Antifungal Activity of Some Plant Essential Oils against *Alternaria brassicicola* (Schw.) Wiltshire

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By

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RECOMMENDATION

This is to certify that Ms. Ritu Gautam has carried out the dissertation work entitled "**Antifungal Activity of Some Plant Essential Oils against *Alternaria brassicicola* (Schw.) Wiltshire**", under my supervision. The entire work is based on the collection of primary data by the student. This result has not been submitted elsewhere for any other academic degree. I, therefore recommend this dissertation for the partial fulfillment of Master's Degree in Botany from Tribhuvan University, Nepal.

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LETTER OF APPROVAL

This is to certify that the dissertation work entitled **Antifungal Activity of Some Plant Essential Oils against *Alternaria brassicicola* (Schw.) Wiltshire** submitted by Ms. Ritu Gautam has been accepted as a partial fulfillment of the requirement for Master of Science in Botany.

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…….

Ritu Gautam

ABSTRACT

The essential oil of six medicinal plants viz. *Zanthoxylum armatum* (DC), *Juniperus recurva* Buch-Ham.ex.D.Don, *Cymbopogan martini* (Roxb) W. Watson, *Cymbopogan citratus* (DC.)Tra, *Mentha arvensis* L. *and Gaultheria fragrantissima* Wall. were assessed in vitro for antifungal activity against, *Alternaria brassicicola*: the causal organism of leaf spot of cauliflower. Pathogenecity test was conformed by inoculating the Pathogen into healthy cauliflower leaf. The assessment of fungitoxicity was carried out by poisoned food technique using five different concentration (2.5 μlml^{-1}, 5 μlml^{-1}, 10 μlml^{-1}, 20 μlml^{-1}, 40 μlml^{-1}) against the test fungi in terms of mycelial growth inhibition. Among the choosen oil samples, oils of *Mentha arvensis, Cymbopogan martini* and *Cymbopogan citratus* were found able to inhibit the mycelial growth of *Alternaria brassicicola* completely.

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ABBERVATIONS

cm	-	Centimeter
m	-	Meter
mm	-	Millimeter
gm	-	Gram
mt	-	Metric Ton
µm	-	Micrometer
^{0}C	-	Degree Celsius
GC	-	Gas chromatography
TLC	-	Thin layer chromatography
CDB	-	Central Department of Botany
PDA	-	Potato Dextrose Agar
MIC	-	Minimal inhibitory concentration
HPPCL	-	Herbs Production and Processing Company Limited
µlml^{-1}	-	Micro-liter per Milliliter
Kcal	-	Kilo calorie
Ha	-	Hectare

CHAPTER-1

1. INTRODUCTION

1.1 Background

Nepal is an agricultural country and more than 80% of the economically active workers in Nepal are involved in agriculture. The farming system is complex and diversified. The agricultural sector has dominated the Nepalese economy for a long time and will continue to be a major source of livelihood for the majority of the farmers. Agriculture is the main occupation in the rural economy. However, agricultural productivity is decreasing each year due to a number of factors. Among them insects, pests and diseases are considered to be the main crop yield depletion factors. Vegetables belonging to family brassicaceae, cucurbitaceae and solanaceae are important due to their nutritional as well as economical values. However, the farmers face losses of heavy yields both in their quality and quantity of these crops due to various diseases. Leaf spot diseases caused by fungal pathogen *Alternaria* spp. inflict serious damage to these crops. Diseases are important factors limiting the production of leafy green vegetables and mainly cause damage by reducing crop quality. Anne (1998) reported *Brassica* crop yields are reduced worldwide by leaf spot (*Alternaria brassicicola* (Schw.) Wiltshire) and black rot (*Xanthomonas campestris* var. *campestris*).

Plant disease is a complex phenomenon and is an interaction among the host, parasite and the environment. The crop protection against pathogen, pest and weeds is a major necessity in our agriculture and forestry. Fungal diseases of plants are primarily controlled by the application of fungicides. It is not an eco-friendly approach to use synthetic fungicides as many of them are reported to have carcinogenic, teratogenic, oncogenic and genotoxic properties (Dalvi and Whittiker, 1995; Hussain *et al*., 2011). Many of these fungicides are bio-hazardous and adversely affect the components of ecosystem. Further, the cost of these fungicides and antibiotics is comparatively high and their constant application results in development of resistance in the pathogens against these fungicides and antibiotics (Nigam *et al*., 1994). Thus use of eco-friendly alternative approaches for the management of plant diseases and human diseases is

suggested (Mishra and Tewari, 1990). A good number of plant derived natural products and its oils are reported to be antifungal and antibacterial in nature (Chattopadhyay *et al.,* 2004; Bhalodia *et al*., 2011).

Medicinal plants are defined as any plant or plant parts that shows curative properties, no matter to which source or country belongs. The use of plants in medicine was developed as far as the development of civilization. Plant diversity serve as renewable natural resources for variety of biologically active chemicals. Medicinal properties such as antifungal, antiviral, antibacterial, anthelminthic, anticancer and others are present in medicinal plant. (Parajuli *et al*.,1998). Medicinal plants contain products such as tannins, alkaloids, quinine, phenols, coumarines in their extracts which are best known for the antimicrobial activities. These compounds are generally present in the roots, stems, bark, leaves, seeds etc.

Essential oils are derived mainly from aromatic plants. Essential oils are also known as essences or volatile oils, complex mixtures of volatile substances biosynthesized by living organisms. They are extracted from the host organism by distillation or extraction with the suitable solvent. They are used in flavor, fragrance food, cosmetics, and toiletry and pharmaceuticals industries. (Rawal, 2001).

Cauliflower *(Brassica oleracea* L.var. *botrytis)* is a herb in the brassicaceae family, related to the cabbage and broccoli and having a whitish undeveloped flower with a large edible head. Typically, only the head (the white curd) is eaten. Cauliflower is one of the most important winter vegetable crops of Nepal (Annonymous, 1987).

Cauliflower queen of the winter vegetables is commercially grown in Nepal. It covers a total area of 29,836 ha and a total production of 399,012 tones (VDD, 2010). It plays an important role in nutrition for the developing countries (Ghimire et al., 1998). The nutritive value of cauliflower as green vegetable claims priority among the first foods of infants (Oowen and Grubbe, 1977).

Cauliflower has long been thought to have medicinal values and therapeutic effects (McDonald, 1971). Cauliflower seeds have contraceptive properties (Singh et al., 2001). The inflorescence extract has been used in the treatments of scurvy, as a blood purifier and as an antacid (McDonald, 1971). It has high concentration of glucothiocyanate, and its extract has been reported to be effective in the inhibition of carcinogenesis in *in vitro* assays (Singh et al., 2001). The potential chemo preventive

agents present in cauliflower are ascorbic acid, carotenoids, tocopherols, isothiocynates, indoles and flavonoids (Singh et al., 2001).

1.2 Nutritional value of cauliflower

Table1: Nutritional content of cauliflower

Contents	**Per 100 g of cauliflower**
Energy	25 Kcal
Carbohydrates	5 g
Sugars	1.9 g
Dietary fibre	2 g
Fat	0.3 g
Protein	2 g
Water	92 g
Thymine (Vit. B_1)	0.05 mg
Riboflavin (Vit. B_2)	0.06mg
Niacin (Vit. B_3)	0.507 mg
Pantothenic acid (B_5)	0.667 mg
Vitamin B_6	0.184 mg
Folate	57µg
Vitamin C	48mg
Vitamin K	15.5µg
Calcium	22 mg
Iron	0.42 mg
Magnesium	15 mg
Phosphorus	44 mg
Potasium	29 mg
Sodium	30 mg
Zinc	0.27 mg

Source: USDA Nutrient Data Base

1.3 Diseases of cauliflower

Cauliflower (*Brassica oleracea* L. var *botrytis*) is one of the most widely cultivated commercial crops. But its production is lost by several diseases. In Nepal cauliflower is infected by many diseases. Some of the diseases that lead to destruction of plant crop are as follows.

Table2: Diseases of cauliflower in Nepal

S.N.	Name of disease	Name of pathogen
1	Stalk rot	*Sclerotinia sclerotiorum* (Lib) de bary
2	Damping off	*Pythium aphanidermatum* (Edson) Fitzp. *Rhizoctonia* spp. *S. sclerotirum* *Phytophthora* spp. *Fusarium* spp.
3	Downy mildew	*Peronspora parasitica* (Pers.) Fr.
4	Alternaria leaf spot	*Alternaria brassicicola* (Schw) Wiltshire *Alternaria brassicae (Berk) Sacc*
5	Club root	*Plasmodiophora brassicae*
5	Black rot	*Xanthomonas campestris* pv. *Campestris* (Pammel) Dowson
6	Soft rot	*Erwinia carotovora* sub sp. *Carotovora*s

Among these diseases, leaf spot of cauliflower is one of the destructive diseases, which is caused by *Alternaria brassicicola* (Schw.) Wiltshire. The fungus basically

infects leaf but can infect all parts of plant. It is of worldwide economic importance resulting occasionally 20-50 % yield reduction in crucifers.

1.4 Test pathogen

A. brassicicola forms dark brown to almost black, circular zonate spots, 1-10 mm in diameter on leaves as necrotic lesion. The spot may increase about 2-3 inches in diameter. Typically the spot surrounded by halo-chlorotic tissues. The spot gradually increase in size in a concentric manner and often coalesce leading to blighted appearance.

The pathogen when cultured in PDA medium, the colonies appears dark, olivaceous brown to dark blackish brown, effuse, amphigenous, velvety and mycelium immersed. Hyphae septate, branched, hyaline at first but later brown or olivaceous brown, inter and intracellular, 1.5-7.5µm thick. Conidiophores arising singly or in groups of 2-12 or more, up to 70 µm long, 5-8 µm thick. Conidia mostly in chains of up to 20 or more, sometime branched, acropleurogenous, pale to dark olivaceous brown, smooth or becoming warted with age , usually tapering slightly towards the apex or obclavate, the basal cell rounded, apical cell more or less rectangular with 1-11, mostly less than 6 transverse septa and usually few but up to 6 longitudinal septa, slightly constricted at the septa, 18-130 µm long, 8 -20 µm thick in the broadest part and the beak is 1/6 of the length of the conidium and is 6-8 µm thick.

1.5 Essential oils

S.N	Oils samples	Local name	Family	Chemical components
1	*Mentha arvensis* L.	Pudina	Labiateae	Mentol, camphene, methone, neomenthol, I-menthol, limonene
2	*Cymbopogan martini* (Roxb.) W. Watson		Poaceae	Myrcene, linalool, geraniol, geranyl acetate, dipentene and limonene
3	*C. citratus* (DC.) Trin.	Pirhe ghans	Poaceae	Myrcene, citronellal, geranyl acetate, geraniol, limonene, citral
4	*Gaultheria*	Dhasingare	Ericaceae	Methyl salicylate, alpha-

	fragrantissima Wall.			pinene,myrcenene,limonene, decta-3-carene
5	*Juniperus recurva* Buch-Ham.ex.D.Don	Dhupi	Cupressaceae	Delta-3-carene, limonene, alpha-pinene,delta-cadiene, elemol,cubenol
6	*Zanthoxylum armatum* DC.	Timur	Rutaceae	1, 8 -cineole *cis*- sabenene hydrate , linalool , terpenin-4-ol , a- terpeniol , cuminaldehyde , thymol and ß- cubebene

1.6 Objectives

- To test the fungitoxicity of the essentisl oils of different plant at different concentration against *Alternaria brassicicola*.
- To determine the minimum inhibitory concentration (MIC) of essential oils used.

1.7 Justification of the study

Cauliflower is known for its nutritional value. It has low fat content but the amount of dietary fibres present in very high amount. Packed with rich nutrients, cauliflower is one of the commonly used flower-vegetable. The flower heads contains numerous health benefiting phyto nutrients such as indole-3-carbinol, sulforaphane etc that help prevent prostate, ovarian and cervical cancers. In recent research, sulforaphane was shown to lower the occurrence of breast tumors in lab animals by almost 40%.

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is an important commercial vegetable grown in Nepal and is one of the highly preferred vegetables in Nepalese kitchen (APP, 1995).

Above importance shows that cauliflower is one of the important vegetable that can flourish the income of farmers if it is grown commercially. Despite of these remarkable advantages, cauliflower is not free from attacks of various diseases/insects,pests' in Nepal. Farmers are always suffered from loss of productivity

of this crop due to diseases, one of the disease is leaf spot disease caused by *Alternaria brassicicola* (Schw.) Wiltshire.

The control of this disease using different fungicides is common method. Many of these fungicides are bio-hazardous and adversely affect the components of ecosystem. Further, the cost of these fungicides and antibiotics is comparatively high and their constant application results in development of resistance in the pathogens against these fungicides and antibiotics (Nigam *et al*., 1994). Essential oils are less likely to be associated with the development of resistance than in the case with fungicides and are less hazardous to the environment and human health than synthetic pesticides (Daferera et al.2003). Using essential oils as a means of controlling diseases is also cheaper compared to the commercially available fungicides and thus are economically more feasible to the farmers, and the health and environmental benefits of using such naturally available products are also worth considering as they have no adverse effects on health and environment.

1.8 Limitation of the study

- The entire work was carried out in limited time for one year only.
- Only one pathogen has been taken for study.
- Essential oil from some selected plants were taken for the study**.**
- TLC and GC were not carried out.
- The antifungal activity of oils was conducted in vitro only.

CHAPTER-2

2. REVIEW OF LITERATURE

Bolakan *et. al* (1983) found foliar disease of *Brassica oleracea* var. *capitata* during study of post harvest disease of fruits and vegetables in Brazil. Disease causing organism from diseased leave was isolated and identified as *Alternaria brassicicola* based on the description given by Ellis (1971). Pathogenecity of the fungus was proved by artificial inoculation on cabbage leaves. Cauliflower, mustard, chinese cabbage, turnip and head of cauliflower were also artificially inoculated by isolate from cabbage. *A. brassicicola* was caused by pathogenic on all crucifer species tested. This is the first record of foliar disease on cabbage caused by *A. brassicicola*.

Pattanamahakul and Strange (1999) isolated *Alternaria* spp. from necrotic lesion on leaves of cauliflower, cabbage, Chinese kale and choi-sum plants of Thailand, and were proved by Koch's postulates to be the causal agent of disease known as dark leaf spot. All isolates of *Alternaria* spp. from the lesion of the diseased plants were identified as *Alternaria brassicicola*, corresponded on morphology to *A. brassicicola* (Wiltshire, 1947).

Tohyama and Tsuda (1995) isolated three species of *Alternaria* from seed samples of *Brassica campestris, B. oleracea* and *Raphanus sativus.* The most frequently detected species were *A. japonica* and *A. alternata* on *B. campestris, A. brassicicola* on *B. oleracea* and *A. japonica* and *A. alternate* on *Raphanus sativus* respectively. *A. brassicicola* isolates from these crops produced nectrotic lesions on all of the crucifers seedling inoculated.

Hemmi and Ishigami (1953) grew *Alternaria brassicicola* in culture at 10^{o}c- 36^{o}c. The optimum temperature for its growth was found at 20^{0}c.It was the causal agent of leaf spot of cabbage and cauliflower head..

Richardson (1970) isolated *Alternaria brassicicola, Alternaria brassicae* and *Plendomis lingam* from 157 Brassica seed samples.

Renu et al. (1980) screened 25 plants for antifungal activity and from the study new petent fungitoxic compounds were obtained. Out of them, *Cestrum diurnum* exhibited

strong activity against *Alternaria solani, A. alternata, A. brassicae,* A. and other fungal species.

Dikshit and Dixit et al. (1982) screened 20 plants for toxicity against *Helminthosporium oryzae*. Essential oil of *Eupatorium capilifolium* inhibited the mycelial growth of *Alternaria alternata* and *Alternaria raphani* by 88% and 100% at 100 ppm respectively.

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

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

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

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

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

Ashok Kumar *et al.* (1998) evaluated 25 botanicals against *Alternaria brassicae,* among them *Solanum xanthocarpum* and *Datura innoxia* at 10 percent concentration completely inhibited the spore germination.

Shenoi *et al.* (1998) evaluated leaf extracts of forty five plants as antifungal agents against *A. alternata* under *in vitro* conditions. They obtained best results with *Thevetia peruviana* and *Lawsonia inermis.*

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

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

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

Muto *et al*. (2005) screened water soluble extracts from fresh and dry tissues of solanaceous plant to evaluate the antifungal activity against the *Alternaria brassicicola* isolates ABA-31 and ABA-104.Only the extracts of *Solanum tuberosum* and *S. nigrum* showed complete inhibition of conidia germination of both isolates of *A. brassicicola* at a concentration of 10% (w/v) from fresh tissues, and 1% (w/v) from dry tissues.

Jasso de Rodriguez et *al.* (2007) evaluated the antifungal activities of ethanol extracts of *Flourensia microphylla, F. cernua and F. retinophylla* against *Alternaria spp. , Rhizoctonia solani* and *Fusarium oxysporium*. The tested plant inhibited the three pathogens at $1500\mu ll^{-1}$.

Feng and Zheng (2007) tested the inhibitory effects of five essential oils (thyme, sage, nutmeg, eucaptus and cassia) *against A. alternata*. The cassia oil and thyme oil exhibited antifungal activity against test fungus. The cassia oil inhibited completely

the growth of *A. alternata* at 300ppm while thyme oil inhibited 62% at 500ppm. Numeg, sage and eucaptus oils did not affect the growth of *A. alternata.*

Pandey *et.al*. (2007) isolated the alkaloid fuyuziphine from *Funaria indica* to test inihibitive effect against spore germination of some plant pathogenic fungi (*Collectotrichum sp., C. gloeosporioides, C. falcatum, Curvularia maculans, C. lunata, Erysiphe cichoracearum, Helminthosporium pennisetti, Oidium erysiphoides, Ustilago cynodontis, Alternaria chieranthi, A. melongenae, A. brassicicola and A. solani*). Germination of most fungi was significantly inihibited at 100-750 ppm concentration.

Sitara *et al* (2008) extracted essential oils from the seeds of neem (*Azadirachta indica*), mustard (*Brassica campestris*), black cumin (*Nigella sativa*) and asafoetida (*Ferula assafoetida*) to evaluate their antifungal activity at 0.5, 0.1 and 0.15% against eight seed borne fungi viz., *Aspergillus niger,A. flavus, Fusarium oxysporum, F. moniliforme, F. nivale, F. semitectum, Drechslera hawiinesis* and *Alternaria alternata*. All the oils extracted except mustard, showed fungicidal activity of varying degree against test species.

Piyo *et.al (*2009) evaluated effectiveness of essential oils of basil (*Ocimum basilicum*) and sweet fenna (*Ocimum gratissimum*) against seven species of rice pathogenic fungi; *Alternaria brassicicola, Aspergillus flavus, Bipolaris oryzae, Fusarium moniliforme,Fusarium proliferatum, Pyricularia grisea* and *Rhizoctonia solani*. The basil oil inhibited mycelia growth of F. *moniliforme, by F. proliferatum, P. grisea* by 100%,49.6% and 100% respectively at concentrarion of 0.6% v/v. While *B.oryzae, A.brassicicola* and *A.flavus* was inhibited by 97.40%, 94.62% and 59.25% respectively at 2% v/v concentration. However, basil essential oil was not effective in controlling *R. solani.* Sweet fennel oil inhibited mycelium growth of all pathogenic fungi at 0.8% v/v concentration.

Suwitchayanon and Kunasakdakul (2009) evaluated the antifungal activity of extracts of clove (*Syzgium aromaticum*) and turmeric (*Curcuma longa*) against crucifer pathogens using soaking method. MIC of clove extract were 1900, 2300 and 470ppm for *Alternaria brassicicola, Fusarium oxysporum and X. campestris* respectively while MIC of turmeric extract were 7500, 13200 and 230ppm respectively.

CHAPTER-3

3. MATERIALS AND METHOD

3.1 Materials

Different equipments, glasswares chemicals, were used in the experiment. And essential oils of six medicinal plants were taken from HPPCL, Kathmandu. All such materials are listed is Appendix-I.

3.2 Methods

The research was carried out in the laboratory of Central Department of Botany, Plant Pathology Unit, Kirtipur.

3.2.1 Collection of Diseased Leaves

During research period the diseased leaf samples of cauliflower were collected from the fields of Machheganhu, Kirtipur.

3.2.2 Preparation of the PDA media

While preparing PDA media, 200gm of peeled potatoes were cut into small pieces and boiled for sometimes in 500ml of distilled water. The cooked potato pieces were filtered by muslin cloth, 20 gm of dextrose was dissolved thoroughly and the volume of filtrate was maintained to 1000ml adding more distilled water. 20gm of agar was added and stirred gently to get thoroughly mixed. The flask was plugged with cotton plug and autoclaves for 30 minutes at 121^0 c in 15 lbs pressure.

3.2.3 Isolation of fungi

The small part of infected leaves were first surface sterilized by 2% sodium hypochlorite solution and repeatedly washed with distilled water and transferred to sterilized Petri plates containing PDA media. The Petri plates were incubated in inverted position at temperature (25±2°C) and observed periodically for the growth of the fungus. After one week, the morphological characteristics of the fungus were studied.

3.2.4 Conidial measurement

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

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

Now the stage micrometer was replaced by a fine slide of the test fungus mounted in cotton blue and lacto-phenol. Size of conidia was measured by determining the number of division of ocular micrometer occupied by conidia of the fungus. Measurement of different conidia was carried out for 10 times to find the particular range of the size of conidia.

3.2.5 Identification of test fungus

The test fungus *Alternaria brassicola* was identified with the help of literature (Ellis,1971) and Singh (1982).

3.2.6 Maintenance of pure culture

The pure culture of test fungus was preserved by sub-culturing the test fungi in sterilized Petri plates containing PDA media and slant, every week.

3.2.7 Pathogenicity test

The potting mixture (sand and soil in the ratio of 5:2) were autoclaved at 121^{o}c in 15 lbs pressures for 30 minutes. The seeds of cauliflower were washed in tap water thoroughly and surface sterilized with 2% sodium hypochlorite solution. The sterilized seeds were planted in pre-sterilized pot filled with pre-sterilizd potting mixture. The pot was watered with sterilized distilled water regularly for proper growth of plant. Equal amount of sterilized potting mixture was kept in six pots. The plants were transplanted into pre-sterilized pot filled with sterilized potting mixture. Regular watering was done for proper growth of plants in each pot. The leaves were thoroughly cleaned with distilled water using moist cotton. Later on, the healthy leaves were washed with ethanol. Inoculum suspension from seven day old culture of

Alternaria brassicicola was prepared in distilled water and sprayed on leaves with the help of sterilized syringe after wounding on the leaves. Similarly controlled plants were sprayed with distilled water. The triplicate was maintained for control and infected plants in each test. After ten days of inoculation, the leaves exhibited symptoms of infection. The symptoms appeared in the inoculated leaf were compared with the symptoms first noted. The symptoms like small, dark brown to almost black, circular, zonate spot noticed on the artificially inoculated leaf. The symptom appear on inoculated leaf were similar to the symptoms first noted from the infected leaf.

3.2.8 Re-isolation

The pathogen was re-isolated from the inoculated plants in PDA media. The colony of pure culture of test pathogen re-isolated in PDA media was effuse, dark, olivaceous brown to dark blackish brown, velvety. Conidia usually tapering slightly towards the apex, pale to dark olivaceous brown colour, 2-5 tranverse septa, conidia constricted at the septa and basal cell rounded. Its characteristics were found to be similar with previously isolated one from infected plant. This proves that the pathogen in both the cases was *Alternaria brassicicola* which causes *Alternaria* leaf spot of cauliflower. Its characteristics were found to be similar with previous one. This proved that the pathogen in both cases was *Alternaria brassicicola* which cause leaf spot disease of crucifers.

3.2.9 Preparation of one week old culture

For testing the antifungal activity of essential oil, the inoculums' disc from one week old culture is required. For preparation of one week old culture, the fungus from pure culture was transferred in sterilized Petri plate containing PDA media and was placed in the incubator at 25^0 C ± 2^0 C. After 7 days the inoculums discs were taken from the culture plate for the further experiment.

3.2.10 Dilution of essential oil

Six different essential oil samples were diluted into 40µlml^{-1}, 20 µlml^{-1}, 10 µlml^{-1}, 5 µlml^{-1} and 2.5 µlml^{-1} with 80% acetone (Rao and Srivastava, 1994). First 0.4 ml of essential oil was added to 9.6 ml of acetone to make 40 µlml^{-1} concentrations. Four sterilized test tube were taken and 5ml of acetone was taken in each test tube to prepare the oils of different concentrations ranging from 20 to 2.5 µlml^{-1}. 20 µlml^{-1}

concentrations were made by adding 5 ml of diluted essential oil from 40 μlml^{-1} to 5 ml of acetone. Similarly, 5 ml of diluted essential oil from each concentration of 20 μlml^{-1}, 10 $\mu lml^{-1,}$ 5 μlml^{-1} was added to 5 ml acetone separately to make 10 μlml^{-1}, 5 μlml^{-1}, 2.5 μlml^{-1} concentration respectively. 5ml of acetone was taken in a pre-sterilized test tube that was regarded as 0 μlml^{-1} concentration of essential oil.

3.2.11 Antifungal assay

The antifungal assay was assessed by using poisoned food technique (Grover and Moore, 1962). 0.5ml of each concentration of essential oil was poured into sterilized Petri plates followed by addition of 9.5 ml of melted PDA. The Petri plates were swirled gently to allow through mixing of the contents. In the control set no oil was maintained. When the media solidified one inoculums disc of 4mm diameter (prepared with the help of sterilized cork borer) of the test fungus was aseptically inoculated upside down at the centre of each Petri plates and incubated at temperature $25^{o}c\pm2^{o}c$ for 7 days. For each control and treatment set 3 replicates were maintained for each test. Average diameter of the fungal colonies was measured on the 7^{th} day of incubation and the percentage of mycelial growth of incubation was calculated separately.

3.2.12 Calculation of Mycelial Growth Inhibition

The antifungal effect of different concentrations of different essential oil was calculated in terms of percentage of mycelial growth inhibition (Rao and Srivastava, 1994).

Mycelial growth inhibition (%) = $\frac{g_c - g_t}{g_c} \times 100$

Where,

g_c = growth of mycelial colony after incubation period in control set

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

3.2.13 Determination of Minimum Inhibitory Concentration (MIC)

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

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

3.2.14 Photographs

The photographs of infected leaf, conidia of test fungus and Petri plates showing inhibition of mycelial growth at different concentration of different essential oils were taken.

CHAPTER- 4

4. RUSULTS

4.1 Isolation of test pathogen

The test pathogen *Alternaria brassicicola* was isolated from the infected leaf of cauliflower. The colony of pure culture of test pathogen isolated in PDA media was effuse, dark, olivaceous brown to dark blackish brown, velvety. Hyphae septate, branched, conidia pale to dark olivaceous brown in colour, mostly in chain, usually tapering slightly towards the apex, 2-5 tranverse septa,1-2 longitudinal septa, conidia constricted at the septa and basal cell rounded. The size of conidia was found (26-53.44×9.46-16.55) µm. These characteristics were resembled with description given by (Ellis, 1971). The characteristics of fungus re-isolated from artificially inoculated leaf in PDA media were found to be similar with previously isolated one from infected leaf of plant.

4.2 Antifungal activity of essential oils

Six different essential oil samples were tested for their antifungal activity against *Alternaria brassicicola* by poisoned food technique. The colony size of the test fungus at different concentrations of essential oils measured on the 7^{th} day of incubation and the inhibitory effects of the oils were calculated. The measurement of the diameter of the colony was done by using the rular and finally the inhibition percentage was calculated. For each concentration, the diameters of triplicates were measured and the mean is calculated. The inhibition percentage was finally calculated in reference to the colony diameter in control set. The percentage of mycelial growth of the test fungus was to be different with different concentrations of the oils used.

Table3: Antifungal activity of oils of *Gaultheria fragrantissima* against *A. brassicicola*

S.N.	Concentration of oils µlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth A-B (mm)	Inhibition%
1	Control	4	65.5	61.5	0
2	2.5	4	63	59	4.06
3	5	4	60	56	8.94
4	10	4	51	47	23.57
5	20	4	49	45	26.82
6	40	4	43	39	36.59

The table shows that the oil of *Gaultheria fragrantissima* inhibited the mycelial growth of *Alternaria brassicicola* by 4.06%, 8.94%, 23.57%, 26.82%, 36.59%, at concentration of 2.5µlml^{-1}, 5 µlml^{-1} , 10 µlml^{-1} , 20 µlml^{-1} and 40 µlml^{-1} respectively.

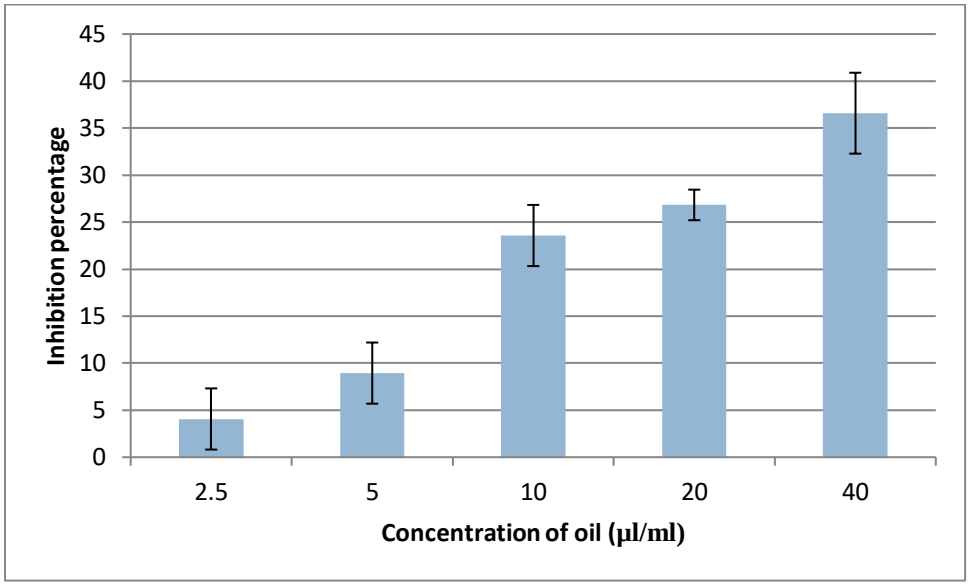

Fig 1:- Antifungal activity of essential oil of *Gaultheria fragrantissima* against *Alternaria brassicicola*

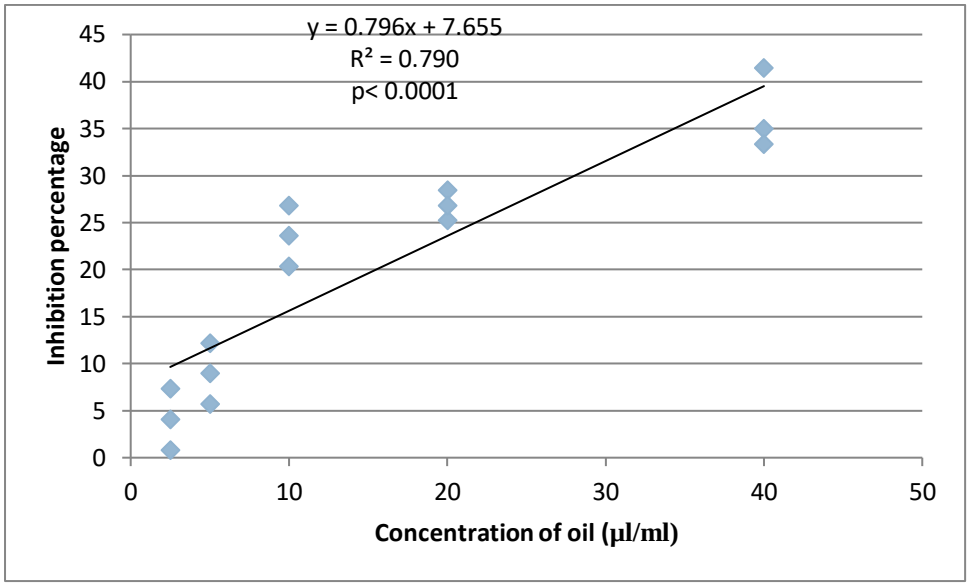

Fig 2: Regression line (linear) showing inhibition percentage of oil of *Gaultheria fragrantissima* at different concentration.

The figure shows that concentration of oil of *Gaultheria fragrantissima* contribute 79% to inhibit the mycelial growth of the fungus(*Alternaria brassicicola*). It also shows that at zero percentage concentration also, the oil can inhibit the mycelial growth of the fungus by 7.655 %, which is significant at less than one percentage level of significance.

Table 4: Antifungal activity of oil of *Mentha arvensis* against *Alternaria brassicicola*

S.N.	Concentration of oils µlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth A-B (mm)	Inhibition%
1	Control	4	82	78	0
2	2.5	4	70	66	15.38
3	5	4	50	46	41.02
4	10	4	4	0	100
5	20	4	4	0	100
6	40	4	4	0	100

The table shows that the oil of *Mentha arvensis* inhibited the mycelial growth *of A. brassicicola* by 15.38%, 41.02%, 100%, 100% and 100% at concentration of 2.5µlml^{-1}, 5 µlml^{-1}, 10 µlml^{-1}, 20 µlml^{-1} and 40 µlml^{-1} respectively.

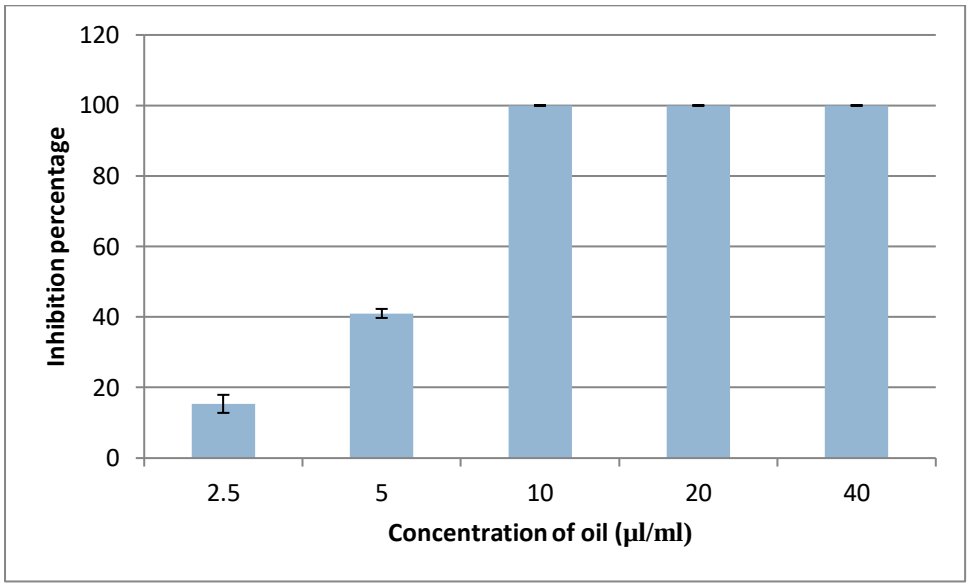

Fig 3:- Antifungal activity of oils of *Mentha arvensis* against *Alternaria brassicicola*

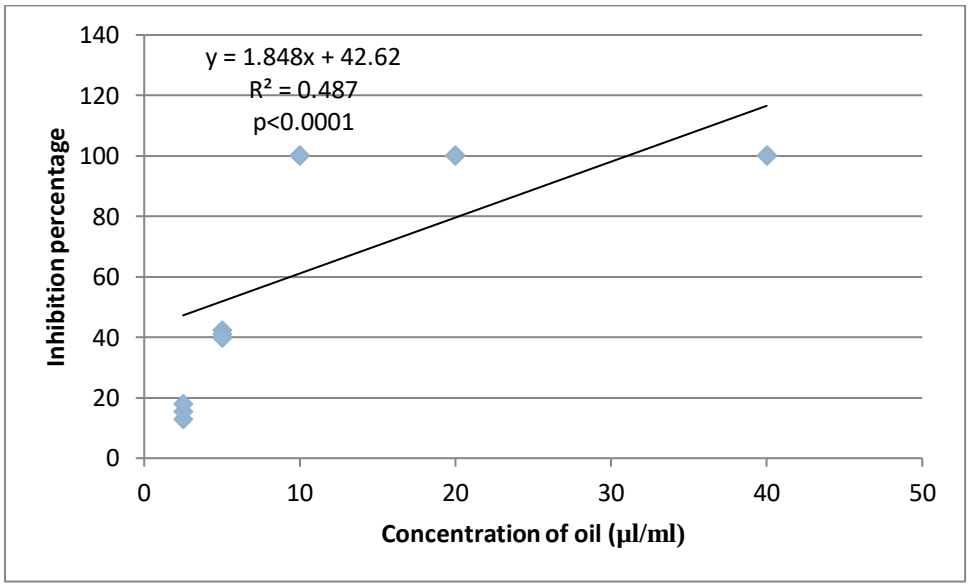

Fig 4 : Regression line (linear) showing inhibition percentage of oil of *Mentha arvensis* at different concentration

The figure shows that concentration of oil of *Mentha arvensis* contribute 48.7% to inhibit the mycelial growth of the fungus (*Alternaria brassicicola*). It also shows that at zero percentage concentration also, the oil can inhibit the mycelial growth of the fungus by 42.62%, which is significant at less than one percentage level of significance.

Table 5:- Antifungal activity of essential oils of *Zanthoxylum armatum* against *Alternaria brassicicola*

S.N.	Concentration of oils μlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth A-B (mm)	Inhibition%
1	Control	4	65	61	0
2	2.5	4	59	55	9.83
3	5	4	57	53	13.11
4	10	4	54	50	18.03
5	20	4	39	35	42.62
6	40	4	23	19	68.85

The table shows that the oils of *Zanthoxylum armatum* inihibited the mycelial growth of *Alternaria brassicicola* by 9.83%, 13.11%, 18.03%, 42.62% and 68.85% at concentration of 2.5μlml^{-1}, 5 μlml^{-1} , 10 μlml^{-1} , 20 μlml^{-1} and 40 μlml^{-1} respectively.

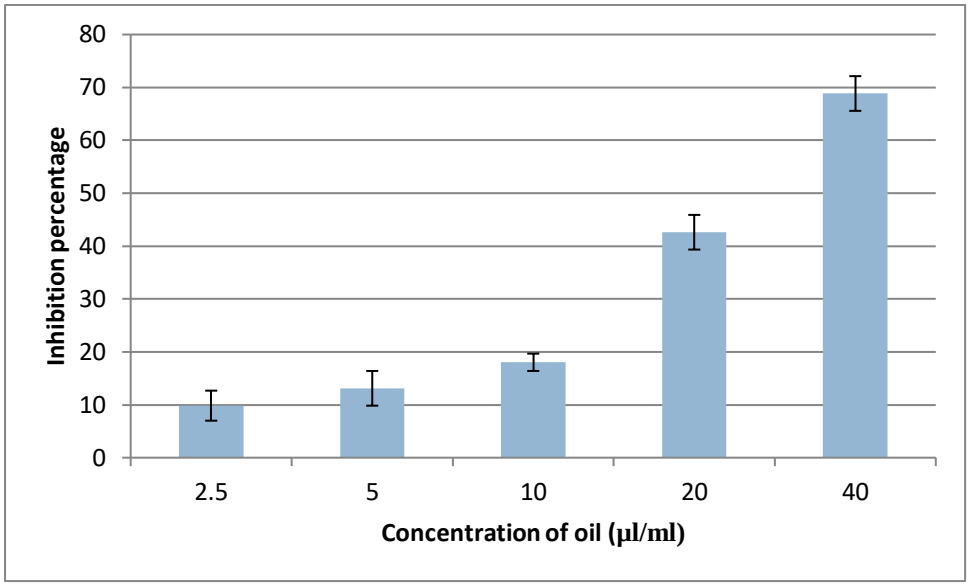

Fig 5: Antifungal activity of essential oil *Zanthoxylum armatum* against *A. brassicicola*

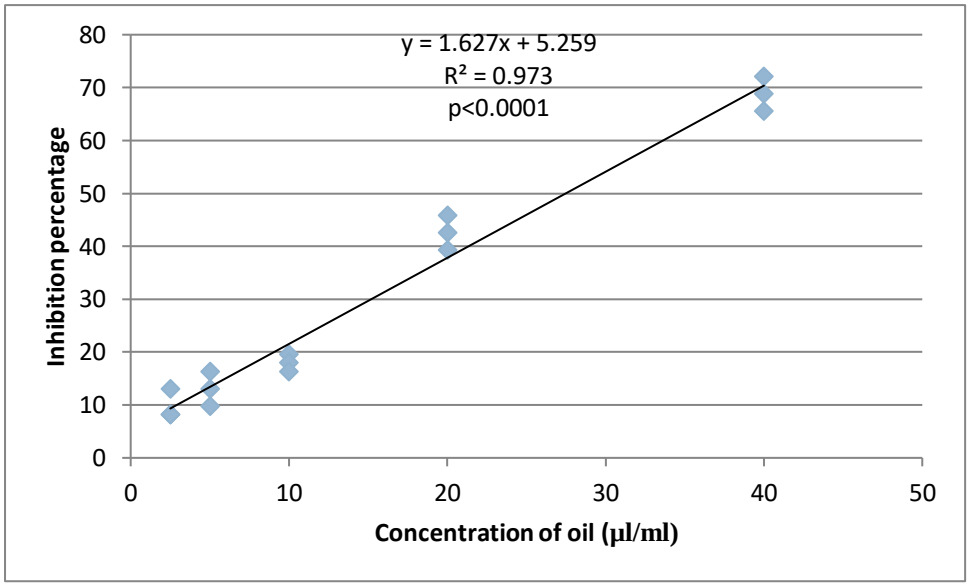

Fig 6: Regression line (linear) showing inhibition percentage of oil of *Zanthoxylum armatum* at different concentration

The figure shows that concentration of oil of *Zanthoxylum armatum* contribute 97.3% to inhibit the mycelial growth of the fungus (*Alternaria brassicicola*). It also shows that at zero percentage concentration also ,the oil can inhibit the mycelial growth of

the fungus by 5.26 %, which is significant at less than one percentage level of significance.

Table 6:- Antifungal activities of essential oils of *Juniperus recurva* against *A. brassicicola*

S.N.	Concentration of oils µlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth (mm) A-B	Inhibition%
1	Control	4	64	60	0
2	2.5	4	62	58	3.33
3	5	4	59	55	8.33
4	10	4	49	45	25
5	20	4	47	43	28.33
6	40	4	42	38	36.66

The table shows that the oil of *Juniperus recurva* inhibited the mycelial growth of*Alternaria brassicicola* by 3.33%, 8.33%, 25%, 28.33% and 36.66% at concentration of 2.5µlml^{-1}, 5 µlml^{-1} , 10 µlml^{-1} , 20 µlml^{-1} and 40 µlml^{-1} respectively.

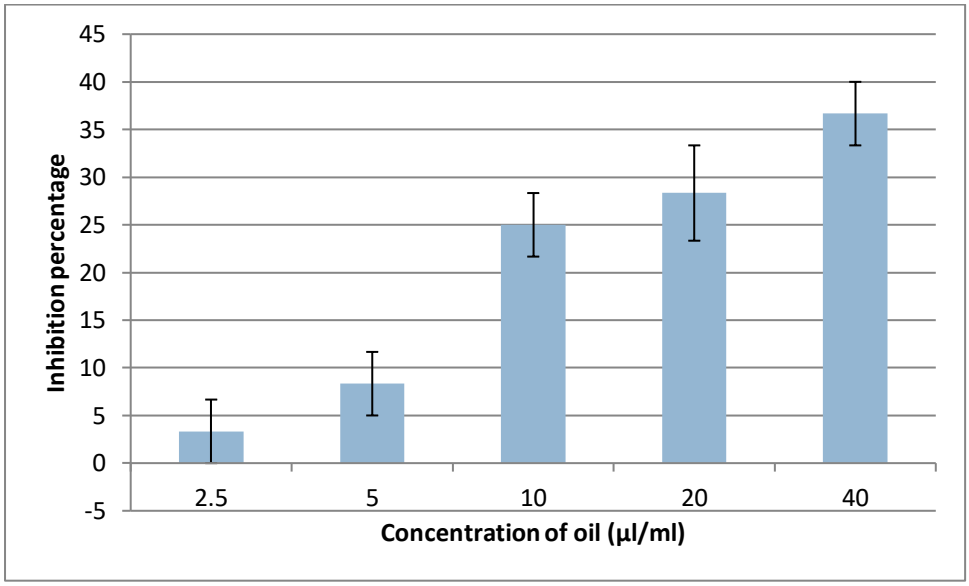

Fig 7: Antifungal activity of essential oil *Juniperus recurva* against *A. brassicicola*

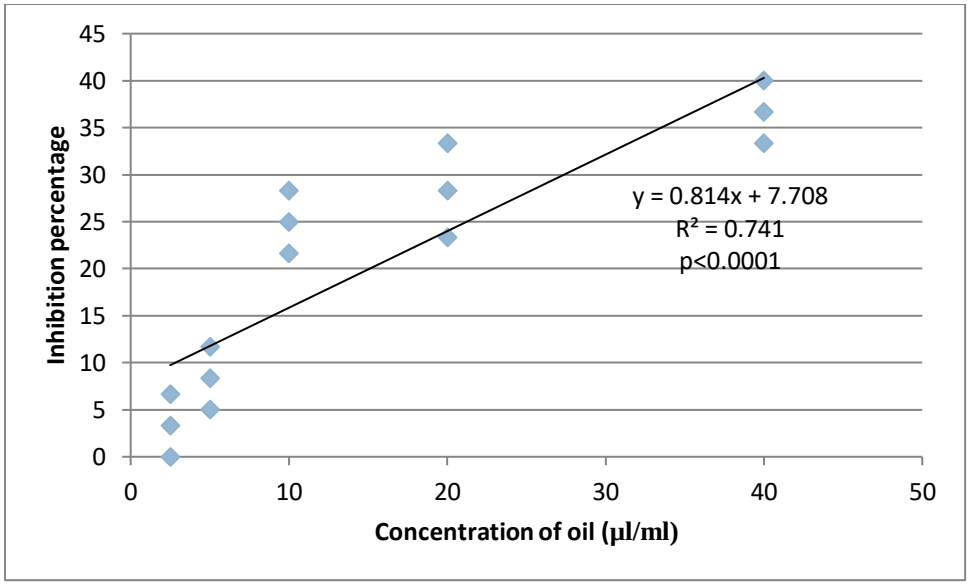

Fig 8 : Regression line (linear) showing inhibition percentage of oil of *Juniperus recurva* at different concentration

The figure shows that concentration of oil of *Juniperus recurva* contribute 74.1% to inhibit the mycelial growth of the fungus (*Alternaria brassicicola*). It also shows that at zero percentage concentration also, the oil can inhibit the mycelial growth of the fungus by 7.70 %, which is significant at less than one percentage level of significance.

Table7: Antifungal activities of essential oil of *Cymbopogan martini* against *A. brassicicola*

S.N.	Concentration of oils µlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth A-B (mm)	Inhibition%
1	Control	4	55	51	0
2	2.5	4	4	0	100
3	5	4	4	0	100
4	10	4	4	0	100
5	20	4	4	0	100
6	40	4	4	0	100

The table shows that the oil of *Cymbopogan martini* inhibited the mycelial growth of *Alternaria brassicicola by* 100%, 100%, 100%, 100% and 100% at concentration of 2.5µlml^{-1}, 5 µlml^{-1} , 10 µlml^{-1} , 20 µlml^{-1} and 40 µlml^{-1} respectively.

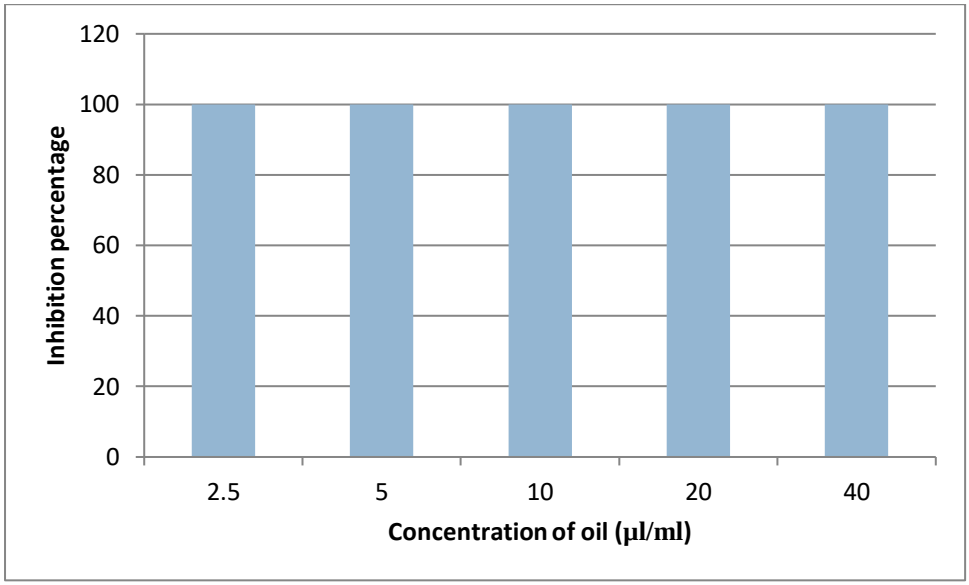

Fig 9: Antifungal activity of oil of *Cymbopogan martini* against *Alternaria brassicicola*.

Table 8: Antifungal activity of *Cymbopogan citratus* against *A. brassicicola*

S.N.	Concentration of oils µlml^{-1}	Inoculum diameter B (mm)	Mean colony diameter A (mm)	Mycelial growth A-B (mm)	Inhibition%
1	Control	4	60	56	0
2	2.5	4	45	41	26.78
3	5	4	26	22	60.71
4	10	4	18	14	75
5	20	4	4	0	100
6	40	4	4	0	100

The table shows that the oil of *Cymbopogan citratus* inhibited the mycelial growth of *Alternaria brassicicola* by 26.78%, 60.71%, 75%, 100% and 100% at concentration of 2.5µlml^{-1}, 5 µlml^{-1} , 10 µlml^{-1} , 20 µlml^{-1} and 40 µlml^{-1} respectively.

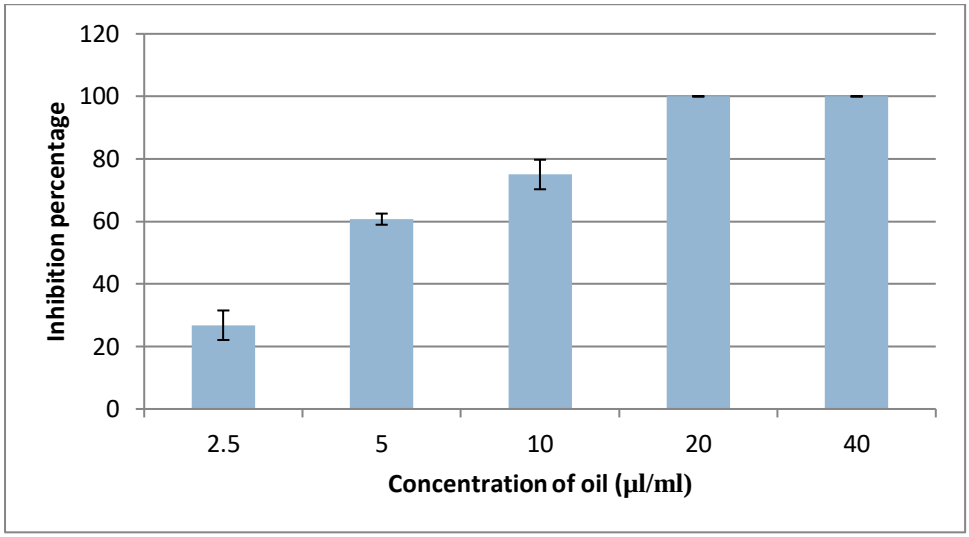

Fig 10 : Antifungal activities of essential oil of *Cymbopogan citratus* against *A. brassicicola*

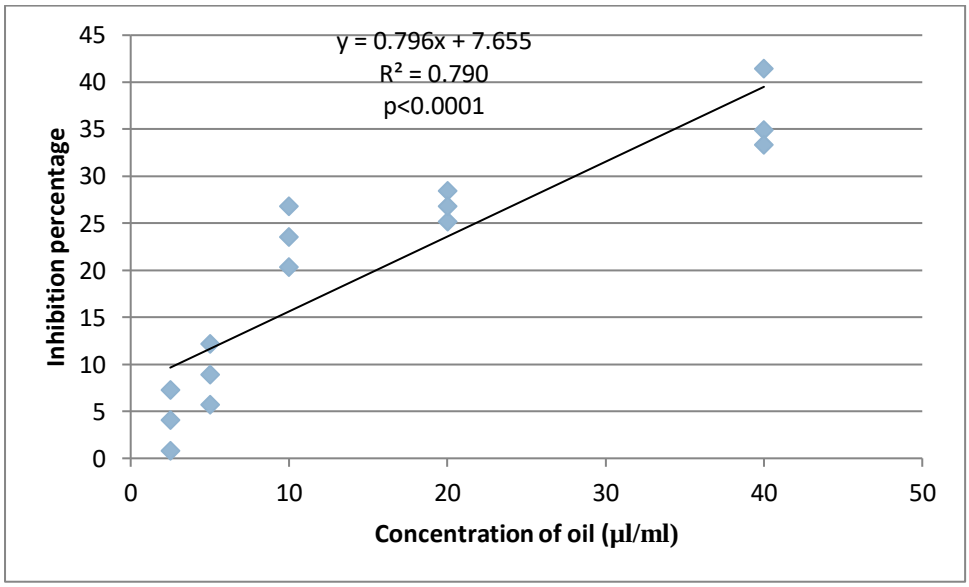

Fig 11: Regression line (linear) showing inhibition percentage of oil of *Cymbopogan citratus* at different concentration

The figure shows that concentration of oil of *Cymbopogan citratus* contribute 79% to inhibit the mycelial growth of the fungus (*Alternaria brassicicola*). It also shows that at zero percentage concentration also, the oil can inhibit the mycelial growth of the fungus by 7.65 %, which is significant at less than one percentage level of significance.

4.3 Minimum inhibitory concentration (MIC) of essential oil

MIC of essential oil can be expressed as the minimum concentration of the extract required for complete (100%) inhibition of mycelial growth of test fungus (Rao and Srivastava, 1994). MIC of essential oil were obtained by poisoned food technique employing different concentrations of oils till the minimum concentration.

Table 9: Minimum Inhibitory Concentration (MIC) of Essential Oil

S.N	**Essential oil**	**MIC(µlml^{-1})**
1	*Mentha arvensis*	10
2	*Cymbopogan martini*	2.5
3	*Cymbopogan citratus*	20

The above table 9 shows that the oil of *Cymbopogan martini* was found to have most lowest value of MIC. The minimum concentration of the oil of this plant required for complete inhibition of mycelial growth of test fungus was found to be 2.5 µlml^{-1}.

4.4 Two Way ANOVA

Two way Analysis of Variance was performed to check the effect of individual factor (concentration and species) and their interaction on antifungal activities. Inhibition percentage data were log transformed before ANOVA test to make the data normal.

Table 10: Two Way ANOVA Table

Source of variation	df	Mean square	F	Sig
Species	4	1.447	91.38	0.000
Concentration	4	1.731	109.341	0.000
Interaction	16	0.049	3.087	0.001

Results indicate that species, concentration and their interaction all have significant effect on inhibition percentage (antifungal activities of different oil).

CHAPTER-5

5. DISCUSSION

Cauliflower is one of the most important commercial vegetable crops of Nepal. The crop is subjected to attack by a number of diseases, of which *Alternaria* leaf spot caused by *Alternaria brassicicola* is one important disease.

In the present study, an attempt was made to study about leaf spot disease of cauliflower caused by *Alternaria brassicicola* , it's isolation, it's pathogenicity test and to study its inhibition of mycelial growth by using essential oils of different medicinal plants.

The pathogenicity of the fungus was established by artificial inoculation of cauliflower leaves with the conidia of the pathogen as Koch's postulates. The symptom of the disease was observed on the leaves after ten days of inoculation. Initially dark brown to almost black, circular zonate spots ,1-10mm in diameter were appeared on leaves as necrotic lesion. These spot increase about 2-3 cm in diameter and spot surrounded by halo-chlorotic tissues. The spot gradually increase in size in a concentric manner and often coalesce leading to blighted appearance. The symptoms appeared on inoculated leaf were similar as the symptoms those observed originally in the cauliflower leaf. The characters of the test pathogen was confirmed by reisolation of the pathogen in the PDA media.

Application of chemical fungicide is a conventional method to control the disease is a conventional method to control the disease caused by fungal pathogens. Tremendous health hazards are reported to occur during the application of fungicides, in field conditions (Alam, et.al, 1999). The use of essential oils to control post harvest fungi and pest is gaining attention because of the increasing public concern over the level of residues in food (Bishop et.al, 1997). Essential oils are likely to be associated with the development of reistance than in the case of fungicides and are less hazardous to the environment and human health than synthetic pesticides (Daferera et.al, 2003). Besides, essential oils can be used as good herbicides since they are highly volatile and do not persist in soil for long time and therefore have little or no mammalian toxicity (Isman, 2000). Many of these fungicides are biohazardous and adversely affect the components of ecosystem. Further, the cost of these fungicides and antibiotics is comparatively high and their constant application results in development

of resistance in the pathogens against these fungicides and antibiotics (Nigam *et al*., 1994).

The present study, some essential oils of aromatic plants have been used for their antifungal activity against the *Alternaria brassiciola,* the pathogen of cauliflower casing leaf spot disease. The nontoxic, non-pollutive and biodegradable nature of these essential oils prompted to exploit these natural products of plants against *Alternaria brassicicola*.

Among the oil sample taken for study,the oil of *Mentha arvensis, Cymbopogan martini and Cymbopogan citratus* were able to inihibit the mycelial growth of *Alternaria brassicicola* completely. The reason for such high toxicity of these essential oil against test fungi may be due to the presence of different fungi-toxic compounds in high proportion.

Among these, the oil of *Cymbopogan martini* was found to be most effective one as it inhibited the growth of mycelium by 100% even at low concentration of $2.5\mu lml^{-1}$. The oil of *Cymbopogan citratus* and *Mentha arvensis* inhibit the mycelial growth of test fungus by 100% at concentration of $10\mu lml^{-1}$ and $5\mu lml^{-1}$ respectively. The inhibition percentage of *Zanthoxylum armatum* was found to be more than 65%. The oils of *Gaultheria fragrantissima* and *Juniperus recurva* were found to be less effective to control the disease.

In the present study, the oils of *Cymbopogan martini* was the most effective in controlling the test fungus. High degree of inhibitory effect on the mycelial growth of the test fungus might be due to presence of higher concentration of active chemicals on the essential oil that could sufficiently suppress the physiological development of the fungus. Bansod and Rai (2008) extracted oil from fifteen medicinal plants for their antifungal activity against *Aspergillus fumigates* and *A. niger*. The oil of *Cymbopogan martini, Cinnamomum zylenicum, Eucalyptus globus and Cymbopogan citratus* showed maximum antimycotic activity. Flori *et al*. (2000) studied antifungal activity of leaf extracts and essential oils of medicinal plants against *Didymella bryoniae*. They reported that the essential oil of *Cymbopogan citrates, Ageretum conizoids* and *Eucalyptus citriodora* provided 100% inhibition of the mycelial growth and germination of spores of *Didymella bryoniae*.

In this study *Gaultheria fragrantissima* and *Juniperus recurva* were found to be less effective in control of the test fungus. One of the reason for different fungi toxicity of various plant extract may be due to their different chemical composition (Rao and Srivastava, 1994). The composition of plant oils and extracts is known to vary according to local climate and environmental conditions (Janseen *et.al.*, 1987; Sivropoulou et.al., 1995). However, the result obtained in controlling plant diseases by using different plant extracts and essential oil may differ as many factors vary between assays. These may include difference in microbial growth, exposure of micro-organism to plant oil or extract, the solubility of oil or oil components and the use and quantity of an emulsifier (Reynolds, 1996).

CHAPTER-6

6. CONCLUTION

It can be concluded form the study that leaf spot disease of cauliflower is caused by the fungus *Alternaria brassicicola*. This was confirmed by isolation of pathogen, conidial measurement and pathogenicity test. From the experiment it was conformed that the fungus was pathogenic to cauliflower.

The study was conducted to test the fungitoxicity of the essential oils of medicinal plants at different concentrations against the *Alterneria brassicocola.* The experiment result shows that essential oil of *Cymbopogan citratus, Cymbopogan martini* and *Mentha arvensis* are able to control the mycelial growth of the test fungi completely under lab condition. Among them, the oil of *Cymbopogan martini* showed highest fungitoxicity of 100% even at low concentration of 2.5µlml^{-1}. The oil of *Cymbopogan citratus* and *Mentha arvensis* completely inhibited the mycelium growth of test fungus at 20µlml^{-1} and 10µlml^{-1} concentration respectively. The oil of *Zanthoxylum armatum, Juniperus recurva and Gaultheria fragrantissima* inhibited the mycelium growth of test fungus by 68.85%, 36.66% and 36.59% at 40µlml^{-1} concentrations respectively.

CHAPTER-7

7. RECOMMENDATIONS

1. This investigation was carried out strictly under the controlled conditions of the laboratory. The result found might not correspond with those when performed in field under the influence of different environmental condition. So it is recommended that further work in the field condition is necessary.
2. These essential oil could be recommended for the protection of cauliflower against the *Alternaria brassicicola*.
3. The oil of *Cymbopogan martini* might be most useful for practical application in controlling leaf spot disease of cauliflower.

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APPENDIX-I

Apparatus and Equipments

Microscope, hot air oven, laminar air flow, incubator, autoclave, slides, coverslips, forceps, needle, inoculating loop, ocular micrometer, stage micrometer, measuring cylinder, cork-borer, test tube, test tube stand, pipette, conical flasks, glass rod, Petri plates, balance, beaker, cotton swabs etc.

Chemical and Reagent

2% Sodium hypochlorite, agar, dextrose, lactophenol, cotton blue, potato, distilled water, sprit, ethanol, 80% acetone etc.

Essential oil samples

Cymbopogan citratus(Dc.) Trin.

Cymbopogan martini (Roxb.)

Mentha arvensis L.

Zanthoxylum armatum Dc.

Juniperus recurva Buch-Ham.ex.D.Don

Gaultheria fragrantissima Dc.

APPENDIX-II

Micrometry

In micrometer, 100 division = 1000µm

1division = $\frac{1000}{100}$ µm

=10 µm

19 division of ocular micrometer coincide with 9 division of stage micrometer.

1 division of ocular micrometer coincides with $\frac{9}{19}$ division of stage micrometer.

∴ 1 division = $\frac{9}{19} \times 10$µm

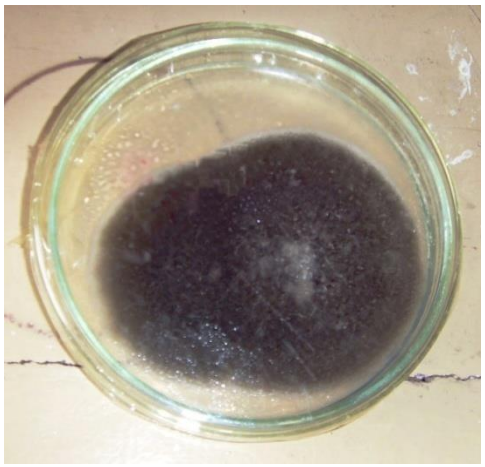
= 4.73 µm

Measurement of conidia size of conidia of *Alternaria brassicicola* (Schw.) Wiltshire

S.N.	**Length**		**Breadth**	
	No. of ocular divisions occupied by conidia (a)	Size of conidia = a × calibration factor for one ocular division i.e., 4.73 µm	No. of ocular divisions occupied by conidia (b)	Size of conidia = b × calibration factor for one ocular division i.e., 4.73 µm
1	5.5	5.5×4.73=26	2	2×4.73=9.46
2	10.2	10.2×4.73=48.25	3.2	3.2×4.73=15.14
3	7	7×4.73=33.11	3	3×4.73=14.19
4	7.5	7.5×4.73=35.47	3	3×4.73=14.19
5	6.4	6.4×4.73=30.27	2.1	2.1×4.73=9.93
6	11	11×4.73=52.03	3	3×4.73=14.19
7	9	9×4.73=42.57	2.5	2.5×4.73=11.82
8	11.3	11.3×4.73=53.44	3.5	3.5×4.73=16.55
9	8	8×4.73=37.84	2.2	2.2×4.73=10.40
10	10	10×4.73=47.30	3	3×4.73=14.19

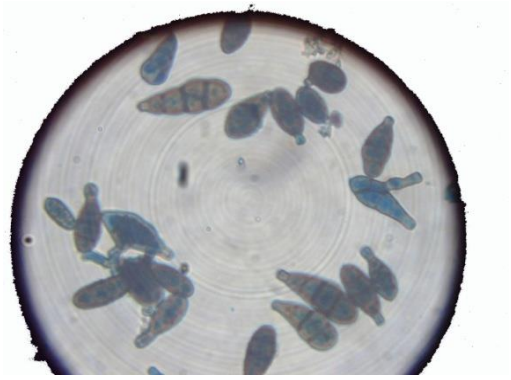
Hence the size of conidia ranges from (26-53.44×9.46-16.55) µm.

PHOTO PLATE-I

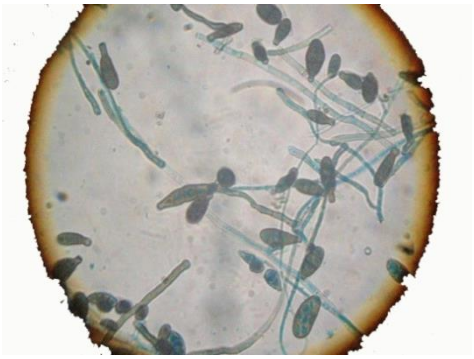


Pure culture of *Alternaria brassicicola*

Infected leafs of Cauliflower



Conidia of the test pathogen

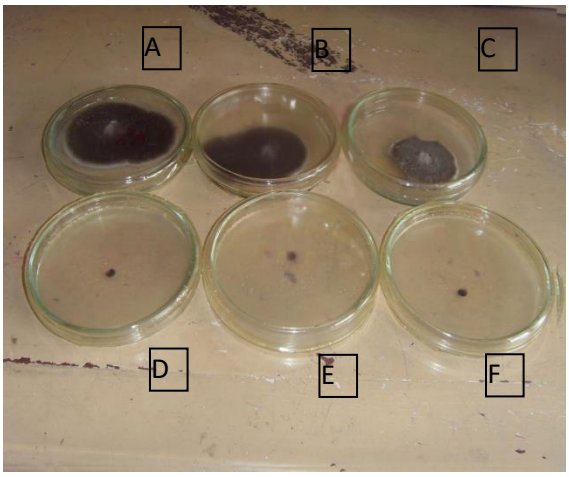


Conidia and conidiophore

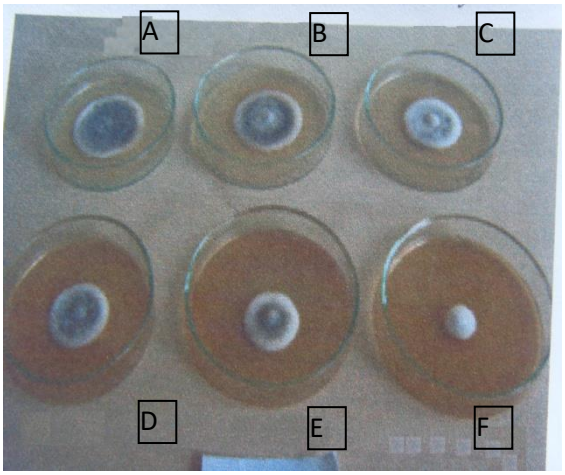
Infected Cauliflower Plant

Controlled Potted Plant

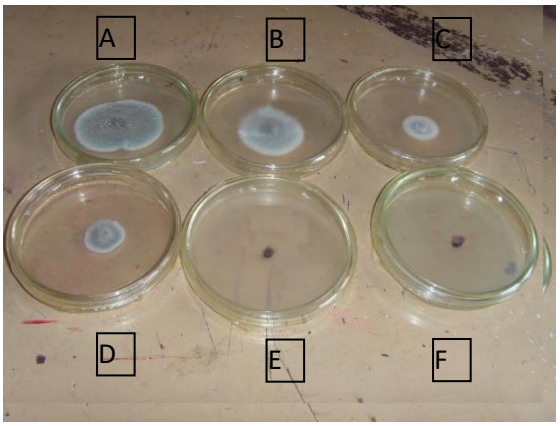
PHOTO PLATE-II



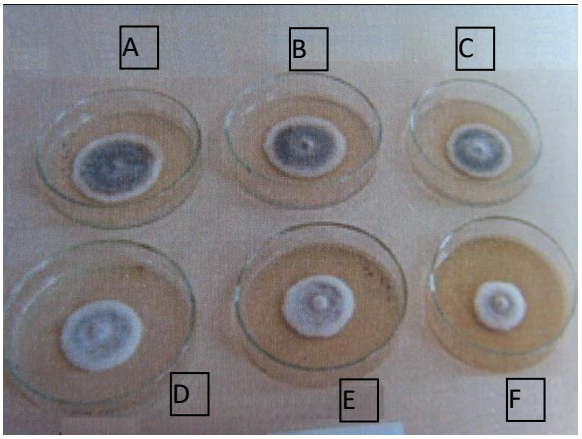
Antifungal activity of *Mentha arvensis*



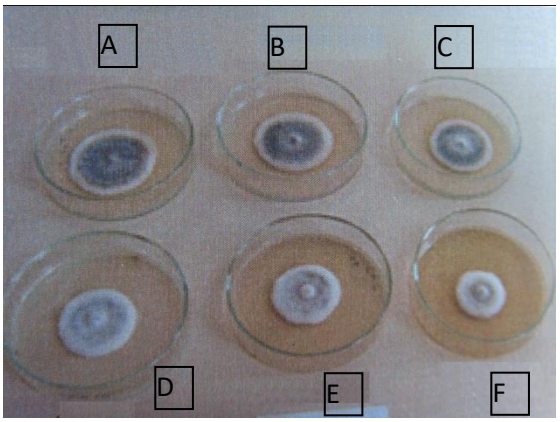
Antifungal activity of *Zanthoxylun armatum*



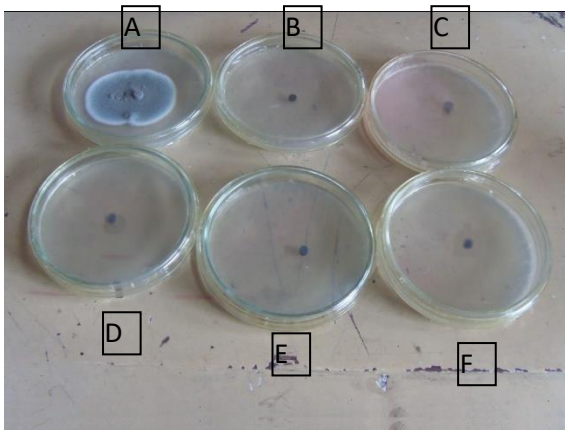
Antifungal activity of *Cymbopogan citratus*



Antifungal activity of *Juniperus recurva*



Antifungal activity of *Gaultheria fragrantissima*



Antifungal activity of *Cymbopogan martini*

A= Control

B= 2.5µlml^{-1}

C= 5µlml^{-1}

D= 10µlml^{-1}

E= 20µlml^{-1}

F= 40µlml^{-1}