CHAPTER ONE
INTRODUCTION

1.1 Background

Nepal a Himalayan kingdom, lies at 80^o 4' E to 88^o 12' E and 26^o 22' N to 30^o 27' N. It has an area of 1,47,181. Sq. km. Despite of its small size, it has diverse forms of altitudinal variations less than 100m in south to 8,848 m in the North. The great varieties of habitat provide the great amount of biodiversity.

About 5,160 species of higher plants have been enumerated by Hara & Williams (1978, 1979) and 5,833 by Koba *et al.* (1994). Among these plant species, medicinal and aromatic plants (MAPs) are the most popular and rich resource of the country. HMGN (1970, 1984) accounted 571 species of MAPs in Nepal. Malla and Shakya (1999) compiled a list of 630 species of MAP from Nepal. Edwards, D.M. (1996) analyzed the current trade status of non-timber forest products (NTFPs) especially the medicinal and aromatic plants. There are about 700 plant species in Nepal that are known to possess medicinal properties (Shrestha, T.B.; 1999). DPR (2001) enumerated the total number of flowering plants to be 6,501 (5,636 species, 206 subspecies, 599 varieties, and 60 forma). It is believed that about 15 percentages (i.e. about 1600 species) of plant species are medicinally used in Nepal (Shrestha K.K. *et al*., 2000).

Most of the aromatic plants possess medicinal properties such as antibacterial, antifungal, antiviral, anthelmintic, anticancer, sedative, laxative, cardiotonic, diuretic, and others. (Parajuli D.P. et al., 1998).

Plants of ethno botanic importance are exploited for their inherited potential by many ethnic groups of Nepal. Oils found in plants are classified as either volatile or fixed oil. Volatile oil contains the mixture of hydrocarbons containing 10 – 15 carbon atoms while fixed oils contain ester of glycerol and long chain aliphatic acids (Manandhar M.D., 2004).

Plant parts, their constituents like essential oils; extracts have been found to be less phytotoxic, more systemic (Fawcett & Spencer 1970) easily biodegradable (Beye,

1978) and as host metabolism stimulatory pesticides (Tripathi R.D. et al, 1980). The volatile fraction from the fresh leaves can be isolated in the form of essential oils through hydrodistillation by Clevenger's apparatus (Clevenger J.F., 1928).

The medicinal property of a plant is due to the presence of active components or secondary metabolites. The main groups of active components are alkaloids, glycosides, saponins, essential oils, mucilage, tannins, bitter principles etc. (Kruger, 1992).

The use of chemical pesticides only is not very effective in crop protection.. Excessive use of pesticides causes severe environmental problem. Foods produced by such farming methods are extremely toxic for people. Latvian ecologists estimate that excessive pesticides use currently results in 14,000 deaths per year in the territories of former Soviet Union. (Enger, E. D & Smith B. F, 2004). Crop protection is necessary for the food safety. Pathogens, pests and weeds are three natural enemies of our agriculture and forestry. Lower as well as higher organisms have been studied for use as biocides to protect plants from these enemies.

Angiospermic plants are reservoir of the chemotherapeutants and provide inexhaustible sources of useful pesticides. (Swaminathan, M.S.1978). Various plant-based oils are capable of controlling several plant diseases. Oils obtained from seeds of several plants such as sunflower, olive, corn and soybeans give excellent control of some plant diseases. (Mehrotra R.S. et al, 2003). Essential oils are even more effective and have more antimicrobial effect. They sometime serve as antiseptic and stimulants, as ingredient in medicines, as a lab reagent, as solvents in the paint industry, as insecticides and as a component of plastics, polishes, pastes, ink, glue and the like (Schery, R.W. 1972).

Essential oils usually occur as mixtures of many oils, evaporate in the contact with air and possess a pleasant taste and strong aromatic odor. They can readily be removed from plant tissues without any change in composition. They are comparatively smaller molecules than other oils ordinarily less than 20 carbon atoms long. Up on crude separation the essence in commonly fixed with smaller quantities of various acids, bases, phenols, ketones, aldehydes and the like, the presence of which may affect the delicate nuance of fragrance so desired in certain oils. Frequently they are considered

hydrolysis product of complex glycosides. They occur in some 60 families and are particularly characteristics of Lamiaceae, Myrtaceae, Composite, Lauraceae and Umbeliferae (Schery, R.W.; 1972). There are more than 20 named species, many hybrids and cultivars of strawberry. There are 7 basic types of chromosomes that they all have in common. Some species are diploid, tetraploid, hexaploid, octoploid or decaploid.

Many diseases attack the fruit, leaves, roots and stem of strawberry. Some of the notable diseases and their pathogens are illustrated below.

Disease caused by virus: Strawberry Crinkle [Strawberry Crinkle Virus (SCV)], Strawberry mild yellow-edge (Strawberry Mild Yellow-edge Virus (SMPEV), Strawberry mottle [Strawberry Mottle Virus (SMV)], Strawberry Vein Banding [Strawberry Vein Banding Virus (SVBV)] (Caulimovirus) etc.

Disease caused by fungi: Anthracnose (*Colletotrichum acutatum*), Downy mildew (*Peronospora potentillae*), Fruit rots (*Cladosporium* sps, *Mucor* sps.), hard brown rot (*Rhizoctonia solani*), Leather rot (*Phytophthora cactorum*). (Fall, J.E., 1951) etc.

Gray mold fruit rot of strawberry is caused by *Botrytis cinerea*. It also attacks the young stem, flower and buds. It attacks the fruit even inside the cold storage. The fungus produces a velvety gray growth on the fruit surface. Abundant powdery dry spores (conidia) are borne on spore-bearing structures in this growth. In high humidity, the surface growth may be cottony and white. (Maas, J.L.;1981).

Since strawberry is eaten full and have no fruit covering peel. The pesticide sprayed over it is directly consumed. So the control of insects and pests by use of essential oils has very great significance.

1.2 Objectives of the Study

The study was carried out with the following objectives.

- To isolate the pathogen from infected strawberry fruit and test it's pathogenicity.

- To test the fungitoxicity of essential oils of test aromatic plants against the test fungus.
- To evaluate comparative efficacies of essential oils of test plant species.
- To calculate the minimum inhibitory concentration (MIC) of the test plant oils.

1.3 Justification of the Study

Strawberry (*Fragaria vesca L)* is one of the important fruit crops. It is rich in vitamins and minerals. Due to it's excellent color and flavor, it is used as food additive. (Sauer, J.D., 1993). Gray mold fruit rot caused by *Botrytis cinerea* is one of the serious diseases of strawberry. It also attacks the fruit in cold storage (Maas, J. L. 1981). Strawberry fruit is eaten whole, so harmful pesticides use may be hazardous for the health of consumer. Essential oils are safe for health, less phytotoxic, more systemic, easily biodegradable and environmentally non-polluting (Parajuli R.R. *et al*., 2005). Control of gray mold fruit rot by essential oil treatment in vitro has not been done yet in Nepal.

1.4 Limitation of the Study

- Only one test fungus was taken.
- Selected plants were used for extraction.
- TLC and GC could not be carried out for screening essential oils.

CHAPTER TWO
LITERATURE REVIEW

2.1 Fungi and Fungitoxicities

Botrytis cinerea pers. Ex. Fr. is the causal agent of gray mold fruit rot of strawberry. (Maas J.L., 1981). *Botrytis cinerea* also attacks the bunch of flowers and fruits of grape *(Vitis vinifera)* and causes gray mold fruit rot. The young plantlets in the nursery are often attacked by *Botrytis cinerea.* A bioorganic substance **'chitosan'** improves development and protects the plantlets of grape (*Vitis vinifera)* against *Botrytis cinerea.* (Barka E. *et al.* 2004*).* Twenty-five plants were screened for antifungal activity and from the study new potent fungitoxic compounds were obtained. Out of them, *Cestrum diurnum* exhibited strong fungitoxic activity against *Botrytis cinerea, Alternaria solani* and other fungal species. (Renu K.M. *et al*., 1980).

Distribution of fungitoxicity was studied at five different growth stages of *Iberis amara* against *Helminthosporium oryzae* by spore germination, poisoned food and modified paper techniques. (Pandey D.K. *et al*. 1983).

Screening of 20 plant species was carried out for their volatile toxicity against *Helminthosporium oryzae.* (Chandra et al. 1982).

Leaves of 25 plant species were screened for their volatile toxicity against the test pathogen *Fusarium lateritium* f. sp. *cajani.* Out of the test plant species *Aegle marmelos, Citrus aurantifolia* and *Mentha arvensis var piperascons* exhibited strong toxicity inhibiting the mycelial growth completely. The volatile constituents from each plant were isolated in the form of essential oil and the fungitoxicity of each oil was tested separately. (Pandey D.K. et al. 1983).

The volatile antifungal action of the leaves of *Melaleuca leucodendeon* was collected as an essential oil by hydrodistillation in Clevenger's apparatus and evaluated for its activity against *Rizoctonia solani* (Dubey et al., 1983).

Distribution of fungitoxicity was studied at five different growth stages of *Iberis amara* against *Helminthosporium oryzae* by spore germination, poisoned food and modified paper techniques (Tripathi N.N. *et al*. 1983).

Essential oil extracted from the leaves of *Aegle marmelos* inhibited the mycelial growth of the test fungus *Rhizoctonia solani* completely at its minimum inhibitory concentration of 3000 ppm. (Renu et al, 1980).

Leaf extracts of 30 species were tested against *Aspergillus flavus,* only the leaf extract of *Anisomeles ovata* showed absolute toxicity. (Upadhaya *et al.*, 1987).

Melia azdirachata, Hydrocotyl asiatica, Allium sativum and *Artemisia indica* were investigated for their antimicrobial activity against some filamentous and non-filamentous fungi and bacteria (Adhikari S., 1988).

Antimicrobial activities of some plant products viz. *Mentha arvensis, Acrus calamus, Zanthoxylum oxyphyllum* and turpentine oil were studied against some fungi and bacteria (Shrestha R. & Sharma A.P., 1988).

The yield of essential oil of *Eupatorium triplinerve* by hydrodistillation was found to be 6 percent. The essential oil showed antifungal & antibacterial activity. (Yadava & Saini, 1990).

The essential oil from *Lipia sioides* exhibited the highest and broad activity against fungi and bacteria among ten essential oils tested. It was active against all the test fungi, including yeast, a dermatophyte and filamentous fungi. (Lemos et al, 1990).

The efficacies of 16 plant extracts were tested against the pathogen of five major rice diseases namely Sheath blight, Blast, Bakane and leaf scald. Out of these test species, *Sapium indicum, Tagetes erecta, Polyalthia longifolia, Leucaena leucocephala* exhibited more than 50 percent antifungal activity (Miah et al., 1990).

In a preliminary microbiological screening, the essential oils of five Sardinian plant viz. *Juniperus oxycedrus* Subsp. *oxycedrus, Spartium junceum, Helichysum italicum sub sp. microphyllum, Inula viscosa* and *Asphodelus microcarpus* showed activity against some species of blastomycetes. (Bonsignore et al. 1990).

Eucalyptus oil exhibited absolute mycotoxicity against *Microsporium gypseum* and *Trichophyton mentagrophytes* at its minimum inhibitory concentration of 400 ppm against *Pyricularia oryzae, Aspergillus niger.* Plants tested showed toxic principles against one or other tested pathogens (Mishra D.N. *et al.*1990).

Essential oils from fresh leaves of nine plants were tested against the test fungus. Essential oil from *Amomum subulatum* exhibited complete inhibition of the fungus at 3000 ppm. Other plants as *Ageratum naustonianum, Alpinia galangal, Artemisia indica, Curcuma longa, Elettaria cardanomomm, Lippia alba* and *Salvia pleneiadamomum* were also tested (Mishra M. & Tiwari S.N., 1990).

The essential oils from the leaves of Aegle marmelos, Blumea lacianata, Blumea mollis, Callistemon lanceolatus, Callistemon polandi, Daucus carota var Sativa, Ocimum gratissimum var. colocimum, Pogostemon bengalensis, Syzygium cuminii and fruitrind oil of Cinnamomum cecidoda pune were tested against some plant and human pathogens (Khanna et al., 1991).

Antifungal activities of 20 medicinal plants were tested against four pathogenic fungi namely *Curvularia lunata, Cylindrocarpon sps, Fusarium solani* and *Myrothecium leucotrichum.* Extracts of all the test plant species were fungitoxic and showed inhibitory effect on the test fungi (Gourinath & Manoharacharya, 1991).

On screening 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 the test fungi inhibiting the mycelial growth completely (Khan & Tripathi, 1994).

Two hundred and nineteen species of aromatic and medicinal plants belonging to sixty plant families were collected from different part of the country. The essential oil content of these plants was determined by hydrodistillation method. Chemical constituents of some of the oils were determined by GC (Shrestha P.M. et al., 1994).

In vitro screening of the selected medicinal plants of Nepal was studied for antimicrobial activities. Extracts of twenty-one species of plants showed activity

against at least two fungal species and three were active against gram-negative bacteria. (Tylor et al. 1995).

Aqueous extract effects of 64 weed species on growth and development of *Alternaria solani, Helminthosporium sativum* and *Rhizoctonia solani* were studied in vitro. Extracts varied in the strength and persistence of their antifungal effects against the three fungal species. (Qusem & Abu-blan, 1996).

Antifungal properties of *Cinnamomum* were studied using well agar diffusion method and filter paper disc diffusion method. (Meharotra et al. 1997). Antimicrobial properties of *Punica granatum* were studied against 13 test organisms (Bhatt, 1998).

Ecological study of *Nardostachys grandifolra* was conducted in three different sites and essential oils were collected by hydrodistillation method. Essential oil content was varied from 0.4 to 1.66% depending up on the geographical location and habitat (Chhetri, 1999).

The study reported that the experimental data from the recovery and the composition of the extract under superficial fluid extraction from *Juniperus communis L.* and their comparisons with those of the essential oils was obtained hydrodistillation. Significant differences were recorded between the supercritical carbon dioxide extract and the distilled oil. The distilled oil was found to be more enriched in monoterpenoid hydrocarbons (Chatopoulou et al. 2002).

The essential oil and several pure sulfur compounds isolated from *Scorodopholeus zenkeri* were tested for antifungal activity using a paper disc method, the poisoned food techniques, a micro atmosphere technique and the measurement of cellular ATP present. The essential oil completely inhibited the growth of all fungi tested including yeasts except *Aspergillus flavus,* was active against the gram-positive bacteria studied but not the gram-negative bacteria. (Koukam et al., 2002).

Antifungal activity of the steam distilled essential oil fraction of *Artemisia douglasiana* was detected by bioautography on silica gel TLC plates against three *Colletotrichum sps.* The yield of essential oil from *Artemisia douglasiana* was about 0.6-0.8% by weight of the dry material including plant stems (Meepagala et al. 2003).

A commonly available lemon plant leaves extract in aqueous or ethanolic extract preparation and essential oil displayed fungitoxic effect against effect against three destructive fungal pathogens viz. *Pyricularia grisea, Aspergilus niger* and *Aspergillus flavus.* (Tiwari et al., 2004).

Fungitoxicity of the essential oils of *Xanthoxylum armatum, Nardostachys grandiflora, Juniperus recurva, Artemisia dubia, A. gmelinii* and *Thymus linearis* were assessed against *Alternaria brassicicola,* the causal agent of leaf spot diseases of cabbage, by poisoned food technique. The fungitoxicity was found to be variable with different plant essential oils. (Parajuli R.R. *et al*. 2005).

Saxena et al. (1983), Singh et al. (1983), Chandra et al. (1982), Dwivedi et al. (1990), Rao and Srivastava (1994), Dubey et al. (1983) and Renu et al. (1980) studied the fungitoxicities of essential oils of different plant species against fungal species.

2.2 Strawberry

Strawberry (*Fragaria vesca L.*) is an important fruit crop. It belongs to the family Rosaceae. Strawberry is native to the regions of Eurasia and North America (Brown, D. 2002). Strawberry fruits are eaten raw or used in making juice, desserts, jam, syrup and wine. Fruits, leaves and roots are also used medicinally. Strawberry is a valuable source of Vitamin C (Sauer, J.D., 1993). Because of it's excellent color, taste and flavor it is widely used as the additives in the food. In the context of Nepal, it is a new crop. In commercial form it is cultivated in Kakani by the Japanese technological assistance. It is also cultivated in Daman, Dakshinkali etc. (NARC Bulletin, 2004). But being a new crop for Nepal, It is not widespread and widely cultivated in Nepal.

2.3 Test fungus

Botrytis cinerea Pers. Ex. is a causal agent of Gray mold fruit rot of strawberry. It may also attack leaves, petioles, flower buds, petals and stem (Maas, J. L.1992). Rot by *Botrytis cinerea* start on any portion of the fruit but is most frequently found in the calyx end or on sides of fruits touching other rotten fruits. Affected tissue turns light brown but remains firm. No distinct line separates diseased tissue from healthy tissue. Completely rotted berries retain their general shape but become tough and dry. The

causal fungus usually produces a velvety growth on the fruit surface. Abundant powdery dry spores (conidia) of the fungus are borne on spore bearing structures (conidiophores) in this growth. In high humidity, the surface growth may be cottony and white with little or no spore production. In some conditions, large black sclerotia may be produced on affected plant parts. (Maas J.L., 1981). In culture, the colonies are gray, effuse and spongy. Conidia are hyaline or brightly colored and are borne in grape like cluster. They are attached singly to sterigmata on simple or branched conidiophores. Sclerotia are produced on diseased plant parts and in old cultures. (Barnes, E.H.1968). Sclerotia are large and black irregular bodies. The size of conidia varies from 5μm- 12.5 μm in diameter but conidiophores are long, up to 5 mm in length. (Barnes, E.H.1968). Sclerotia are large and black irregular bodies. The size of conidia varies from 5μm- 12.5 μm in diameter but conidiophores are long, up to 5 mm in length. The perfect stage of the pathogen is *Botryotinia fuckeliana*. The classification of *Botrytis cinerea* is as follows,

Kingdom - Mycetae
Division - Eumycota
Sub- division - Duteromycotina
Class - Hyphomycetes
Order - Moniliales
Family - Moniliaceae
Genus - *Botrytis*
Species - *B. cinerea* (Martin, 1961)

The classification of *Botryotinia fuckeliana* is as follows,

Kingdom - Fungi
Phylum - Ascomycotina
Sub- Phylum - Pezizomycotina
Class - Leotiomycetes
Order - Helotiales
Family - Sclerotinicaeae
Genus - *Botrytionia*
Species - *B.fuckeliana* (De Bary & Whetzel, 1945).

2.4 Test Plant Species

Five following plant species were taken for the assessment of fungitoxicity.

2.4.1 *Artemisia gmelinii* Web. Ex. Stechm. (Asteraceae)

Description: *Artemisia gmelinii* Web. Ex. Stechm, gregarious aromatic perennial herb of drier areas, erect, finely hoary stems 30-120 cm, shrubby below. Leaves 2-3 times cut into very fine slender segments, green or gray to whitish hoary. Flower heads comparatively large, 3-4 cm across, broadly hemispheric with many yellow disk florets, short stalked, nodding in rather slender spike like clusters. Involucral bracts-hoary, with broad transparent papery margins (Polunin & Stainton, 1984).

Uses: It is ground to make powder and half spoon full is mixed with the cup of boiled water. Two to three times a day after meal is prescribed for fever. It is also used in cough and cold. (Polunin & Stainton, 1984).

Distribution: W C Nepal, alt: 2800-4300m. Afghanistan to C Nepal, N. Asia, dry stony slopes; common in Ladakh and Lahul (Polunin & Stainton, 1984; Press et al; 2000).

2.4.2 *Thymus linearis* Benth (Lamiaceae)

Description: *Thymus linearis* Benth. Small tufted, strong aromatic herb, spreading stems up to 20 cm in height, leaves tiny, elliptic oblong, entire, gland dotted- up to 8 mm, flowers purple, in small whorls, crowded in to a dense terminal clusters. Calyx 2 lipped, upper lip 3 lobed, lower with 2 narrow bristles and throat with shaggy hairs, corolla 6 mm or more weakly lipped, upper lip entire, lower 3- lobed, Bracts minute (Polunin & Stainton, 1984).

Uses: Ten grams of plant is boiled in two cups of water and half cup of decoction is drunk at night for eye infection. It is powdered on stone and mixed with chilly and salt to use as pickle (Polunin & Stainton, 1984).

Distribution: W C Nepal, alt: 2400-4500m, Afghanistan, Pakistan Himalayas (Kashmir to Nepal), India, China, Japan (Polunin & Stainton), 1984; Press et al.; 2000).

2.4.3 *Artemisia indica* Willd (Asteraceae)

Description: *Artemisia indica* Linn. Perennial, shrubby, aromatic, 0.61m- 2.43m high. pubescent or villous, stems leafy, paniculately branched. Lower leaves 5 cm- 10cm x 2.5 cm- 5cm, petioled, ovate in outline, with stipule- like lobes at the base, deeply pinnatisect, the lobes entire, toothed or white-tomentose beneath. Upper leaves smaller. 3- fid or entire, lanceolate. Heads 0.317cm- 0.4233cm long, ovoid or subglobose, solitary or 2 or 3 together, sessile or very shortly pedicelled, outer female flowers very slender, inner female fertile, involucral- bracts villous and with acute, the inner oblong; Achenes oblong- ellipsoid, minute (Cooke, 1967).

Uses: It is used as the green manure and the mulch in the agricultural fields because of its allelopathic properties. Root extract is used against the fever. Leaf extract used against diarrhoea.

Distribution: CE alt. 300-2400 m. Himalaya, India, Myanmar, Thailand, S. China, Japan.

2.4.4 *Tanacetum gracile* Hook f. and Thomas (Compositae)

Description

Hoary- pubescent, stems many from a woody stock, slender 0.3m-0.60 m corymbosely branched above, branches slender, spreading leaves 1.27 cm- 2.54 cm palmately compound, 2- pinnatisect, segments very slender, heads 0.42 cm in diameter in small corymbs terminating in to the long slender branches, involucral Bracts broadly oblong, scarious, glaborous, pale, receptacle conical, achenes- obovoid with a terminal cupula. (Cooke, 1967).

Uses

Ornamental and religious use. Boiled with water, taken in fever and cold. Root extract taken in constipation.

Distribution

CEN Nepal. Himalaya (Kashmir to Nepal). China (Sichuan), alt. 3000m – 4400m (Shrestha KK and Sutton DA, 2000).

2.4.5 Murraya koenigii syn. Bergera koenigii (Rutaceae)

Description

Small tree, up to 4m high. Leaves 10-25 cm, leaflets 13-23, ovate, 1-4 × 1-1.5 cm.

Base asymmetrically rounded or crenate, margins minutely crenate, apex, acuminate, and surface black gland dotted. Calyx 1mm. Petals 5 × 1.5 mm. Stamens 4-5 mm, style 5mm. Fruit ovoid, crimson, found on open land. (Shrestha KK and Sutton DA, 2000).

Uses

Used as the condiments, spices for the excellent flavor. Used in curry and pickles, so it is also called commonly as the curry plant.

Distribution

Nepal (WCE, 150-1450 m), Himalaya (Uttar Pradesh to Sikkim), India, Sri Lanka, Myanmar, Indo- China, China.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

Different equipments, glassware and chemicals were used in performing the research work. All materials used are listed in the Appendix I.

3.2 Methods

The research was carried out with the following steps.

3.2.1 Collection of Test Plant Species

Three different plant species (*Thymus linearis*, *Artemisia gmelinii* and *Tanacetum gracile)* were collected from Manang and the two plant species i.e. *Murraya koenigii* and *Artemisia indica* were collected from the CDB garden.

3.2.2 Herbarium Preparation and Identification

The collected plant species were washed with clean water, left to remove moisture, and then pressed inside the paper sheet in between blotting papers. The paper sheets were changed in a regular interval of a day until the herbaria became dry. The specimens were pasted on herbarium sheets with tag no and labeling. Then the herbarium specimens were identified in the central department of Botany, Tribhuvan University.

3.2.3 Processing of Samples

The collected plant species were processed for hydrodistillation of essential oils by the following steps.

3.2.3.1 Shade Drying

The collected plants were cut into smaller pieces and spread under the shade at room temperature and turned up and down to hasten drying.

3.2.3.2 Storage of Samples

The completely dried samples were packed in waterproof bags. Then the samples were taken for hydrodistillation turn by turn to extract essential oil.

3.2.3.3 Isolation of Essential Oils

50 gm of shade-dried sample of plant species was surface sterilized with 0.1% mercuric chloride solution followed through washing with distilled water. The sample was then pulverized in distilled water and subjected to hydrodistillation for 6-8 hours in Clevenger's apparatus. The volatile fractions condensed after hydrodistillation exhibited two distinct layers an upper aromatic layer of essential oil and a lower colorless aqueous layer. The aromatic layer was collected and dried over anhydrous Sodium Sulphate and stored at low temperature (10^{o} C). Following Rao and Srivastava (1994).

3.2.3.4 Determination of Yield of Essential Oil

50 gm of each dried plant sample was hydrodistilled with 500 ml water for extracting essential oil. The volume of the oil extracted was noted down. The yield of essential was calculated in terms of percentage using following formula.

$$\% \text{ of essential oil} = \frac{\text{Volume of essential oil}}{\text{Wt. of sample}} \times 100$$

3.2.4 Dilution of Essential Oil

Essential oil was diluted into different concentration with 80% Acetone (Rao and Srivastava). The oil was diluted to 20,000 ppm, 10,000 ppm, 5,000 ppm, 2,500 ppm, 1,250 ppm and 625-ppm concentrations. 0.2 ml of essential oil was mixed with 19.8 ml acetone to make 20,000-ppm concentration. Six sterilized plastic containers were taken and 5 ml acetone was poured in each container. 5 ml of diluted essential oil from 20,000 ppm was poured to 5 ml acetone to form 10,000 ppm. In such a way the further concentrations of 5,000 ppm, 2,500 ppm, 1,250 ppm and 625 ppm of essential oil was made. 5 ml acetone was taken in a separate container and that was regarded as 0 ppm essential oil concentration.

3.2.5 Media Preparation

Glasswares were cleaned thoroughly in cleaning mixture and finally with distilled water. The glasswares were dried and then wrapped with paper and sterilized in hot air oven for 2 hours at 160^{o} C. Potato dextrose agar (PDA) media was prepared and poured into Petri plates and culture tubes aseptically (Annex-I).

3.2.6 Isolation of *Botrytis cinerea* (Test fungus)

Infected fruits were collected from the horticultural farm of the National Agricultural Research Council NARC. *Botrytis cinerea* is a cool loving fungus. It also attacks the fruits under refrigeration in the cold store (Maas J.L. 1981). At the temperature around 3^{o}C, growth of the other fungal species greatly reduces but the *Botrytis cinerea* continues to grow. By the help of sterilized needles and forceps, some piece of fungal colony was transferred aseptically on a Petri plates containing PDA media. It was incubated in inverted position in an incubator at aseptic condition for five days. After five days, the mycelial growth was observed under the compound microscope to study the characteristics of the pathogen.

3.2.7 Maintenance of Five Days Old Culture

For assessing fungitoxicity in each case Inoculum disc of 5 days old fungal culture was used. To get five days old fungus, the fungus from pure culture was inoculated into PDA media and then incubated in incubator at 25 ± 2^{o} C. After 5 days, the experiment for fungitoxicity was conducted.

3.2.9 Assessment of Fungitoxicity

The fungitoxicity of the essential oils were assessed by poisoned food technique.

3.2.9.1 Poisoned Food Technique

0.5 ml from every concentration of oil was taken in a pre sterilized and cooled Petri plate. 9.5 ml-melted culture medium was poured on that with gently swirling to mix the contents thoroughly. The essential oil was replaced by equal volume of acetone in control set. After the medium solidified inoculum disc (4mm) of the test fungus was

aseptically inoculated upside down on the medium in the center of each plate. The plates were then incubated at 25 ± 2^{o} C for five days following Bocher (1938) cited in Rao and Srivastava (1994).

3.2.10 Calculation of inhibition or the cessation of complete mycelial growth.

After 5 days of the experiment of by poisoned food technique, the average diameters of the colonies of the test fungus in every concentration were measured. Fungitoxicity was assessed in triplicates in each concentration of essential oils. Fungitoxicity was assessed in terms of percentage inhibition of mycelial growth of test fungus (Arora & Dwivedi 1979) Cited in Rao and Srivastava, 1994).

The gradually increased concentration of essential oil was placed on the Petri plate. The colony also gradually kept on decreasing. At the certain concentration, cessation of complete growth was observed. It is termed as the minimum inhibitory concentration.

$$\% \text{ Inhibition of mycelial growth} = \frac{g_c - g_t}{g_c} \times 100$$

g_c = growth of mycelial colony after incubation **in control set** (diameter of colony-diameter of Inoculum disc).

g_t= Growth of mycelial colony after incubation period **in treatment set** (Diameter of colony – diameter of Inoculum disc).

3.2.11 Spore Measurement

Ocular micrometer was placed inside the eyepiece of a compound microscope and calibrated by superimposing the graduation of stage micrometer. The number of divisions of ocular micrometer coinciding with the number of divisions in stage micrometer was noted down and calculated the calibration factor applying the formula:

$$\text{One ocular division} = \frac{\text{No. of division on stage micrometer}}{\text{No. of division of ocular micrometer}}$$

Then the stage micrometer was replaced by a fine slide of *Botrytis cinerea* mounted in lacto phenol. Size of conidia and conidiophores were measured by determining the no. of divisions of ocular micrometer (Annex- II). In each measurement, readings were taken. Measurement was carried out to find the range and size of different parts of the fungus.

3.2.12 Pathogenicity Test

For carrying out the pathogenicity test, firstly the infected fruits were collected and characteristic symptoms were noted down. Then *Botrytis cinerea* was isolated in PDA as pure culture. Inoculum from the pure culture was transferred to the healthy fruits. When incubated at the temperatures of 23^{o}C, the characteristic symptoms were produced, which were found to be similar with the symptoms on fruits previously collected from the field. The fungus was isolated and its characters were compared with the previously isolated fungus.

3.2.13 Identification of Fungus

From the pathogenicity test, the disease was proved to be caused by *Botrytis cinerea.* The characteristic features of the fungus were identified with the help of standard literature.

3.2.14 Photography

Photographs of herbaria of test plant samples, infected fruits and fungal structures were taken. Besides, inhibitions of mycelial colony in different concentrations by different essential oils were also subjected to photography.

CHAPTER FOUR
EXPERIMENT AND OBSERVATION

4.1 Assessment of Fungitoxicity of Essential Oils

Fungitoxicity of each essential oil was assessed in different concentrations against *Botrytis cinerea* by poisoned food technique. All essential oils were diluted into similar degree of concentration; essential oils of different plant species showed differential efficacies in inhibiting the mycelial growth.

4.1.1 Essential Oil of *Thymus linearis*

Table 1

Table Showing Growth Inhibition of *Botrytis cinerea* by Essential Oil of *Thymus linearis*

Inoculum size: 4 mm in diameter

Oil Concentration	Size of Colony Diameter (mm)			
	Obs. 1	Obs. 2	Obs. 3	Mean
Control	90	90	87.5	89.16
625ppm	80	85	80	81.66
1,250ppm	75	70	73	72.66
2,500ppm	50	45	45	46.66
5,000ppm	14	15	13	13
10,000ppm	No growth	No growth	No growth	No growth

.

Table 2

Fungitoxicity of E. oil of *Thymus linearis* against *Botrytis cinerea*

Concentration of E. oil (ppm)	Treatment set					
	Control Set	625	1,250	2,500	5,000	10,000
Colony size (mm)	89.16	81.66	72.66	46.66	13	0
Inoculum size (mm)	4	4	4	4	4	4
Mycelial growth (mm)	85.16	77.66	68.66	42.66	9	0
Growth Inhibition %	0	8.81	19.38	50.48	89.43	100

Minimum Inhibitory concentration (MIC) = 10,000ppm

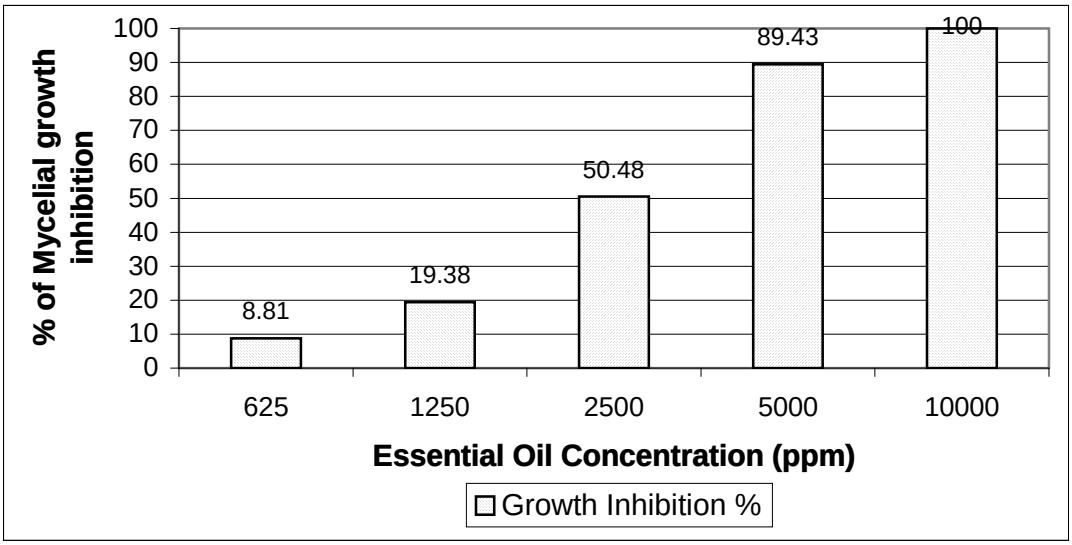

Fig. 1: Fungitoxicity of E. oil of *Thymus linearis* against *Botrytis cinerea*

Essential oil of *Thymus linearis* showed mycelial growth inhibition as 8.81%, 19.38%, 50.48%, 89.43% and 100% at 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm and 10,000 ppm oil concentrations against *Botrytis cinerea* respectively.

4.1.2 Essential Oil of *Tanacetum gracile*

Table 3

Table Showing Growth Inhibition of *Botrytis cinerea* by Essential Oil of *Tanacetum gracile*

Inoculum size: 4 mm in diameter

Oil Concentration	Size of Colony Diameter (mm)			
	1	2	3	Mean
Control	90	90	90	90
625ppm	80	75	82	79
1,250ppm	75	75	75	75
2,500ppm	70	72	70	90.66
5,000ppm	50	52	45	49.00
10,000ppm	14	13	10	12.33

Table 4

Fungitoxicity of E. oil of *Tanacetum gracile* against *Botrytis cinerea*

Concentration of E. oil (ppm)	Treatment set					
	Control Set	625	1,250	2,500	5,000	10,000
Colony size (mm)	90	80	75	70	50	14
Inoculum size (mm)	4	4	4	4	4	4
Mycelia growth (mm)	86	76	71	66	46	10
Growth Inhibition %	0	12.7	17.41	22.48	47.67	90.31

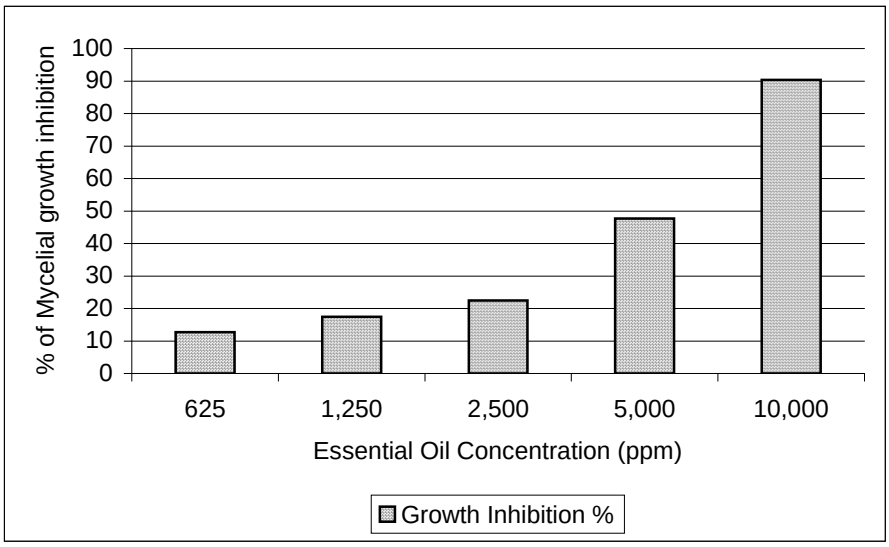

Fig. 2: Fungitoxicity of E. oil of *Tanacetum gracile* against *Botrytis cinerea*

Essential oil of *Tanacetum gracile* showed mycelial growth inhibition as 12.7%, 17.41%, 22.48%, 47.67%, and 90.31% respectively at 628ppm, 1280ppm, 2,500ppm, 5,000 ppm and 10,000ppm essential oil concentrations against *Botrytis cinerea* respectively.

4.1.3 Essential Oil of *Artemisia gmelinii*

Table 5

Table Showing Growth Inhibition of *Botrytis cinerea* by Concentrations of Essential Oil of *Artemisia gmelinii*

Inoculum size: 4 mm in diameter

Oil Concentration (ppm)	Size of Colony Diameter (mm)			
	1	2	3	Mean
Control	90	90	90	90
625ppm	75	74	74	74.83
1,250ppm	70	71	70	70.83
2,500ppm	65	67	64	65.33
5,000ppm	54	53	53	53.33
10,000ppm	38	34	35	35.66
12,500ppm	32	31.5	33	32.166
25,000ppm	22	22	21	21.66
50,000ppm	10	9	10	9.66
75,000ppm	0	0	0	0

Table 6

Fungitoxicity of E. oil of *Artemisia gmelinii* against *Botrytis cinerea*

Concentration of E. oil (ppm)	Treatment set									
	Control Set	625	1,250	2,500	5,000	10,000	12,500	25,000	50,000	75,000
Colony size (mm)	90	74.33	70.33	65.33	53.33	35.66	32.166	21.66	9.66	0
Inoculum size (mm)	4	4	4	4	4	4	4	4	4	4
Mycelia growth (mm)	86	70.33	66.33	61.33	49.33	31.66	28.16	17.66	5.66	0
Growth Inhibition %	0	17.63	22.29	28.68	42.63	63.18	67.24	79.46	93.41	100

Minimum Inhibitory concentration (MIC) = 75,000ppm

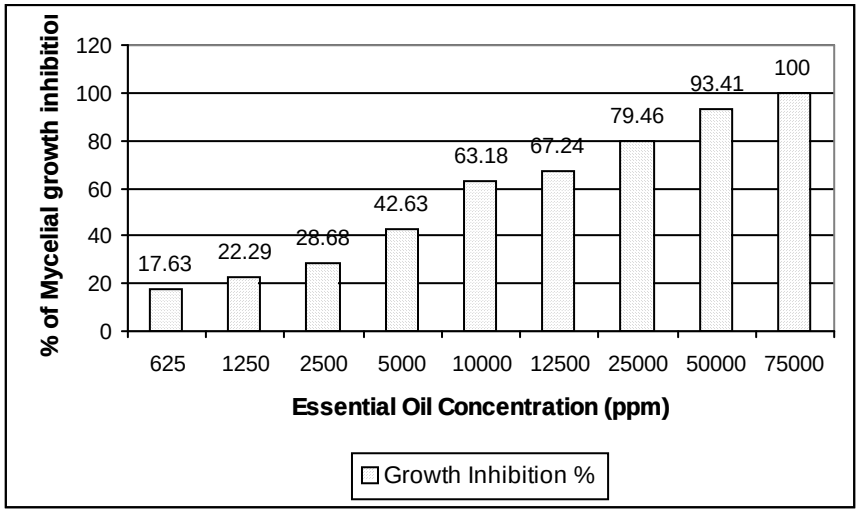

Fig. 3: Fungitoxicity of E. oil of *Artemisia gmelinii* against *Botrytis cinerea*

Essential oil of *Artemisia gmelinii* showed mycelial growth inhibition as 17.63%, 22.29%, 28.68%, 42.63%, 63.18%, 67.24 %, 79.46%, 93.41% and 100% at the concentrations of 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm, 10,000 ppm, 12,500 ppm, 25,000 ppm, 50,000 ppm and 75,000 ppm respectively.

4.1.4 Essential Oil of *Artemisia indica*

Table 7

Table Showing Growth Inhibition of *Botrytis cinerea* by Concentrations of Essential Oil of *Artemisia indica*

Inoculum size: 4 mm in diameter

Oil Concentration	Size of Colony Diameter (mm)			
	1	2	3	Mean
Control	90	90	90	90
625ppm	75	78	80	77.66
1,250ppm	60	59	63	60.66
2,500ppm	53	50	56	53
5,000ppm	40	35	37	37.33
10,000ppm	21	25	19	21.66
20,000ppm	12	13	12	12.33
40,000ppm	5	4	6	5
60,000ppm	0	0	0	0
80,000ppm	0	0	0	0

Table 8

Fungitoxicity of E. oil of *Artemisia indica* against *Botrytis cinerea*

Concentration of E. oil (ppm)	Treatment set									
	Control Set	625	1,250	2,500	5,000	10,000	20,000	40,000	60,000	80,000
Colony size (mm)	90	77.66	60.66	53	37.33	21.66	12.33	5	0	
Inoculum size (mm)	4	4	4	4	4	4	4	4	4	4
Mycelia growth (mm)	86	73.66	56.66	49	33.33	17.66	8.33	1	0	
Growth Inhibition %		14.34	34.5	43.02	61.24	79.46	90.31	98.88	100	100

Minimum Inhibitory concentration (MIC) = 60,000ppm

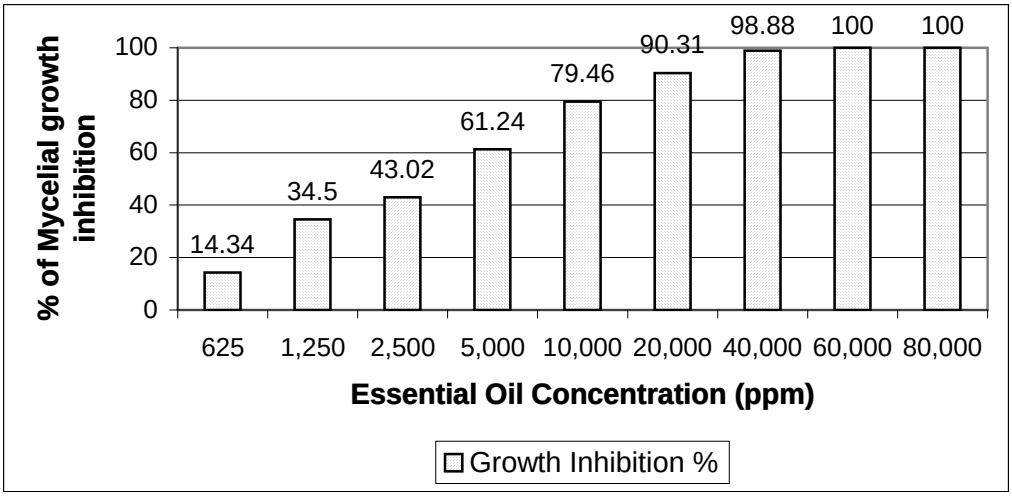

Fig. 4: Fungitoxicity of E. oil of *Artemisia indica* against *Botrytis cinerea*

Essential oil of *Artemisia indica* showed mycelial growth inhibition as 14.34%, 34.5%, 43.02%, 61.24%, 79.46%, 90.31%, 98.88% and 100% respectively at 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm, 10,000 ppm, 20,000 ppm, 40,000 ppm, and 60,000 ppm respectively.

4.1.5 Essential Oil of *Murraya koenigii*

Table 9

Table Showing Growth Inhibition of *Botrytis cinerea* by Concentrations of Essential Oil of *Murraya koenigii*

Inoculum size: 4 mm in diameter

Oil Concentration	Size of Colony Diameter (mm)			
	Obs. 1	Obs. 2	Obs. 3	Mean
Control	90	90	90	90
625ppm	85	85	87	85.66
1,250ppm	81	80	81	80.66
2,500ppm	75	78	76	76.33
5,000ppm	70	71	72	71
10,000ppm	59	60	62	60.33

Table 10

Fungitoxicity of E. oil of *Murraya koenigii* against *Botrytis cinerea*

Concentration of E. oil (ppm)	Treatment set					
	Control Set	625	1,250	2,500	5,000	10,000
Colony size (mm)	90	85.66	80.66	76.33	71	60.33
Inoculum size (mm)	4	4	4	4	4	4
Mycelia growth (mm)	86	81.66	76.66	72.33	67	56.33
Growth Inhibition %	0	5.04	10.8	15.89	22.09	34.5

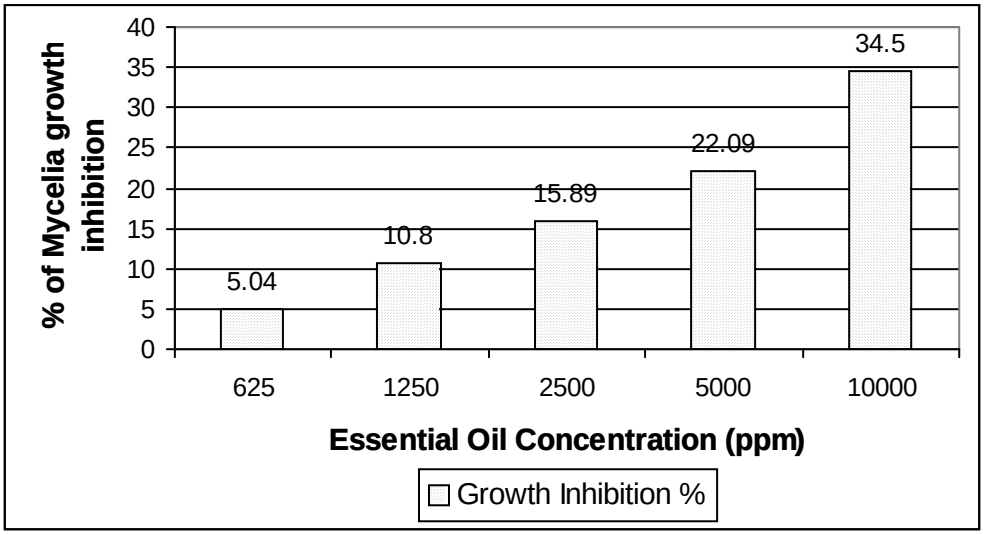

Fig. 5: Fungitoxicity of E. oil of *Murraya koenigii* against *Botrytis cinerea*

Essential oil of *Murraya koenigii* showed mycelial growth inhibition as 5.04%, 10.8%, 15.89%, 22.09 % and 34.5% at 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm and 10,000 ppm respectively.

4.2 Pathogenicity Test

The pathogen mainly attacks the flowers and fruits. The thalamus mainly attacked which result in flower and fruit fall. Part of the fruit turns light brown but remains firm. The causal fungus usually produces a velvety gray growth on the fruit surface. The pathogen was isolated in the pure culture. From the pure culture, slide was prepared. The fungal colony appeared cottony white earlier but gray later, effuse, hyphae septate, branched and hyaline. Conidiophores arise from the mycelium, not in specialized spore producing structures. Conidiophores tall, stout and dark; branch irregularly or dichotomously and have globose basal cell. Several short, dark, septate, spore-producing branches are produced near the apex of each conidiophore. Each branch has a terminal ampulla on which conidia develop synchronously on short, fine denticles. Conidia (5µm -12.5µm) are usually smooth, unicellular, colorless or pigmented and dry. They were ellipsoid and globoid. The characters of the fungus isolated from the strawberry fruit resembled to that of *Botrytis cinerea* (Maas, J.L. 1981).

The fungus was inoculated to the healthy; surface sterilized fruits in the petridishes and enclosed beakers. After one week, the inoculated fruits develop the characters of gray mold fruit rot but the uninoculated fruits remain fresh. From the mold-developed fruits, the fungus was isolated and observed under the microscope.

CHAPTER FIVE
RESULT AND DISCUSSION

5.1 Result

Botrytis cinerea (Test fungus) was isolated from infected fruits of strawberry. All the essential oils were assessed for their antifungal activities against *Botrytis cinerea* and calculated the percentage of inhibition of mycelial growth.

5.1.1 Fungitoxicities of different essential oils

Differential essential oils showed different fungitoxicities. Percentage inhibitions of mycelial growth varied with different oil concentrations. Percentage inhibition of mycelial growth of *Botrytis cinerea* were found 8.81%, 19.38%, 50.48%, 89.43% and 100% by *Thymus linearis;* 12.7%, 17.41%, 22.48%, 47.67%, and 90.31% by *Tanacetum gracile* 17.63%, 22.29%, 28.68%, 42.63% and 63.18% by *Artemisia gmelinii;* 14.34%, 34.5%, 43.02%, 61.24% and 79.46% by *Artemisia indica;* 5.04%, 10.8%, 15.89%, 22.09% and 34.5% by *Murraya koenigii* at 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm and 10,000 ppm essential oil respectively.

Furthermore percentage inhibitions of mycelial growth of *Botrytis cinerea* were found 67.24%, 79.46%, 93.41% and 100% at the concentrations of 12,500 ppm, 25,000 ppm, 50,000 ppm and 75,000 ppm respectively by the essential oil of *Artemisia gmelinii;* 90.31%, 98.88% and 100% respectively at the concentrations of 20,000 ppm, 40,000 ppm and 60,000 ppm respectively by the essential oil of *Artemisia indica.* The minimum inhibitory concentration (MIC) of *Thymus linearis* is found to be 10,000 ppm. In the same way the MIC of *Artemisia gmelinii* and *Artemisia indica* are 75,000 ppm and 60,000 ppm respectively.

Table 11

Fungitoxicities of Different Essential Oils in Different Concentrations

S.N.	E. Oil Concentration (ppm)	Mycelial growth inhibition (%)				
		Artemisia indica	*Artemisia gmelinii*	*Tanacetum gracile*	*Thymus linearis*	*Murraya koenigii*
1.	625	14.34	17.63	12.7	8.81	5.04
2.	1,250	34.5	22.29	17.41	19.38	10.8
3.	2,500	43.02	28.68	22.48	50.48	15.89
4.	5,000	61.24	42.63	47.67	89.43	22.09
5.	10,000	79.46	63.18	90.31	100	34.5
6.	12,500	-	66.67	-	-	-
7.	20,000	90.31	-	-	-	-
8.	25,000	-	79.46	-	-	-
9.	40,000	98.33	-	-	-	-
10.	5,0000	-	93.41	-	-	-
11.	60,000	100	-	-	-	-
12.	75,000	-	100	-	-	-
13.	80,000	100	-	-	-	-

5.2 Discussion

The present study has been carried out to isolate the causal agent of gray mold fruit rot of strawberry (*Botrytis cinerea)* and to control the fungus with essential oils of *Artemisia indica*, *Artemisia gmelinii*, *Thymus linearis, Tanacetum gracile* and *Murraya koenigii.* The main objective of the study is to evaluate the comparative fungitoxicities of the essential oils of the test plant species against *Botrytis cinerea*, to evaluate the fungitoxicity of essential oils of test aromatic plants and to calculate the minimum inhibitory concentration (MIC) of test plant oil.

5.2.1 Extraction of Essential Oils

Three plant species (*Artemisia gmelinii, Thymus linearis and Tanacetum gracile)* were collected from Manang as part of the NUFU funded project. Two other plant species (*Artemisia indica* and *Murraya koenigii)* were collected from the garden of

central department of Botany TU. To obtain essential oils, the plants were hydrodistilled using Clevenger's apparatus as done by Tripathi *et al.*(1983). 50 gm dry weight of plant was mixed with 500 ml water and hydrodistilled in the Clevenger's apparatus for 5 hours. Then the essential oil was carefully isolated and preserved below the temperature of 10^{o} C.(Clevenger, 1928).

5.2.2 Yield of Essential Oil

The yield of essential oil is calculated according to the following formula.

$$\text{Yield of essential oil} = \frac{\text{Volume of essential oil}}{\text{Weight of dried plant}} \times 100\%$$

(a) *Thymus linearis*

$$\text{Yield of essential oil} = \frac{\text{Volume of essential oil}}{\text{Weight of dried plant}} \times 100\%$$

$$= \frac{0.8}{50} \times 100\%$$
$$= 1.6\%$$

In the same way, the essential oil content of *Artemisia gmelinii, Tanacetum gracile, Artemisia indica* and *Murraya koenigii* are found to be 2.4%, 0.8%, 1.6% and 0.4% respectively.

5.2.3 Isolation of Fungus

The causal agent of the gray mold fruit rot of strawberry is *Botrytis cinerea.* The infected fruit shows velvety gray growth on the fruit surface. Abundant powdery spores are borne on the conidiophores; the surface growth is seen to be white and cottony, with or without the spore production. Under some conditions, large black sclerotia are formed. Conidiophores arise from the mycelium. Conidiophores long (up to 5mm), stout and dark, branch irregularly or dichotomously and have a globose basal cell. Short, dark, septate and spore producing branches are produced near the apex of each conidiophore. Conidia (5 µm – 12.5 µm) are usually smooth, unicellular,

colorless or pigmented and dry. They are ellipsoid, ovoid or globoid. Black, irregularly shaped sclerotia, 1-5 µm across, may be produced in culture and in the field on decayed fruit and other plant parts.

5.2.4 Assessment of Fungitoxicities of Different Oils

Different essential oils showed variable antifungal activities at different concentrations against *Botrytis cinerea.* The fungitoxicity was assessed in terms of percentage of mycelial growth inhibition.

The growth of mycelium was inhibited by 8.81%, 19.38%, 50.48%, 89.43% and 100 % by *Thymus linearis,* 17.63%, 22.29%, 28.68%, 42.63%, and 63.18% by *Artemisia gmelinii,* 14.34%, 34.5%, 43.02%, 61.24% and 79.46% by *Artemisia indica*, 5.04%, 10.8%, 15.89%, 22.09% and 34.5% by *Murraya koenigii*,12.7%, 17.41%, 22.48%, 47.67% and 90.31% by *Tanacetum gracile* at 625 ppm, 1,250 ppm, 2,500 ppm, 5,000 ppm and 10,000 ppm essential oil respectively.

MIC (minimum inhibitory concentration) of essential oil from three species of plants has been found. MIC of *Thymus linearis* is 10,000 ppm, i.e. at 10,000 ppm concentration; there is complete inhibition of colony growth of *Botrytis cinerea*. Similarly, MIC of essential oils of *Artemisia indica* and *Artemisia gmelinii* are found to be 60,000 ppm and 75,000 ppm respectively.

5.2.5 Comparative Fungitoxicities of Different Essential Oils

Thymus linearis showed the highest inhibition (100%) followed by *Tanacetum gracile* (90.31%), *Artemisia indica* (79.46%), *Artemisia gmelinii* (63.18%), and *Murraya koenigii* (34.5%) at 10,000-ppm essential oil concentration respectively.

Thymus linearis showed the highest inhibition (89.43%), followed by *Artemisia indica* (61.24%), *Tanacetum gracile* (61.24%), *Artemisia gmelinii* (42.63%) and *Murraya koenigii* (22.09%) at 5,000-ppm essential oil concentration respectively.

Thymus linearis showed the highest inhibition (50.48%), followed by *Artemisia indica* (43.02%), *Artemisia gmelinii* (28.68%), *Tanacetum gracile* (22.48%) and *Murraya koenigii* (34.5%) at 2,500-ppm concentration of essential oil respectively.

Artemisia indica showed the highest inhibition (34.5%), *Thymus linearis* (19.38%), *Artemisia gmelinii* (22.29%), *Tanacetum gracile* (17.41%) and *Murraya koenigii* (10.8%) at 1,250-ppm concentration of essential oil respectively.

Artemisia gmelinii showed the highest inhibition (17.63%) followed by *Artemisia indica* (14.34%), *Tanacetum gracile* (12.7%), *Thymus linearis* (8.81%) and *Murraya koenigii* (5.04%) at 625-ppm essential oil concentration respectively.

Essential oils in different concentrations showed differential fungitoxicities. Essential oil of each plant species was diluted in (80%) acetone into 5 different concentrations viz. 10,000 ppm, 5,000 ppm, 2,500 ppm, 1,250 ppm and 625 ppm. However for finding out the minimum inhibitory concentration (MIC) of some essential oil, the oil was diluted to the concentrations of 12,500 ppm, 20,000 ppm, 25,000 ppm, 40,000 ppm, 50,000 ppm, 60,000 ppm, 75,000 ppm and 60,000 ppm and 80,000 ppm.

The difference in fungitoxicity at same concentration in different essential oils may be due to different chemical composition of the oils (Singh et al, 1983).

This study is the overall study. The result obtained from the study may not be true forever because the concentration of various ingredients in essential oil of the same plant species varies due to several factors such as growth stage, ecological factors etc. (Gulati, 1982).

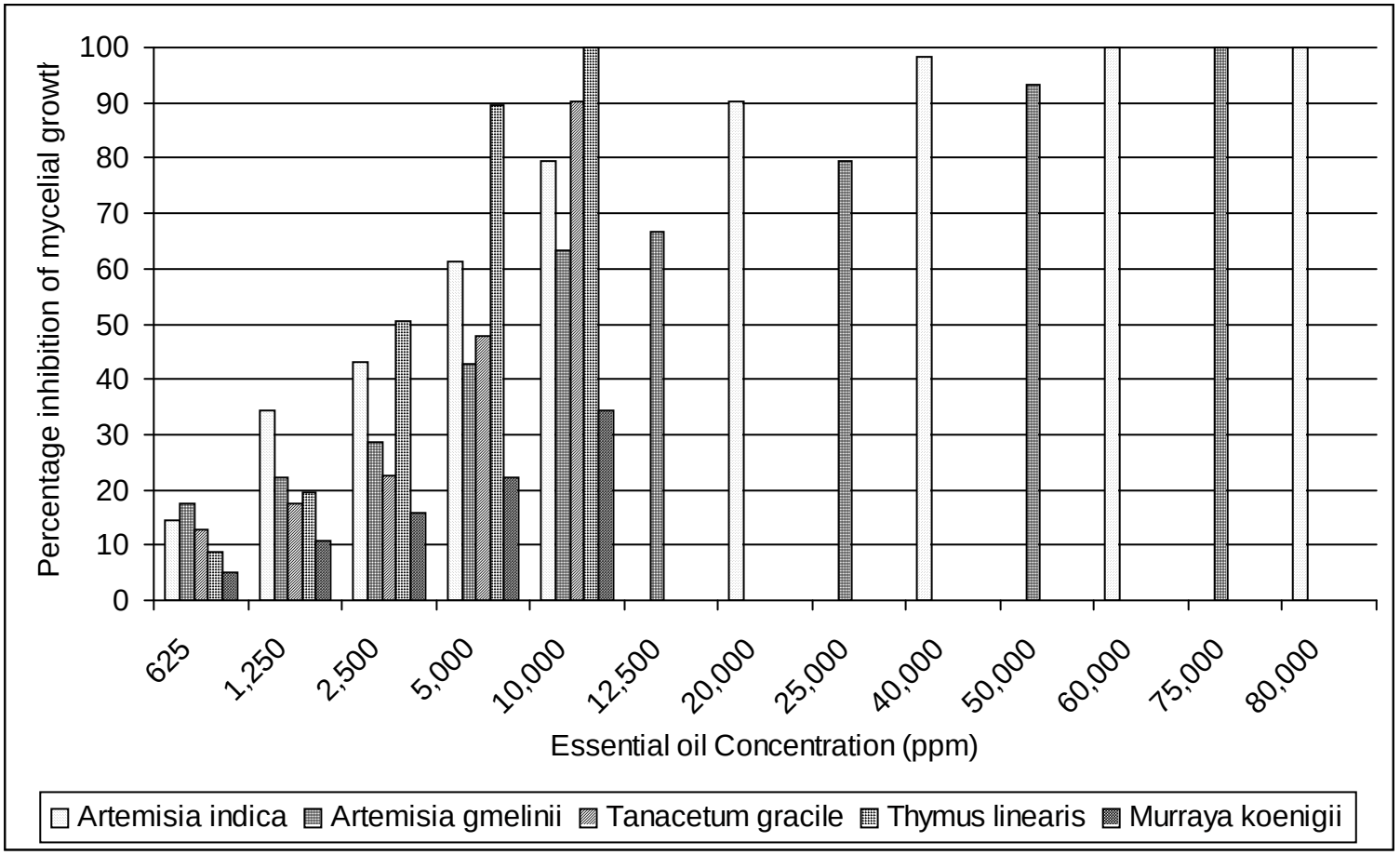

Fig. 6: Comparative Fungitoxicities of Different Essential Oils of Different Plant Species in Different Concentrations Against *Botrytis cinerea*

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From the research it could be concluded that the test plant harbored the fungitoxic principles that inhibited the mycelial growth of *Botrytis cinerea.* Out of five test plant essential oils, *Thymus linearis, Artemisia gmelinii, Tanacetum gracile* and *Artemisia indica* inhibited the mycelial growth with well marked linearity but the oil of *Murraya koenigii* did not inhibit the growth significantly.

Minimum inhibitory concentration (MIC) of essential oil of 3 plant species was found out. A perusal literature showed that determination of the MIC of essential oil against *Botrytis cinerea* has not yet been done in Nepal. So this is the first experiment to study fungitoxicity and to find minimum inhibitory concentration of essential oils against *Botrytis cinerea.*

6.2 Recommendation

- Thin layer chromatography (TLC) and Gas chromatography (GC) can be carried out for screening essential oils.
- This work has been strictly done under controlled environment. So the result found might not correspond with those when performed in natural field under the influence of a number of physical factors like solubility, pH and buffer, photolysis of constituents, temperature etc. Hence a rigorous study in the field is recommended.
- The fungitoxicity of essential oils of more plant species on different pathogens is recommended.

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APPENDIX

Materials

Glasswares, test tubes, culture tubes, funnel, pipettes, conical flasks, glass rods, petridishes, inoculating needles, ocular micrometer, stage micrometer, slides, inoculating loop, forceps, scissors, burner, beakers, cover slip, muslin cloth, cotton, plastic containers etc.

Instruments and Appliances

Spirit lamp, laminar airflow, microscope, hot air oven, incubator, refrigerator, Clevenger's apparatus, autoclave, photographic camera, balances etc.

The Test Plant Species

Thymus linearis, Artemisia gmelinii, Tanacetum gracile, Artemisia indica and Murraya koenigii.

The Test Fungus

Botrytis cinerea isolated from the strawberry fruit.

Required chemicals

Distilled water, 80% acetone, cotton blue, lactophenol, potatoes, Dextrose, Agar, ice, chromic acid etc.

ANNEX- I

Preparation of Media (PDA)

Composition

Peeled potatoes – 200 gms.

Dextrose – 20 gms

Agar – 20 gms

Distilled water – 1000 ml

Peeled and sliced 200 gms potato was boiled in 500 ml of clean water for 15 minutes. The cooked potato chips were filtered by muslin cloth squeezing out all liquid. 500 ml water was heated in another flask and added 20 gms agar bit by bit to dissolve on well. 20 gm dextrose was added to the potato extract. Then, both the contents of two different containers were mixed together and well shaken. Thus, 1000 ml of potato dextrose agar medium was prepared. The flasks were cotton plugged and sterilized by autoclaving for 15 minutes in 15 lb pressure at 121^{o}C. Then, media was poured in different sterilized culture tubes and Petri plates in aseptic conditions.

ANNEX- II

Micrometry

In micrometer, 100 divisions = 1000 μm

Or, 1 division = 1000 /100 = 10 μm

57 divisions of ocular micrometer coincided with 11 divisions of stage micrometer.

i.e. 57 divisions of ocular micrometer = 11 divisions of stage micrometer

1 division of ocular micrometer = $\frac{11}{57}$ division of stage micrometer

or, 1 division of OM = $\frac{11}{57} \times 10\mu m$

Measurement of Conidia

S.N.	Length (μm)	Breadth (μm)
1.	6.5	7
2.	5	6.5
3.	9	12.5
4.	7	10.5
5.	9.5	12.5
6.	5	6.5
7.	8	10.5
8.	9	12.5
9.	6	7
10.	5.5	6.5