CHAPTER-ONE

1. INTRODUCTION

1.1 General Background

Nepal is rich in biodiversity due to the different habitats and favorable climatic and geographical condition. Among different useful plant species, medicinal and aromatic plants (MAPs) are the most popular and rich resources of the country. According to an estimate there are over 2000 species of plants in Nepal which are known to be potentially useful, including about 1600-1900 species commonly used for medicinal purposes (Shrestha *et.al.* 2000; Baral & Kurmi 2006; Ghimire 2008).

Most of the MAPs possess the properties like antibacterial, antifungal, antiviral, anthelmintic, anticancer, sedative, laxative, cardiotonic, diuretic and others (Parajuli *et al;* 1998).

Certain plants contain products such as alkaloids, tannins, quinines, coumarines, phenolic compounds in their extracts and exudates and they are known for antifungal activities. The presence of antifungal compounds in plant tissues has long been recognized as an important factor of disease resistance (Fawcett and Spencer, 1966 & 1970).

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 and bitter principles etc (Kruger, 1992).

Essential oils are derived mainly from aromatic plants. Essential oils are also known as essences or volatile oils, synthesized by living organisms. They are extracted with the suitable solvents. They are used in flavor, fragrance food, cosmetics, toiletry and pharmaceutical industries (Rawal, 2001).

Angiospermic plants are reservoir of the chemotherapeutants and provide inexhaustible sources of useful pesticides (Swaminathan, 1978). Various plant based oils are capable of controlling several plant diseases. Oils obtain from seeds of several plants such as sunflower, olive, corn and soybeans give excellent control of some plant diseases (Mehrotra and Agrawal, 2003). Essential oils have been more effective and have more

antimicrobial effect. They sometime serve as antiseptic and stimulants, as ingredient in medicines, as a laboratory reagent, as solvents in the paint industry, as insecticides and as a component of plastics, polishes, pastes, ink, glue and the likes (Schery, 1972).

Nowadays, extensive use of chemicals or fungicide in agriculture damage the non targeted parts of the plants and lead to the environmental pollution (Duru *et al.,* 2003). The control of the plant diseases by these chemicals are replaced by the use of the naturally available plant extracts and exudates which are proved to be less toxic and environmentally non-pollutive (Carkir *et al.,* 2004).

1.2 Post Harvest Disease

Post Harvest Disease **(**PHD) is one of the most destructive diseases of fruits in storage and transit. According to U.S Department of Agricultural Research Survey in 1965 the annual loss of fresh fruits and vegetables in developing countries have been estimated to be in the range of 5-20% or more of the harvest (Mehrotra and Aggarwal, 2003).

Surface rot is one of the most serous post harvest disease prevalent in tropical and subtropical region. It is caused by a variety of fungi, yeasts and bacteria. (Srivastava and Tandon, 1971)

Table - List of fungal pathogens associated with major post harvest diseases of papaya

Name of Diseases	**Causal organisms**
Anthracnose	*Colletotricum gleosporiodes C. Capsci.*
Stem end rot	*Botryodiplodia theobromae, phomopsis sp.*
Fusarium fruit rot	*Fusarium sp.*
Rhizopus rot	*Rhizopus stolonifer*
Stemphylium rot	*Stemphylium lycopersici*
Phomopsis rot	*Phomopsis sp.*

Source: Pertanika (2008)

1.3 Causal organism

The surface rot of papaya is caused by *Fusarium oxysporum schlecht*

1.3.1 Symptoms

Invasion occurs at the point of injury caused by insect or bird feeding, mechanical or growth cracks. At first appearance of soft dark green water soaked lesions which later on develop into a watery soft rot on the skin of fruits. Clusters of white cottony mycelium seems like wrapped on infected fruits. Intense surface rot or decay of the fleshy tissue give bad smell. The disease spread among the fruits during the storage and transit. Prolonged periods of wetness or high relative humidity are conducive to surface rot development (Srivastava and Tandon, 1971).

1.3.2 Diseases Cycle

The following steps are involved in disease cycle, wounds are the most common ways of infection, harvest bruises, freezing injury and insect wounds are predisposing factors. The organism lives over in decayed refuse serves as primary inoculum. Abundant moisture is essential for invasion. High relative humidity is essential for progress of the disease. The conidia are dispersed by rains, winds, insects, contacts etc. The secondary infection takes place by means of conidia. (Srivastava and Tandon, 1971).

1.3.3 Control Measures

For the control measures of disease following ways are adopted. Proper handling of the crop. Bruising should be avoided. Storage should be done at the lowest temperature. Fruit refuse should be destroyed. (Srivastava and Tandon, 1971).

1.4 Objectives of the study

The main objectives of the study are listed as follows:

- To isolate the pathogen from infected papaya and test its pathogenicity.
- To study and compare the antifungal activities of essential oils and extracts of different aromatic plants *(Mentha arvensis, Cymbopogon flexuosus, Xanthoxylum armatum, Acorus calamus and Cinnamomum tamala)* against the test fungus *Fusarium oxysporum.*

- To determine the minimum inhibitory concentration (MIC) of the essential oils and extracts against the test fungus.

1.5 Justification of the study

Papaya is one of the important edible and commercial fruit of Nepal. Surface rot of papaya caused by *Fusarium oxysporum* mainly effect in storage. The use of plant extract and oil to control surface rot could be one of the cheapest, having no side effect and effective methods of disease management. Plant extracts and oils are less toxic, more systemic (Fawcett and spencer, 1970) easily biodegradable host metabolism stimulatory fungicide (Dixit, 1978) and non toxic to flora and fauna.

1.6 Limitation of the study

- The study was carried out in limited time for one year only.
- Only single pathogen *Fusarium oxysporum* causing surface rot of papaya was selected for the experiment.
- Essential oil and extract of only five plant species were used for assessing their antifungal activities against the test fungus by poisoned food technique.
- The antifungal activity of essential oil and extracts was conducted in lab scale (in vitro) only.

CHAPTER-TWO

2. LITERATURE REVIEW

Antifungal activity of essential oil of *Cinnamomum tamala, Nardostachys grandiflora, Eucalyptus citrodora, Cymbopogon citrates, Menthe arvensis* and *Acorus calamus* were assessed against *Fusarium oxysporum,* the causal agent of dry rot of potato. Out of six test plants, oil of *Eucalyptus citriodora* showed the highest fungitoxicity (100%) at the concentration of 20.0 « lml^{-1} (Poudel, 2009).

Fungitoxicity of essential oil and extract of *Cymbopogon flexuosus, Mentha arvensis, Valeriana jatamansi, Amomum subulatum and Zanthoxylum armatum* were assessed against *Fusarium solani.* The causal agent of *Fusarium* rot of pinted gourd out of five test plants, essential oil and extract of c*ymbopogon flexuosus* was found more effective in its fungitoxic properties (100%) at 5.0 « lml^{-1} and 50 « lml^{-1} (Wagle 2007).

Inhibitory activity of some plant essential oils of *Thymus linearis, Zanthoxylam recurva, Artemisia indica* were assessed against the fungus *Glomerella* cingulata, the causal agents of bitter rot of apple by poisoned food technique. The fungitoxicity was found to be variable with different plant essential oil; *Thymus linerais* and *mentha arvensis* were found more effective in its fungitoxic properties and showed the highest fungitoxicity (100%) at 5000 ppm. (Kuinkel, 2007). Fungitixicity of essential oils of *Thymus linearis, Zanthoxylum armatum, Nardostachys grandiflora, Juniperus recurva, Artemisia dubia, and Artemisia gamelini* were assessed against *Alternaria barssicicola* the causal agents of leaf spot diseases of cabbage by poisoned food technique (Parajuli, 2005). Post harvest disease (sour rot) of tomato and its control by some local plant extracts of *Allium sativvm, Azadirachta indica, Artemisia dubia, Capsiam annum, Eupatorium adenophorum,* the extract of *Allium Sativum*(66.66%), *Artemisia dubia* (83.3%) and *Eupatorium adenophorum* (50%) has shown the highest antifungal activities (Shrestha *et al,* 2005). Assessment for antifungal activities of plant essential oils of *Thymus linearis, Tanacetum gracile, Clinopodium umbrosum* and *Elsholtzia erosotachya, Zanthoxylum armatum* and *Cinnamomum tamala* were used against *Bipolaris sorokininana.* The essential oils of *Thymus linearis* showed highest fungitoxicity (100%) 10000 ppm (Manandhar, 2005). A commonly available lemon plant leaves extract in aqueous or ethanolic extract

preparation and essential oil displayed fungitoxic effect against three destructive fungal pathogen viz, *Pyricularia grisea*, *Aspergillus niger* and *Aspergillus flavus* (Tiwari *et al,* 2004). Essential oil product have a powerful smell because they are highly volatile and many of them vaporize quickly even at room temperature. Fumigants are also volatile, a property that allows them quickly evaporate, leaving no residue. Antifungal activity of the stem distilled fraction of *Artemisia douglasiana* was detected by bioautography on silica gel TLC plates against three *Colletotrichum* sps (Meepagala *et al,* 2003).

The distilled oil was found to be more enriched in monoterpenoid hydrocarbons (Chatopoulou *et al* 2002). Essential oil and several pure sulfur compounds isolated from *Rodopholeus zenkeri* were tested for antifungal activity using a paper disc method, the poisoned food technique, a micro atmosphere technique and the measurement of cellular ATP present (Koukam *et al* 2002). Thirty two essential oils were extracted from higher plants and tested against *Aspergillus flavus, Aspergillus niger, Alternaria alternata* etc. The oil showed 100% inhibition in mycelial growth of *Alternaria alternata* at 600 ppm oil concentration (Kuwar and Tripathi, 2002). The essential oil of *Cinnamomum tamala* in 500ppm and 1000ppm can inhibit 30% are 56.6% of mycelial growth of *Aspergillus flavous* respectively. Similarly the oil can inhibit mycelial growth of *Aspergillus* niger less than *Aspergillus flavus* i.e 23% at 500 ppm and 50% at 1000 ppm (Kuwar and Tripathi, 2002)

The extract of *Thuja, Vinea,* lower dosage of *Cinnamonmm* oil and higher dosage of clove oil exhibited the inhibition of *Fusarium moniliforme* (Rai et al. 2002) Aqueous extract effects of 64 weed species on growth and development of *Alternaria solani, Heminthosporium sativum* and *Rhizo ctonia solani* were studied in vitro contracts varied in the strength and persistence of their antifungal effects against the three fungal species (Qusem and Abublan 1996). On screening the extracts of different parts of 122 higher plants for their volatile antifungal activity against *Aspergillus niger* and *Curvularia uvoides* the lower bud extract of *Ayzygium aromaticum* exhibited absolute toxicity against both the test fungi inhibiting the mycelial growth completely (Khan and Tripathi, 1994).

Antifungl activities of 20 medicinal plants were tested against four pathogenic fungi namely *Curvularia lunata, Cylindrocarpon sp., Fusarium Solani* and *Myrothecium leucotrichum.* Extracts of all the plant species were fungitoxic and showed inhibitory effect on the test fungi (Gourinath and Manoharacharya, 1991). In a preliminary microbiological screening, the essential oils of five sardian plant viz. *Juniperus oxycedrus subsp. oxycedrus, Spartium junceum, Helichysum italium subsp. microphyllum, Inula viscose* and *Asphodlus microcarpu*s showed activity against some species of blastomycetes (Bonsignore et al 1990). The essential oil of *Lippia alba* exhibital absolute inhibition of mycelial growth of *Macrophomina phaseolina* at 2000 ppm following poisoned food technique. The oil was found to be non phytotoxic in nature (Dwivedi & Kishore, 1990).

Eucalyptus oil exhibited absolute mycotoxicity against *Microspornum roseum* and *Trichophyton mentagrophytes* at its minimum inhibitory concentration of 400 ppm against *Pyricularia oryzae* and *Aspergillus niger* (Mishra, et al. 1990). Essential oil from fresh leaves of the nine plants were tested against the fungus *Pyricularia grisea, Aspergillus niger* and *Aspcrgillus flavus*. Essential oil from *Amomun subulatum* exhibited complete inhibition of the fungus at 3000 ppm. Other plants as *Ageratum naustonianum Alpinia galangal, Artemisia indica, Curcuma longa, Elettaria cardanomomum Lippia alba and Salvia pleneiacamomum* were also tested against the fungus. (Mishra and Tiwari, 1990)

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

Antimicrobial activities of some plant products Viz. *Mentha arvensis, Acorus calamus,* and *Zanthoxylum oxyphylum* and turpentine oil were studied against some fungi and a bacteria (Shrestha and Sharma 1988). Leaf extract of 30 species were tested against *Aspergillus flavus*, only the leaf extract of *Anisomeles ovata* showed absolute toxicity (Upadhaya *et al* 1987)

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* 1985). The volatile antifungal action of the leaves of *Melaeuca leucodendeon* was collected as an essential oil by hydrodistilliation in Clevenger's apparatus and evaluated for its activity against *Rhizoctonia solani* (Dubey et al 1983)

Leaves of 25 plant species were screened for their volatile toxicity against the test pathogen of *Fusarium lateritium f.sp. cajani.* Out of the test plant species *Aegle marmelos, Citrus aurantifolia* and *Mentha arvensis var piperascens* 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 *et.al,* 1983).

The essential oil from the seed kernels of *Putranjiva roxburghii* wall was found toxic against *Helminthosporium oryzae*. It was found that *Alternaria brassicae, Alternaria alternata, Alternaria solani* and *Alternaria raphani* were inhibited by 100% at 500 ppm essentia oil concentration (Saxena *et. al,* 1983).

Apples coated with mustard oil, paraffin wax and castor oil checked the infection of a large number of pathogens (Sumbali & Mehrotra, 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 *et al,* 1983 (i)).

The essential oil of the rhizome of *Alpinia galanga* exhibited the highest toxicity to the mycelial growth of *Helminthosporium oryzae.* The volatile oil was fungistatic at MIC of 0.4% in the medium. The oil controlled brown spot disease on detached paddy leaves and had no adverse effect on seed germination and growth of paddy seedling (Tripathi *et al,* 1983 (ii)).

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

Volatile constituents from the root wood of *Cedrus deodara* exhibited strong fungitoxicity against the fungus *Helminthosporium oryzae.* The oil inhibited the mycelial growth of *Alternaria brassicae, Alternaria raphani* and *Alternaria solani* by 100% at 3000 ppm concentration (Dikshit & Dixit 1982).

Hot water dip at 50°c for 5 minutes controlled various post harvest rots caused by *Trechothecium rosuem, Minilinia laxa, Glomerella cingulata Penicillium expansum* (Kaul & Munjal, 1980).

The minimum inhibitory concentration (MIC) of the *Cestrum diurnum* against *Helminthosporium oryzae, Helminthosporium carbonum, Helminthosporium graminium, Helminthosporium maydis,* and *Helminthosporium turcicum* was found at 0.7% (Renu *et al,* 1980).

Papaya (*Carica papaya L.*) is a herbaceous crop. In some parts of the world especially Australia and West Indies, it is known as papaw, or pawpaw, papaye (French), payaya (Spinish). In Brazil the usual name is mamao. It was nicknamed "tree melon" by Europeans (Wikipedia encyclopedia).

The papaya is belived to be native to southern Mexico and neighboring central America. It is now present in every tropical and subtropical country of the world.

It is a large herb reaching 20 -30ft in height with a hellow green or deep purpal stem roughened by leaf scars at the base. The leaves emerge directly from the upper part of the stem in a spiral on horizontal petioles, the blade divided into 5-9 segments and a prominent yellowish ribs and veins. Both stem & leaves contain latex.

The 5 petalled unisexual or bisexual flowers are fleshy, waxy and slightly fragrant. The fruit is melon like oval or club-shaped.

There are several cultivars varieties such as Kamiya, Mexican Red, Mexican Yellow, Solo, Sunrise (sunrise solo), sunset (sunset solo), vista solo Waimanalo etc.

Fruit cultivation plays a great role in the economic development of the country. The natural condition favor the cultivation of several kinds of fruits in Nepal. The total fruit cultivation area is 103652.8 hectares in Nepal. From that area 686213 metric ton fruits are produced. The productivity is 10 metric ton per hector. The cultivation area of papaya is 2763.9 hectors. The total production is 30226.8 metric ton and the productivity is 14.2 metric ton/hector. Terai region is good place for the cultivation of papaya. (Varshik Pragati Bibaran, 2065/066)

Table: Diseases of papaya

S.N.	Diseases	pathogen
1	Bacterial canker	*Erwinia sp.*
2	Bacterial wilt	*Pseudomonas solanacearum*
3	Black rot	*Erwinia cypripedii*
4	Alternaria fruit spot	*Alternaria alternata*
5	Anthracnose	*Colletorichum gloeosporioides*
6	Damping off	*Pythium debaryanum*
7	Dry rot	*Phoma caricoe- papayae*
8	Fusarium fruit rot	*Fusarium sp.*
9	Leaf spot	*Cercospora papayae*
10	Phytopthora blight	*Phytophthora palmivora*
11	Powdery mildew	*Erisiphe sp*
12	Rhizopus soft rot	*Rhizopus stolonifer*
13	Stem rot	*Fusarium solani*
14	Verticillium wilt	*Verticillium dahliae*
15	Wet fruit rot	*Phomopsis sp.*

Source: plant pathology online.

The genus *Fusarium* was erected by link (1809) for species with fusiform, nonseptate spores borne on a stroma and was based on *Fusarium roseum*. The serious wilts, such as panama disease of bananas caused by *F. oxysporum*, are amongst the most devastating plant diseases in the world. The microconidiophore structure provides the easiest means of separating *F. oxysporum* from *F. solani* (Booth, 1997).

The culture of *F. oxysporum* are pale, salmon violet to pale slate, on media. Mycelium is striate, felted to floccose; microconidia always present are unicellular or bicellular, ellipsoidal to allantoid and borne on lateral phialides, macoconidia are falcatre and of the elegans type in *F. oxysporum*. Chlamydospores intercalary or terminal on short lateral branches solitary or in chains, hyaline smooth to rough walled (Booth, 1997).

The surface rot of papaya is caused by *Fusarium oxysporum schlecht* which comes under class deuteromycetes and order moniliales. The fungal colony appears like a white cottony mass. The mycelium is hyaline branched and produces large no of both microconidia and macrocondia on separate phialids or conidiophores. Microcondia are smaller oval single or absent of septa and ranges in size from 13.67-18.31 x 7.00-9.00 « m while macroconidia are larger falcate or sickle shaped, with 3-5 septa, and ranges in size from 30.12-40.10 x 3.98-6.02 « m. In adverse condition the mycelium also produces large number or spherical terminal or intercalary thick walled chlamydospores and ranges in size of 8.12-12.34 « m. in diameter (Booth, 1997).

CHAPTER-THREE

3. MATERIALS AND METHODS

3.1 Materials

Causal organism (*Fusarium oxysporum)* isolated from infected papaya collected from local market of Kathmandu was considered as the material for this research. The different scientific equipments, glassware's and chemicals were used in performing the research work. All materials used are listed in the appendix I.

3.2 Methods

3.2.1 Collection of host and isolation of test fungus

During research period, the infected papaya was collected from market and fungus (*Fusarium oxysporum*) was isolated in pathology laboratory.

3.2.2. Collection of test plant species

During this research period, plant samples of *Cinnamomum tamala* (Buch.Ham.) Necs and Eberm brought from Dhankuta district and *Acorus calamus* L from Dudhpokhari Kathmandu was processed for the extraction of essential oil on laboratory of plant pathology of Central Department of Botany. Remaining three samples of essential oils of *Zanthoxylum armatum*, *Mentha arvensis* and *Cymbopogon flexuosus* were taken from HPPCL. Similarly the crude extracts of aromatic plants were made by grinding on mortar with pistal on the pathology labrotary of CDB. The oils and crude extracts were tested against the fungus *Fusarium oxysporum*. The preliminary antifungal action of oils and extracts was then determined. In case of positive activity, MIC (minimum inhibitory concentration) of all essential oils and extracts was determined against the test fungus.

The collected plant species (*Cinnammum tamala* and *Acorus calamus*) were processed for the hydro distillation of essential oils in the following steps. The collected plants were cleaned and then removal of grasses, mud etc were done. The plants were then spread under the shade in the room temperature and turned up and down to hasten drying. The collected plants were cut into smaller pieces and packed in water proof bags. Then the samples were taken in hydro distillation turn by turn for extraction of essential oils.

3.2.3 Extraction of essential oils and extracts

50 gm of shade dried sample of plant species were surface sterilized with 0.1% of mercuric chloride solution followed by through washing with distilled water. The sample was then pulverized. The hydro distillation of plant samples was carried out for 6-8 hours in clevenger's apparatus in 500 ml water.

The volatile fractions condensed after hydro distillation exhibited two distinct layers an upper aromatic layer of essential oil and a lower colorless aqueous layer. The aromatic layer was collected and dehydrated over anhydrous sodium sulphate and stored at ($<10^{\circ}$c) low temperature (Rao and Srivastava, 1994).

10 gram of fresh plant sample were surface sterilized with 0.1% of mercuric chloride solution followed by through washing with distilled water. Then it was crushed in mortar with pistal. Solution of crushed materials was filtered through muslin cloth and kept in centrifuging machine for three minutes. These extract were treated as pure solution considering as a hundred percent. In this way volume of extract were obtained.

3.2.4. Determination of yield of essential oils

Hydro distillation of fifty gram of each dried plant sample was done for extraction of essential oil. The volume of oil extracted was noted down. The yield of the essential oil was calculated in terms of % using following formula.

$$\% \text{ of essential oil} = \frac{\text{Volume of essential oil}}{\text{Weight of sample}} \text{ X } 100$$

a. *Cinnamomum tamala*

$$\text{Yield of essential oil} = \frac{\text{Volume of essential oil}}{\text{Weight of sample}} \text{ X } 100$$

$$= \frac{0.5}{50} \times 100\%$$

$$= 1\%$$

In the same way the essential oil content of *Acorus calamus* is found to be 1%

3.2.5 Dilution of essential oils and crudes extract

Essential oils were diluted into different concentration with 80% acetone. The oils were diluted into 0.625, 1.25, 2.5, 5.0 10.0, 20.0, 40.0 «*l* ml^{-1}. Each crude extracts were diluted into different concentrations of 0.625, 1.25, 2.5, 5.0, 10.0 , 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 «*l* ml^{-1} with distilled water. Then different concentrations of each oil and extract was labeled and stored at low temperature (<10 $^{\circ}$ c).

3.2.6 Media preparation

Potato Dextrose Agar (PDA) medium was used for the experiment. For preparing 1000ml of PDA media, the following ingredients are required in following amount.

Potato - 200gm Distilled water - 1 liter

Dextrose - 20gm

Agar - 20gm

200gm of peeled potato was washed with clean water. Then cut into small pieces. These pieces were boiled in about 500 ml of water till the pieces were soft to touch. The juice of boiled potato was filtered by the help of muslin cloth on conical flask and added required amount of Agar and Dextrose and shaked the solution finally the volume in total was made to 1000ml by adding distilled water, then PDA media was autoclaved to get sterilized.

3.2.7 Isolation and Identification of the test fungus (*Fusarium oxysporum*)

Infected papaya were collected from the local markets of Kathmandu Valley. Some pieces of fungal colony from the infected papaya was transferred aseptically on a petri plate containing PDA for one week. After one week the growth of fungal colony were observed in petri plate and colony of the culture was observed under the compound microscope and studied the characteristics of the pathogen. The characteristic features of the fungus which resembled with the *Fusarium oxysporum* were identified with the help of standard literature (Booth, 1997).

3.2.8 Maintenance of the pure culture

The pure culture of *Fusarium oxysporum* was preserved by the sub culturing in several plants and plates containing PDA media.

3.2.9 Preparation of one week old culture

For testing the antifungal activity of the essential oil and extract inoculum disc from one week old culture is required. For preparation of one week culture, the fungus from pure culture was inoculated into PDA and after seven days the inoculum disc was taken from the culture for further experiment.

3.2.10 Assessment of toxicity of oil and extract against Fungal pathogen

The toxicity of the oil and extract was assessed by using the poisoned food technique (Grover and Moore, 1962).

Poisoned food technique

It is the way of testing the antifungal efficacy of oil and extract by poisoning the media with oil and extract.

In this technique, 0.5 ml of each concentration of oil and extract was taken in presterilized cooled petriplate and 9.5 ml of PDA media was poured on that and gently swirled to mix the contents thoroughly. In control set the essential oil was replaced by equal volume of Acetone (80%) and the control set of crude extract was replaced by equal volume of distilled water. The inoculum disc (4mm in diameter) taken from the periphery of 7 days old culture of the test pathogen was placed aseptically in the centre of each plate and turned upside down in its position. The plates were then incubated at $25 \pm 2^{\circ}$c for 7 days. All experiments were revised thrice. Diameter of fungal colony of treatment and control sets were measured in the mutually perpendicular directions on the 7 days. The percentage inhibition of mycelial growth of test fungus was calculated separately. (Grover and Moore, 1962)

3.2.11 Calculation of percentage of mycelial growth inhibition

Fungitoxicity was assessed in triplicates in each concentration of essential oils and extracts fungitoxicity was assessed in terms of percentage inhibition of mycelial growth of test fungus (Rao and Srivastava, 1994).

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

Where, gc-growth of mycelial colony after incubation in control set i.e. diameter of colony in control set - diameter of inoculum disc.

gt- growth of mycelial colony after incubation period in treatment set i.e. diameter of the colony in treatment set - diameter of inoculum disc.

3.2.12 Determination of minimum inhibitory concentration (MIC) of essential oil and extract

The minimum inhibitory concentration (MIC) is the concentration of any substance in the external medium which just inhibits cell division of a normal cell.

MIC has been expressed as the minimum dose of the essential oil and extract required for complete (100%) inhibition of mycelial growth of the test fungus (Rao and srivastava, 1994)

3.2.13 Pathogenicity Test

For carrying out the pathogenecity test, the infected fruits of papaya were collected and symptoms were noted down, the *Fusarium oxysporum* was isolated in PDA media as pure culture. Inoculum from the pure culture was transferred to the healthy fruits. When incubated at $25 \pm 2^{\circ}$c for 7 days the characterstics symptoms were produced which were found to be similar with the symptoms on fruit previously collected. The fungus was isolated and its character was compared with previously isolated fungus.

3.2.14 Identification of the fungus

From the pathogenicity test, the disease was proved to be caused by *Fusarium oxysporum*. The characteristics features of the fungus were identified with the help of standard literature (Booth, 1997)

3.2.15 Measurement of Conidia Size

Ocular Micrometer was placed inside the eye piece of a compound micros cope and calibrated by superimposing the gradations of stage micrometer. The number of division of ocular micrometer coinciding with the number of divisions in stage micromerter was noted down and calculated the calibration factor applying the formula.

$$\text{One ocular division} = \frac{\text{No of division of stage micrometer}}{\text{No of division of ocular micrometer}} \text{ X } 10 \text{ « m}$$

Then, the stage micrometer was replaced by a five slide of *Fusarium oxysporum* mounted on lactophenol. Size of the macro, micro conidia and *Chlamydospore* were measured by determining the number of divisions of ocular micrometer (Appendix-II). In each measurement was carried out to find the range of size of the conidia.

3.2.16 Photography

The photo of infected material (fruit) and fungal structures were taken. Besides inhibitions of mycelial colony in different concentration by different essential oils and extracts were also subjected to photography.

CHAPTER - FOUR

4. RESULT

4.1 Isolation of test pathogen

First the infected papaya were collected from the local market of Kathmandu. By the help of sterilized needles and forceps, some pieces o fungal colony from infectd papaya was transferred aseptically on a petriplate containing PDA media. After one week the culture grown were sub-cultured again and again till the pure culture is obtained.

The colony of pure culture of test pathogen isolated in PDA media was white colony which later converts into grey to brown in colour. The diameter of colony reached about 52.50mm after 7 days at 25^0c. Conidia are of two types smaller oval shaped microconidia and larger sickle shaped with 3-5 septa. macroconidia.

The size of microconidie was found 13.67-18.31 x 7.00-9.00 « m and macroconidia was found 30.12-40.10x 3.98-6.02 « m which is comparatively slightly longer than size of conedia described by c. booth. (1997)

Table: Measurement of diameter of colony of test fungaus after 7 days.

S.N.	diameter of colony (mm)	mean diameter (mm)
1	45mm	52.50mm
2	60mm	

4.2 Pathogenicity Test

For carrying out the pathogenecity test, the infected fruits of papaya were collected and symptoms were noted down, the *Fusarium oxysporum* was isolated in PDA media as pure culture. Inoculum from the pure culture was transferred to the healthy fruits. When incubated at $25 \pm 2^{\circ}$c for 7 days the characterstics symptoms were produced which were

found to be similar with the symptoms on fruit previously collected. The fungus was isolated and its character was compared with previously isolated fungus.

4.3 Assessment of fungitoxicity of essential oil and extracts

Fungitoxicity of each essential oils and extracts was assessed in different concentrations against *Fusarium oxysporum* by poisoned food technique. Essential oils and extracts of different plant species showed different efficacies in inhibition of the mycelial growth.

Antifungal activity of different oils and extracts against test Fungus

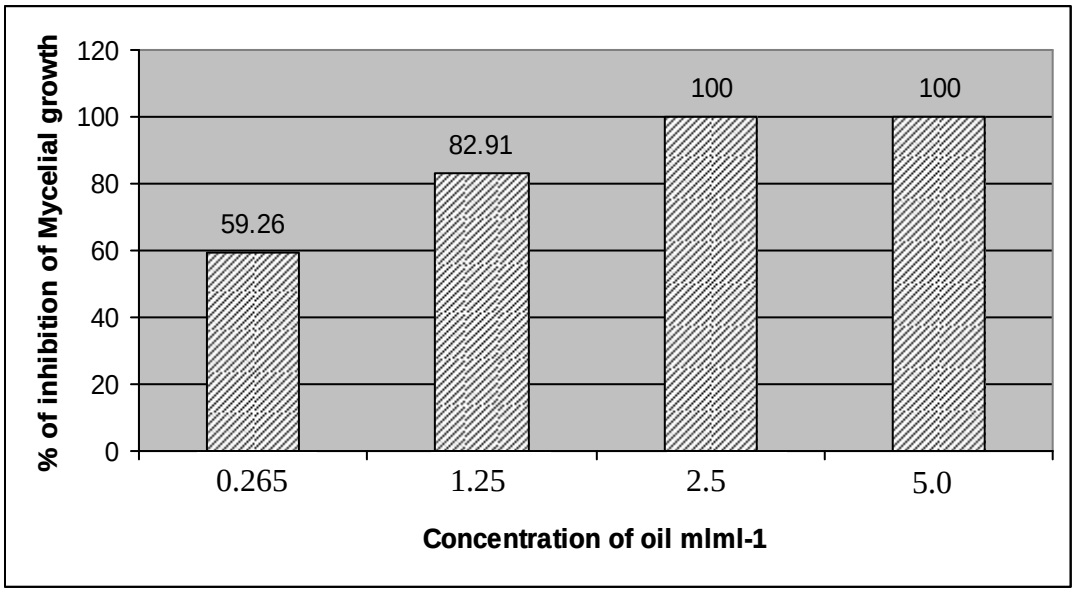

Fig. 1 Percentage of antifungal activity of *Cymbopogon flexuosus* oil against *Fusarium oxysporum*.

Minimum inhibitory concentration (MIC) = 2.5 « l/ml

Essential oil of *Cymbopogon flexuosus* showed mycelial inhibition as 0%, 59.26%, 82.97%, 100% and 100% at 0.625, 1.25, 2.5 and 5.0 « lml^{-1} oil concentration against *F. oxysporum* respectively.

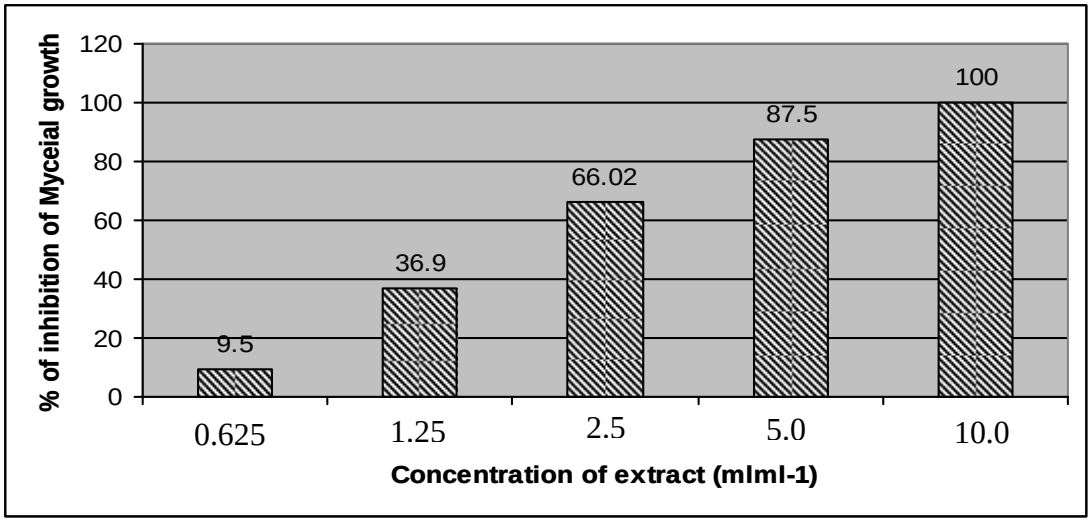

Fig - 2 Percentage of antifungal activity of *Cymbopogon flexuosus* extract against *Fusarium oxyporum*

Minimum inhibitory concentration (MIC) = 10 « lml^{-1}

Extract of *Cymbopogon flexuosus* showed myclial inhibition as 0% , 9.5%, 36.9%, 66.07%, 87.5%, 100% and 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 « lml^{-1} extract concentration against *Fusarium oxysporum* respectively.

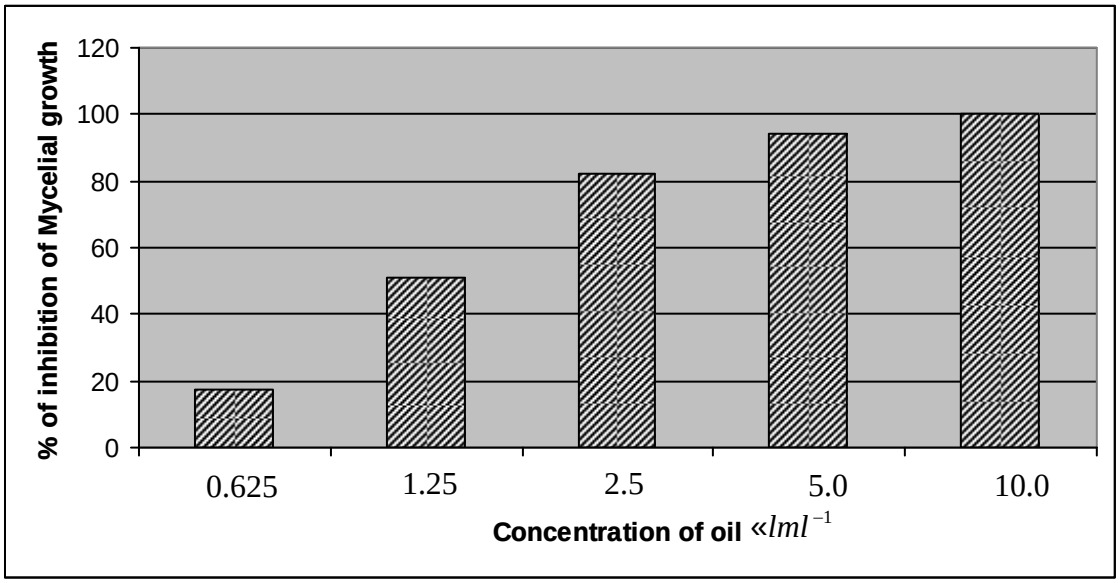

Fig - 3: Percentage of antifungal activity of *Mentha arvensis* oil against *Fusarium oxysporum*.

Minimum inhibitory concentration (MIC) = 10 « lml^{-1}

Essential oil of *Mentha arvensis* showed mycelial inhibition as 0%, 17.07%, 51.12%, 82.12% 94.31% and 100% at 0, 0.625, 1.25, 2.5, 5.0 and 10.0 « lml^{-1} oil concentrations against *Fusarium oxysporum* respectively.

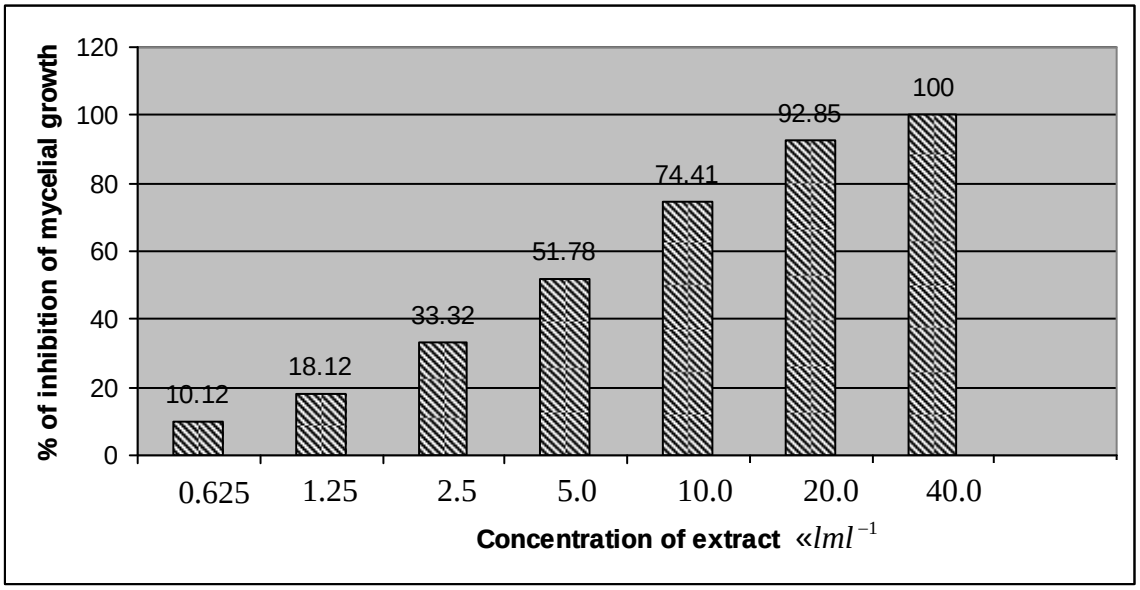

Fig - 4: Percentage of antifungal activity of *Mentha arvensis* extract against *F. oxysporum.*

Minimum inhibitory concentration (MIC) = 40 « lml^{-1}

Extract of *Mentha arvensis* showed mycelial inhibition as 0%, 10.12%, 18.12%, 33.92%, 51.78%, 74.41%, 92.85%, and 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20, 40 « lml^{-1} extract concentration against *F. oxysporum* respectively.

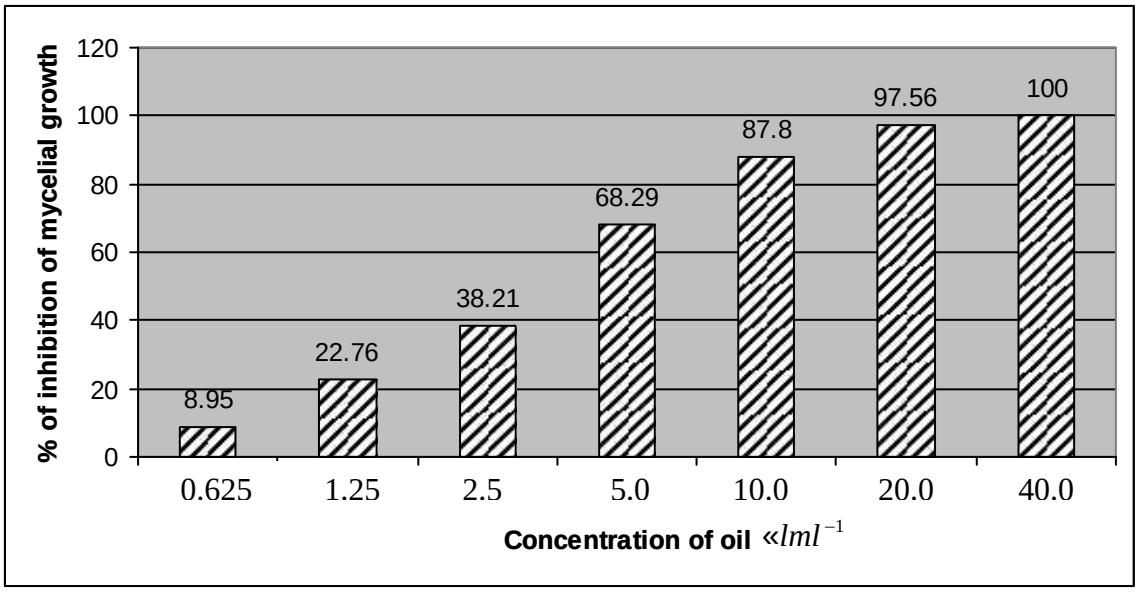

Fig-5: Percentage of antifungal activity of *Acorus calamus* oil against *F. oxysporum.* Minimum inhibitory concentration (MIC) = 40 « ml^{-1}

Essential oil of *Acorus Calamus* showed mycelia inhibition as 0%, 8.95%, 22.78%, 38.21%, 68.29%, 87.80%, 97.56% and 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 Ml ml-1 oil concentration against *F. oxysporum* respectively.

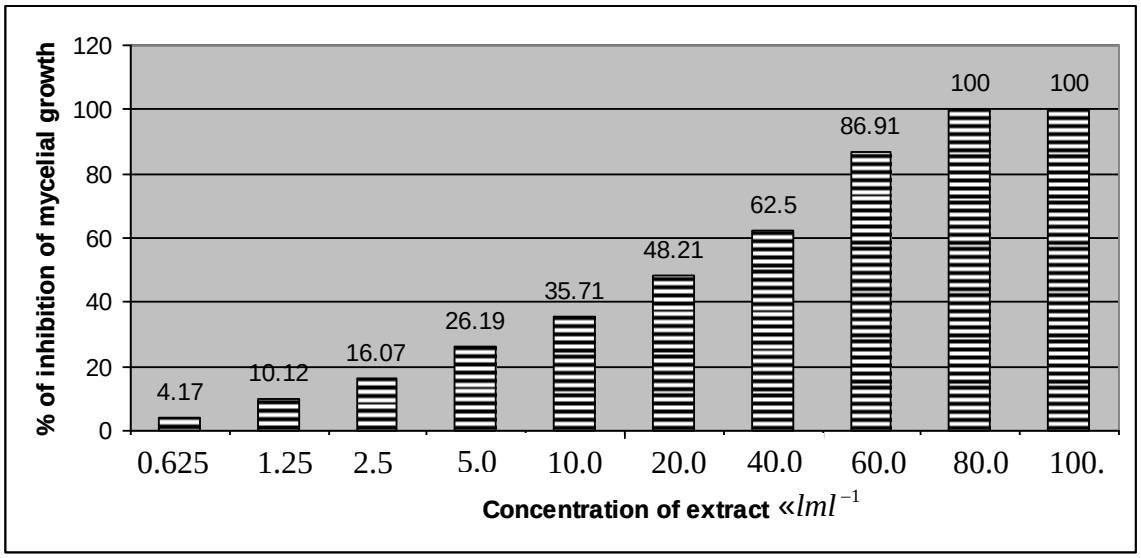

Fig - 6: Percentage of antifungal activity of *Acorus calamus* extract against *F. oxysporum.*

Minimum inhibitory concentration (MIC) = 80 « l ml^{-1}

Extract of *Acorus calamus* showed my celial inhibition as o%, 4.17%, 10.12%, 16.07%, 26.19%, 35.71%, 48.21%, 62.5%, 86.91%, 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80, « lml^{-1} extract concentration against *F. oxysprum* respectively.

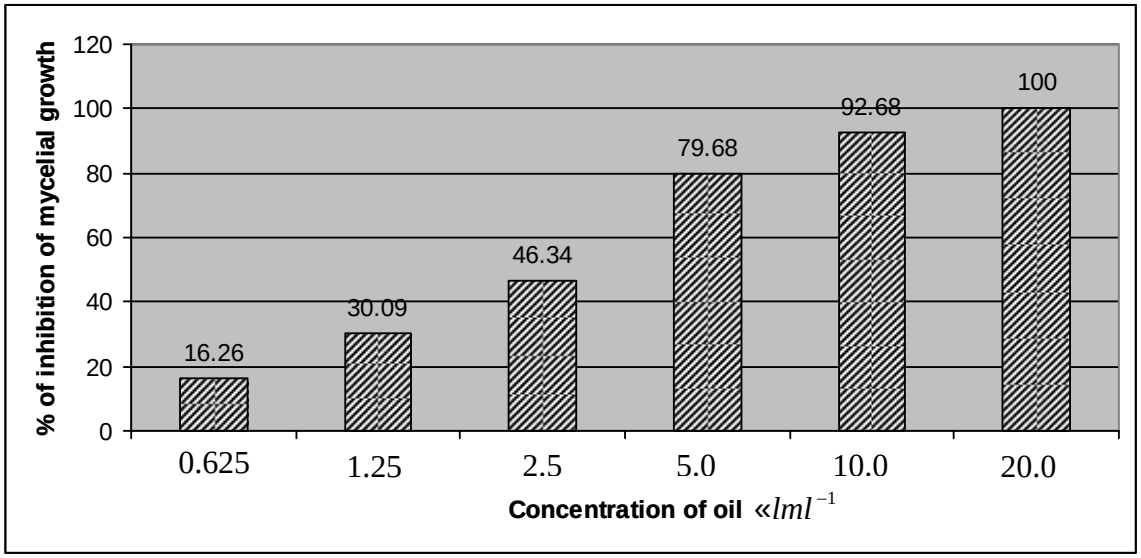

Fig - 7: Percentage of antifungal activity of *Xanthoxylum armatum* oil against *Fusarium oxysporum.*

Minimum inhibitory concentration (MIC) = 20 « lml^{-1}

Essential oil of *Xanthoxylum armatum* showed mycelial inhibition as 0%, 16.26%, 30.09%, 46.34%, 79.68%, 92.68% and 100% at o.625, 1.25, 2.5, 5.0, 10.0 20.0 « lml^{-1} oil concentrations against *Fusarium oxysporum* respectively.

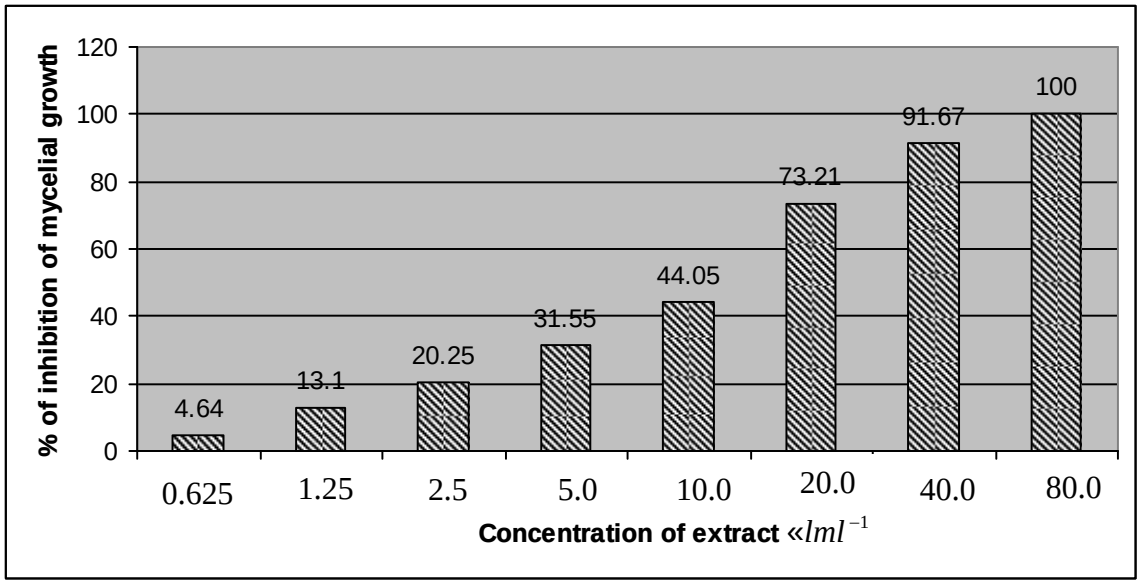

Fig - 8: Percentage of antifungal activity of *Xanthoxylum armatum* extract against *Fusamum oxysporum.*
Minimum inhibitory concentration (MIC) = 80.0 « lml-1

Extract of *Xanthoxylum armatum* showed mycelial inhibition as 0%,4.64%, 13.10%, 20.25%, 31.55%, 44.05%, 73.21%, 91.67%, 100%,at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 mlml-1 extract concentration against *F. oxysporum* respectively.

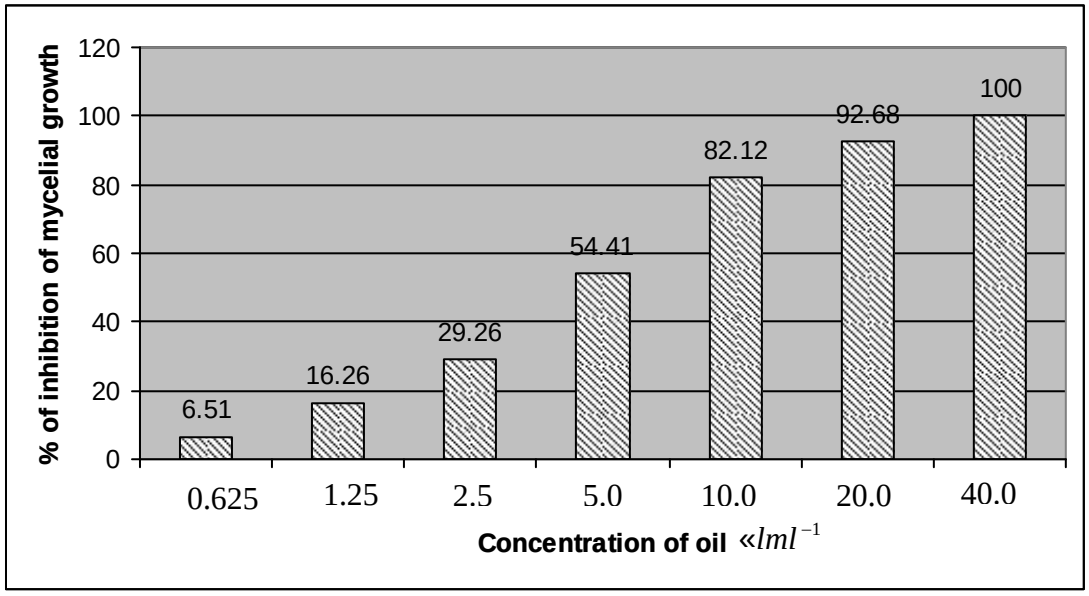

Fig - 9: Percentage of antifungal activity of *Cinnamomum tamala* oil against *Fusarium oxysporum.*

Minimum inhibitory concentration (MIC) = 40.0 « lml-1

Essential oil of *Cinnamomum tamala* showed mycelial inhibition as 0%, 6.51%, 16.26%, 29.26%, 50.41%, 82.12%, 92.68%, and 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0 « lml^{-1} oil concentration against *Fusarium oxysporum* respectively.

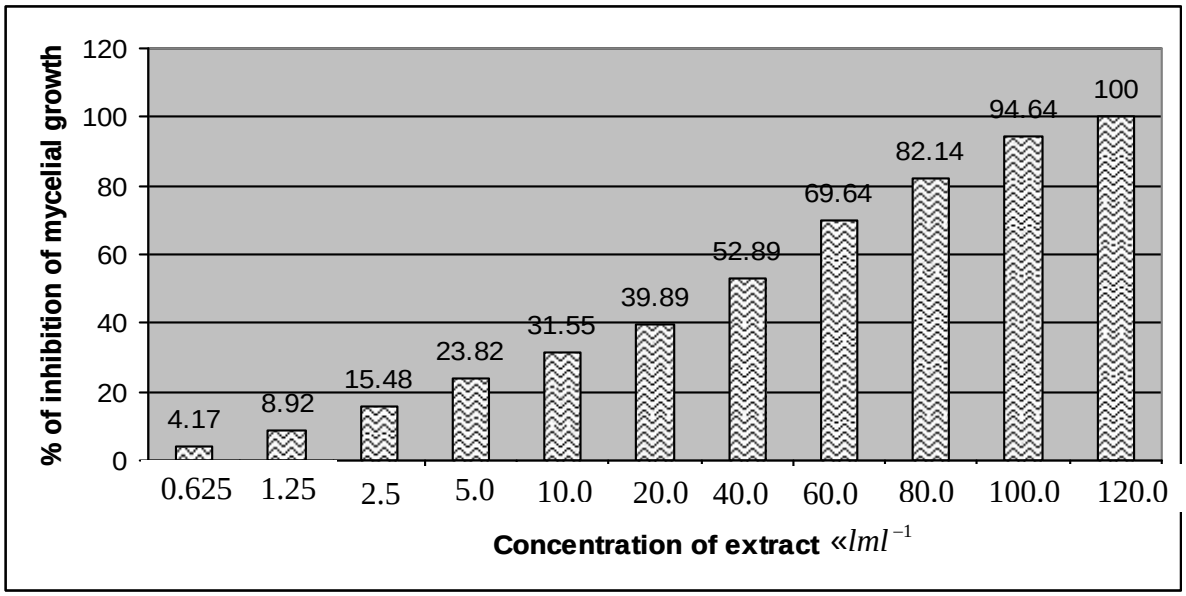

Fig 10: Percentage of antifungal activity of *Cinnamomum tamala* extract against *Fusarium oxysporum.*

Minimum inhibitory concentration (MIC) = 40.0 « lml^{-1}

Extract of *Cinnamomum tamala* showed mycelial inhibition as 0%, 4.17%, 15.48%, 23.82%, 31.55%, 39.89%, 52.98%, 69.64%, 82.14%, 94.64%, and 100% at 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0,80.0,100.0 and120.0 « lml^{-1} extract concentration against *Fusarium oxysporum* respectively.

RESULT

Minimum inhibitory concentration (MIC) of oils and extracts of 5 aromatic plants (*Cymbopogon flexuosus, Mentha arvensis, Acorus calamus, Xanthoxylum armatum and Cinnamomum tamala*) were achieved through poisoned food technique.

Table 11: MIC of Essential Oils and Extracts

S.N	Aromatic plants	MIC (« lml-1)	
		Oil	**Extract**
1.	*Cymbopogon flexuosus*	2.5	10
2.	*Zanthoxylum armatum*	20	60
3.	*Acorus calamus*	40	80
4.	*Mentha arvensis*	10	40
5.	*Cinnamomum tamala*	40	120

All the five oils and extracts obtained from five aromatic plants showed positive results in inhibiting the mycelial growth of *Fusarium oxysporum.* All the essential oils and extracts showed their different potential of fungitoxicity.

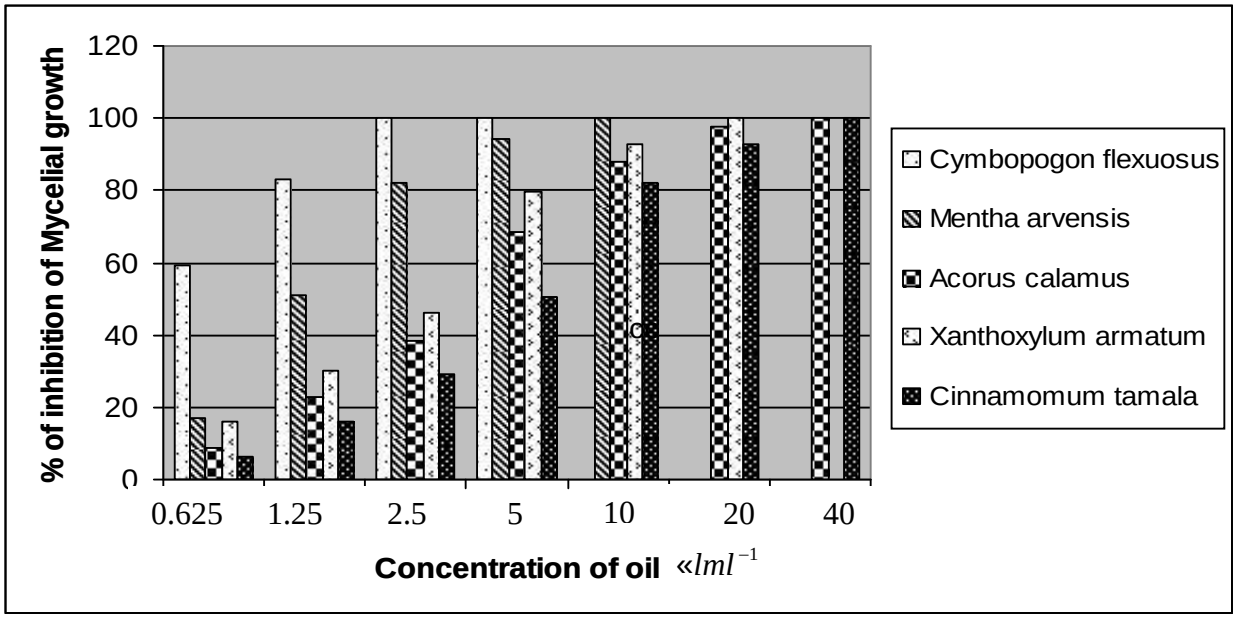

Fig:11 Fungitoxicities of different essential oils in different concentration

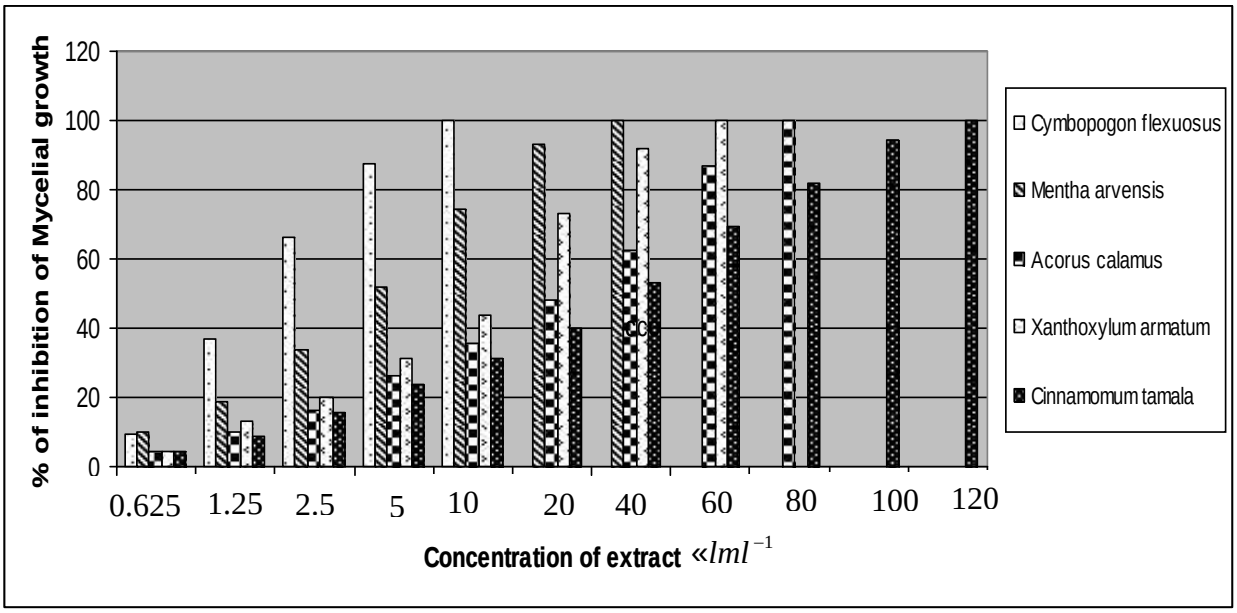

Fig: 12 Fungitoxicities of different extracts in different concentrations

CHAPTER-FIVE

5. DISCUSSION

In the present dissertation an attempt was made to study about the surface rot of papaya caused by *Fuasarium oxysporum*, its pathogenicity test and to observes its control measures by using different concentration of oils and extract of 5 different aromatic plants.

The isolated pathogen was identified with the help of standard literature C. Booth, (1997) and under guidance of professors and teachers of Central Department of Botany, NARC and CHC. The isolated pathogen showed similar characteris described by C. Booth (1997) However the size of conidia of the pathogen was found slightly larger than the size of conidia given by C. Booth (1997). The reasons may be due to the variable places and climate.

The pathogencity test was conducting according to kochs postulates. This test is one of the basic method for confirmation the test pathogen to cause the surface rot of papaya the symptoms appeared on inoculated fruit were similar as the symptoms those observed originally on the papaya fruit.

The present study has been carried out to isolate *Fusarium oxysporum* the causal agent of surface rot of papaya and to control the fungus using essential oils and extracts of 5 different aromatic plants e.g; *Cymbopogon flexuosus, Mentha arvensis, Acorus calamus, Xanthoxylum armatum* and *Cinnamomum tamala.* The main objective of the study is to calculate the comparative fungitoxicitics of the essential oils and extracts of the test plant species against *Fusarium oxysporum* and to find out the minimum inhibitory concentration (MIC) of each oil and extract against the test fungus.

Since the surface rot is an important disease of papaya. It should be protected from fungal pathogen *Fusarium oxysporum* by biological method with an aim to control with no side effects. The study of antifungal efficacy of *Cymbopogon flexuosus, Mentha arvensis, Acorus calamus, Xanthoxylum armatum* and *Cinnamomum tamala* was carried out in vitro.

The difference in fungitoxicity at same concentration in different essential oil and extracts may be due to different chemical composition of the oils and extracts cited in Rao and Srivastava (1994).

Cymbopogon flexuosus showed highest antifungal activity followed by *Mentha arvensis*, *Xanthoxylum armatum, Acorus calamus* and *Cinnamomum tamala* as shown in table 11. Also the essential oil showed more fungitoxic effect as compared to extract of each aromatic plant at the same concentration.

In comparison with other researchers the MIC of oils and extracts of *C. flexuosus* *M. arvensis* and *X. armatum* are 5 and 50 « lml^{-1}, 10 and 100 « lml^{-1} and 10 and 100 « lml^{-1} respectively (Wagle, 2007). The MIC of oils of *M. arvensis* and *X. armatum* are 5 and 12.5 « lml^{-1} respectively (Kuinkel, 2007). The MIC of oils of *C. flexuosus* and *A. calamus* are 40 and 40 « lml^{-1} respectively (Poudel, 2009). The MIC of oils of *X. armatum* and *C. tamala* are 40 and 40 « lml^{-1} respectively (Manandhar, 2005).

CHAPTER-SIX

6. CONCLUSION

From this research it can be concluded that the test plants showed the fungitoxic effect that inhibits the mycelial growth of *Fusarium oxysporum.* Out of 5 test plants the essential oil and extract of *Cymbopogon flexuosus* were found more effective in its fungitoxic properties, and showed 100% inhibition of mycelial growth of the test fungus at 2.5 and 10 « lml-1 respectively.

Similarly, *Mentha arvensis, Xanthoxylum armatum, Acorus calamus* and *Cinnamomum tamala* were found effective and showed 100% mycelial growth inhibition at 10 « lml^{-1}, 20 « lml^{-1}, 40 « lml^{-1} and 40 « lml^{-1}oil concentration and at 40 « lml^{-1}, 60 « lml^{-1}, 80 « lml^{-1} and 120 « lml^{-1} extract concentration respectively.

A perusal literature showed that the similar experiment for the control of *Fusarium oxysporum* using essential oils and extracts has not yet been done in Nepal. So this is the first study to assess fungitoxicities of essential oils and extract against *Fusarium oxysporum*.

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 a number of physical factors like temperature, ph, photolysis of constituents, buffer, solubility etc. Hence a rigorous study in the commercial field is recommended.

The surface sterilization of fruit should be done before or in the storage.

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APPENDIX-1

Materials Used For the Study

Apparatus and Equipment:

Test tubes	Culture tubes
Funnel	Pipettes
Conical flasks	Glass rod
Petri dishes	Inoculating needles
Ocular micrometer	Glass slides
Inoculating loop	Forceps
Scissors	Burner
Beakers	Cover slip
Muslin cloth	Cotton
Measuring cylinder	Borers
Plastic Containers	Sprit lamp
Laminar airflow	Microscope
Hot air oven	Incubator
Refrigerator	Autoclave
Photographic camera	Balances

Aromatic Plant Samples:

a. Zanthoxylum armatum Dc.

Family: Rutaceae

Nepali name: Timur

Description:

Spinuous shrub or small tree with corkybark, up to 3m. High, with pinnately compound leaves, leaflets elliptic lanceolate with small yellow flower in short bnanched lateral cluster (Rajbhanlari, 2001) Flowers commonly 1mm. Unisexed, calyx with 6-8 acute lobes, petal absent, stamen 6-8, much larger than calyx in male flower, leaf stalk narrowly winged, leaf 2-6 pairs about toothed, sparsely gland dotted ripe capsule, 3-4 mm gobular red wrinkled aromatic seed, shining black (Plounin and Stainton, 1984)

Distribution:

Western, Central and Eastern Nepal, attitude 1100-2500m. Himalaya (Kashmir to Bhutan), North India to china, Taiwan, Philippines (press *et al* 2000)

Parts Used

Fruit

Uses:

Decoction of fruit is used in cold treat constipation and as anthelmintic for cattles. Seeds are extremely used in preparation of tooth powder. Seeds are used as a spice and treat stomach disorder. Fruits were taken to cure in digestion. Powdered Fruit is taken internally as stigmachic and also used curry as well as antimicrobial spice in mushroom cooking Bark is used as fish poison (Acharya, 1996; Bhattrai 1990; Parajuli, 2000)

b. Mentha arvensis L.

Family: Labiatae

Nepali name: Pudina

Description

Aromatic herbs, strongly scented, erect or diffuse herb, root stock creeping.Leaves nearly sessile, lanceolate, ovate and pubscent.

Flower small, in large whorls crowed in axillarly and terminal cylindrical tapering spikes, calyx, hairy, bell shaped, actually 5 toothed, corolla- tube in the calyx limb, erect, 4lobes, lobes nearly equal stamen 4, protruding filament nacked.

Distribution:

Central Nepal, alt 1200-2000m. Europe, west to china, North America, Artic to Himalayas, south east Asia to Hawaii and Australia, throughout Africa, cosmopolitan (Press et.al 2000)

Partsused

Leaves

Uses:

Flavoring of food, essential oil used in perfumery pharmacy and ornamental (Stainton, 1984)

c. Cymbopogon flexusous sheud

Family: Graminae

Nepali name: Kagati ghans

Description:

Aromatic herb, odour same as lemon so called lemon grass, average 3m in height, bushy, leaf 125 cm long and 9.7 cm broad, scrate, small hairy thin long.

Distribution

Found in 150-1500m altitude in Nepal. Also found in west Indies, India, china, Bengal etc.

Parts Used

Including leaves all the parts of above ground

Use:

It is used as grasses. For making herbal tea. For making soap, sweets, perfume. For killing pests. To cure eye disease and also oil can be obtaind after distillation process from leaf. Leaf contains 80% citrol.

d. Acorus calamus Linnaeus

Family : Acoraceae

Nep. Name : Bojo

Description :

Calamus is a hardy, perennial herb of marshy places, 6 feet tall, aromatic, rhizome stout, pinkish, leaves $\frac{3}{4}$ inch wide a prominent midrib, spadix stout to 4 inches long.

Distribution: It is found near swamps and banks in Europe, Asia, and North America.

Uses:

The root is antifungul, antiperiodic, anti-rheumatic, antispasmodic, aromatic, cardiac, carminative, diaphoretic, febrifuge, stimulant, stomachic, tonic and vermifuge. It is also used internally in the treatment of digestive problems gastralgia and diarrhea, cough, bronchial asthma, depression and epilepsy (Manandhar, 2002).

e. Cinnamomum tamala (Buc- Ham) Nees and Eberm.

Family : Lauraceae

Nepali name -Tej pat

Description :

A moderate size evergreen tree with ovate- lanceolate long-pointed leathery leaves, leaves 10-17cm long, glabrous opposite and with pale yellowish flowers in terminal and axillary flowers are 3 nerved and small, unisexual, perianth 7 mm long with oblong lobes and a short tube, silky- aired, fertile stamens 9, ovary hairy with slender style, leaves short- stalked glaucous beneath with 3 conspicuous nearly parallel veins arising from near the base, the leaf-tip often curved leaves bright pink when young in spring, aromatic when crushed. Fruit black,succulent, ovoid 12mm.

Distribution :

WEC between 1000-2100m in Mahabharat region of Nepal. Kashmir to Bhutan, Burma, North India, East to china. Taiwan, Philippines, lesser Sunda Islands, found in forests.

Uses :

Astringent, stimulant and carminative, find application in rheumatism colic, diarrhoed and scorpion-sting.

The Test Fungus:

Fusarium oxysporum isolated from infected papaya.

Chemicals and Reagent

Distilled water	80% Acetone
Cotton blue	Lacto phenol
Potatoes	Dextrose
Agar	Sprit
Ice	0.1% Mercuric chloride

Anhydrous sodium sulphate etc.

Apparatus used in oil and extract extraction:

For Oil :	**For Extract**
Clevenger's apparatus	Mortar
Round bottom flask	Pistal
Condenser	Test tubes
Ice	Centrifuge
Bucket	Muslin cloth

Appendix-2

Micrometry

In stage micrometer, 100divisions= 1000 « m or, 1 divisions = $\frac{1000}{100}$ = 10 « m

45 divisions of ocular micrometer coincided with 70 divisions of stage micrometer.

i.e. 45 divisions of ocular micrometer = 70 divisions of stage micrometer.

1 divisions of ocular micrometer = $\frac{70}{45}$ division of stage micrometer

Or, 1 division of 0.M = $\frac{70}{45} \times 10$

= 15.55 « m

Measurement of Macroconidia

S.N	Length (« m)	Breadth (« m)
1	30.12	3.98
2	32.0	4.01
3	35.0	6.02
4	31.5	4.21
5	36.2	5.0
6	40.1	5.51
7	37.3	4.00
8	39.0	5.31
9	33.4	4.82
10	38.1	6.02

Measurement of Microconidia

S.N	Length (« m)	Breadth (« m)
1	15.55	7.00
2	13.67	7.68
3	14.68	8.55
4	17.21	7.98
5	15.56	7.12
6	13.67	9.00
7	18.31	9.00
8	14.02	7.12
9	15.55	8.00
10	16.31	8.34

Measurement of Chlamydospore:

S.N	Diameter (« m)
1	10.1
2	8.12
3	9.41
4	11.21
5	12.34
6	11.55

Appendix - 3

1 « lL^{-1} = 1 ppm
1 « lml^{-1} = 1000 ppm

1 ppm concentration of solution means,

10,00,000ml of acetone contains = 1mlof oil

For, 10,000 ppm,

10,00,000ml of aceeton contains: 10,000ml oil

or, 100ml of aceeton contains: 1ml oil

1ml oil +100ml acetore = 101ml (stock solution)

or, 0.1ml oil +10ml acetone = 10.1 ml (stock solution)

For, 5,000 ppm / 5 « lml^{-1}

5ml of stock solution (10,000 ppm) + 5ml of acetone = 10 ml (stock solution)

For 2500 ppm / 2.5 « lml^{-1}

5ml of stock solution (5000 ppm) + 5ml acetone = 10 ml (stock solution)

For 1250 ppm / 1.25 « lml^{-1}

5ml of stock solution (2500 ppm) + 5ml acetone = 10 ml (stock solution)

For 625 ppm / 0.625 « lml^{-1}

5ml of stock solution (1250 ppm) + 5ml acetone = 10 ml (stock solution)