

**ANTIFUNGAL EFFECTS OF SOME PLANT ESSENTIAL  
OILS AGAINST *Alternaria alternata* (Fr.) Keissl. AND  
*Aspergillus niger* van Tiegh. FROM GRAPES**

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## ABSTRACT

The essential oil of six plants viz. *Murraya koenigii* (L.) Spreng., *Eucalyptus citriodora* Hook., *Artemisia indica* Willd., *Cinnamomum camphora* (L.) J. Presl., *Cinnamomum tamala* (Buch.-Ham.) Nees and Eberm and *Lantana camara* L. were assessed in vitro for the antifungal activity against *Alternaria alternata* (Fr.) Keissl. and *Aspergillus niger* van Tiegh., causing postharvest rots in grapes. The test fungi were isolated from infected grapes obtained from local markets. The essential oils were extracted through hydrodistillation process using Clevenger oil extracting apparatus. For screening of antifungal activity treatments at concentration of 20, 40, 80, 160 and 320 µl/ml and controls were set to determine percentage inhibition of mycelial growth of two test fungi using poisoned food technique. All the tested oils exhibited significant antifungal effect ( $P < 0.05$ ) over tested fungi. Among all six essential oils, *Cinnamomum camphora* showed the most effective antifungal activity against *Aspergillus niger* which inhibited the mycelial growth by 81.58 % and 100 % at 20 and 80 µl/ml oil concentration respectively. Furthermore, *Cinnamomum tamala* showed best antifungal effect in controlling *Alternaria alternata* among all six oils which inhibited the mycelial growth by 93.11 % at 20 µl/ml and by 100 % at 80 µl/ml oil concentrations.

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## LIST OF ABBREVIATIONS

Alt.	= Altitude
cm	= centimeter
CBY	= Commercial Baking Yeast
CDB	= Central Department of Botany
EOs	= Essential oils
ft	= feet
FAO	= Food and Agriculture Organization
FDD	= Fruit Development Directorate
Fig.	= Figure
gm	= gram
gm/ml	= gram per milliliter
GC-MS	= Gas Chromatography Mass Spectrometry
Kcal	= Kilocalorie
m	= meter
mt	= milliton
mt/ha	= million/hectare
Mql	= millions of quintals
mha	= Thousands of hectares
Mhl	= Millions of hectoliters
masl	= Meter above sea level
mg	= milligram
mg/l	= milligram per liter
MIC	= Minimum inhibitory concentration
NE	= Northern and Eastern
Neg.	= Negative
Pos.	= Positive
PDA	= Potato Dextrose Agar
ppm	= parts per million

S.N.	= Serial number
μg	= microgram
μg/ml	= microgram per milliliter
μl/ml	= microliter per milliliter
μl/l	= microliter per liter
T.U.	= Tribhuwan University
MIC	= Minimum inhibitory concentration
WCE	= Western, Central and Eastern
° C	= Degree Celsius
WP	= Wettable powder

# CHAPTER - ONE

## 1. INTRODUCTION

### 1.1. General Background

The agro-ecological conditions of Nepal are very much suitable for the successful cultivation of large number of fruit species. As of 2067/68, about 831576 mt of fruits produced from 111882 hectare of area whereas the productivity is 10.5 mt/ha. There is an increasing trend in area coverage and fruit production but the yield per hectare is low in comparison with other fruit producing countries. Fruit Development Directorate (FDD) was first established as Horticulture Section in 1955, which evolved as Fruit Development Section in 1966, Fruit Development Division in 1990 and the directorate in 2000. FDD is the central body responsible for the development of fruits, coffee, tea and ornamental crops in the country (FDD, 2015).

The Eurasian grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world (Mattia *et al.*, 2008). *Vitis vinifera* (common grape vine) is native to the Mediterranean region, central Europe and Southwestern Asia, from Morocco and Portugal north to south Germany and east to northern Iran. It has 5000 to 10,000 varieties but only a few are commercially important (Wine and Spirits Education Trust, 2012).

The grape plant is a vigorous vine of the family Vitaceae. The European grape *Vitis vinifera* (L.) is a bisexual whereas some native North American *Vitis* species are monoecious. Multiple buds are produced laterally on the previous season's cane and flowers are wind pollinated. It is a lianous species growing to about 32 m tall, with flaky bark. The leaves are alternate, palmately lobed, 5-20 cm long and broad. The fruit is a berry, known as a grape. The fruit clusters mature about 5-7 months after bud burst. The grape is eaten fresh, processed to make wine, or dried to produce raisins. The grapes may be green, red or purple (Anonymous, 2015). Grape flowers are borne on an inflorescence or flower cluster. The main axis of the cluster is the rachis, and the individual flowers are attached to the rachis by the pedicel or cap stem. Prominent branches arising from the rachis stem are referred to as shoulders. The peduncle attaches the cluster to the vine, extending from the shoot to the first branch of the rachis. At harvest, the rachis framework typically accounts for 2 to 5 percent of cluster fresh weight (Dokoozlian, 2000).

Major components found in grapes are; Phenolics such as Anthocyanins (skin of berries), hydroxycinnamic acids (in the pulp) and proanthocyanidins (seeds). Trans-resveratrol is a phytoalexin produced against growth of fungal pathogens such as *Botrytis cinerea* and delta-vinifera is another grapevine phytoalexin produced against *Plasmopara viticola* (Anonymous, 2015). Grapes are known for their high phytochemicals content. Phenolic compounds isolated from grapes include catechins, epicatechin, procyanidin and some dimers and trimers. These may prevent a number of chronic diseases and cancers. Numerous studies have indicated that grape consumption may be beneficial in reducing the plasma concentration of cholesterol and preventing atherosclerosis (Hashemi, 2014).

Grapes are highly digestible and have a number of therapeutic properties: firstly, they are laxative and diuretic. They are useful in fighting dyspepsia, hemorrhoids, stones in the urinary tract and bile ducts. They also activate liver functions, ease digestion, help reduce the cholesterol level of the blood and eliminate uric acid. Grapes are also disinfectant and antiviral, aid the nervous system and are useful in processes that demineralize the body such as pregnancy and nursing (FAO, 2005).

Table grape (*Vitis vinifera* L.) is a nonclimacteric fruit with a relatively low rate of physiological activity. Postharvest decay caused by *Rhizopus stolonifer*, *Aspergillus niger*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Lasioidiplodia theobromae* and *Phomopsis viticola* is the major cause of rapid and extensive deterioration of table grapes (Nelson, 1979; Barkai-Golan, 2001; Lichter *et al.*, 2002, cited by Sukatta *et al.*, 2008).

Grape is one of the world's most widely grown fruit crops in relatively warm temperate-zone climates. World vineyards reached a total area surface (including areas not yet in production or harvested) of 7,528 Mha. World production of grapes in 2012 was estimated to be about 691 Mql. In 2012, the biggest grape producing countries are China, Italy, USA, France, Spain, Chile, India, Argentina and Iran respectively. Since 2000, the world's grape production has gone up somewhat. It is not a great increase, from 648 Mql to 691 Mql in 2012. It shows, 7% increase over 12 years (OIV, 2013).

The common varieties of grapes grown in Nepal are; Himrod (seedless), Stuben (black colored), Kyoho, Olympia and Muscat Bailey A. Fruit cultivation is popular and commercialized in Nepal. Although, grape cultivation is not so widespread and its

commercial cultivation is very rare. In Kathmandu valley, 1 in 10 houses grow grapes in their kitchen garden. Mostly Stuben variety is grown in Kathmandu and in some other parts of Nepal like Banke, Kavre, Bhaktapur etc. The meteorological data shows that; low level of humidity and rainfall and high level of sunshine is suitable for grape cultivation. The potential areas for grape cultivation are Mustang, Kathmandu, Banke, and some places in inner Terai. The climatic conditions of Western Nepal are favorable for grape cultivation than Eastern Nepal (FDD, 2014).

According to general survey, 70-80 % grapes sold in common fruit centers of Kathmandu valley are imported from India. The most common Indian variety found in Nepalese market is 'Perlet', which is light green to light yellow in color. These grapes gets easily infected by fungus due to lack of post-harvest management.

Essential oils are natural aromatic compounds found in the seeds, bark, stems, roots, flowers, and other parts of plants. They can be both beautifully and powerfully fragrant. In addition to giving plants their distinctive smells, essential oils provide plants with protection against predators and disease and play a role in plant pollination. Essential oils are non-water based phytochemicals made up of volatile aromatic compounds. Although they are fat soluble, they do not include fatty lipids or acids found in vegetable and animal oils. Pure, unadulterated essential oils are translucent and range in color from crystal clear to deep blue. In addition to their intrinsic benefits to plants and being beautifully fragrant to people, essential oils have been used throughout history in many cultures for their medicinal and therapeutic benefits (Lawless, 2013).

Essential oils have become an integral part of everyday life. They are used in a great variety of ways: as food flavorings, as feed additives, as flavoring agents by the cigarette industry, and in the compounding of cosmetics and perfumes. Furthermore, they are used in air fresheners and deodorizers as well as in all branches of medicine such as in pharmacy, balneology, massage, and homeopathy. In recent years, the importance of essential oils as biocides and insect repellents has led to a more detailed study of their antimicrobial potential. Essential oils are also good natural sources of substances with commercial potential as starting materials for chemical synthesis (Baser and Buchbauer, 2009).

Essential oil bearing plants constitute a rich source of bioactive chemicals, which have been reported to have various antifungal properties. These chemicals are often active

against a limited number of species, including the specific target species. They are also biodegradable and non-toxic (Adebayo *et al.*, 2013). Naturally occurring biologically active compounds from plants are generally assumed to be more acceptable and less hazardous than synthetic compounds and represent a rich source of potential disease-control agents (Tripathi *et al.*, 2008).

## **1.2. Post-Harvest Disease and Grapes**

Post-harvest disease account for substantial losses of fruits, vegetables and other plant products during the transit and storage process (Mahmoudi *et al.* 2012). Postharvest diseases may start before or after harvesting. Plants or fruits infected in the field may not develop symptoms until stored. Once in storage, infections continue to develop on the fruits and vegetables. Wounds, cuts, or bruises caused during harvesting are common entry points for bacteria and fungi (Coates and Johnson, 1997).

The most important losses in agricultural production which involve the greatest costs on the farm economy occur postharvest. It is estimated that worldwide between 10 and 40% losses of agricultural produce occur postharvest (Enyiukwu *et al.*, 2013). Postharvest losses have been estimated to be of the order of 20 to 30% for fresh fruits and vegetables and could exceed 50% under adverse conditions in Nepal. Losses in fruits result from harvesting at an improper stage of maturity, improper methods of harvesting, packaging, transportation and storage (Rosa, 2006).

Post-harvest berry infection is primarily caused by conidial infection at or after veraison (De-Kock and Holz, 1991) although some authors suggest it may happen at the flower stage (Nair and Allen, 1993). The fungus remains quiescent in the developing fruit, with symptoms only appearing on the mature fruit. It is thought that loss of berry resistance is due to the decreasing ability of the maturing berry flesh to synthesize antimicrobial stilbenes and also due to the fall in proanthocyanidin concentration during development (Creasy and Coffee, 1988). Berry cracking in certain cultivars also encourages infection. The primary cause of post-harvest loss in table grapes is grey mould disease or *Botrytis cinerea* (Pearson and Goheen, 1988; Snowdon, 1990). Some other important fungal post-harvest diseases of table grapes include Aspergillus rot (*Aspergillus niger*) which doesn't grow below 5°C, Alternaria rot (*Alternaria alternata*), blue mold rot (*Penicillium* spp.), Rhizopus rot (*Rhizopus oryzae*; *R. stolonifer*), anthracnose (*Elsinoe ampelina*, *Glomerella cingulata*) and others (Snowdon, 1990).

### 1.3. Nutritional value of Grapes

Grapes are known to have various kinds of nutritional substances.

Table 1: Nutritional content of Grapes

<b>Nutrients Content</b>	<b>Value per 100 g</b>
Energy	57 kcal
Protein	0.81 g
Total lipid (fat)	0.47 g
Carbohydrate, by difference	13.39 g
Fiber, total dietary	3.9 g
Water	84.29 g
Calcium, ca	37 mg
Iron, Fe	0.26 mg
Magnesium, Mg	14 mg
Phosphorus, P	24 mg
Potassium, K	203 mg
Sodium, Na	1 mg
Zinc, Zn	0.11mg
Vitamin C, total ascorbic acid	6.5 mg
Riboflavin	1.500 mg
Vitamin A, RAE	3 µg

Source: *USDA Nutrient Data Base*

#### 1.4. Diseases of Grapes

Table 2: Major diseases of Grapes

S.N.	Name of disease	Name of pathogen
1.	Anthraco nose	<i>Gloeosporium ampelophagum</i> (Pass.) Sacc.
2.	Botrytis bunch rot	<i>Botrytis cinerea</i> Pers.
3.	Downy Mildew	<i>Plasmopara viticola</i> (Berk. and M.A. Curtis) Berl. and De Toni
4.	Powdery Mildew	<i>Uncinula necator</i> (Schwein.) Burriil
5.	Alternaria rot	<i>Alternaria alternata</i> (Fr.) Keissl.
6.	Aspergillus rot	<i>Aspergillus niger</i> Tiegh.
7.	Leaf Rust of grapes	<i>Phakopsora eu vitis</i> Y. Ono
8.	Black rot	<i>Phyllosticta ampel icida</i> Engelm.
9.	Leaf spot	<i>Cercospora viticola</i> (Ces.) Sacc.

#### 1.5 Objectives of the study

##### General objective:

- 1) To test the in-vitro antifungal effect of the essential oils of six plants against two rot fungi of grapes namely *Alternaria alternata* and *Aspergillus niger*.

##### Specific objectives:

- 1) To isolate pathogens from infected grapes and test its pathogenicity.
- 2) To determine percentage of extraction of essential oils from six test plants.
- 3) To study and compare the antifungal activities of essential oils against the test fungi, *Alternaria alternata* and *Aspergillus niger*.

## **1.6 Rationale**

Grape is one of the commonly known fruit plant. It is beneficial fruit with high medicinal values. Due to lack of proper post-harvest management, several rots affect the grapes in storage condition. Post-harvest disease account for substantial losses of fruits, vegetables and other plant products during the storage process (Mahmoudi *et al.*, 2012). Post-harvest losses have been estimated to be of the order of 20 to 30% for fresh fruits and could exceed 50% under adverse conditions in Nepal (Rosa, 2006). *Alternaria* rot caused by *Alternaria alternata* and *Aspergillus* rot caused by *Aspergillus niger* significantly deteriorate the grapes during storage. Various types of hazardous contact fungicides are used for controlling post-harvest diseases. These fungicides may cause oncogenic effects. Naturally occurring biologically active compounds from plants are generally assumed to be more acceptable and less hazardous than synthetic compounds and represent a rich source of potential disease-control agents (Tripathi *et al.*, 2008). Hence, the use of plant essential oils for the control of these diseases may be suitable and practically applicable method. The oils have greater antifungal potency than synthetic fungicides and the oils don't exhibit any phytotoxic effects on the fruit peel so, they can act as potential source of ecofriendly botanical fungicide.

## **1.7 Limitations of the study**

- 1) Only two test pathogens were taken.
- 2) The antifungal activity of essential oil was conducted in vitro only.
- 3) GC-MS of the essential oils was not carried out.
- 4) MIC of essential oils were not identified.

## CHAPTER - TWO

### 2 LITERATURE REVIEW

Moleyar and Narasimham (1986) evaluated fifteen essential oil components for antifungal activity towards five spoilage-causing fungi. Unsaturated aldehydes (citral, cinnamic aldehyde and citronellal) followed by geraniol, an unsaturated alcohol showed MIC at 100 µg/ml toward *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium digitatum*. Menthol, a terpene alcohol was most inhibitory to *Rhizopus stolonifer* and *Mucor* sp. with a MIC at 200 µg/ml. In agar medium citral, cinnamic aldehyde, citronellal, geraniol and menthol failed to show complete inhibition of *A. niger*, *F. oxysporum* and *P. digitatum*, but showed effect against *R. stolonifer* and *Mucor* sp. than in liquid medium.

Mishra and Tiwari (1990) studied the antifungal activities of essential oil of nine plants against the fungus *Pyricularia grisea*, *Aspergillus niger* and *Aspergillus flavus*. Essential oil from *Amomum subulatum* exhibited complete inhibition of the fungus at 3000 ppm.

Dube *et al.* (1990) studied the antifungal and insect repellent activity of essential oil of *Zanthoxylum alatum*. The oil showed effective repellent activity against insect *Allacophora foveicollis* and antifungal activity against 24 fungi, including aflatoxin producing strains of *Aspergillus flavus* and *A. parasiticus* at a minimum dose of  $2.0 \times 10^3$  µl/l. High temperature, prolonged storage and increased inoculum did not affect fungistatic property of the oil.

Mishra and Dubey (1994) evaluated antifungal activities of some essential oils against *Aspergillus flavus*. The oil of *Cymbopogon citratus* exhibited fungitoxicity against test fungus. The oil showed MIC at 1000 ppm along with its fungistatic nature, wide fungitoxic spectrum, non-phytotoxic nature and superiority over synthetic fungicides. They concluded the effectiveness of the oil of *Cymbopogon citratus* against storage fungi.

Jobling (2000) studied the effect of tea tree oil on the growth of Botrytis from grapes. Tea tree oil showed antibacterial and antifungal properties. The oil concentrations between 100 and 500 ppm prevented the growth of this fungus when it was grown in laboratory.

Koukam *et al.* (2002) investigated the antifungal activity of essential oil and several pure sulfur compounds isolated from *Scorodopholeus zenkeri* against some bacteria and fungi. The essential oil completely inhibited the growth of all fungi tested including yeasts except *Aspergillus niger*. It showed active effect against gram-positive bacteria studied but not the gram-negative bacteria.

Tiwari *et al.* (2004) studied antifungal effects of leaves extract and essential oil of common lemon plant (*Citrus medica*) against three destructive fungal pathogens *Pyricularia grisea*, *Aspergillus niger* and *Aspergillus flavus*.

Dharmagadda *et al.* (2004) studied the antibacterial and antifungal effects of essential oils of *Lantana camara*, *Ocimum sanctum* and *Tagetes patula* leaves. *Lantana camara* oil compared to *Ocimum sanctum* and *Tagetes patula* was found effective in inhibiting the growth of fungi, *Aspergillus niger* and reducing the growth of other fungi. Higher susceptibility of fungi to *Lantana camara* oil compared to other oils might be due to higher concentrations of active terpenoides. Similarly, *Ocimum sanctum* and *Tagetes patula* has higher antibacterial activity compared to *Lantana camara* oil, because their oils contain eugenol.

El-Zemity and Ahmed (2005) studied antifungal activity of 15 essential oils, caraway, clove, fennel, thyme, chenopodium, cinnamon, geranium, rosemary, matrecary, rose, garlic, eucalyptus, lemon and peppermint and their monoterpenoidal constituents against six plant pathogenic fungi (*Rhizoctonia solani*, *Macrofomina phaseolina*, *Fusarium oxysporum*, *Helminthosporium* sp., *Alternaria alternata* and *Diplodia* sp.). The result showed that caraway, clove, fennel and thyme oils exhibited potential inhibition effect of the tested fungi. Among the monoterpenoidal constituents, thymol, chlorothymol and carvacrol showed the highest fungicidal activity compared with the other essential oil components used.

Manandhar (2005) studied the antifungal activities of plant essential oils of *Thymus linearis*, *Tanacetum gracile*, *Clinopodiumum brosum*, *Elsholtzia erostachya*, *Zanthoxylum armatum* and *Cinnamomum tamala* against *Bipolaris sorokiniana*. Among the six plants, oil of *Thymus linearis* showed highest fungitoxicity (100%).

Sharma and Tripathi (2006) studied the effects of essential oil of *Citrus sinensis* against *Aspergillus niger*. The oil inhibited the mycelial growth at 2.5 and 3.0 µg/ml of oil in Potato Dextrose Broth and Agar medium, respectively. The main changes observed under oil treatment were loss of cytoplasm in fungal hyphae, and budding of hyphal tip. GC-MS studies of the oil revealed the presence of 10 chemical constituents. The major component of the oil was Limonene (84.2%). Antifungal activity of oil is due to presence of limonene.

Ozcan and Chalchat (2006) studied chemical composition and antifungal effect of anise (*Pimpinella anisum*) fruit oil against some fungi. The oil showed most effective inhibitory activity against mycelial growth of the fungus *Aspergillus parasiticus* followed by *Aspergillus niger* and *Alternaria alternata*.

Helal *et al.* (2006) had investigated effects of essential oil of *Cymbopogon citratus* on growth, lipid content and morphogenesis of *Aspergillus niger*. They determined the ultra-structural modification of *A. niger* hyphae after treatment with *C. citratus* essential oil. They reported that the oil causes decreased hyphal wall and also caused plasma membrane disruption, mitochondrial structure disorganization and decreased in lipid content.

Lee *et al.* (2007) studied the effects of 39 essential oils. Five essential oils showed inhibitory activities against mycelial growth of at least one Phytopathogenic fungus. *Origanum vulgare* essential oil inhibited mycelial growth of all of the five fungi tested. *Eucalyptus citriodora* and *Cuminum cyminum* oils have a potential as antifungal preservatives for the control of storage diseases of various crops.

Kuinkel (2007) evaluated the antifungal activity of some plant essential oils of *Thymus linearis*, *Zanthoxylum recurva*, *Mentha arvensis* and *Artemisia indica* against the fungus *Glomerella cingulate*, the causal agent of bitter rot of apple by poisoned food technique. The oil of *Thymus linearis* and *Mentha arvensis* showed the highest fungitoxicity (100%) at 5000 ppm.

Kishore *et al.* (2007) evaluated antifungal activity of clove oil, Cinnamon oil and five essential oil components (citral, eugenol, geraniol, limonene and linalool) against 14

phytopathogenic fungi. Citral completely inhibited the growth of *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium moniliforme*, *F. pallidoroseum*, and *Phoma sorghina* in paper disc agar diffusion assays. Cinnamon oil, citral and clove oil as low as 0.01% (v/v) inhibited the spore germination of *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis* by > 90% in vitro. Limonene and linalool showed least antifungal activity against test fungi.

Feng and Zheng (2007) tested the inhibitory effects of five essential oils (thyme, sage, nutmeg, eucalyptus and cassia) against *Alternaria alternata*. The cassia oil and thyme oil exhibited antifungal activity against test fungus. The cassia oil inhibited completely the growth of *A. alternata* at 300 ppm while thyme oil inhibited 62% at 500ppm. They recommended EOs as an alternative to chemicals for post-harvest phytopathogenic fungi on fruits or vegetables.

Ziedan and Farrag (2008) surveyed antifungal activity of essential oils from leaves of Peppermint and sweet basil against post-harvest decay of peach caused by 3 fungi *Rhizopus stolonifer*, *Monilinia fructicola* and *Aspergillus niger*. The oil of peppermint and sweet basil showed antifungal effect against fungal pathogens. *R. stolonifer* and *M. fructicola* were completely inhibited by crude oils at dose of 30 µl/400 cm<sup>3</sup> air in a closed system. The findings showed the possibility of using crude of peppermint and sweet basil oils as alternative components to chemicals for preservations of stored fruits.

Tripathi *et al.* (2008) evaluated antifungal effects of 26 essential oils against *Botrytis cinerea*. The essential oils of the ten plants viz. *Chenopodium ambrosioides*, *Eucalyptus citriodora*, *Eupatorium cannabinum*, *Lawsonia inermis*, *Ocimum canum*, *O. gratissimum*, *O. sanctum*, *Prunus persica*, *Zingiber cassumunar* and *Z. officinale* were found to exhibit absolute fungitoxic activity (100% growth inhibition). The MIC values of values of *O. sanctum*, *P. persica* and *Z. officinale* were found to be 200, 100 and 100 ppm (mg/l) respectively. The oils were thermo stable, and exhibited a wide range of fungitoxicity against 15 other post-harvest fungal pathogens. The essential oils showed practical applicability in controlling grey mould of grapes caused by *B. cinerea* during storage. The *O. sanctum* and *P. persica*-oil-treated grapes enhancement of storage life up to 5 and 4 days respectively. *Z. officinale*-oil-treated grapes showed storage life of

6 days. The oils did not exhibit any phytotoxic effect on the fruit peel. Therefore, they recommended oils as a potential source of ecofriendly botanical fungicide.

Sukatta *et al.* (2008) studied antifungal effect of essential oil from clove and cinnamon against 6 fungi causing post-harvest decay of grapes: *Aspergillus niger*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae*, *Phomopsis viticola* and *Rhizopus stolonifer* by inverted petriplate method. The clove oil showed MIC against all the test fungi at 200, 200, 400, 800, 200 and 200 mg/ml, respectively whereas cinnamon oil showed MIC at 50, 100, 200, 200, 100 and 800 mg/ml respectively.

Sitara *et al.* (2008) evaluated the antifungal effect of essential oils extracted from the seeds of neem (*Azadirachta indica*), mustard (*Brassica campestris*), black cumin (*Nigella sativa*) and asafoetida (*Ferula assafoetida*) were evaluated for 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 hawaiiensis* and *Alternaria alternata*. Fungicide, Ridomyl gold (MZ 68% WP) was used for comparison. All the oils extracted except mustard, showed fungicidal activity of varying degree against test species. Of these oils, Asafoetida oil at 0.1% and 0.15% significantly inhibited the growth of all test fungi except *A. flavus* and *Nigella sativa* oil at 0.15 was also effective but showed little fungicidal activity against *A. niger* followed by neem, Ridomyl gold and mustard oils.

Abd-Alla *et al.* (2008) studied antifungal effects of yeast (*Saccharomyces cerevisiae*), *Candida tenuis* and the commercial baking yeast of *S. cerevisiae* mixture (CBY) and peppermint oil, melon and rose essential oils against *Botrytis cinerea*, *Rhizopus stolonifer* and *Alternaria alternata*, the causal agents of tomato fruit decay. CBY showed highest inhibitory effect on growth of tested pathogenic fungi than two yeast isolates. Peppermint oil showed superior inhibitory effect against the growth of tested pathogenic fungi followed by rose and melon oils, respectively.

Poudel (2009) studied antifungal activity of essential oil of *Cinnamomum tamala*, *Nardostachys grandiflora*, *Eucalyptus citriodora*, *Cymbopogon citratus*, *Mentha arvensis* and *Acorus calamus* 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 µl/ml.

Piyo *et al.* (2009) evaluated activities of essential oils of basil (*Ocimum basilicum*) and Sweet Fennel (*Ocimum gratissimum*) against seven species of rice pathogenic fungi; *Alternaria brassicicola*, *Aspergillus flavus*, *Bipolaris oryzae*, *Fusarium moniliforme*, *Fusarium proliferatum*, *Pyricularia arisea* and *Rhizoctonia solani*. The basil oil inhibited mycelia growth of *F.moniliforme*, *F. proliferatum* and *P. grisea* by 100%, 49.6% and 100% respectively at concentration of 0.6% v/v. At concentration of 2.0% v/v, the oil inhibited *B. oryzae*, *A. brassicicola* and *A. flavus* by 97.40%, 94.62% and 59.25%, respectively. However, basil essential oil was not effective in controlling *R. solani*. Sweet Fennel Oil could inhibit mycelium growth of all pathogenic fungi at 0.8% v/v concentration.

Hadizadeh *et al.* (2009) studied antifungal effects of essential oils from some medicinal plants like nettle (*Urtica dioica*), thyme (*Thymus vulgaris*), eucalyptus (*Eucalyptus* spp.), Rue (*Ruta graveolens* L.) and common yarrow (*Achillea millefolium*) against *Alternaria alternata* on tomato. Nettle oil showed highest inhibition effect against the test fungus among all.

Abdolahi *et al.* (2009) screened effects of essential oils from sweet basil, fennel, summer savory and thyme plants against mycelial growth of *Botrytis cinerea* under in vitro condition and on fungal decay and quality sensors of table grape (*Vitis vinifera* L.) under in vivo conditions. The essential oils showed high inhibitory effect on mycelial growth of *B. cinerea*. Under in vivo assays, thyme and summer savory oils reduced fungal decay on table grape after 60 days of storage.

Maqbool *et al.* (2010) studied the antifungal effect of Cinnamon oil against *Colletotrichum musae*, causal organism of anthracnose in bananas during storage. The oil showed significant ( $P<0.05$ ) inhibition of mycelial growth and conidial germination of test fungus. At 0.4% concentration oil exhibited best effect in inhibiting mycelial growth and conidial germination (83.2%). The oil treatments of 0.3% and 0.4% delayed the onset of disease incidence up to 21 days in bananas during storage.

Dagostin *et al.* (2010) evaluated the antifungal ability of sage (*Salvia officinalis*) extract for controlling grapevine downy mildew under green house and field conditions. Sage extract effectively controlled grapevine downy mildew. According to them, sage could be a promising alternative to copper in organic viticulture.

Siramon *et al.* (2012) evaluated antifungal activities of leaf essential oils from *Eucalyptus camaldulensis* from Thailand against 9 fungal strains. The fungi were *Aspergillus niger*, *Cladosporium cladosporides*, *Chaetomium globosum*, *Penicillium citrinum*, *Fomitopsis palustris*, *Trametes versicolor*, *Fusarium oxysporum*, *Thanatephorus cucumeris* and *Rhizopus oryza*. The results showed that *Eucalyptus camaldulensis* leaf oils provided 100% inhibition of the mycelial growth of *T. cucumeris* (5 mg/ml), and *C. globosum* (10 mg/ml). There was no inhibition effect against *Rhizopus oryzae* even at the concentration of 10 mg/ml.

Passos *et al.* (2012) studied the chemical composition of essential oils of two very similar species of the Verbenaceae family (*Lantana camara* and *L. radula*). They revealed that the main components of essential oil of *L. camara* were germacrene-D (19.8%) and E-caryophyllene (19.7%), while those of *L. radula* were E-caryophyllene (25.3%), phytol (29.2%) and E-nerolidol (19.0%). The oils of *L. radula* were more fungistatic than the oils of *L. camara* against the fungus *Corynespora cassiicola*.

Mahmoudi *et al.* (2012) evaluated the effects of *Zataria multiflora* essential oil at different concentrations (0, 100, 200, 300 and 500 ppm) on the growth rate and morphogenesis of *Alternaria alternata* in *in-vitro* and *in-vivo* conditions on tomato fruits. The oil inhibited mycelial growth of test fungus at concentrations of 500 and 200 ppm. Similarly, oil exhibited 100 % inhibition of conidial germination at 300 ppm concentration. *In vivo* *Z. multiflora* oil at 500 ppm effectively reduced the percentage of decayed tomatoes. They concluded that essential oils can be used as alternative for control of post-harvest phytopathogenic fungi on fruits or vegetables.

Behdani *et al.* (2012) studied the antifungal activity of 13 essential oil against apple gray mold. The oils of Anise, Cumin, Caraway, Ammin, Pennyroyal, Thyme and Cinnamon at all concentrations of 250, 500, 750 µl/l showed significant effect against

*Botrytis cinerea* on PDA culture. They found that antifungal activity increases parallel with raising concentrations of plant essential oils.

Sharma *et al.* (2013) evaluated antifungal effects of essential oil extracted from Ginger (*Zingiber officinale*). Ginger rhizome oil was found effective against a range of bio-deteriorating storage fungi, *Aspergillus flavus*, *Penicillium expansum*, *Alternaria alternata* and *Fusarium oxysporum*. The oil showed MIC at 500 ppm in respect to all fungi. At higher concentration of 1000 ppm of oil was fungicidal in action.

Moghtader (2013) evaluated antifungal activity of *Mentha piperita* essential oil and its comparison with synthetic menthol on *Aspergillus niger*. The main oil content from the plants of *M. piperita* was 3.26% (v/w). They identified 23 compounds in essential oil of *M. piperita*. The major components were menthol (38.33%), menthone (21.45%) and menthyl acetate (12.49%). For study of antifungal activity, the essential oil was tested against *A. niger* by disc diffusion method via average inhibition zone. The essential oil from *M. piperita* at 1 and 1/2 oil dilutions exhibited a strong antifungal activity than gentamycin (8 mg/ml) antibiotic on *A. niger* and exhibited a strong synthetic menthol at 10% dilution. Antifungal activity of oil is due to high amount of menthol and menthone.

Kocic-Tanackov and Dimic (2013) studied antifungal activity of essential oils in the control of food-borne fungi growth and mycotoxin biosynthesis in food. They showed that EOs in antifungal and antimycotoxigenic protection of food can be applied as surface protection, as an addition to the modified atmosphere packaging or as an addition to food.

Hussain *et al.* (2013) investigated the inhibitory effects of 5 essential oils (ajwain oil, clove oil, garlic oil, neem oil and olive oil) against test fungi (*Aspergillus niger*, *A. flavus*, *Paecilomyces variotii*, *Penicillium* sp., *Grifola* sp. and *Trichoderma* spp.). The oil from neem, ajwain and clove showed effective activity against test fungi. They supported the potential use of essential oils for natural wood protection against decay mold fungi infestation for surface treatment of fumigation of wood products.

Reddy *et al.* (2014) studied the effects of 15 different medicinal and aromatic plants against *Aspergillus niger*, *A. flavus*, *Chaetomium globosum*, *Cladosporium cladosporoides*, *Nigrospora sphaerica*, and *Penicillium* sp. using poisoned food

technique. Among the 15 plants studied 60% of the plants had antifungal activity. The extract from *Cymbopogon citratus*, *C. caesius* showed maximum activity. *Balanites aegyptica*, *Cassia occidentalis*, *Hyptis suaveolens*, *Indigofera barberi*, *Cassia tora*, *C. senna*, *Eucalyptus citriodora* and *Corollocarpus apigaeus* did not exhibit antifungal activity at the condition studied.

Mahilrajan *et al.* (2014) studied the effects of plant essential oils against three palmyrah leaf decay fungi *Aspergillus niger*, *A. flavus* and *Penicillium sp.* Neem and castor oils showed no significant ( $P>0.05$ ) antifungal activity while citronella and camphor oils showed significantly different antifungal activity compared with control. Camphor oil was found to be highly antifungal and most effective against *Aspergillus niger* and *A. flavus*, compared with *Penicillium sp.*

Ibrahim and Ebady (2014) evaluated the antifungal activity of some plant extracts of tarragon (*Artemisia dracunculus*), rosemary (*Rosemarinus officinalis*) and thyme (*Thymus vulgaris*) and the essential oil of oregano (*Origanum vulgare*) against many fungi including *A. niger*, *A. flavus*, *Penicillium spp.*, *Rhizopus spp.* and *Fusarium spp.* Oregano essential oil showed a very strong antifungal activity against *Fusarium spp.* (MIC: 0.8 mg/ml), *A. niger* (MIC: < 1 mg/ml) and *Penicillium spp.* (MIC: 4.5 mg/ml). Application of essential oils or ethanolic rosemary extract at different concentrations controlled post-harvest spoilage of tomatoes for 4 weeks.

Atul (2014) studied the effect of essential oil of *Abutilon indicum* and *Lantana camara* against *Aspergillus niger*, *A. nidulans*, *Rhizopus nigricans*, *Cladosporium herbarium* and *Penicillium digitatum*. Both oils exhibited and showed different degree of antifungal activity against the test fungi.

Mahato *et al.* (2015) studied the antibacterial and antifungal effects of essential oil, alcoholic and aqueous extracts of leaves of *Eucalyptus* against fungal and bacterial pathogens of plant as well as animal through agar well diffusion. The results were found to be positive on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Alternaria alternata*, *Penicillium chrysogenum* and *Malassezia furfur*. The essential oil shows higher inhibition levels as compared to aqueous and alcoholic extract of root and leaves.

## CHAPTER-3

### 3 MATERIALS AND METHODS

#### 3.1. Materials

Various equipment, glassware and chemicals were used in performing the experiment. All those materials are listed in the Annexes.

#### 3.2. Methods

##### 3.2.1 Collection of Test Plant Species

Leaves of *Cinnamomun tamala*, *Cinnamomum camphora* and *Murraya koenigii* were collected from garden of Central department of Botany, TU. Similarly leaves of *Eucalyptus citriodora*, *Artemisia indica* and *Lantana camara* were collected from around TU area, Kirtipur.

##### 3.2.2 Herbarium preparation and identification

The collected plant species were pressed inside the paper sheet in between blotting papers. The paper sheets were changed in a regular interval of a day till the herbaria became dry. The specimens were pasted on herbarium sheets with tag number and labeling. Then the herbarium specimens were identified in CDB, T.U.

##### 3.2.3 Processing of samples

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

###### 3.2.3.1 Shade drying

The collected plant's leaves were cut into small pieces and spread under the shade at the normal room temperature and turned up and down regularly to hasten drying.

###### 3.2.3.2 Storage of samples

The well dried samples were packed in water proof bags. Then, the samples were taken for hydrodistillation turn by turn for the extraction of essential oil.

###### 3.2.3.3 Extraction of essential oils

100 gm shade dried leaves were surface sterilized with 0.1% mercuric chloride solution and washed thoroughly with distilled water. The leaf sample was then pulverized and subjected to hydro distillation for 6-8 hours in Clevenger's apparatus in 1000 ml water. Two distinct layers, upper aromatic layer of essential oil and lower colorless aqueous

layer were observed. The aromatic layer was collected and dehydrated over anhydrous sodium sulphate and stored at (<10°C) temperature (Rao and Srivastava, 1994).

### **3.2.4 Determination of percentage yield of essential oils**

After hydrodistillation, the volume of the oil extracted was noted down. The yield of essential oil was calculated in terms of percentage using following formula;

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

### **3.2.5 Media Preparation**

Potato Dextrose Agar (PDA) media was prepared for the culture of test fungal pathogens. For preparing 1000 ml of PDA media, 200 gm peeled potato was washed and cut into small pieces. Then, Potato was boiled in about 1 liter distilled water till it become soft to touch. The potato extract was filtered with the help of muslin cloth into conical flask. 20 gm Agar and 20 gm Dextrose was added into conical flask and stirred gently. Final 1000 ml volume was made by adding distilled water. Then, PDA was autoclaved for 15-20 minutes at 15 lb. pressure and 121°C temperature.

### **3.2.6 Isolation and Identification of test fungi (*Aspergillus niger* and *Alternaria alternata*)**

Infected grapes were collected from (Kalimati tarkari bazaar) Kalimati fruit and vegetable center of Kathmandu valley. Some pieces of fungal colony from infected grapes were transferred aseptically on the petriplate containing PDA media for one week. After one week, the growth of fungal colony was observed. The pathogens were identified by observing under compound microscope and comparing their microscopic characters using the standard literature (Ellis, 1971; Watanabe, 2010).

#### **3.2.6.1 *Aspergillus niger* van Tiegh.**

*Aspergillus niger* van Tiegh. is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mould on certain fruits and vegetables such as grapes, apricots, onions, and peanuts, and is a common contaminant of food (Samson, 2001). This pathogen can be serious problem when grapes are marketed at ambient temperatures (Mandal and Dasgupta, 1983; Cited by Barkai-

Golan, 2001). The fungus survives on plant debris in the soil at high temperature (25-30 °C) and airborne spores infect punctures, splits and stem-end fractures. Infection occurs only in mature berries; young berries are resistant to infection even when wounded (Barkai-Golan, 2001).

The pathogen when cultured in PDA medium, the colonies appears black with white reverse. Hyphae, septate hyaline. Conidial heads are large (up to 3 µm × 15-20 µm in diameter), globose, dark brown becoming radiate and tending to split into several loose columns with age. Conidiophores are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia were globose to subglobose (3.63-7.26 × 3.63-7.26) µm in diameter, dark brown to black and rough-walled.

Systemic position (Martin, 1961)

Kingdom - Mycetozoa  
Division - Eumycota  
Sub-division - Duteromycotina  
Class - Hyphomycetes  
Order - Moniliales  
Family - Moniliaceae  
Genus - *Aspergillus*  
Species - *A. niger*

### **3.2.6.2 *Alternaria alternata* (Fr.) Keissl.**

*Alternaria alternata* is an important post-harvest pathogen of grapes, in which infection is often initiated at the stem-end (Swart and Holz, 1994). It is a common pathogen in wine grapes and has been recorded in 80% of the fruits collected in Argentina (Magnoli *et al.*, 2003). *Alternaria alternata* causes rot of stored table grapes (Harvey, 1955), particularly those in cold storage (Swart and Holz, 1991). The disease is characterized by fairly firm, superficial lesions which often occur on berries near the pedicel. Lesions are tan at first, become dark brown to black with age and remain localized. Under humid conditions provided by cold transit, fluffy gray tufts of fungus often occur on rachis and pedicels, occasionally without causing visible lesions. Symptoms of *Alternaria* bunch rot on export grapes are only evident at the end of prolonged cold storage (Swart and Holz, 1991).

The pathogen when cultured in PDA medium, the colonies appears dark ash colored centers with fluffy margins. Hyphae septate, branched, hyaline at first but later brown or olivaceous brown. Conidiophore septate, branched arising singly or in groups pale to mid-olivaceous or golden brown in color. Conidia mostly in chains of up to 20 or more or sometimes single, golden brown or olive oil, obclavate, obpyriform, ovoid or ellipsoidal with cylindrical pale beak not more than one third of the length of the conidium. Conidia were  $(25.41-65.34 \times 7.26-14.52)$   $\mu\text{m}$  with 3 to 7 transverse septa and produced 5 to 10 germ tubes.

Systemic position (Martin, 1961)

Kingdom - Mycetae  
Division - Eumycota  
Sub-division - Duteromycotina  
Class - Hyphomycetes  
Order - Moniliales  
Family - Dematiaceae  
Genus - *Alternaria*  
Species - *A. alternata*

### 3.2.7 Maintenance of pure culture

The pure culture of *Aspergillus niger* and *Alternaria alternata* were preserved by subculturing in PDA media and incubated at  $25 \pm 2^\circ\text{C}$ . Similarly, Agar slant inoculated with fungi were stored at ( $< 10^\circ\text{C}$ ) for the preservation of their vigor and long term availability.

### 3.2.8 Dilution of essential oil

Six different essential oils were diluted into different concentrations of 20, 40, 80, 160 and 320  $\mu\text{l/ml}$  with 60 % Acetone (Rao and Srivastava, 1994)-with slight modification. For preparing 5 ml oil of each concentration the following method was implemented using Formula;  $V_1S_1=V_2S_2$ . For instance, for preparing oil concentration of 20  $\mu\text{l/ml}$ , 4.9 ml Acetone was added to 100  $\mu\text{l}$  of the stock oil.

### 3.2.9 Assessment of Fungitoxicity

The fungitoxicity of the essential oils were assessed by 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. At first, 1ml of each concentration of essential oil was poured into sterilized petriplates followed by addition of 9 ml of melted PDA. The petriplates were swirled gently to allow proper mixing of the contents. After the media solidified, each petriplate was then inoculated aseptically upside down at the center by a 4 mm diameter of the test fungus. Positive and negative control set were also maintained. In positive control set, 20 µg/ml concentration of Fungicide namely Mancozeb 75 % WP was used. Similarly, in negative control set only 60% Acetone was used. Five replicates were maintained for each control and treatment set for each test. The inoculated petriplates were incubated for one week at temperature 25±2°C. Average diameter of the fungal colonies was measured in mutually perpendicular directions on the seventh day of incubation. Then, percentage inhibition of mycelial growth of test fungus was calculated separately (Grover and Moore, 1962).

### 3.2.10 Calculation of mycelial growth inhibition

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

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

Where,  $g_c$  = growth of mycelial colony after incubation in control set (Diameter of colony in control set- diameter of Inoculum disc).

$g_t$  = growth of mycelial colony after incubation period in treatment set (Diameter of colony in treatment set-diameter of Inoculum disc).

### 3.2.11 Pathogenicity test

For conducting pathogenicity test, the infected grapes were collected and symptoms were noted down. *Aspergillus niger* and *Alternaria alternata* were isolated in PDA separately as pure culture. Fresh and healthy grape fruits were collected and inoculated with test fungi separately. Then inoculated grapes were incubated at 25±2 °C for 1 week. After 1 week, characteristics symptoms were produced which were found to be similar with the symptoms on fruits previously collected. The fungi were isolated and were studied under compound microscope for the comparison with previously isolated fungus.

### 3.2.12 Identification of the fungus

From the pathogenicity test, the isolated fungus were studied and identified as *Aspergillus niger* and *Alternaria alternata* with the help of standard literature (Ellis, 1971; Watanabe, 2010).

### 3.2.13 Measurement of conidia size

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

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

After that, the stage micrometer was replaced by a fine slide of the test fungus mounted in cotton blue and lacto-phenol. Size of conidia of both fungi was measured by determining the number of divisions of ocular micrometer occupied by conidia of the fungus. Measurement of different conidia was carried out for 10 times for finding the appropriate range of the size of conidia.

### 3.2.14 Photography

The photographs of plants, infected grapes, fungal structure under compound microscope and petriplates showing inhibitions of mycelial growth at different concentrations of plant essential oils were taken.

### **3.2.15 Data Analysis**

Excel 2013 was used for entering data, drawing charts and required graphs. The data were analysed with the help of ANOVA followed by Post-Hoc; Bonferroni test at ( $P < 0.05$ ) using Software, SPSS version 20.

## CHAPTER- FOUR

### 4. RESULTS

#### 4.1 Determination of yield of essential oils

The hydrodistillation process was carried out for the extraction of essential oils. The percentage yield was calculated and represented in the graph.

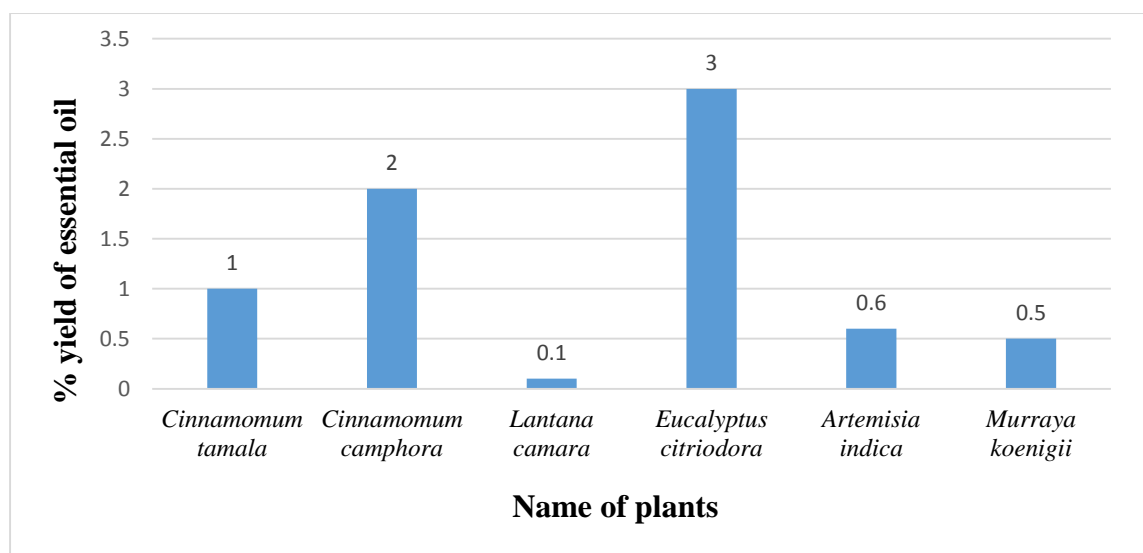


Fig 1: Percentage yield of essential oil of test plant species.

The percentage yield of essential oil of *Cinnamomum tamala*, *Cinnamomum camphora*, *Lantana camara*, *Eucalyptus citriodora*, *Artemisia indica* and *Murraya koenigii* was found to be 1%, 2%, 0.1%, 3%, 0.6% and 0.5% respectively.

#### 4.2. Antifungal Bioassay of essential oils

Six different essential oils were tested for their antifungal activity against *Alternaria alternata* and *Aspergillus niger* by poisoned food technique. The colony size of the test fungi at different concentrations of essential oils measured on the seventh day of incubation and the inhibitory effects of the oils were calculated. The diameter of the colony was measured by using measuring scale and finally the inhibition percentage was calculated. For each concentration, the diameters of five plates were measured and then mean was calculated. The inhibition percentage was finally calculated in reference to the colony diameter in control set (Negative control).

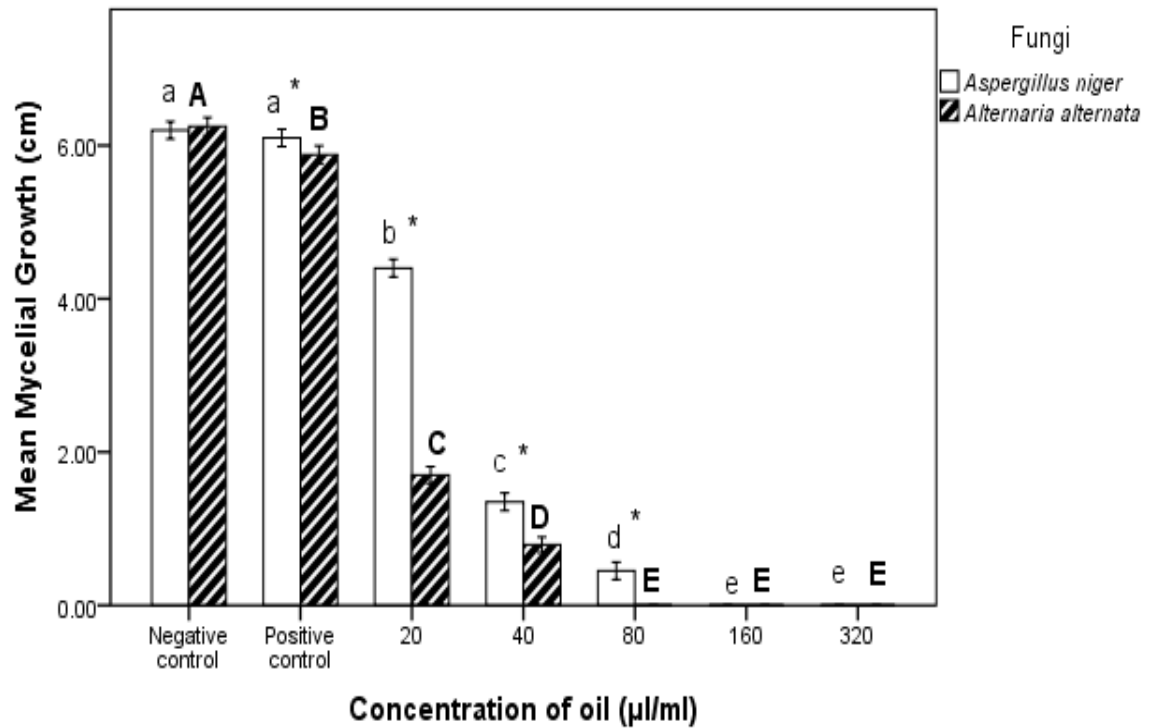


Fig 2: Antifungal effect of *Artemisia indica* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The *Artemisia indica* oil showed significant antifungal activity over both tested fungi. It was more effective against *Alternaria alternata* in comparison with *Aspergillus niger*. At 20 and 40 µl/ml oil concentration the mycelial growth of *Aspergillus niger* was relatively higher than that of *Alternaria alternata*. The oil showed some mycelial growth upto 80 µl/ml concentration against *Aspergillus niger* whereas it completely inhibit the growth of *Alternaria alternata* at the same concentration. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

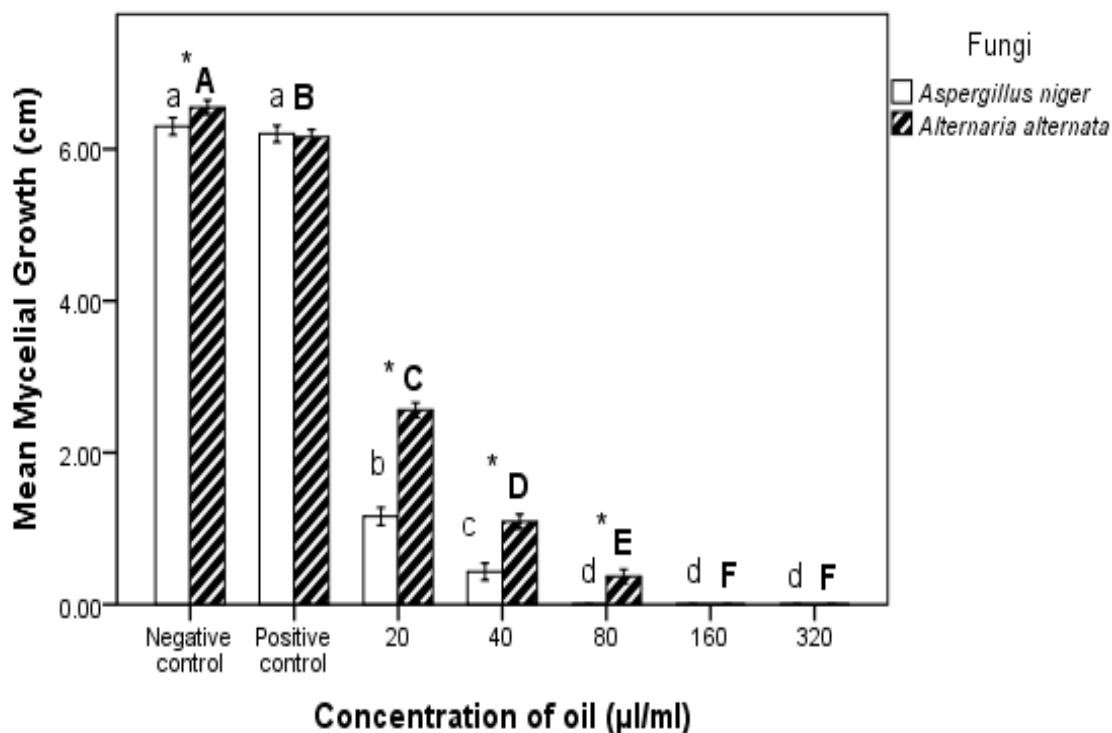


Fig 3: Antifungal effect of *Cinnamomum camphora* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The *Cinnamomum camphora* oil showed significant antifungal activity over both tested fungi. It was more effective against *Aspergillus niger* in comparison with *Alternaria alternata*. At 20 and 40 µl/ml oil concentration the mycelial growth of *Alternaria alternata* was relatively higher than that of *Aspergillus niger*. The oil showed some mycelial growth upto 80 µl/ml concentration against *Alternaria alternata* whereas it completely inhibit the growth of *Aspergillus niger* at the same concentration. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

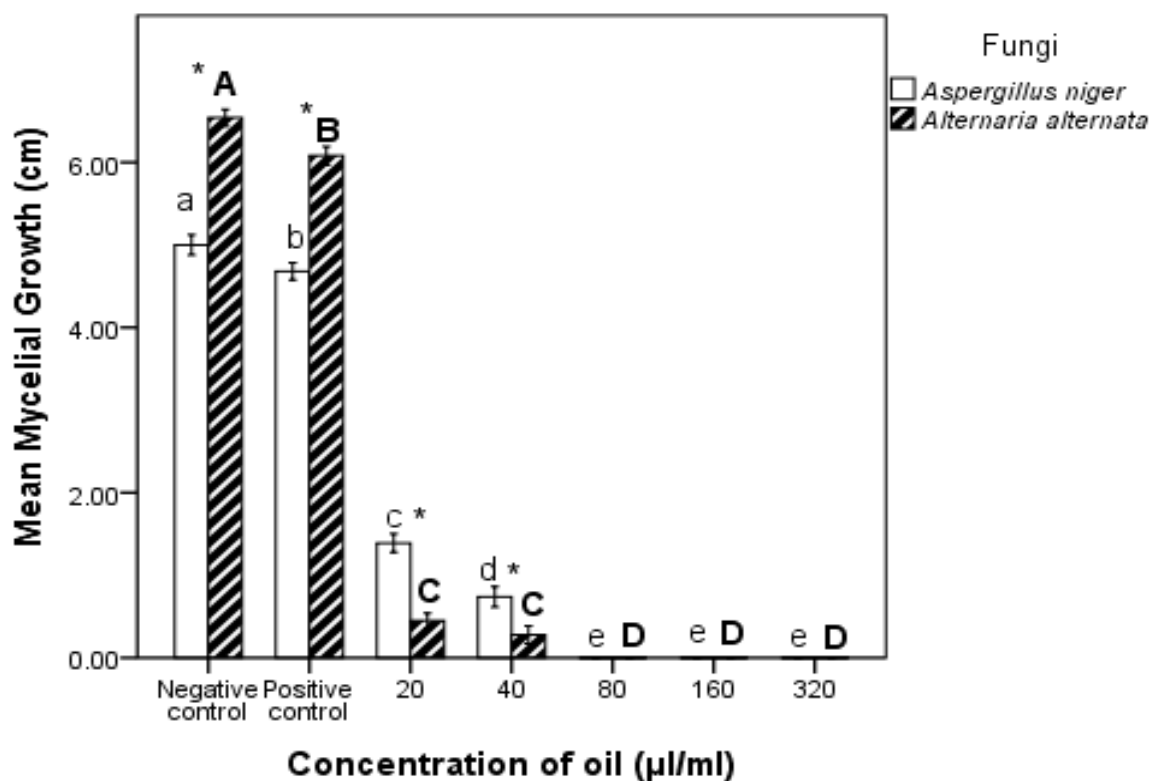


Fig 4: Antifungal activity of *Cinnamomum tamala* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The *Cinnamomum tamala* oil showed significant antifungal activity over both tested fungi. The oil has better effects over *Alternaria alternata* than *Aspergillus niger*. At 20 and 40 µl/ml oil concentration the mycelial growth of *Aspergillus niger* was relatively higher than that of *Alternaria alternata*. The oil showed complete inhibition of mycelial growth of both fungi at 80 µl/ml concentration. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

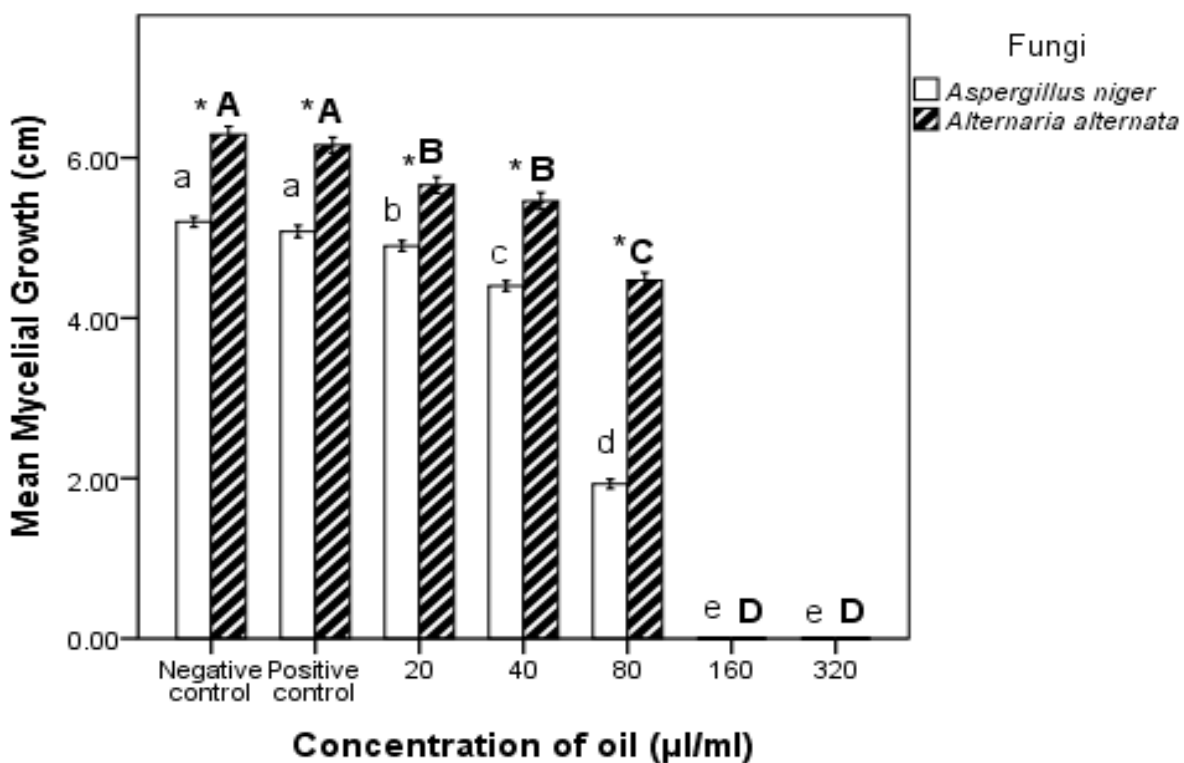


Fig 5: Antifungal activity of *Eucalyptus citriodora* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The *Eucalyptus citriodora* oil showed significant antifungal activity over both tested fungi. It was more effective against *Aspergillus niger* in comparison with *Alternaria alternata*. The oil showed complete inhibition of mycelial growth of both fungi at 160 µl/ml concentration. At 20, 40, 80 µl/ml oil concentration the mycelial growth of *Alternaria alternata* was relatively higher than that of *Aspergillus niger*. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

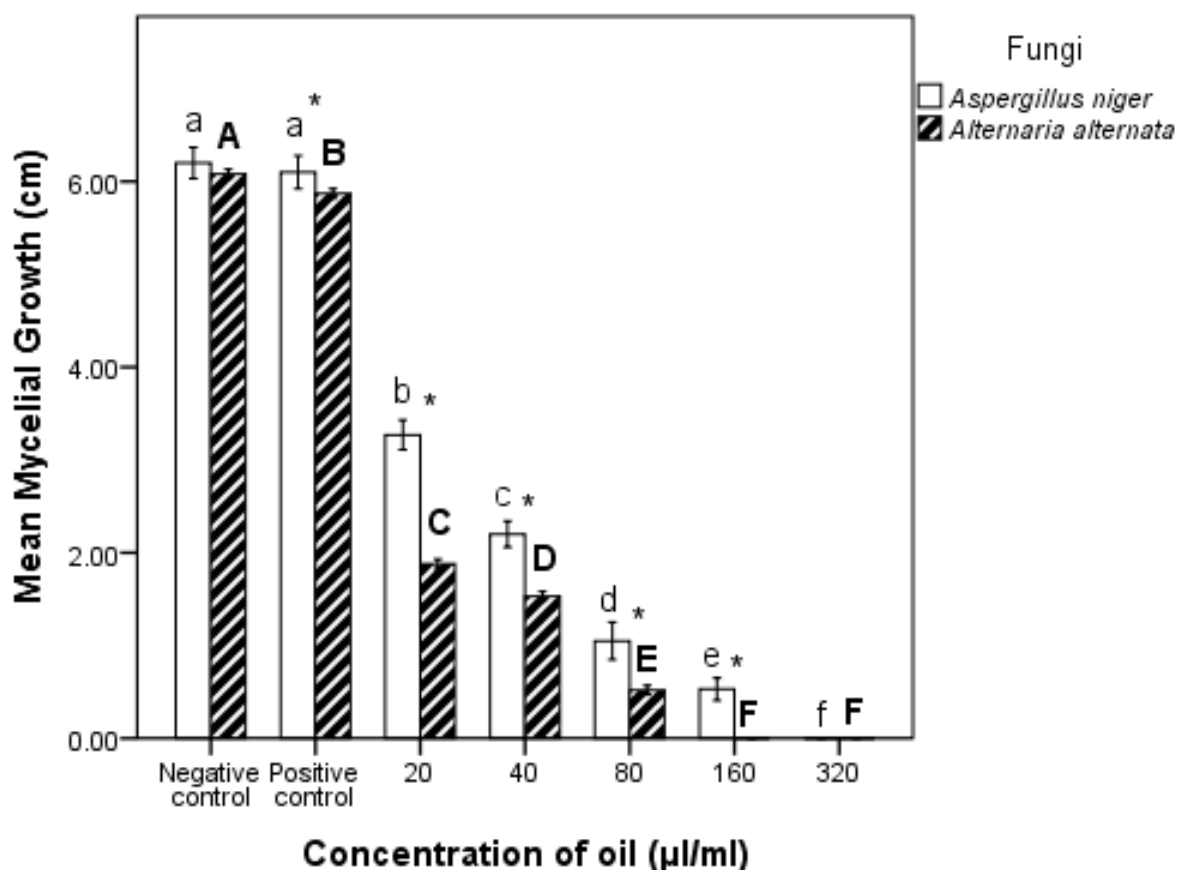


Fig 6: Antifungal activity of *Lantana camara* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The *Lantana camara* oil also showed significant antifungal activity over both tested fungi. It was more effective against *Alternaria alternata* in comparison with *Aspergillus niger*. The oil showed some mycelial growth upto 160 µl/ml concentration against *Aspergillus niger* whereas it completely inhibit the growth of *Alternaria alternata* at 160 µl/ml concentration. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

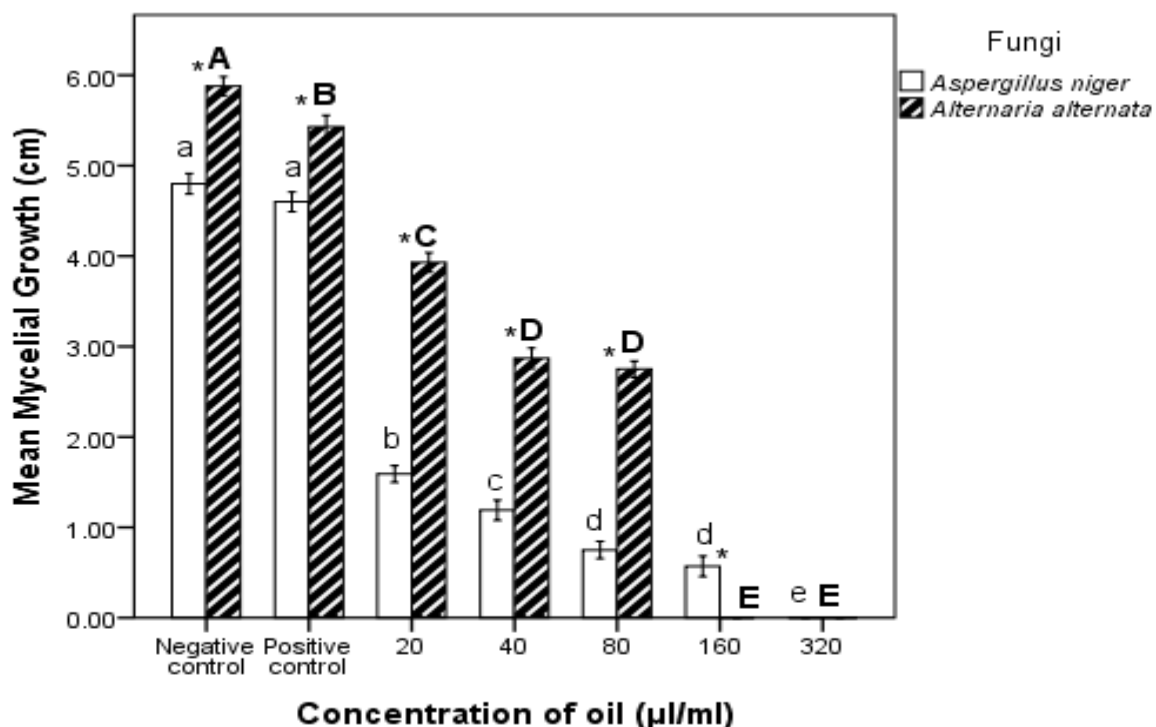


Fig 7: Antifungal activity of *Murraya koenigii* against *Aspergillus niger* and *Alternaria alternata*.

The mean values sharing same alphabet (small letter for *Aspergillus niger* and bold capital letter for *Alternaria alternata*) are not significantly different from each other according to Bonferroni test at ( $P < 0.05$ ), “\*” indicates the significant difference between the two fungi at different concentration based on T-test ( $P < 0.05$ ).

The oil of *Murraya koenigii* showed the significant activity over both tested fungi. It exhibited some mycelial growth upto 160 µl/ml concentration against *Aspergillus niger* whereas it completely inhibited the growth of *Alternaria alternata* at the same concentration. But at 20, 40 and 80 µl/ml concentrations *Alternaria alternata* shows greater colony size than that of *Aspergillus niger*. The positive control (20 µg/ml fungicide) was comparatively very less effective for both fungi than oil at 20 µl/ml concentration.

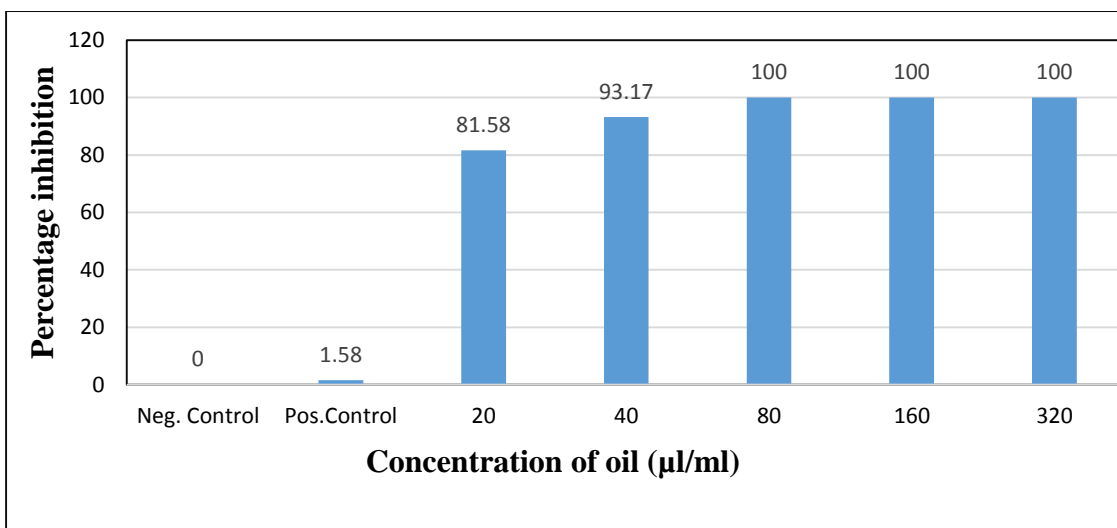


Fig. 8: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Cinnamomum camphora* oil.

The graph shows that the oil of *Cinnamomum camphora* inhibited the mycelial growth of *Aspergillus niger* by 81.58%, 93.17%, 100%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

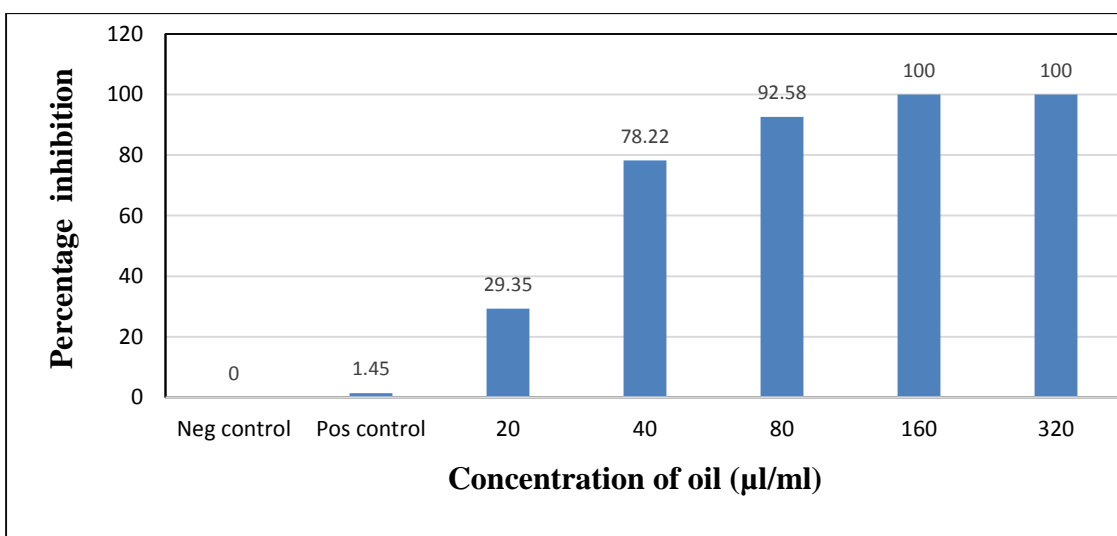


Fig. 9: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Artemisia indica* oil.

The graph shows that the oil of *Artemisia indica* inhibited the mycelial growth of *Aspergillus niger* by 29.35%, 78.22%, 92.58%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

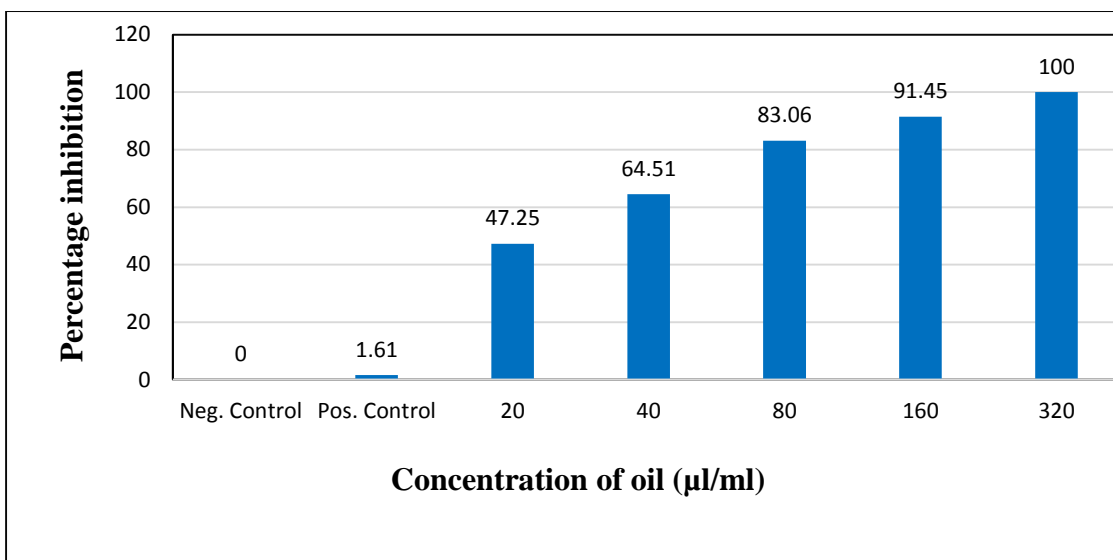


Fig. 10: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Lantana camara* oil.

The graph shows that the oil of *Lantana camara* inhibited the mycelial growth of *Aspergillus niger* by 47.25%, 64.51%, 83.06%, 91.45% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

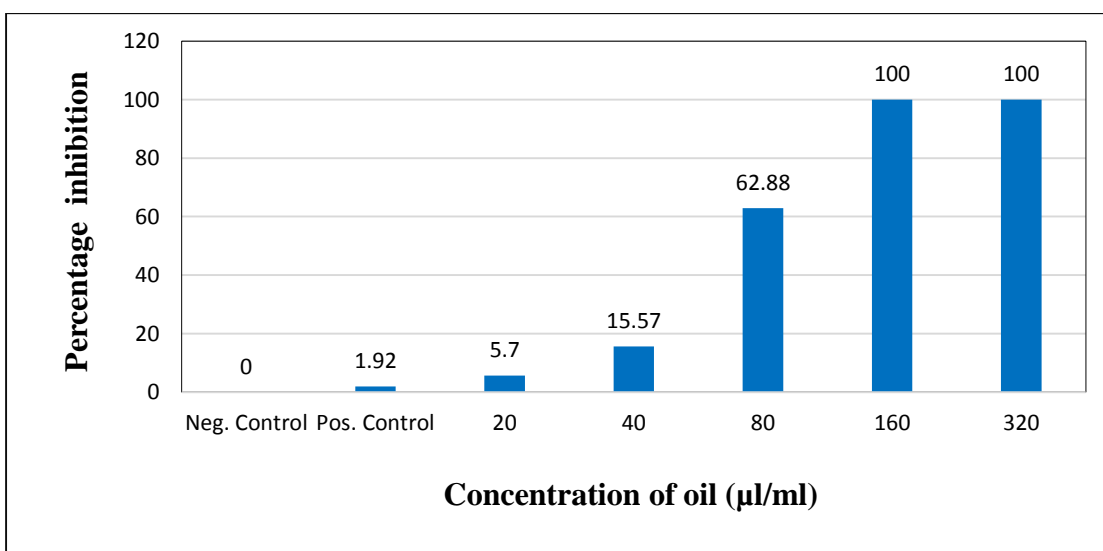


Fig. 11: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Eucalyptus citriodora* oil.

The graph shows that the oil of *Eucalyptus citriodora* inhibited the mycelial growth of *Aspergillus niger* by 5.7%, 15.57%, 62.88%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

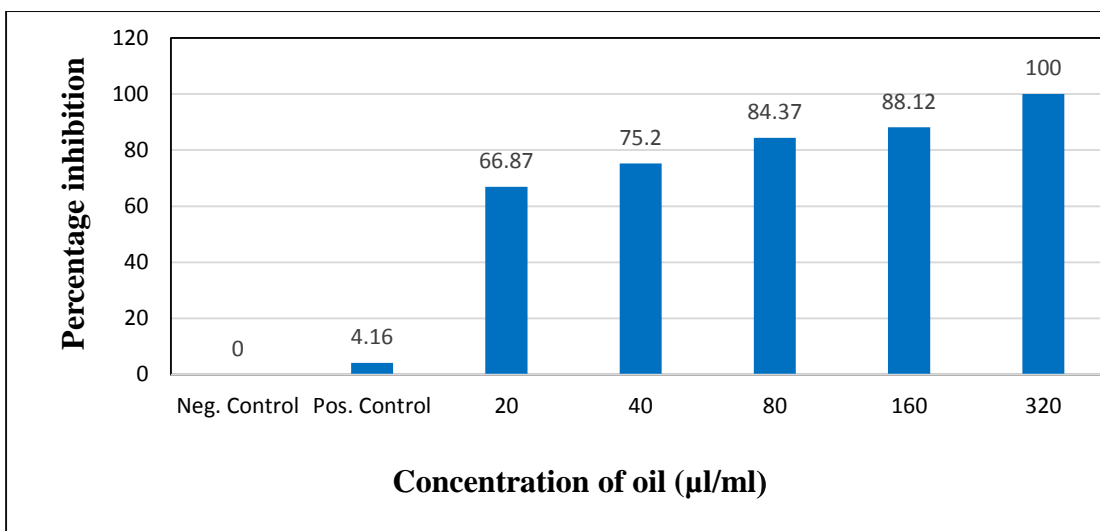


Fig. 12: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Murraya koenigii* oil.

The graph shows that the oil of *Murraya koenigii* inhibited the mycelial growth of *Aspergillus niger* by 66.87%, 75.2%, 84.37%, 88.12% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

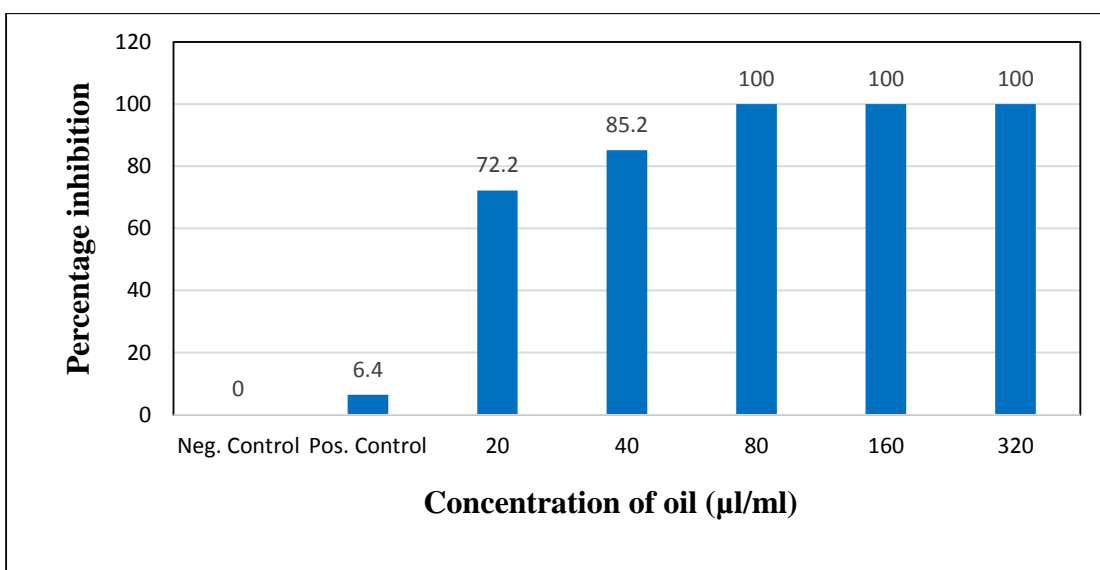


Fig. 13: Percentage inhibition of mycelial growth of *Aspergillus niger* at different concentrations of *Cinnamomum tamala* oil.

The graph shows that the oil of *Cinnamomum tamala* inhibited the mycelial growth of *Aspergillus niger* by 72.2%, 85.2%, 100%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

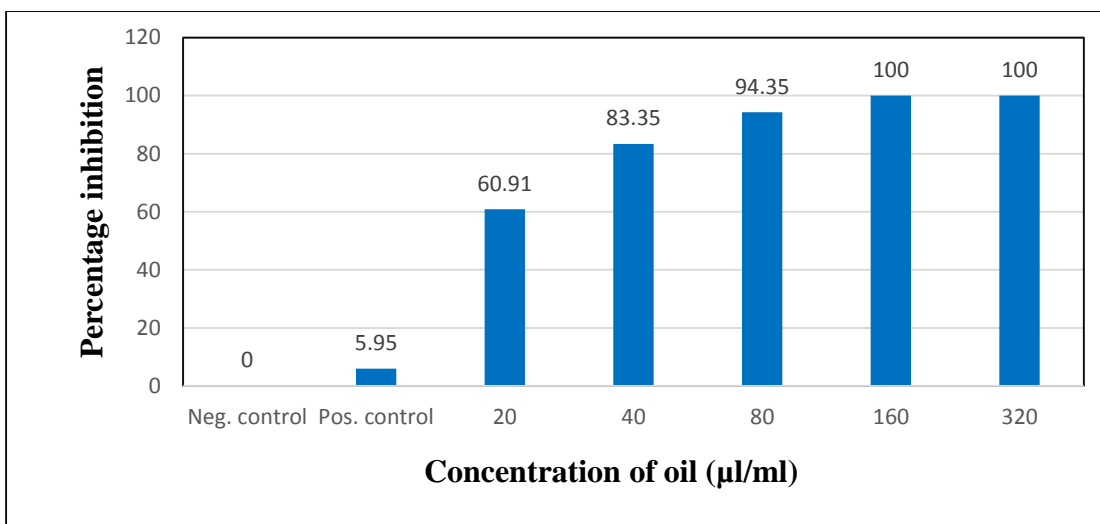


Fig. 14: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Cinnamomum camphora* oil.

The graph shows that the oil of *Cinnamomum camphora* inhibited the mycelial growth of *Alternaria alternata* by 60.91%, 83.35%, 94.35%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

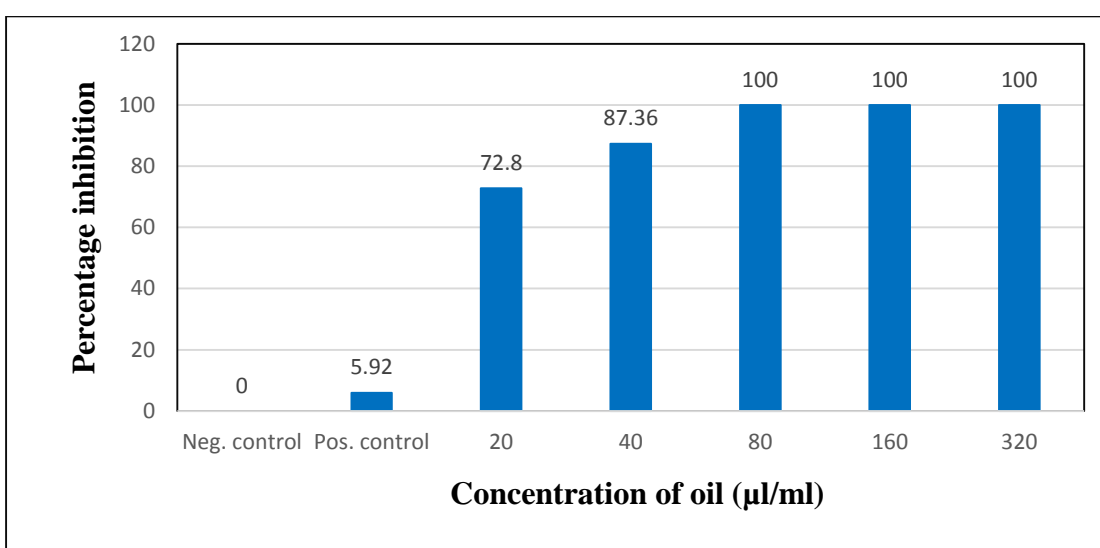


Fig. 15: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Artemisia indica* oil.

The graph shows that the oil of *Artemisia indica* inhibited the mycelial growth of *Alternaria alternata* by 72.8%, 87.36%, 100%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

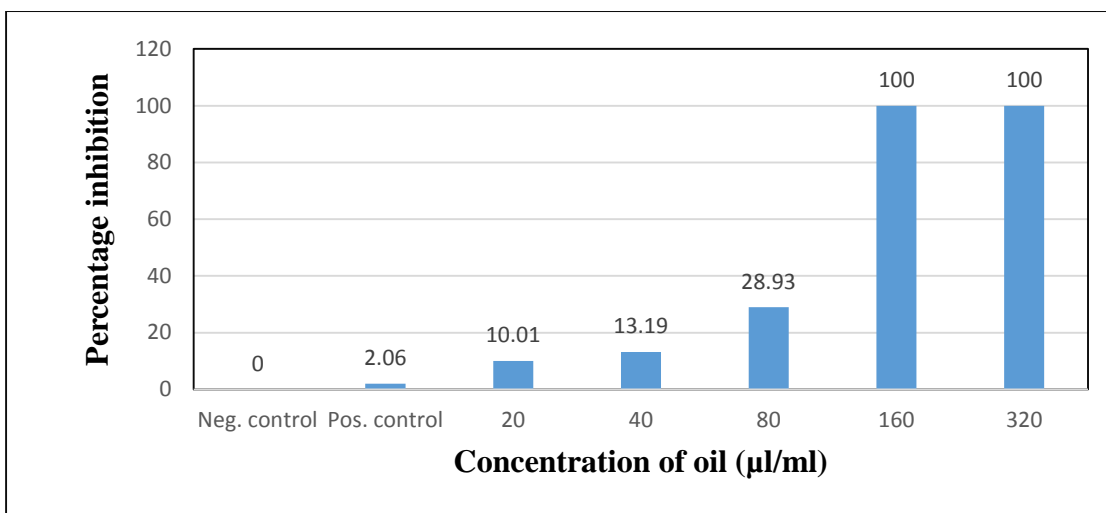


Fig. 16: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Eucalyptus citriodora* oil.

The table shows that the oil of *Eucalyptus citriodora* inhibited the mycelial growth of *Alternaria alternata* by 10.1%, 13.19%, 28.93%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

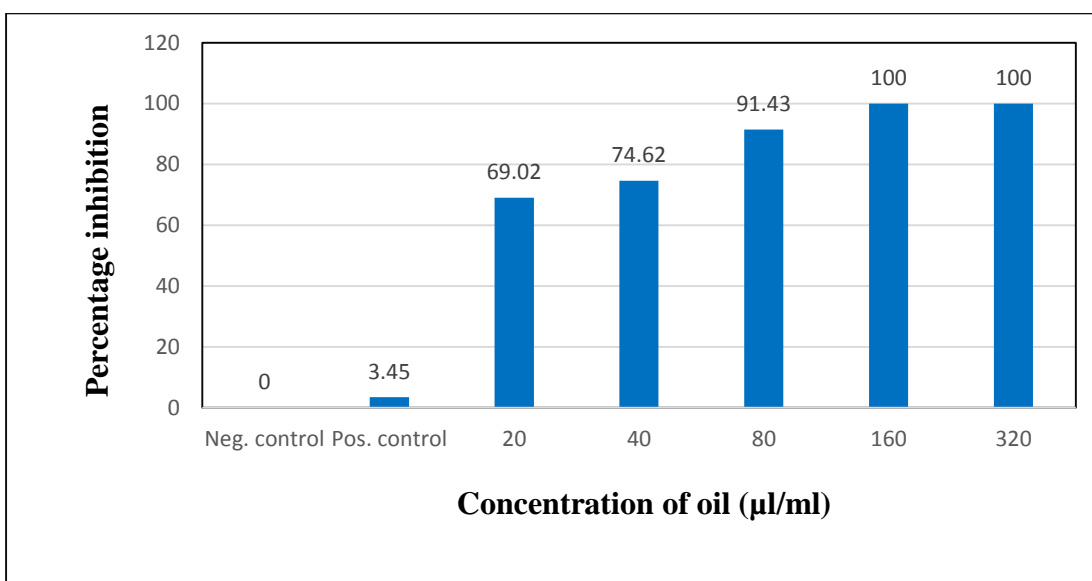


Fig. 17: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Lantana camara* oil.

The graph shows that the oil of *Lantana camara* inhibited the mycelial growth of *Alternaria alternata* by 69.02%, 74.62%, 91.43%, 100% and 100% at concentration of 20  $\mu$ l/ml, 40  $\mu$ l/ml, 80  $\mu$ l/ml, 160  $\mu$ l/ml and 320  $\mu$ l/ml respectively.

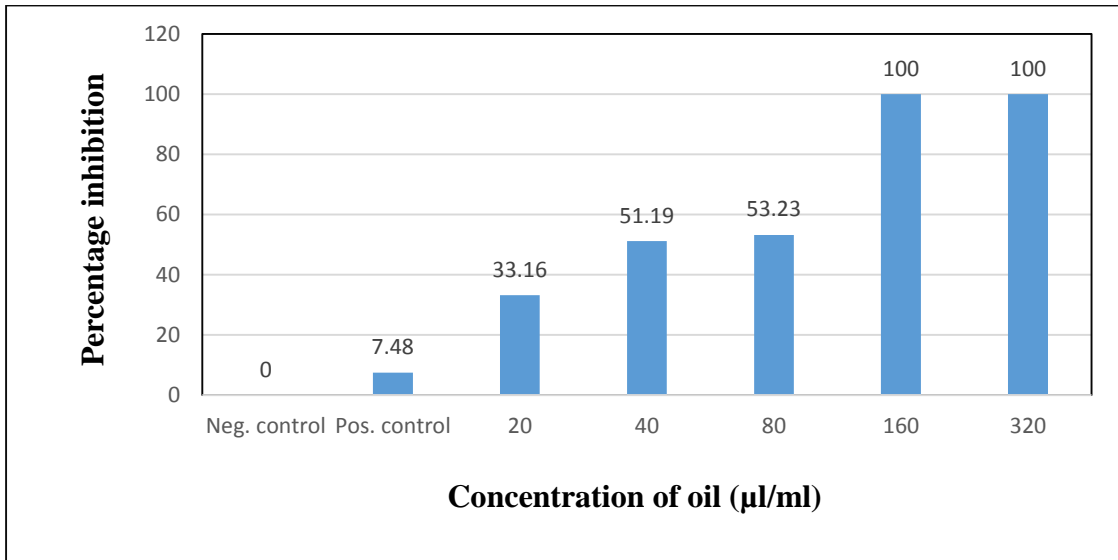


Fig. 18: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Murraya koenigii* oil.

The table shows that the oil of *Murraya koenigii* inhibited the mycelial growth of *Alternaria alternata* by 33.16%, 51.19%, 53.23%, 100% and 100% at concentration of 20  $\mu$ l/ml, 40  $\mu$ l/ml, 80  $\mu$ l/ml, 160  $\mu$ l/ml and 320  $\mu$ l/ml respectively.

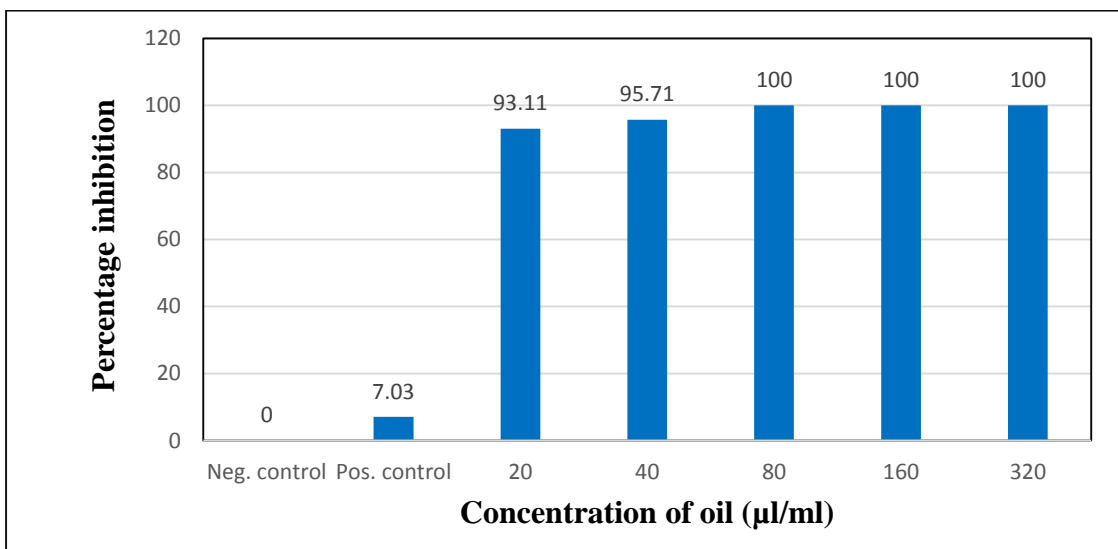


Fig. 19: Percentage inhibition of mycelial growth of *Alternaria alternata* at different concentrations of *Cinnamomum tamala* oil.

The graph shows that the oil of *Cinnamomum tamala* inhibited the mycelial growth of *Alternaria alternata* by 93.11%, 95.71%, 100%, 100% and 100% at concentration of 20 µl/ml, 40 µl/ml, 80 µl/ml, 160 µl/ml and 320 µl/ml respectively.

### Two way ANOVA

Table 3: ANOVA for mycelial growth of *Aspergillus niger* at different oil concentrations.

Source	Sum of square	Degree of freedom (df)	Mean square	F	Sig.
Plant(Oil type)	143.222	5	28.644	4.200	0.005
Concentration of oil	1155.513	6	192.586	28.240	0.000
Interaction	204.590	30	6.820	873.915	0.000

Table 4: ANOVA for mycelial growth of *Alternaria alternata* at different oil concentrations.

Source	Sum of square	Degree of freedom (df)	Mean square	F	Sig.
Plant(Oil type)	105.550	5	21.110	3.979	0.007
Concentration of oil	1198.935	6	199.823	37.664	0.000
Interaction	159.160	30	5.305	685.613	0.000

Two way ANOVA results for both the test fungi shows that plant species (oil type), concentration of oil and their interaction all have significant effect on mycelial growth of the test fungi.

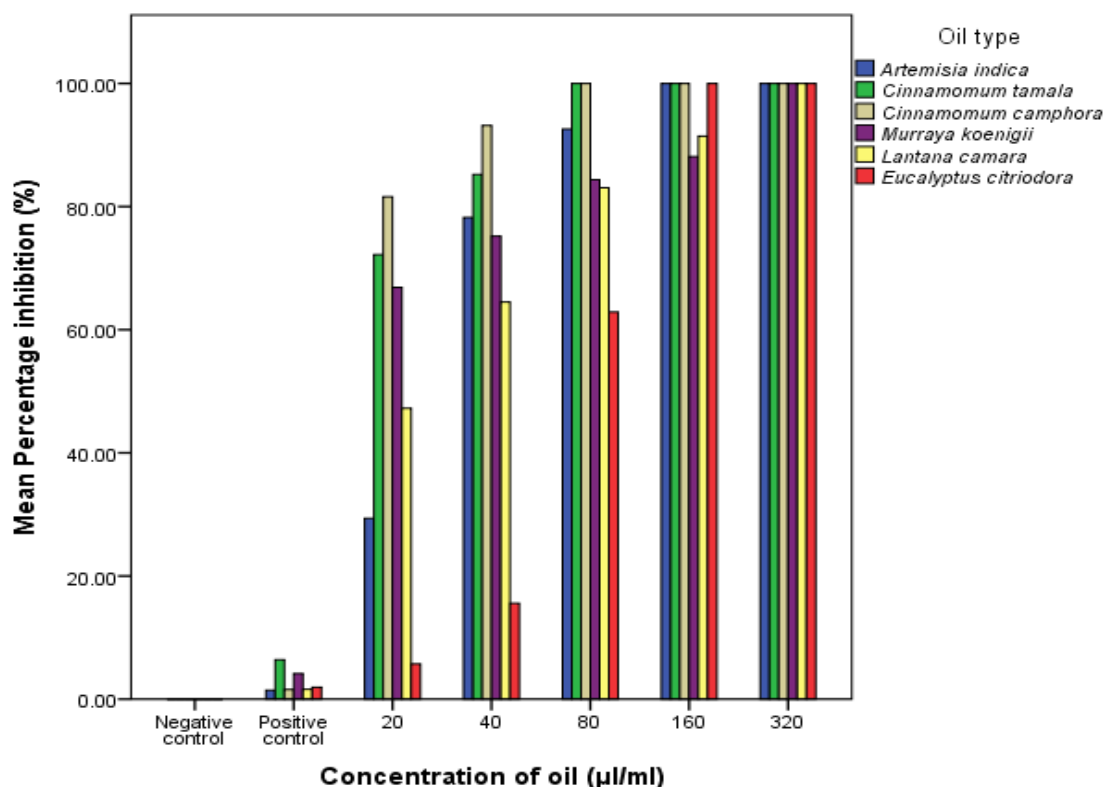


Fig. 20: Fungitoxicities of different essential oils at different concentrations against *Aspergillus niger*.

Among all the six essential oils, *Cinnamomum camphora* showed the most effective antifungal activity against *Aspergillus niger*.

At 20 µl/ml concentration, *C. camphora* showed the highest inhibition (81.58%), followed by *C. tamala* (72.2%), *Murraya koenigii* (66.87%), *Lantana camara* (47.25%), *Artemisia indica* (29.35%) and *Eucalyptus citriodora* (5.7%) respectively. Similarly, at 40 µl/ml concentration, *C. camphora* showed the highest inhibition (93.17%), followed by *C. tamala* (85.21%), *Artemisia indica* (78.22%), *Murraya koenigii* (75.2%), *Lantana camara* (64.51%), and *Eucalyptus citriodora* (15.57%) respectively.

Meanwhile, at 80 µl/ml oil concentration, *C. camphora* and *C. tamala* showed 100% inhibition of mycelial growth followed by *Artemisia indica* (92.58%), *Murraya koenigii* (84.37%), *Lantana camara* (83.06%) and *Eucalyptus citriodora* (62.88%) respectively. At 160 µl/ml concentration, *Artemisia indica* and *Eucalyptus citriodora* oil also showed 100 % inhibition of mycelial colony.

At, 320 µl/ml concentration rest of the oil showed 100 % inhibition of the mycelial colony.

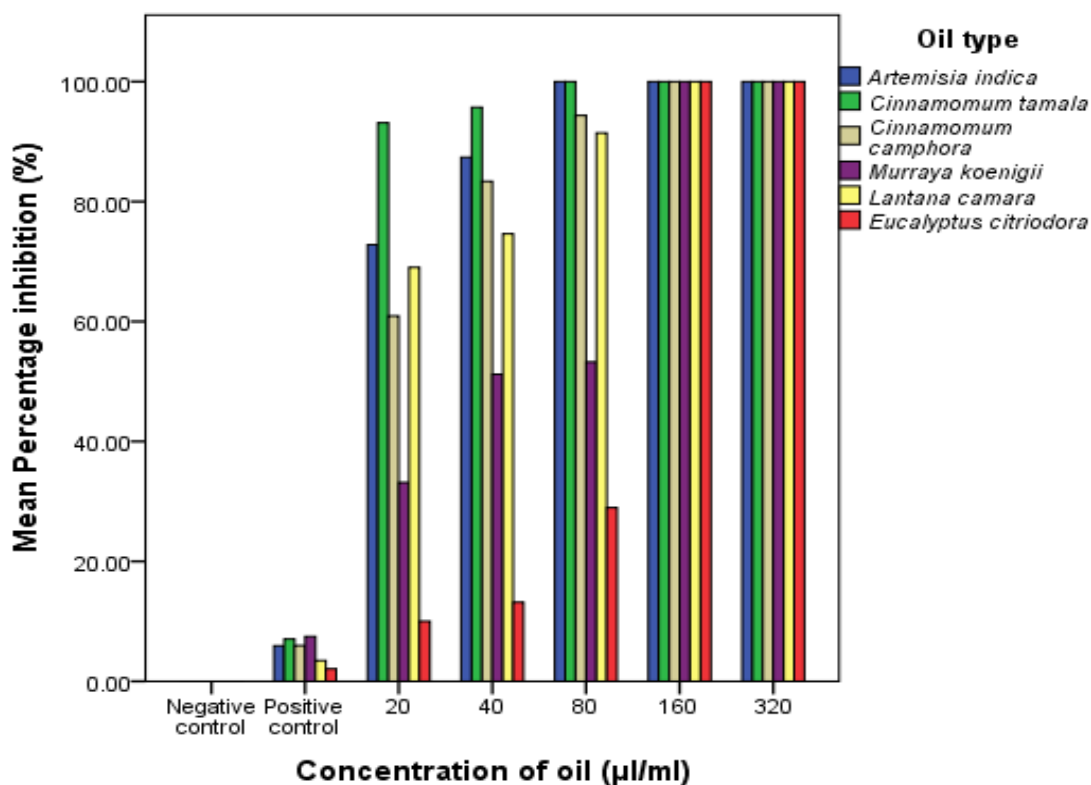


Fig. 21: Fungitoxicities of different essential oils at different concentrations against *Alternaria alternata*.

*Cinnamomum tamala* showed best activity whereas *Eucalyptus citriodora* showed least antifungal effect in controlling *Alternaria alternata* among all six oils.

At 20 µl/ml oil concentration, *C. tamala* showed highest inhibition (93.11%) followed by *Artemisia indica* (72.8%), *Lantana camara* (69.02%), *C. camphora* (60.91%), *Murraya koenigii* (33.16%) and *Eucalyptus citriodora* (10.01%) respectively.

*C. tamala* showed highest inhibition (95.71%) followed by *Artemisia indica* (87.36%), *C. camphora* (83.35%), *Lantana camara* (74.62%), *Murraya koenigii* (51.19%) and *Eucalyptus citriodora* (13.19%) at 40 µl/ml oil concentration respectively.

Similarly, *C. tamala* and *Artemisia indica* showed the highest inhibition (100%) followed by *C. camphora* (94.35%), *Lantana camara* (91.43%), *Murraya koenigii* (53.23%) and *Eucalyptus citriodora* (28.93%) at 80 µl/ml oil concentration respectively.

At 160 µl/ml oil concentration, rest of the oils also showed 100% inhibition of mycelial growth of *Alternaria alternata*.

## CHAPTER – FIVE

### 5. DISCUSSION

In the present study an attempt was made to study about two fruit rot diseases of grapes caused by *Alternaria alternata* and *Aspergillus niger* in post-harvest condition, to test their pathogenicity and to examine antifungal activity of six plants essential oils against these fungi.

Nowadays, application of chemical compounds is considered as the most inexpensive and common method in plant disease control. However, their adverse effects on human health and the environment, promoted man to produce natural pesticides (Hayes and Laws, 1991). Biologically active compounds found in plants appear to be more adaptable, acceptable and safer than synthetic compounds and display a wealthy source of potential pathogens control agents (Tripathi *et al.*, 2008). Essential oils are made up of many different volatile compounds and the makeup of the oil quite often varies between species. It seems that the anti-fungal and anti-microbial effects are the result of many compounds acting synergistically. This means that the individual components by themselves are not as effective (Jobling, 2000). The use of herbal essence in the control of postharvest fruit diseases has been proposed as a new method in recent years. These compounds not only haven't side effects, but also due to antioxidant properties, increase fruit quality and storage periods (Behdani *et al.*, 2012).

The study demonstrated that all six essential oils had considerable antifungal effect at different concentrations on growth rate of two test fungi. All essential oils were found significantly ( $P < 0.05$ ) affecting the growth of both fungi after seven days of incubation. The *Cinnamomum camphora* oil was proved to be the most effective against *Aspergillus niger*. The oil showed complete inhibition of mycelial growth at 80  $\mu\text{l/ml}$  concentration. The oil contains camphor as main component (68%) and linalool, the second most important (9%) which may be the reason for their effective antifungal activity (Frizzo *et al.*, 2000; Chen *et al.*, 2013). Similar experiment was carried out by (Mahilrajana *et al.*, 2014). According to them camphor and citronella oil caused 100% inhibition of *Aspergillus niger* at 5, 10 and 15 ml/dl.

In this study, *Cinnamomum tamala* showed best antifungal activity against *Alternaria alternata* among all six oils. *C. tamala* inhibited the mycelial growth of *Alternaria alternata* by 93.11% at 20  $\mu\text{l/ml}$  and by 100 % at 80  $\mu\text{l/ml}$  oil concentration.

Phenylpropanoids have been reported to be major component (about 66%) in *C. tamala* leaf oil. The marker phenylpropanoids include eugenol, cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate and cinnamic acid. In addition several other components such as B-caryophyllene, spathulinol, sesquiterpenoids and viridiflorene etc. are also present in appreciable quantity in the oil. Presence of these chemical moieties in *C. tamala* leaf oil may be accounted for the observed antifungal properties (Pandey *et al.*, 2012).

The *Artemisia indica* oil exhibited complete inhibition of mycelial growth over *Aspergillus niger* at 160 µl/ml concentration in the present research. But, Satyal *et al.* (2012) examined that *Artemisia indica* was marginally active against *Aspergillus niger* with MIC of 625 µl/ml in their study. The antifungal activity of *Artemisia indica* oil may be due to high concentration of ascaridole (15.4%), a compound that has demonstrated broad bioactivity according to them.

Among many essential oils studied by (Tripathi *et al.*, 2008), *Murraya koenigii* and *Eucalyptus citriodora* showed 80% and 100% mycelial growth inhibition respectively of *Botrytis cinerea*, causing grey mould of grapes. According to Hadizadeh *et al.* (2009), *Eucalyptus citriodora* oil exhibited low antifungal activity against *Alternaria alternata* ranging from 8.7-20.7% at 1500 and 2000 ppm concentration, respectively. In the present study, *Murraya koenigii* exhibited 100% growth inhibition at 160 and 320 µl/ml oil concentration against *Alternaria alternata* and *Aspergillus niger* respectively. And, *Eucalyptus citriodora* exhibited 100% growth inhibition at 160 µl/ml oil concentration against both fungi. The essential oils from leaves of *Murraya koenigii* contains higher concentration of α-pinene, δ-sabinene, β-caryophyllene, α-copaene, β-ocimene and cadinene and many carbazole alkaloids which is responsible for antifungal activities against a range of plant pathogenic fungi. Biological activity of essential oils starts most likely, affecting cell membrane structure and functions. Because of their lipophilicity, many of their components can accumulate in membranes being able to disrupt its structure and to affect transport processes and other membrane-associated events such as signal transmission, and ATP synthesis particularly in microorganisms (Einhellig, 1995).

Dharmagadda *et al.* (2004) reported that *Lantana camara* oil compared to *Ocimum sanctum* and *Tagetes patula* was found effective in inhibiting the growth of fungi, *Aspergillus niger* and reducing the growth of other fungi. The oil completely inhibited

the growth of *A. niger* at 40 µl and 60 µl concentrations. Higher susceptibility of fungi to *Lantana camara* oil compared to other oils might be due to higher concentrations of active terpenoides. Comparatively, in present study the oil concentration was 320 µl/ml for 100% inhibition of the fungus *Aspergillus niger*.

The difference in fungitoxicity at same concentration in different essential oils may be due to different chemical composition of the oils (Singh *et al.*, 1983). The results from the present study may not be true and accurate forever because the concentration of various constituents in essential oils of same plant species varies due to several factors such as growth stage, ecological and environmental factors etc. (Gulati, 1982).

In summary, this study confirms that many essential oils possess *in vitro* antifungal activity. However, if plant oils are to be used for preservation of fruits during storage, issues of safety and toxicity will need to be addressed. This obviously justifies the use of above essential oils in controlling plant pathogenic fungi.

## CHAPTER- SIX

### 6. CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusion

This study concludes that plant essential oils used in the present research can be promising in management of post-harvest diseases of grapes especially in controlling two rots fungi namely *Aspergillus niger* and *Alternaria alternata*.

Different essential oils of the selected plant shows significant control of these fungi. The oil of *Cinnamomum camphora* and *Cinnamomum tamala* showed the most effective antifungal activity against *Aspergillus niger* and *Alternaria alternata* respectively. The results suggest their possible use as an alternative or complements to synthetic compounds.

Further studies are needed to mark the optimal type of essential oil, concentration and method of use of these natural and safe products for protecting and increasing shelf life of fruits in storage conditions.

#### 6.2 Recommendations

- 1) This research was conducted under controlled laboratory conditions (in vitro). So, further research can be carried out in the field (in vivo) conditions so that actual effect of oils can be noted under the influence of various physical factors prevalent in the field.
- 2) These essential oil could be recommended for the protection of grapes against fruit rots. The used oils from *Cinnamomum camphora* and *Cinnamomum tamala* is recommended for practical applications instead of synthetic fungicides.
- 3) Essential oils contain active phytochemicals exhibiting effective antifungal activity. Therefore, phytochemical studies over the tested essential oil can be made for the determination of active chemical compounds present on them.
- 4) Similar type of oils can be examined over other varieties of plant pathogens.

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## ANNEXES

### Materials used for the study

#### Apparatus and Equipment:

Test tubes, funnel, conical flasks, beakers, Petri dishes, culture tubes, funnel, pipettes, glass rods, inoculating needles, ocular and stage micrometer, glass slides, cover slip, inoculating loop, forceps, scissors, tape, burner, muslin cloth, cotton, measuring cylinder, laminar air flow, hot air oven, refrigerator, digital camera, borers, spirit lamp, compound microscope, incubator, autoclave, weighing balance, Clevenger's apparatus, Round bottom flask, Bucket, Mortar and Pistal.

#### Chemicals and reagents:

Distilled water, Cotton blue, Agar, Dextrose, Potato, 60% Acetone, Lacto phenol, Sprit, 0.1% Mercuric chloride, anhydrous sodium sulphate.

#### Test plant species:

a) *Murraya koenigii* (L.) Spreng. (Family: Rutaceae)

#### Botanical Description:

Small tree, up to 4m high. Leaves 10-25 cm, leaflets 13-23, ovate, 1-4×1-1.5 cm. Base asymmetrically rounded or crenate, margins minutely crenate, apex, acuminate, and surface black gland dotted. Calyx 1mm. Petals 5×1.5 mm. Stamens 4-5 mm, style 5mm. Fruit ovoid, crimson, found on open land (Press *et al.*, 2000).

#### Uses:

Antiemetic, antidiarrhoeal, dysentery, febrifuge, blood purifier, tonic, stomachic, flavoring agent in curries and chutneys. The oil is used externally for bruises, eruption, in soap and perfume industry. It possess antioxidant, antibacterial, antifungal, larvicidal, anticarcinogenic, hypoglycemic, antilipid peroxidative, hypolipidemic and antihypertensive activity (Ajay *et al.*, 2011).

**Chemical components:**

Alkaloids viz., mahanine, koenine, koenigine, koenidine, girinimbiol, girinimibine, koenimbine, O-methyl murrayamine A, O-methyl mahanine, isomahanine, bismahanine, bispyrayafoline and other phytoconstituents such as coumarin glycoside viz., scopotin, murrayanine, calcium, phosphorus, iron, thiamine, riboflavin, niacin, vitamin C, carotene and oxalic acid (Ajay *et al.*, 2011).

**Distribution:**

Nepal (WCE, 150-1450 m), Himalaya (Uttar Pradesh to Sikkim), India, Sri Lanka, Myanmar, Indo- China, China (Press *et al.*, 2000).

**b) *Eucalyptus citriodora* Hook. (Family: Myrtaceae)****Botanical Description:**

Evergreen tree 24-40 m high with tall straight trunk 0.6-1.3 m in diameter. Bark smooth, gray, peeling off in thin irregular scales or patches and becoming mottled. Twigs slender, slightly flattened, light green or brown. Leaves simple, alternate, narrow lance shaped, about 10-20 cm long, 1-2.5 cm wide, apically acuminate, basally acute, entire, glabrous, thin, light green on both surfaces, with many fine parallel straight veins. Inflorescence Corymbs terminal 5-6 cm long at leaf base, branched. Flowers many, 3-5 on equal short stalks (umbels) from ovoid buds 8-12 mm long, 5-8 mm wide. Stamens many, thread like white and 6 mm long (Little, 1983).

**Uses:**

Antiseptic and is used all over the world as a respiratory decongestant, for relieving colds, coughs, bronchitis, flu, pneumonia, headache and sore throats. Also, analgesic, anti-inflammatory, antimicrobial, acaricidal, larvicidal, fungicidal and phytotoxic effects. (Husain and Ali, 2013).

**Chemical components:**

Citronellal,  $\beta$ -citronellol, isopulegol and citronellyl acetate (Mejdoub and Katsiotis, 1998).

**Distribution:** Cultivated in sub-tropical regions, east to west (MFSC, 2007).

**c) *Artemisia indica* Willd. (Family: Asteraceae)**

**Botanical Description:**

*Artemisia indica* Linn. Perennial, shrubby, aromatic, 0.61m- 2.43m high. Pubescent or villous, stems leafy, paniculately branched. Lower leaves 5 cm-10 cm x 2.5 cm- 5 cm, petioled, ovate in outline, with stipule- like lobes at the base, deeply pinnatisect, the lobes entire, toothed or white-tomentose beneath. Upper leaves smaller. 3-fid or entire, lanceolate. Heads 0.31 cm- 0.42 cm long, ovoid or subglobose, solitary or 2 or 3 together, sessile or very shortly pedicelled, outer female flowers very slender, inner female fertile, involucrel-bracts villous and with acute, the inner oblong; Achenes oblong- ellipsoid, minute (Cooke, 1967).

**Uses:**

Diarrhea, dysentery, conjunctivitis, wounds, cuts, cough, sinusitis anthelmintic, abdominal pains, asthma, diuretic, ringworm (Manandhar, 2002).

**Chemical components:**

Ascaridole (15.4%), isoascaridole (9.9%), trans-p-mentha-2, 8-dien-1-ol (9.7%), and trans-verbenol (8.4%) (Satyal *et al.*, 2012).

**Distribution:**

Nepal (CE, 300-2400 m); India, Himalaya, Burma, Thailand, S. China, Japan (Press *et al.*, 2000).

**d) *Cinnamomum camphora* (L.) J. Presl (Family: Lauraceae)**

**Botanical Description:**

Evergreen tree potentially to 20 m (65 ft). Twigs green or reddish green; all vegetative parts glabrous; cut stems and bruised leaves giving off a strong aroma of camphor. Leaves simple, alternate; blades entire but may have wavy margins, mostly ovate, 4-10 cm (1.5-4 inch) long and 2-5 cm (0.8-2 inch) broad, glossy green above, duller green below, with impressed glands below at major veins. Flowers small, greenish white to cream, in loose panicles on branchlets of season; 6 petaloid parts; 12 stamen parts,

usually 5-9 fertile stamens plus smaller sterile staminodes. Fruits small, subglobose drupes, black, seated on persistent floral tubes (Joker, 2000).

**Uses:**

Used as antiseptic, analgesic, counterirritant, antipruritic, rubefacient, abortifacient, antimicrobial, aphrodisiac, contraceptive and lactation suppressant (Zuccarini, 2009).

**Chemical components:**

Camphor, Linalool, Eucalyptol, alpha.4-trimethyl, propanoate, Caryophyllene, alpha.-Caryophyllene, Naphthalene, Phytol, Tetratetracontane, Oxirane, hexadecyl, and so on (Kai-fu *et al.*, 2009).

**Distribution:**

Nepal (WCE, 450-2100 m); Himalayas (Kasmir to Bhutan), NE India (Assam, Meghalaya), Bangladesh, Sri-Lanka (Shrestha and Joshi, 1996)

e) *Cinnamomum tamala* (Buch.-Ham.) Nees and Eberm. (Family: Lauraceae)

**Botanical Description:**

It is a perennial or small evergreen tree, attaining 8-12 meters height and girth of 150 cm. Stem rough with gray-brown, soft wrinkled bark which produces mucilage. Terminal bud small, sericeous, 2 bud scales. Leaves are large, 12-20 cm long and 5-8 cm broad, ovate-lanceolate, thick leathery, acuminate, coriaceous, glabrous, shining green above and glaucous beneath, opposite, sub-opposite or alternate and short stalked. Petiole slender, 0.8-1.8 cm long. It has bisexual flowers, but on same plant (monoecious). Flowers whitish, numerous, small, in axillary, cymes and terminal pubescent panicles, pedicels are as long as calyx. The plant produce flowers in last week of March or first week of April. The fruit is an ellipsoidal drupe and seeds require approximately one year attaining maturity. Ripe fruits are dark purple in color and contain single seed (Sharma *et al.* 2009, cited by Sharma and Nautiyal, 2011).

**Uses:**

As carminative, anti-flatulent, diuretic and in cardiac disorders (Showkat *et al.* 2004, cited by Sharma and Nautiyal, 2011). “Ayurveda” describes the use of leaves in the treatment of ailments such as anorexia, bladder disorders, and dryness of mouth, coryza,

diarrhea, nausea and spermatorhea (Kapoor, 2000, cited by Sharma and Nautiyal, 2011).

**Chemical components:**

A-pinene, camphene, myrcene, limonene, eugenol, p-cymene, methyl eugenol, eugenol acetate and methyl ether of eugenol (Smith *et al.*, 2002; Saino *et al.*, 2003, cited by Sharma and Nautiyal, 2011).

**Distribution:**

WCE Nepal alt. 450-2000 masl; Himalaya (Kashmir to Bhutan), Assam, Khasia (Press *et al.*, 2000).

**f) *Lantana camara* L. (Family: Verbenaceae)**

**Botanical Description:**

An undershrub. Branches 4-angled, sometimes prickly. Leaves simple, opposite, petiolate, ovate, acuminate, crenate, rugose, up to 8 cm long and 6.5 cm broad. Flowers in capitate head, bracts leafy, conspicuous, exceeding the calyx. Corolla-lobes 4-5, spreading, orange, pink or yellow. Stamens 4, didynamous, inserted within the corolla-tube; anther 2-celled, broadly oblong. Style short. Stigma oblique, Sub-capitate. Fruit a drupe, fleshy, or nearly dry and black (Press *et al.*, 2000).

**Uses:**

*Lantana camara* leaves are boiled and applied for swellings and pain of the body. Its bark is astringent and used as a lotion in cutiginous eruptions, leprous ulcers. Alkaloidal fractions, obtained from leaves, have been found to lower blood pressure, accelerate deep respiration and stimulate intestinal movements in experimental animals (Singh *et al.*, 1996).

**Chemical components:**

E-Caryophyllene,  $\alpha$ -Humulene, Germacrene-D, Bicyclogermacrene, Phytol,  $\alpha$ -Copaene,  $\beta$ -Elemene, Tetradecane, etc (Passos *et al.*, 2012).

**Distribution:**

400-1300 m; Himalaya (Nepal), India, Burma, China, Indo-China, Malaya. Native of America, widely naturalised in Nepal, India and other parts of Asia (Press *et al.*, 2000).

## Micrometry

In micrometer, 100 divisions = 1000  $\mu\text{m}$

$$\text{Or, 1 division} = \frac{1000}{100} = 10 \mu\text{m}$$

11 divisions of ocular micrometer coincided with 4 divisions of stage micrometer.

i.e. 11 divisions of ocular micrometer = 4 divisions of stage micrometer

$$\text{Or, 1 division of ocular micrometer} = \frac{4}{11} \text{ division of stage micrometer}$$

$$\text{Or, 1 division of ocular micrometer} = \frac{4}{11} \times 10 \mu\text{m} = 3.63 \mu\text{m}$$

$\therefore$  Calibration factor = 3.63  $\mu\text{m}$

### Measurement of Conidia of *Aspergillus niger*

S.N.	Length ( $\mu\text{m}$ )	Breadth ( $\mu\text{m}$ )
1.	3.63	3.63
2.	7.26	3.63
3.	7.26	7.26
4.	5.445	7.26
5.	7.26	3.63
6.	5.445	3.63
7.	7.26	5.445
8.	3.63	3.63
9.	7.26	5.445
10.	5.445	7.26

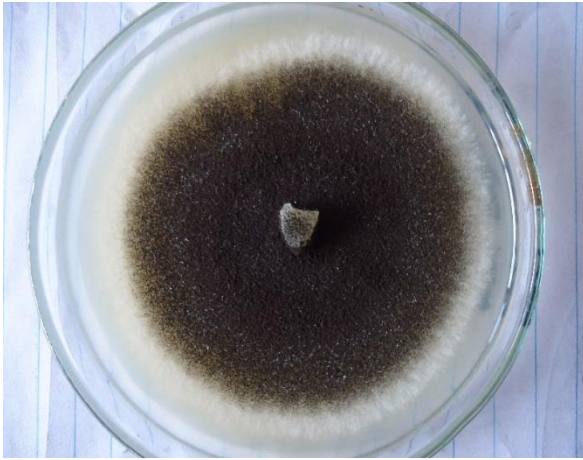
Hence size of conidia of *Aspergillus niger* ranges from (3.63-7.26 $\times$ 3.63-7.26)  $\mu\text{m}$ .

**Measurement of Conidia of *Alternaria alternata***

S.N.	Length ( $\mu\text{m}$ )	Breadth ( $\mu\text{m}$ )
11.	29.04	7.26
12.	25.41	7.26
13.	65.34	14.52
14.	50.82	10.89
15.	29.04	7.26
16.	32.67	10.89
17.	36.3	10.89
18.	25.41	7.26
19.	32.67	10.89
20.	39.93	10.89

Hence size of conidia of *Aspergillus niger* ranges from (25.41-65.34 $\times$ 7.26-14.52)  $\mu\text{m}$ .

## PHOTO PLATES



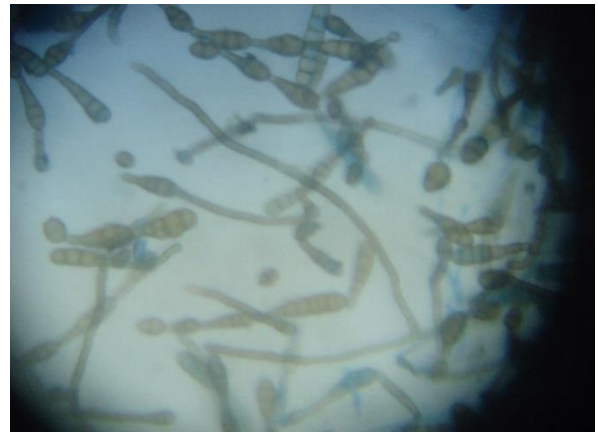
Pure culture of *Aspergillus niger*



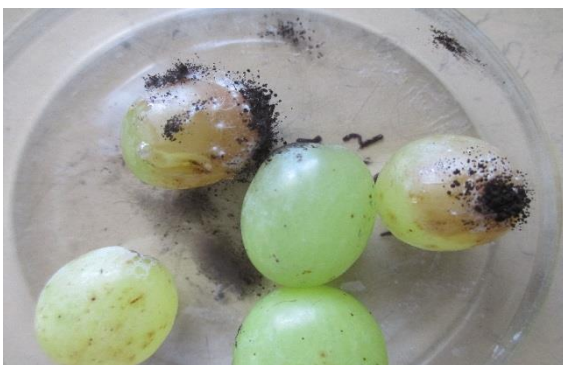
Pure culture of *Alternaria alternata*



Conidial head and conidiophore of  
*Aspergillus niger*



Conidia and conidiophore of *Alternaria alternata*



Grapes infected with *Aspergillus niger*



Grapes infected with *Alternaria alternata*



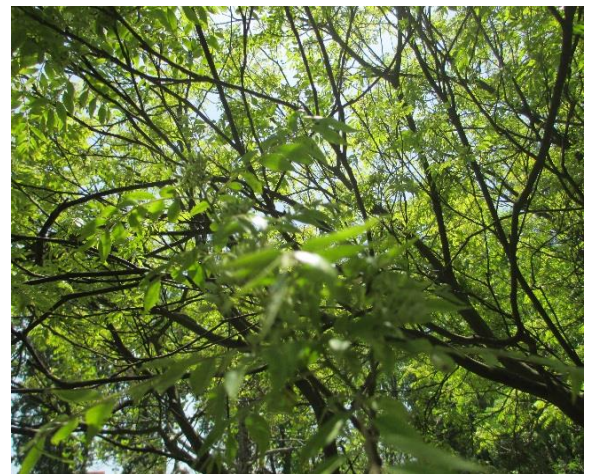
*Eucalyptus citriodora*



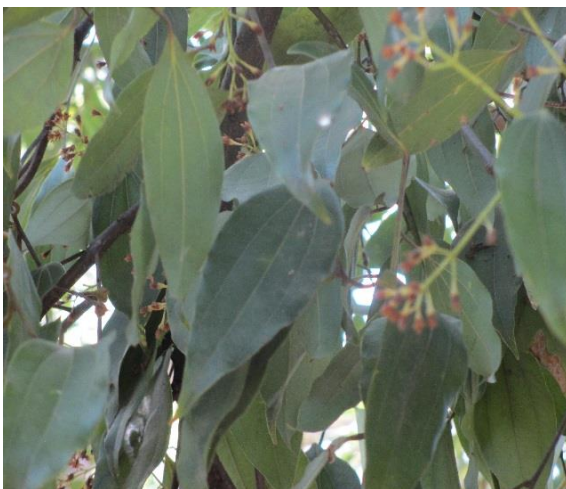
*Artemisia indica*



*Lantana camara*



*Murraya koenigii*



*Cinnamomum tamala*



*Cinnamomum camphora*



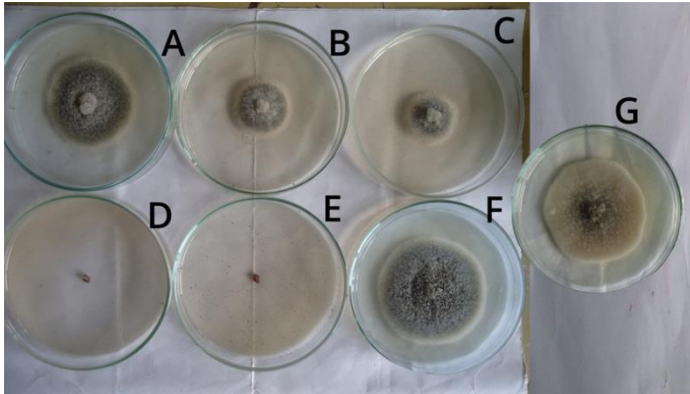
Clevenger's apparatus



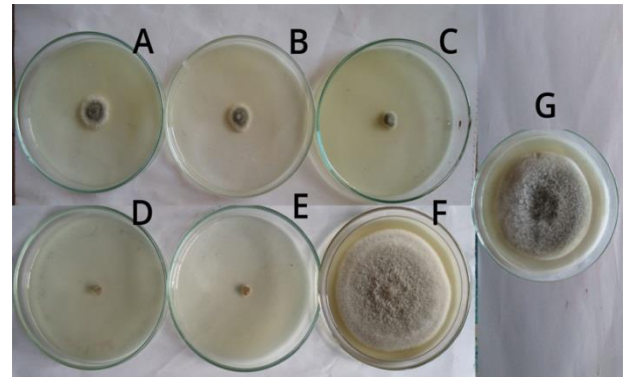
Used fungicide (Mancozeb)



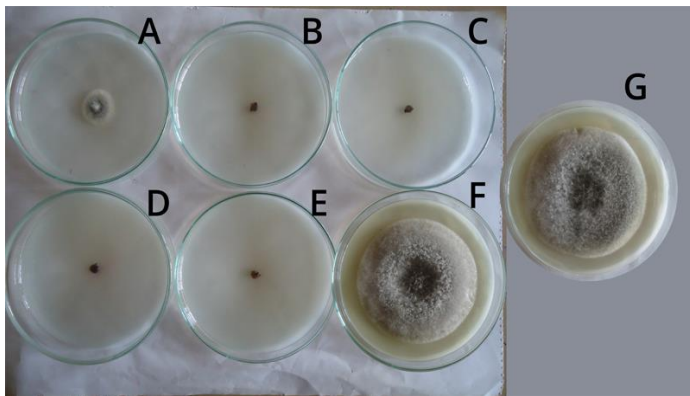
Working on laminar air flow at pathology lab, CDBTU.



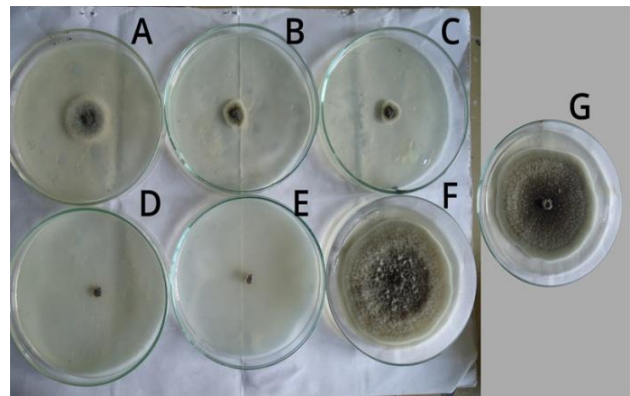
Antifungal activity of *Murraya koenigii*



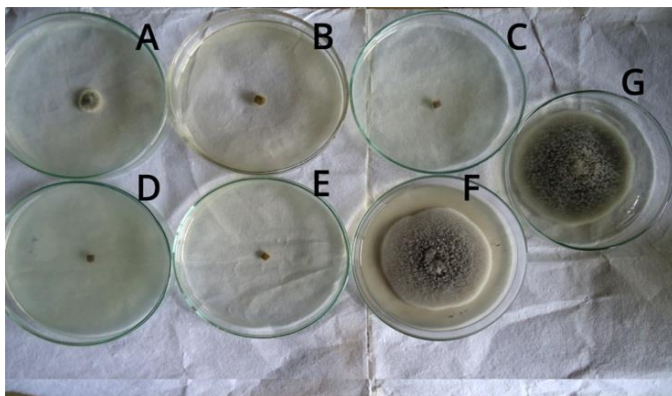
Antifungal activity of *Lantana camara*



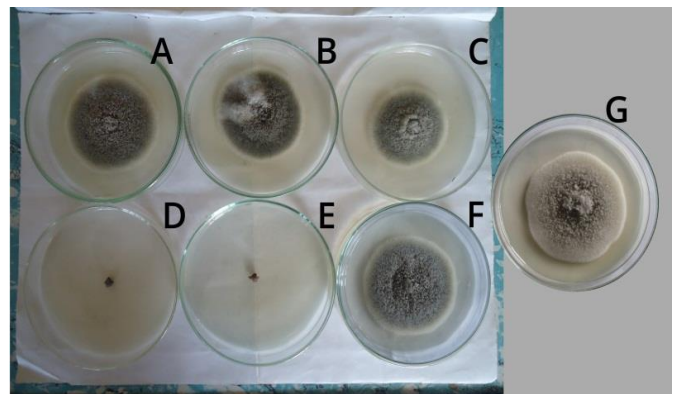
Antifungal activity of *Artemisia indica*



Antifungal activity of *Cinnamomum camphora*



Antifungal activity of *Cinnamomum tamala*



Antifungal activity of *Eucalyptus citriodora*

Antifungal activities of six essential oils against *Alternaria alternata* at different concentrations.

A = 20  $\mu$ l/ml

C = 80  $\mu$ l/ml

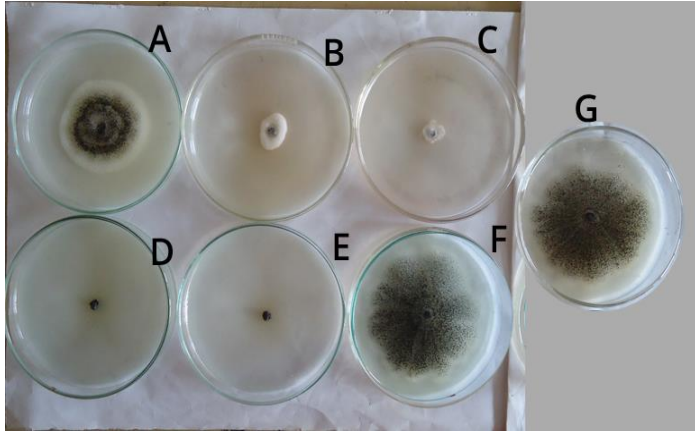
E = 320  $\mu$ l/ml

G = Pos. control

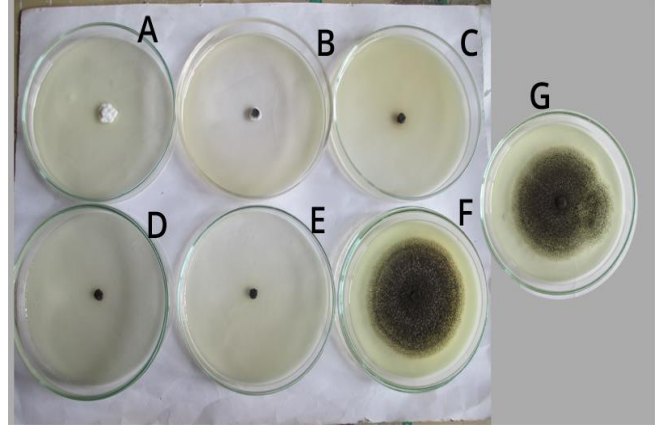
B = 40  $\mu$ l/ml

D = 160  $\mu$ l/ml

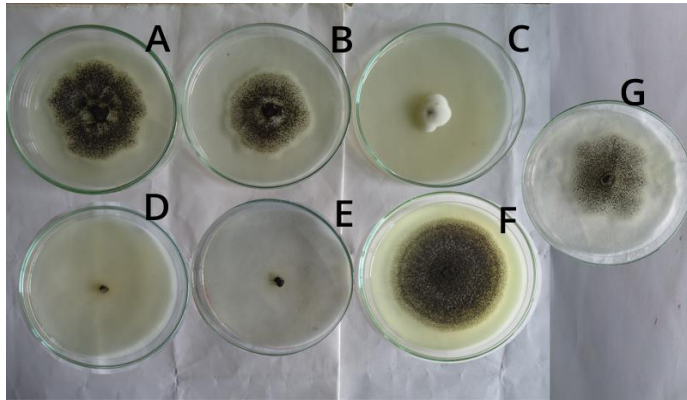
F = Neg. control



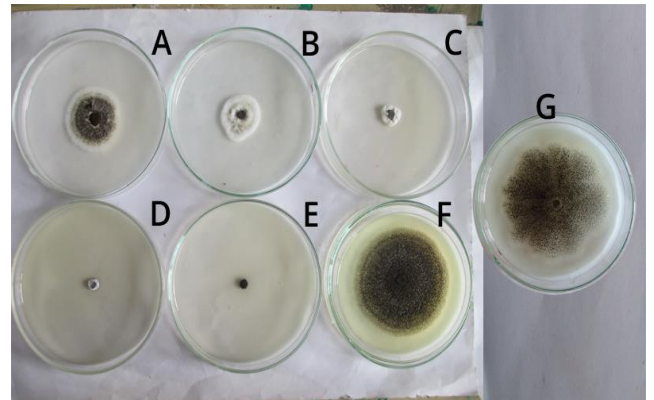
Antifungal activity of *Artemisia indica*



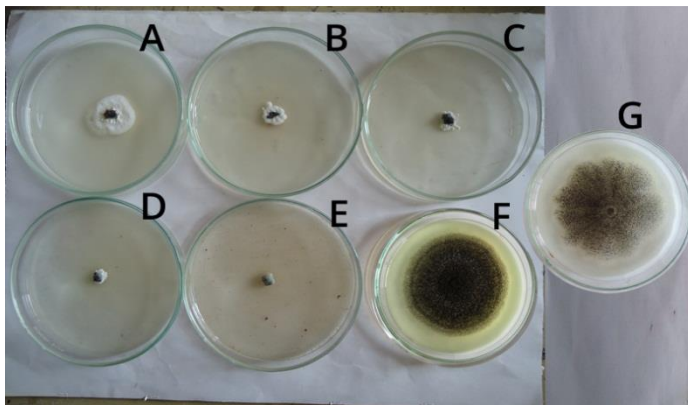
Antifungal activity of *Cinnamomum camphora*



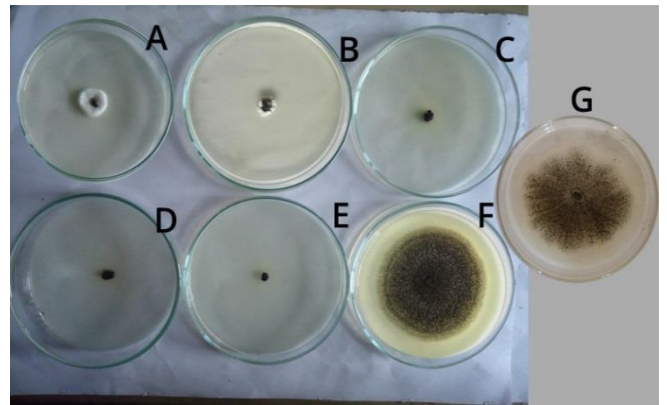
Antifungal activity of *Eucalyptus citriodora*



Antifungal activity of *Lantana camara*



Antifungal activity of *Murraya koenigii*



Antifungal activity of *Cinnamomum tamala*

Antifungal activities of six essential oils against *Aspergillus niger* at different concentrations.

A = 20 µl/ml

C = 80 µl/ml

E = 320 µl/ml

G = Pos. control

B = 40 µl/ml

D = 160 µl/ml

F = Neg. control