*Equisetum diffusum*	-0.93**	P < 0.001

The correlation analysis revealed the negative effects of the plant extracts on the mycelial growth of the test fungus. All the correlation coefficients for those plant extracts against colony diameter of mycelial growth of test fungus were found highly significant with negative correlation coefficient ($p<0.001$). The negative correlation coefficient indicates that mycelial growth of test fungus is decreased with the increase of concentration of extracts of test plants.

CHAPTER- 5

5. DISCUSSION

In the present dissertation, an attempt was made to study about leaf blight disease of garlic caused by *Stemphylium vesicarium* (Wallr.)Simmons, it's pathogenicity test and to observe its control measures by using extracts of different medicinal plants and biological control by using *Trichoderma* spp.

During the survey of diseased plants in Bhaktapur area on the month of mid January, garlic plants were infected in most of the field. The leaf parts were severely damaged by the disease. According to farmers, severe attack started from February and last till late spring. According to them the size of cloves of diseased garlic plant also smaller than normal plants. Because of mostly leafy parts are affected by this disease, most of farmers, who depended on vegetables for their income source were economically affected.

The isolated pathogen was identified with the help of Standard literature (Yu and Cho,2001) and under guidance of Professors and teachers of Central Department of Botany and NARC. The isolated pathogen showed similar characters described by Yu and Cho, 2001. However the size of conidia of the pathogen was found slightly smaller than size of conidia given by Yu and Cho, 2001. The reason of this may be due to variable places and climates.

The pathogenicity test was conducted as Koch's postulates. This test is one of basic method for confirmation of the test pathogen to cause the leaf blight in garlic. The symptoms appeared on inoculated leaf were similar as the symptoms those observed originally in the garlic leaf. The characterization of the test pathogen was confirmed by reisolation of the pathogen in PDA media.

Application of chemical fungicide is a conventional method to control the disease caused by fungal pathogens. Tremendous health hazards are reported to occur during the application of fungicides in field conditions (Alam *et al*., 1999). The present study

was carried out for the protection of environment and save the health hazards of the animal kingdom.

Among the test plants, the extracts of *Cuscuta reflexa*, *Syzygium aromaticum* and *Allium sativum* were able to inhibit the growth of the mycelium of the test fungus completely. Among these, the extract of *Cuscuta reflexa* was found to be most effective one as it inhibited the growth of mycelium by 100% even at low concentration of 20%. The inhibition percentage of mycelium growth of the test fungus by extract of *Cinnamomum zeylanicum* and *Solanum xanthocarpum* were found to be more than 80%. The extract of *Phyllanthus emblica, Cinnamomum camphora* and *Equisetum diffusum* were found to be less effective to control the disease.

In this experiment, *Cuscuta reflexa* was found as most effective in controlling the mycelia growth of the pathogen as the percentage of mycelia growth inhibition by its extract were found to be 100% even at low concentration of 20%.

In this experiment, the extract of *Cuscuta reflexa* was the most effective in controlling the test fungus. The reason behind its high degree of inhibitory effect on the mycelial growth of the test fungus might be due to the presence of higher concentration of active inhibitory chemicals on the plant extract that could sufficiently supress the physiological development of the fungus. The similar finding by Anjum *et al.* (2003) who tested the antibacterial and antifungal activity from the crude extract of *C. reflexa* towards Gram negative (*Pseudomonas aereginosa* and *Escherichia coli*) and Gram positive (*Bacillus subtilis* and *B. licheniformis*) and fungi (*Aspergillus niger*) and found high degree of inhibitory action of the plant over the fungus also supports the present finding of this fungus.

Similarly the extract of *Syzygium aromaticum* was also found to be effective against the growth of test fungus. The result of the present study is found to be corresponding to the work of Cavaleiro *et al.* (2009). They had analyzed the composition and the antifungal activity of clove essential oil, obtained from *S. aromaticum* and showed high contents of eugenol (85.3%) that inhibited the growth of mycelium of all the tested fungus, *Candida and Aspergillus*. Therefore, the presence of active inhibitory

chemical eugenol, present in *S. aromaticum* might be the cause of this fruitful finding in this dissertation.

Another best extract out of eight medicinal plants that shows inhibition of mycelium growth by 100% is extract of *Allium cepa*. The similar finding by Amirrajab *et al.* (2006) who tested the extract of *Allium cepa* against *Malassezia furfur* (25 strains), *Candida albicans* (18 strains), other *Candida* sp.(12 strains) as well as 35 strains of various dermatophyte species and found to be able to inhibit growth of all tested fungi. This result indicates that *Allium cepa* might be promising in treatment of fungal associated disease.

The extract of *Solanum xanthocarpum* exhibited preferential growth inhibitory activity against *Stemphylium vesicarium* by 88.88%. This effectiveness of *S. xanthocarpum* matches with the investigation of Devi *et. al* (2007), who isolated chemical component of carpesterol and steroidal glycosides from extract of fruit of *S. xanthocarpum* and tested against *Aspergillus niger* and *Trichoderma viride*. They found inhibitory effect on the growth of test fungi. Therefore presence of chemical components, carpesterol and steroidal on the extract of *S. xanthocarpum* might be cause of inhibition of mycelial growth of *S. vesicarium.*

In this study, the extracts of *Phyllanthus emblica*, *Cinnamomum camphora* and *Equisetum diffusum* were found to be less effective in control of the test fungus. One of the reason for different fungitoxicity of various plant extract may be due to their different chemical composition (Rao and Srivastava, 1994). The composition of plant oils and extracts is known to vary according to local climate and environmental conditions (Janseen *et al*., 1987; Sivropoulou *et al*., 1995). However, the result obtained in controlling plant diseases by using different plant extracts and essential oil may differ as many factors vary between assays. These may include difference in microbial growth, exposure of micro-organism to plant oil or extract, the solubility of oil or oil components and the use and quantity of an emulsifier (Reynolds, 1996). Besides, some sort of experimental difficulties, time of incubation, incubation temperature etc. also could play a role in the mycelia growth of the test fungus.

Besides different medicinal plant extracts, in this dissertation, *Trichoderma* spp. was tested as biological control agent to inhibit the growth of test fungus. For this, the suspension of *Trichoderma* spp. and the test fungus were plated in petri dishes containing PDA. The mycelial growth of test fungus was totally inhibited due to overgrowth of *Trichoderma* sp. The isolate of *Trichoderma* spp. grew considerably faster on PDA than did the pathogens, in the same conditions of culture (Cigdem and Merih, 2004). Therefore their rapid colonization of substrates helps in removing pathogen and the numerous mechanisms that *Trichoderma* spp. have evolved in attacking other fungi and enhancing plant and root growth (Ozbay and Newman, 2004). Trichoderma spp. exhibited 100% growth inhibition of the pathogen under *in-vitro* condition. Chakraborty and Chatterjee (2008), found that *Trichoderma* spp. produced fungal cell wall degrading enzymes, chitinase and β-1,3- glucanase which were main cause of inhibitory effect of the test fungus. Therefore this result signifies that the fungus *Trichoderma* spp. is one of the potent agent for the biocontrol of plant pathogen.

CHAPTER 6

6. CONCLUSION

It can be concluded from the study that leaf blight disease of garlic is caused by the fungus *Stemphylium vesicarium* (Wallr.) Simmon. This was confirmed by isolation of pathogen, conidial measurement and pathogenicity test.

Furthermore the study was conducted to test the fungitoxicity of the extracts of medicinal plants at different concentration against the test fungus. The most of the extracts of the test plants were effective in the diseased control. Among them, the most effective plant species is *Cuscuta reflexa* showed highest fungitoxicity of 100% even at low concentration of 20%. Besides this, the extract of *Syzygium aromaticum* and *Allium cepa* completely inhibited the mycelium growth at 100% concentration. Fungitoxicities of the remaining plant extracts *Cinnamomum zeylanium, Solanum xanthocarpum, Phyllanthus emblica, Cinnamomom camphora and Equisetum diffusum* was 96.0%, 88.86%, 68.08%, 37.25% and 36.17% at 100% concentration. Thus, the extract of *C. reflexa, S. aromaticum* and *A. cepa* were found the best plant extracts for controlling the disease of 'Leaf blight of garlic' caused by *Stemphylium vesicarium* (Wallr.) Simmons.

The present study is useful to overcome the harmful effects caused by chemicals used by farmers. The plant extracts are less toxic that can help to raise the economical condition of farmers as well as sound environmental condition.

The interaction between *Trichoderma* spp. and the test fungus in PDA media has concluded that *Trichoderma* spp. grows faster than *S. vesicarium* . It was found that *Trichoderma* spp. rapidly colonize the substrate, thus suppressing the growth of test fungus. Therefore *Trichoderma* spp. is found to be efficient biocontrol agent to inhibit the growth of test fungus, *S. vesicarium*.

CHAPTER - 7

7. RECOMMENDATIONS

- This research work has been strictly done under controlled environment (*in-vitro*) so the result found might not correspond with those when performed in commercial field under the influence of different physical factors. Hence a rigorous study in the commercial field is recommended.
- The extract of *Cuscuta reflexa* might be most useful for practical application in controlling plant diseases.
- *Trichoderma* spp. can be commercially produced to prevent development of several soil pathogenic fungi.

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APPENDIX – I
Materials Used for the Study

Plant samples

- *Phyllanthus emblica* L.
- *Syzygium aromaticum* L.
- *Cinnamomum camphora* L.
- *Solanum xanthocarpum* Schrad and Wendl
- *Cuscuta reflexa* Roxb.
- *Allium cepa* L.
- *Equisetum diffusum* D.Don
- *Cinnamomom zynlanicum* B.

Test fungus

- *Stemphylium vesicarium* Wallr.

Apparatus and Equipments

- Conical flask
- Test tube
- Pipette
- Stand
- Measuring cylinder
- Glass slide
- Petridishes
- Inoculating loop
- Forceps
- Borers
- Cotton swabs
- Cover slips

- Mortar and Pistil
- Autoclave
- Hot air oven
- Incubator
- Laminar air flow chamber
- Microscope
- Balance
- Beaker
- Heater

Chemical and Reagent

- Sprit
- Distilled water
- Sodium hypochlorite (2%)
- Ethanol
- Cotton blue
- Lacto phenol

Appendix- II

Nutritional content of garlic

Contents	**Nutritional value (per 100 g of raw garlic)**
Carbohydrates	33.06g
Sugars	1.00g

Dietary fibers	2.1g
Fat	0.5g
Protein	6.39g
β carotene	5mg
Thiamin(Vit.B1)	0.2mg
Riboflavin (Vit.B2)	0.11mg
Pantothenic (Vit.B5)	0.596mg
Vitamin B6	1.235mg
Folate (Vit.B9)	3mg
Vitamin C	31.2mg
Calcium	181mg
Iron	1.6mg
Magnesium	25mg
Phosphorus	153mg
Potassium	401mg
Sodium	17mg
Zinc	1.16mg
Manganese	1.672mg
Selenium	14.2 mcg

Source: USDA Nutrient Date Base

APPENDIX – III

Top Ten Garlic Producers-11 June 2008

Country	**Production (Tonnes)**	**Footnote**
People's Republic of China	12,088,000	F
India	645,000	F
South Korea	325,000	F
Russia	254,000	F

United States	221,810	
Egypt	168,000	F
Spain	142,400	
Argentina	140,000	F
Myanmar	128,000	F
Ukraine	125,000	F
World	15,686,310	A
No symbol= official figure, P= official figure, F=FAO estimate, * = unofficial/semiofficial/mirror data C = calculated figure, A= aggregate (may include official, semiofficial or estimates)		

Source: Food and Agricultural Organization of United Nations: Economic and Social Department: The Statistical Division.

APPENDIX – IV
Micrometry

In micrometer, 100 division = 1000µm
1 division = 1000/100 µm = 10µm
41 division of ocular micrometer coincide with 20 division of stage micrometer.
1 division of ocular micrometer coincide with 20/41 division of stage micrometer

=20/57 x 10µm = 4.87µm

Table 1 : Measurement of length and breadth of conidia.

S.N.	Length		Breadth	
	No. of ocular divisions Occupied by Conidia (a)	Size of conidia= a × calibration factor for one ocular division i.e. 4.87 µm	No. of ocular divisions Occupied by Conidia (b)	Size of conidia= b × calibration factor for one ocular division i.e. 4.87 µm
1.	5.9	5.9x4.87=28.73	3.0	3.0x4.87=14.61
2.	5.0	5.0x4.87=24.35	3.8	3.8x4.87=18.50

3.	5.0	5 0x4.87=24.35	3.3	3.3x4.87=16.07
4.	4.8	4.8x4.87=23.37	2.9	2.9x4.87=14.12
5.	5.1	5.1x4.87=24.83	4.7	4.7x4.87=22.88
6.	4.8	4.8x4.87=23.37	3.0	3.0x4.87=14.61
7.	7.0	7.0x4.87=34.09	5.0	5.0x4.87=24.35
8.	8.2	8.2x4.87=39.93	4.5	4.5x4.87=21.91
9.	6.0	6.0x4.87=29.22	3.9	3.9x4.87=16.99
10.	9.1	9.1x4.87=44.31	3.0	3.0x4.87=14.61

Thus, the size of measured conidia is (23-44x14-24) µm