

# Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources

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(MSA-2018-1232)



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**Identifying Plant Inhibitors against *Alternaria mali* from  
Diverse Plant Sources**

**Mariya**  
(MSA-2018-1232)



**Thesis**  
Submitted to  
**Faculty of Agriculture**  
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**Master of Science in Agriculture**  
**(Plant Pathology)**

**2021**



*Dedicated  
To  
My Parents*

**Sher-e-Kashmir**  
**University of Agricultural Sciences and Technology of Kashmir**  
**Division of Plant Pathology, Faculty of Agriculture, Wadura**  
**193201**

**Certificate – I**

This is to certify that the thesis entitled, “**Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources**” submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture (Plant Pathology)**, to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Mariya (Regd. No. MSA-2018-1232)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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This is to certify that the thesis entitled, “**Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources**” submitted by **Ms. Mariya (Regd. No. MSA-2018-1232)** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture (Plant Pathology)** was examined and approved by the Advisory Committee and External Examiner on .....

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**ABSTRACT**

A number of diseases like scab, *Alternaria* leaf blotch, Marssonina, sooty blotch, fly-speck and a number of post-harvest diseases pose a threat to the apple industry in Kashmir. The occurrence of *Alternaria* leaf blotch in J&K was reported by Shahzad *et al.* (2002) and the disease is prevalent in almost all the apple growing districts of Kashmir valley (Sofi *et al.*, 2013). *Alternaria* leaf blotch was considered a disease of minor importance in comparison to apple scab. However, the disease resulted in epidemic during summer of 2013 and huge yield losses were reported. The present study was undertaken in search of alternatives to chemical fungicides for management of this disease. This study was devised to evaluate the antifungal efficacy of seven locally available plant extracts against *Alternaria mali*. Aqueous, Ethanol and Methanol extracts of the seven test plants each were evaluated at three concentrations of 500ppm, 1000ppm and 2000ppm *in vitro* for mycelial inhibition using Poisoned food technique and also for spore germination inhibition. Furthermore the current investigation was aimed at the phytochemical profiling of the most effective plant extract obtained in this study through Liquid Chromatography cum Mass Spectroscopy. All the seven botanicals inhibited both the mycelial growth and conidial germination of *Alternaria mali*, however, with varying efficacy. Among the seven botanicals tested, 100 per cent

mycelial inhibition was recorded in case of clove methanol extract at both 1000ppm and 2000ppm concentrations. It also showed highest inhibition of 89.3 % in case of spore germination at concentration 2000ppm. The clove ethanol and aqueous extracts also showed promising results. The phyto-chemical profiling of the highly effective botanical obtained in this study revealed a number of compounds like eugenol, quercetin, chlorogenic acid, luteoline etc. Most of the detected compounds were either flavonoids and poly-phenols. Certain compounds detected like imidazole and eugenol have known antifungal properties. Further, molecular docking approach was undertaken to identify key inhibitor molecules. Among the compounds detected through LC-MS/MS diosmin, rhein and quercetin proved to be the top 3 in terms of affinity towards the enzyme lanosterol 14-alpha demethylase. The present study conclusively demonstrates that Clove is a good source of various phyto-chemicals like poly-phenols, flavonoids, alkaloids and various anti-microbial compounds. This preliminary finding warrants an in-depth analysis of the usefulness of these compounds against *Alternaria mali* for a possible drug discovery in future.

**Keywords:** Alternaria leaf blotch, Antifungal, Plant extracts, Clove, Eugenol, Diosmin

Signature of Student

Dated \_\_\_\_\_

Signature of Major Advisor

Dated \_\_\_\_\_

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***Mariya***

***Place:** Wadura, Sopore*

***Dated:***

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## Chapter-1

### INTRODUCTION

Apple is a rosaceous fruit tree, belonging to genus *Malus*. It is the most widely grown fruit in the world and is cultivated in temperate regions of both northern and southern hemispheres of the world for its high economic value. The genus has five sections including 122 species and subspecies. Natural varieties of cultivated apple belong to *Malus pumila* Mill. while its hybrid varieties belong to *Malus domestica* Bork. Taxonomically apple is a dicotyledonous tree belonging to family Rosaceae with subfamily Maloideae (former Pomideae) having basic chromosome number 17.

It is mainly grown in the European countries, North America, New Zealand, Australia, China and Japan. The commercial cultivation of apple fruit in India is confined to North Himalayan hill region comprising the states of Jammu & Kashmir, Himachal Pradesh and Uttaranchal and to a limited extent to the states of Arunachal Pradesh, Sikkim, Nagaland, Meghalaya and Manipur covering a total area of 0.3 million ha with an annual production of 2.32 million tonnes (Anonymous, 2018a). Of this area and production, Jammu & Kashmir State accounts for 0.16 million ha and 1.88 million tonnes respectively (Anonymous, 2018b).

Worldwide, apples are affected by a large number of diseases caused by pathogenic fungi, bacteria, oomycetes and viruses. Fungi cause many different diseases, including root rots, leaf spots, leaf blights, blossom blights, fruit decay, fruit spots, defoliation, trunk, branch and twig cankers (Grove *et al.*, 2003).. Alternaria leaf blotch, a fungal disease of apple was first reported in USA in 1924 by Roberts and the disease since then has been reported from most apple producing countries. It has been observed in most apple cultivars, however Delicious cultivars are worldwide most susceptible (Kishore and Sharma, 2005). The disease is characterized by small circular brown or blackish brown spots,

which gradually enlarge to 2-5 mm in diameter and are bordered by a dark brown to purple margin (Bulajic and Babovic, 1997). Fruit spot symptoms are characterized by small slightly sunken, light to medium brown spots on mature fruit. The disease causes severe defoliation, reduced tree vigor and a reduced yield in the following seasons (Sharma, 2000). Worldwide, different *Alternaria* species have been associated with Alternaria leaf blotch and fruit spot on apple. The most commonly cited causal agent of Alternaria leaf blotch on apple is *Alternaria mali* (Filajdic and Sutton, 1991).

In Jammu and Kashmir, a number of diseases like scab, Alternaria leaf blotch, Marssonina, sooty blotch, fly-speck and a number of post-harvest diseases pose a major threat to the apple industry. The occurrence of Alternaria leaf blotch in J&K was reported by Shahzad *et al.* (2002) and the disease is prevalent in almost all the apple growing districts of Kashmir valley (Sofi *et al.*, 2013). Alternaria leaf blotch was considered a disease of minor importance in comparison to apple scab. However, the disease resulted in epidemic during summer of 2013, and about 40-60 per cent yield loss was reported (Anonymous, 2013). This epidemic was attributed to climate change (high temperature coupled with prolonged rains), absence of disease forecasting system in the valley and also to the fact that currently used fungicides do not provide satisfactory level of disease control (Bhat *et al.*, 2015). Therefore, a need arises to search for alternatives to chemical fungicides for management of this disease.

Natural plant products have the potential as safe alternatives for chemical fungicides in plant disease management. In present scenario due to the increased demand for organic products, there has been a change from the use of conventional systems to organic systems using alternative approaches for disease management (Hwang *et al.*, 2009). Botanicals are substantial sources to replace synthetic fungicides as these are environmentally safe and have recently gained importance due to their efficacy against phytopathogens and cost effectiveness (Kokab *et al.*, 2018).

Plants have ability to synthesize aromatic secondary metabolites like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins (Cowan, 1999). These groups of compounds show antimicrobial effect and serve as plant defence mechanisms against pathogenic microorganisms (Das *et al.*, 2010). At present, scientists are investigating for plant products of antimicrobial properties. Several researches have been conducted on plant extracts and oils to find out such alternatives and valuable results have been obtained (Ayoub and Niazi, 2001). It would be advantageous to standardize methods of extraction and *in vitro* antimicrobial efficacy testing so that the search for new biologically active plant products could be more systematic. Natural products play a highly significant role in new drug discovery (Rout *et al.*, 2009). Secondary metabolites from natural sources are often preferred as they show more drug likeness and biological friendliness than totally synthetic molecules (Koehn and Carter, 2005), making them good candidates for drug development (Balunas and Kinghorn, 2005). Therefore, the search for plant based antimicrobials compounds to replace synthetic chemicals has become important.

To date neither the efficacy of plant extracts against *Alternaria* leaf blotch has been studied nor have the methods of extraction been standardized. Therefore, it has become imperative to undertake the studies aiming at evaluating various plant extracts against this pathogen and standardizing the extraction solvents.

In view of searching for alternatives to synthetic chemicals for the management of *Alternaria* leaf blotch of apple, the present study was undertaken with the following objectives:

1. To study the antifungal activity of different plant extracts against *Alternaria mali*.
2. To figure out the probable bioactive compounds in the most effective plant extract.

## Chapter–2

### REVIEW OF LITERATURE

Nature has bestowed us with numerous medicinally important plants and more than 2,50,000 higher plant species are having potential antimicrobial properties. During last several decades researchers have evaluated numerous plant extracts against plant pathogens and valuable results have been achieved (Zaker, 2016). Plant extracts provide attractive alternative to currently used synthetic fungicides as regards controlling phytopathogenic fungi, since they are a rich source of inhibitory bioactive molecules (Wink, 1993). They are often active against a limited number of specific target pests, are biodegradable into non-toxic products and thus have the advantage of being less hazardous for the ecosystem and therefore more acceptable (Kim *et al.*, 2003). These biologically active natural products have the potential to replace synthetic fungicides. Therefore, recent efforts have been directed towards screening more plant for potential antimicrobial properties and also identify and isolate novel bioactive molecules.

#### 2.1 Effect of Aqueous Plant extracts against Fungal Pathogens

Balamurugan (2014) assessed aqueous extracts of leaf of *Citrus aurantifolia* *in vitro* for inhibitory activity against *Macrophomina phaseolina*. The antifungal activity was determined by poisoned food technique. The extract inhibited the mycelial growth. Further an increase in the concentration of extract resulted in an increased inhibition.

Dev *et al.* (2016) studied *in vitro* the antifungal activity of aqueous extracts of nine plants *viz.*, Calotropis, Lantana, Lemon grass, Nagadhale, Neem, Pongamia, Simarouba, Subabul and Tulsi against pathogen *Colletotrichum gleosporioides* causing anthracnose of pomegranate. The extracts were tested at three different concentrations (10%, 20% and 30%) by poisoned food technique. Among the nine plant extracts, maximum per cent inhibition of mycelial growth

(68.28%) was recorded in Nagadhale leaf extract followed by Pongamia extract (61.31%) and Simarouba leaf extract (60.07%).

Fatima *et al.* (2019) assessed the antifungal activity of the crude aqueous extract of *Boswellia carteri* *in vitro* at three different concentrations (1%, 2.5% and 5%) against three fungal strains namely *Alternaria alternata*, *Fusarium solani* and *H. rostratum*. Results showed that the crude aqueous extract of *B. carteri* inhibited the growth of all the selected fungal species. The percentage of mycelial growth of the tested fungi decreased as the concentration of the aqueous extract increased from 1 to 5 per cent. Moreover the treated *A. alternata* samples were analyzed for morphological changes using scanning electron microscopy (SEM) which showed shrunken hyphae, membrane disintegration, and distorted conidial structures compared to the untreated fungal cells.

Hans and Sharma (2017) conducted an experiment to evaluate the bio-efficacy of seven plant extracts under *in vitro* conditions against *Alternaria alternata*, causal agent of Mouldy core, core rot of apple. The inhibitory effect of the aqueous extracts of botanicals were assessed against the pathogen by poisoned food technique. Among the plant extracts *Dodonia viscosa* showed the maximum inhibitory effect (66.55%).

Jagtap *et al.* (2013) tested a total of eleven aqueous plant extracts (Ashoka, Babhul, Bael, Parthenium, Neem, Beshram, Mehandi, Tulsi, Turmeric, Onion and Lantana) *in vitro* against the pathogen *Colletotricum capsici* causing leaf spot of turmeric by poisoned food technique. Overall percent inhibition of growth of the fungus ranged from 7.30 to 76.15 per cent. *Polyalthia longifolia* recorded maximum inhibition of 76.15 per cent at 15 per cent concentration followed by rhizome extract *Curcuma longa* with 66.88 per cent and *Allium cepa* 63.96 per cent. Whereas, *Aegale marmelos* and *Parthenium hysterophorus* showed minimum mean inhibition of 20.86 and 28.66 per cent, respectively.

Khalse *et al.* (2017) conducted an experiment to evaluate the effect of

three aqueous plant leaf extracts namely eucalyptus, datura and lantana leaf extract @ 10 per cent concentration *in vitro* and *in vivo* against *Alternaria brassicae* causing leaf spot of cabbage. The percent mycelial growth inhibition recorded was (58.69%) (54.96%) and (52.17%) for eucalyptus, lantana and datura respectively as compared to treated propiconazole (86.95%) and untreated control (00.00%). The plant extracts were tested under field conditions. Among the treatments minimum disease intensity per cent was recorded in eucalyptus leaf extract @ 10 per cent (25.60%) followed by datura leaf extract @ 10 per cent (28.13%), lantana leaf extract @ 10 per cent (25.73%) as compared to propiconazole (treated check) @ 0.05 per cent (25.08%) and untreated control (34.05%).

Kumar and Singh (2017) evaluated six plants extracts viz. *Datura stramonium* (Dhatura) green fruit extract, *Azadirachta indica* (Neem) seed kernel extract, *Allium sativum* (Garlic) bulb extract, *Eucalyptus spp.* (Eucalyptus) dry leaf extract, *Crotalaria juncea* (Sunhemp) seed extract, *Euphorbia hirta* (Bara Dudhi) whole plant extract at 1, 2, 3, 4 and 5 percent concentrations against *Alternaria solani* causing Early blight in tomato. Out of six aqueous plant extracts tested against *A. solani in vitro*, most effective plant extract was *Allium sativum* @ 5 per cent which exhibited maximum inhibition in mycelium growth (45.15%) followed by *Crotalaria juncea* @ 5 per cent (44.40%), while minimum inhibition in mycelial growth was recorded in *Euphorbia hirta*.

Ravikumar *et al.* (2013) evaluated aqueous extracts of 39 plants for antifungal potential against *Alternaria solani*, causing early blight of tomato at 4 per cent concentration by poison food technique. Out of these, 13 plant extracts significantly reduced the mycelial growth of the pathogen. Inhibition rate of above 20 per cent was shown by seven plant extracts namely *Crotalaria trichotoma* (36.6%), *Citrus aurantifolia* (27.3%), *Azadirachta indica* (23.7%), *Polyalthia longifolia* (23.3%), *Datura metel* (21.3%), *Muntingia calabura* (20.09%) and *Oxalis latifolia* (20.09%).

Salhi *et al.* (2017) investigated the antifungal properties of aqueous extracts of four plant species (*Artemisia herba alba*, *Cotula cinerea*, *Asphodelus tenuifolius*, and *Euphorbia guyoniana*) at concentrations 10 and 20 per cent against fungal species *Fusarium graminearum* and *Fusarium sporotrichioides*. All the four plants inhibited the mycelial growth of both the *Fusarium* species. Maximum inhibition was exhibited at 20 per cent concentration of the extracts with *Asphodelus tenuifolius* extract most effective against *Fusarium graminearum*, while *Fusarium sporotrichioides* mycelium growth was significantly inhibited by *Euphorbia guyoniana*.

## **2.2 Effect of Organic solvent Plant extracts against Fungal Pathogens**

Gatto *et al.* (2011) investigated *in vivo* and *in vitro* activity of aqueous methanol extracts (80%) of nine wild herbaceous species (*Borago officinalis*, *Orobancha crenata*, *Plantago coronopus*, *P. lanceolata*, *Sanguisorba minor*, *Silene vulgaris*, *Sonchus asper*, *Sonchus oleraceus* and *Taraxacum officinale*) against post-harvest fungal pathogens *Botrytis cinerea* and *Penicillium expansum*. Out of the nine extracts *S. minor*, *O. crenata* and *P. coronopus* showed the highest efficacy. *S. minor* significantly reduced *in vitro* conidial germination of *B. cinerea* and *P. expansum* with the per cent inhibition of 93 and 47 per cent respectively.

Hassanein *et al.* (2008) tested leaf extracts of neem (*Azadiracta indica*) and chinaberry (*Melia azedarach*) extracted by ethanol, ethyl acetate and water against two tomato fungal pathogens at 5, 10, 15 and 20 per cent concentrations and found that both ethanol and ethyl acetate extracts of neem leaves at a concentration of 20 per cent completely suppressed the growth of *Fusarium oxysporium* and inhibited *Alternaria solani* by 62.77 per cent.

Patel *et al.* (2014) investigated the antifungal activity of ethanolic and aqueous extract of *Moringa oleifera* against strains *Saccharomyces cerevisiae*, *Candida albicans* and *Candida tropicalis* using agar well diffusion method. The extracts showed inhibitory activity against *Saccharomyces cerevisiae* and

*Candida tropicalis* with largest zone of inhibition produced against *Saccharomyces cerevisiae*.

Rodino *et al.* (2014) investigated *in vitro* the antifungal activity of the ethanolic and aqueous extracts of four locally available medicinal plants from Romania namely absinth (*Artemisia absinthium*), rosemary (*Rosmarinus officinalis*), jimson weed (*Datura stramonium*) and cocklebar (*Xanthium strumarium*) against the plant pathogenic fungus *Alternaria alternata*. The extracts presented antifungal potential demonstrated by the inhibition of the mycelial growth. The ethanolic extracts showed a higher antifungal activity than the aqueous extracts, for all the tested variants.

Wijesundara *et al.* (2015) evaluated the antifungal activity of ethanolic extract of *Croton aromaticus* leaves *in vitro*, against mycelial growth and spore germination of postharvest fungal pathogens isolated from banana (*Colletotrichum musae*, *Lasiodiplodia theobromae*), papaya (*Colletotrichum gleosporioides*, *L. theobromae*) and mango (*Alternaria alternata*, *Pestalotiopsis mangiferae*, *L. theobromae*). Inhibitory effect of the ethanolic extract was investigated by well diffusion method with different concentrations (1, 5, 10, 30, 50, 100, 200 and 300 mg ml<sup>-1</sup>) along with positive control (Captan) and negative control (DMSO). Significant inhibitory effects were exhibited by the extract against *C. musae*, *P. mangiferae*, *A. alternata* and *C. gleosporioides*.

Zaheer *et al.* (2012) investigated the mycotoxic potential of root, shoot and leaf aqueous, methanol and n-hexane extracts of *Parthenium hysterophorus* against *Fusarium solani* causing wilt in potato plant. It was seen that growth of *F. solani* was greatly inhibited at 1 and 2 per cent concentrations of aqueous and methanol leaf and stem extracts while n-hexane extract at concentration of 3 and 4 per cent was found effective. Among root extracts, higher concentrations of aqueous and n-hexane exhibited more promising results by causing fungal biomass reduction of 85 and 74 per cent respectively; whereas methanol extracts were effective even at lower concentrations.

Zaker and Mosallanejad (2010) evaluated pure methanol and methanol water extracts of five plants namely peppermint, eucalyptus, lavandula, Russian knapweed and datura(at 5,10 and 15 per cent concentrations *in vitro*) for their antifungal ability against *Alternaria alternata*, causing Alternaria leaf spot of potato. Fungicide mancozeb 0.2 per cent was used for comparison. Methanol extracts of peppermint(15%), lavandula(15%), peppermint(10%) and eucalyptus(15%) effectively inhibited the mycelial growth while majority of methanol water extracts had either less or no effect. Spore germination of *A. alternata* was also significantly reduced by methanol extracts in comparison to methanol water extracts which had very less effect in this regard. Findings from this study confirmed that plant extracts can be used as less hazardous natural fungicides in controlling plant pathogenic fungi.

### **2.3 Identification of the Phyto-constituents responsible for the Anti-fungal activity**

The most commonly used plant products for plant disease management include essential oils and extracts (Burt, 2004). These products may have fungicidal or fungistatic activity on plant pathogens or they can create conditions unfavourable for establishment and multiplication of pathogenic microorganisms on host plants (Scheuerell and Mahaffee, 2002).

Natural products contain advanced chemical novelty compared to chemically synthesized products, and for this reason, studies have been conducted to discover new bioactive compounds in plants (Ma *et al.*, 2015). Plants synthesize aromatic secondary metabolites like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins (Cowan, 1999). These groups of compounds show antimicrobial properties and serve as plant defence mechanism against pathogenic microorganisms and have the potential to replace synthetic fungicides (Das *et al.*, 2010).

Clove represents a good source of bioactive compounds with eugenol as

the main bioactive compound, and among phenolic compounds gallic acid is found in abundance (Cortes-Rojas *et al.*, 2014)

Rijai *et al.* (2017) conducted the secondary metabolites profiling from stem bark of Kolowe (*Chydenanthus excelsus*). Analysis of the secondary metabolites was done with two approaches, chemical methods (use of reagents) and spectrum analysis of compounds by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Class of secondary metabolites analysed were flavonoids, steroids, triterpenes, phenolics, tannins, carbohydrates, and saponins.

Fathoni *et al.* (2017) conducted phytochemical screening to identify nonvolatile compounds in clove from Manado by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Several compounds were identified and characterised as asquinic acid, monogalloylglucose, gallic acid, digalloylglucose, isobiflorin, biflorin, ellagic acid, hydroxygallic acid, luteolin, quercetin, naringenin, kaempferol, isorhamnetin, dimethoxyluteolin, and rhamnetin.

Yusoff *et al.* (2020) conducted antifungal assay and phytochemical screening of *Vernonia amygdalina* extract against *Botrytis cinerea*. The dichloromethane extract of *Vernnonia amygdalina* was found most effective and was screened for the bioactive compounds. A total of 23 compounds were identified in *V. amygdalina* dichloromethane extract using GCMS analysis. The top five major compounds identified were squalene (16.92%), phytol (15.05%), triacontane (11.31%), heptacosane (7.14%), and neophytadiene (6.28%).

Chimahali *et al.* (2019) evaluated the whole plant extract of *Gloriosa superba* (Flame lily) for antifungal potential against fungal pathogens *Rhizopus oryzae*, *Mucor Sp.*, *Aspergillus niger*, *Candida krusei* and *Candida albicans* followed by its phytochemical profiling. The different solvents used for extraction of phytochemicals were dimethyl sulfoxide, ethyl acetate, and ethanol. The phytochemical screening of extracts indicated the presence of carbohydrates,

reducing sugars, proteins, amino acids, steroids, flavonoids, terpenoids, saponins, alkaloids, tannins, and phlobatannins.

Muthukumaran *et al.* (2016) evaluated methanolic leaf and bark extract of *Solanum trilobatum* against two fungal isolates *Aspergillus brasiliensis* and *Aspergillus fumigatus* by agar-well diffusion method. The leaf extract of *Solanum trilobatum* showed better antifungal activity in comparison to the bark extract. The methanol leaf and bark extract of *Solanum trilobatum* was further screened for the presence of bioactive compounds. The phytochemical screening of the extracts revealed the presence of Phenols, tannins, saponin, flavonoids, steroids, and trepenoid.

Dar *et al.* (2017) in their study evaluated the aqueous and methanolic extract of *Solanum nigrum* (Black nightshade) for antifungal potential against four fungal strains, *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus* and *Penicillium chrysogenum*. The highest antifungal potential was exhibited by the methanolic extract against *Saccharomyces cerevisiae* and *Candida albicans* while the aqueous extract exhibited the highest antifungal potential against *Saccharomyces cerevisiae* followed by *Candida albicans* and *Aspergillus fumigatus* at the concentration of 100 mg/ml. The extracts were further evaluated for the presence of phytochemicals which revealed various secondary metabolites like alkaloids, saponins, flavonoids, phenols and volatile oils.

Gaspar *et al.* (2018) identifies eugenol as the abundant bioactive compound in whole clove buds. The other major compounds identified were eugenyl acetate and  $\beta$ -caryophyllene.

Yassin *et al.* (2020) reported that Gas chromatography-mass spectrometry (GC-MS) analysis of clove methanolic extract revealed that eugenol was the main bioactive compound followed by eugenyl acetate and trans-caryophyllene. The novel compounds detected were musk ketone,  $\alpha$  cubebene, chavicol etc.

## Chapter-3

### MATERIALS AND METHODS

The present investigation “Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources” was undertaken in the laboratory of Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Faculty of Agriculture, Wadura during 2019-20. The details of materials and methodologies followed during the course of investigation are described as follows:

#### 3.1 Isolation of the pathogen

The leaves showing typical *Alternaria* leaf blotch symptoms (Plate-1) were collected and were brought to the laboratory and examined under microscope for the associated pathogen followed by isolation of pathogen. The isolation of causal agent was done by tissue bit transfer method (Sicard *et al.*, 1997). The infected leaf samples were cut into small pieces from the advancing margins of lesions and the healthy portions. The pieces were surface sterilized with 0.1 per cent mercuric chloride for 30 seconds and washed thrice serially with sterile distilled water. Surface sterilized leaf bits were then aseptically placed on solidified and cooled PDA (Potato Dextrose Agar) medium in Petri plates under aseptic conditions in Laminar-air-flow cabinet. Inoculated plates were incubated at  $25\pm 1^{\circ}\text{C}$  to maintain its growth.

#### 3.2 Purification and maintenance of pathogen

The pure culture of the fungus was obtained by single spore transfer method (Jhonston and Booth, 1983). Diluted spore suspension of the pathogen was prepared in sterilized distilled water containing 8 to 10 spores per microscopic field at low power. One ml of such suspension was spread uniformly on 2 per cent solidified water agar plates and incubated at  $25\pm 1^{\circ}\text{C}$  and regularly observed under compound microscope for spore germination. The germinating spores along with agar disc were lifted with the help of a sterilized inoculation

needle, transferred to PDA medium plates under aseptic conditions and incubated at 25±1°C. The pure cultures thus obtained, were maintained by repeated sub-culturing at an interval of 30 days for further studies. The stock cultures in PDA slants were stored at 4°C in refrigerator.

### **3.3 Identification of the pathogen**

The pathogen was identified on the basis of the cultural and morphological characteristics.

### **3.4 Pathogenicity test**

Fresh healthy leaves of apple were brought to the laboratory of Plant Pathology and thoroughly washed with 0.1 per cent mercuric chloride for 15 seconds and then washed in distilled water thrice. The leaves were then dried within the folds of blotting paper. These were placed in large Petri-dish of 90 mm with the base of the Petri dish covered with blotting paper and also lined with thin layer of cotton and moistened with distilled water to create a moist chamber. The leaves were inoculated with drops of spore suspension of the pathogen with first causing slight injury to the leaves with a sterilised needle in the form of a pin prick. Thereafter placed in BOD incubator at 25±1°C for development of symptoms. Suitable control using water drop without the spores was also maintained.

### **3.5 *In vitro* evaluation of the plant extracts against the causal pathogen *Alternaria mali***

The following seven plant extracts were evaluated *in vitro* for their efficacy against the pathogen causing *Alternaria* leaf blotch of apple by using poisoned food technique (Nene and Thapliyal, 2002):



**Plate 1: Alternaria symptoms visible on apple leaves**

**Table 3.1: List of Plants and Plant parts used**

S No.	Scientific Name	Common Name	Plant Part used
1.	<i>Allium sativum</i>	Garlic	Cloves (seed)
2.	<i>Zingiber officinalis</i>	Ginger	Rhizome
3.	<i>Datura</i> sp.	Jimson weed	Leaves
4.	<i>Mentha arvensis</i>	Mint	Leaves
5.	<i>Curcuma longa</i>	Turmeric	Rhizome
6.	<i>Syzygium aromaticum</i>	Clove	Floral buds (cloves)
7.	<i>Artemisia absinthium</i>	Sweet wormwood	Whole plant

### 3.5.1 Collection of Plant materials

Seven plant species were selected to evaluate their efficacy against *Alternaria mali* after considering their potential to inhibit the plant pathogens based on the available literature (Table-1). The plants were collected from different sources.

### 3.5.2 Preparation of Aqueous plant extracts

The selected whole plant / plant parts were shade dried or in some cases oven dried and made into powder by the electric blender. 16gms of ground powder of plant material was mixed with sterile distilled water (150ml) in a conical flask to make a suspension. The resultant suspension was then heated to 100° C. Thereafter the flask was plugged tightly. This suspension was kept in a shaker incubator at temperature 25 for a period of 4 days for better extraction of phytochemicals (Plate-2). Afterwards it was filtered using Whatman's No. 1 filter paper. The filtered extracts were dried in the hot air oven at 60° C till the solvent was evaporated completely (Ravikumar and Rajkumar, 2013).

### **3.5.3 Preparation of Ethanolic and Methanolic plant extracts :**

The selected whole plant / plant parts were shade dried or in some cases oven dried and made into powder by the electric blender. 16gms of ground powder of plant material was mixed with sterile solvent (150ml) in a conical flask to make a suspension. Thereafter the flask was plugged tightly. This suspension was kept in a shaker incubator at temperature 25 for a period of 4 days for better extraction of phytochemicals. Afterwards it was filtered using Whatman's No. 1 filter paper. The filtered extracts were dried in the hot air oven at 60° C till all the solvent was evaporated completely (Zaker and Mosallanejad, 2010).

### **3.5.4 Reconstitution of the extract :**

One gram of the extract was weighed and dissolved in 5ml of sterile distilled water to make a stock concentration of 2,00,000 ppm and stored in plastic tubes in the refrigerator at 4° C for further studies (Plate-2). From this stock solution three different concentrations viz., 500ppm, 1000ppm and 2000ppm were made.

### **3.5.6 Antifungal activity assay of the plant extracts**

#### **3.5.6.1 Determination of mycelial growth inhibition by Poisoned food technique**

The efficacy of the plant extracts vis-a-vis the extraction method against *Alternaria mali in vitro* was determined on the basis of mycelial inhibition through Poisoned food technique (Nene and Thapliyal, 2002). From the stock solution with concentration 2,00,000ppm desired volume of the plant extract was taken with a pipette and added to 50 ml sterilized PDA medium in conical flasks to make the required final concentrations of 500ppm, 1000ppm and 2000ppm. Twenty ml of PDA poisoned with the plant extract was poured into sterilized Petri plates under aseptic conditions (in laminar air flow) and allowed to solidify. For each treatment combination PDA plates containing the plant extracts were inoculated aseptically in the centre with 5 mm mycelia discs of young culture



**Plate 2: Preparation of the Plant extracts**

A) Plants kept to dry; B) Ground plant powder; C) Plant powder dissolved in various solvents; D) Flasks in shaker incubator; E) Suspension being filtered; F) Extract left after solvent evaporation; G) Reconstituted product

(7days old) of *Alternaria mali*. Control plates without any plant extracts were also maintained. The experiment was laid out in a completely randomized design (CRD) with three replications in each treatment. In addition, 0.15% (1500ppm) mancozeb was also used as a check. The inoculated Petri plates were incubated at 25±1°C in an incubator. The colony diameters were measured after 5 days.

The fungitoxicity of the extract in terms of per cent inhibition of the mycelial growth was calculated using the formula given by Vincent (1947)

$$I = \frac{C - T}{C} \times 100$$

Where,

I= Percent inhibition;

C= Colony diameter in control plate

T= Colony diameter in treated plate

### **3.5.6.2 Determination of spore germination inhibition**

For determination of per cent spore germination inhibition, slide germination technique was used (Mohana and Raveesha, 2007). Desired volume of the plant extracts from the stock solution were taken and required concentrations of 500ppm, 1000ppm and 2000ppm were prepared. Simultaneously a spore suspension containing about 10-20 spores per microscopic field at low power was prepared from young culture of the pathogen. From the prepared final concentration of the plant extracts one drop of the same was taken with a dropper and placed in the cavity slides and allowed to dry. After drying a drop of spore suspension was placed on that particular cavity slide. In addition, a control was also maintained. This arrangement was placed in a larger Petri dish of 90mm containing moistened blotting paper at the base. It was then placed and incubated overnight at 25±1°C. The experiment was laid out in a completely randomized design (CRD) with three replications in each treatment. The observations on germination of spores were recorded the next day by placing the

cavity slides under microscope at 100X resolution. Per cent germination inhibition for each plant extract was calculated using the formula given by Vincent (1947) as:

$$I = \frac{C - T}{C} \times 100$$

Where,

I= Percent spore germination inhibition

C= Number of spores germinated in control

T= Number of spores germinated in treated

### **3.6 Identification of the bioactive compounds**

The most effective plant extract that was obtained was subjected to high resolution Liquid Chromatography-cum-Mass Spectrometry (HR-LCMS/MS) for identification of the various secondary metabolites present in the plant extract. This experiment was outsourced and was done at The Indian Institute of Technology, Bombay.

### **3.7 Binding affinity calculation through CB-Dock**

In order to elucidate the binding affinity of selected molecules towards the enzyme lanosterol 14-alpha demethylase, which is the key enzyme in sterol biosynthesis in fungi, molecular docking approach was employed. Crystal structure coordinates of lanosterol 14-alpha demethylase of *Candida albicans* was fetched from Protein Data Bank possessing PDB ID: 5V5Z (Keniya *et al.*, 2018). Maestro software was used for checking missing residues in the structure. Junk items were removed from the structure using Chimera software (Pettersen *et al.*, 2004). Coordinates of ligands were retrieved from PubChem and were subsequently docked independently against lanosterol 14-alpha demethylase. Docking was done using the AutoDock Vina based CB-Dock (Liu *et al.*, 2020). This web based server predicts five active sites as a default and performs docking using autodock vina (Trott and Olson, 2010). The correct pose of the ligand and

correct active site was confirmed using the information of co-crystallized ligand. The Vina Score indicating the binding affinity was extracted for the most appropriate pose from the output file (Labbé *et al.*, 2015). More negative the value of Vina Score more is the affinity of ligand towards the lanosterol 14-alpha demethylase (Trott and Olson, 2010).

## Chapter-4

### EXPERIMENTAL FINDINGS

The findings of the present investigation on “Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources” are presented as under.

#### 4.1 Isolation and Identification of the pathogen

Isolation of the pathogen was done from apple leaves showing typical *Alternaria* symptoms by Tissue-bit method following standard pathological techniques. The culture of the fungus obtained on the PDA media had a velvety light olivaceous growth. The cultural characteristics of the fungus such as colony colour, colony texture and radial growth recorded on the seventh day of inoculation revealed light to dark olivaceous colony colour with greenish or brownish tinge, velvety in appearance and 45mm in diameter after 7 days of inoculation (Plate-3). Morphological characters like mycelium color, septation of conidia and conidiophore studied using microscopy revealed pale brown conidia with obclavate shape having both transverse and longitudinal septation with a short beak (Plate-3).

#### 4.2 Pathogenicity test

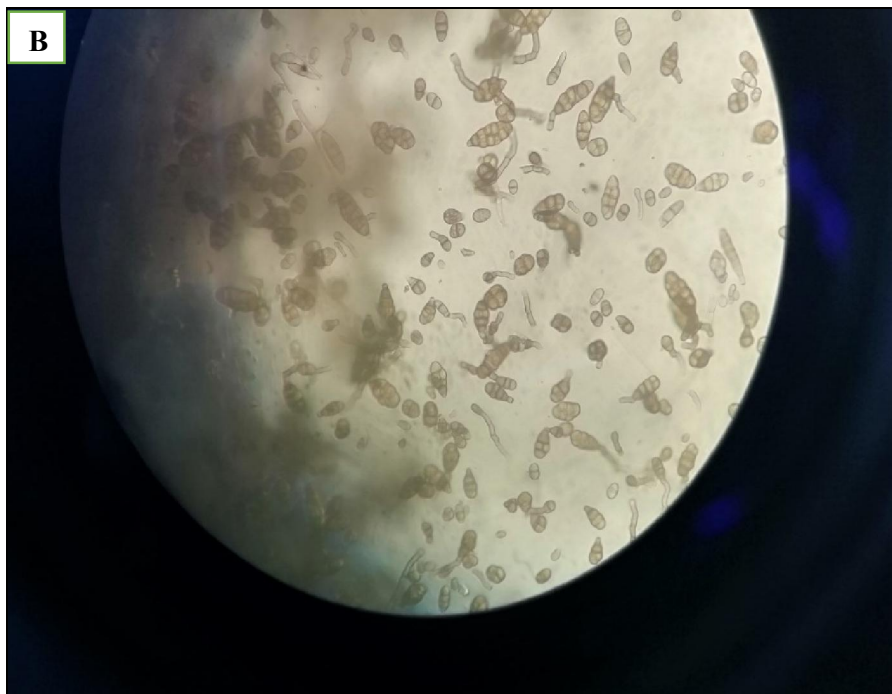
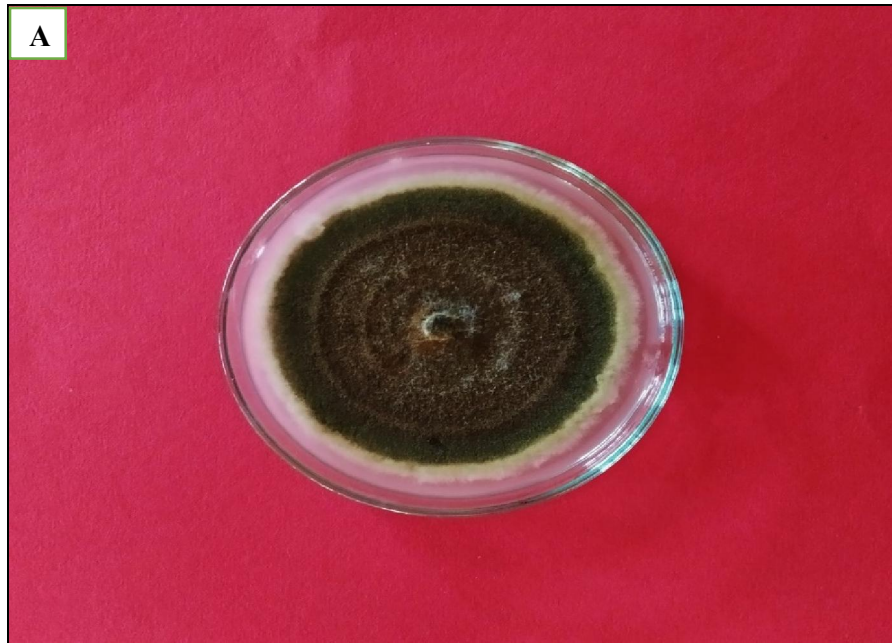
The pathogenicity of *A. mali* (Roberts) was established on wounded detached leaves of apple cv. “Red Delicious”. The characteristic symptoms of disease appeared after 72 hours of the inoculation (Plate-4).

#### 4.3 Antifungal activity assay of the plant extracts

##### 4.3.1 *In vitro* evaluation of the effect of plant extracts on the radial growth of *Alternaria mali*

##### 4.3.1.1 Effect of the Aqueous Plant extracts on mycelial growth of *Alternaria mali*

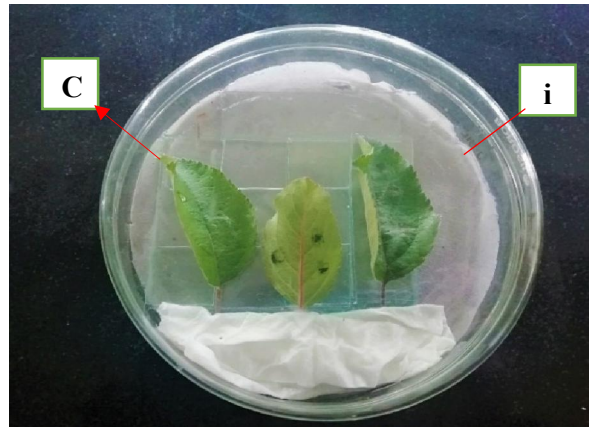
Antifungal activity of seven aqueous plant extracts was tested *in vitro* by Poisoned Food Technique on the mycelial growth of *Alternaria mali*. The results presented in Table 4.1 revealed that the inhibition of mycelial growth of



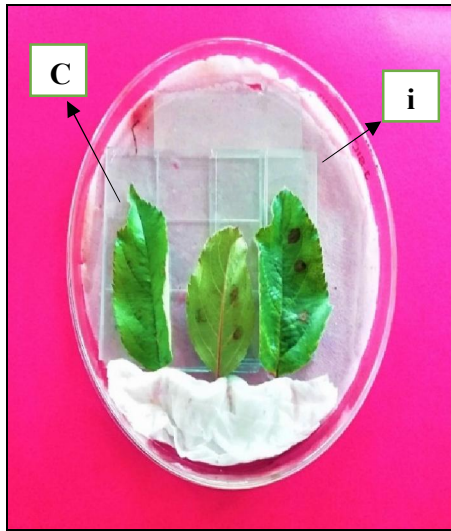
**Plate 3: Cultural and Morphological characteristics of *Alternaria mali***

A) Velvety olivaceous colony(10 days old culture)

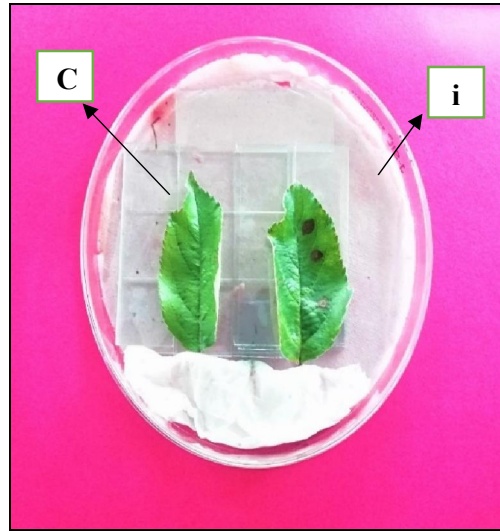
B) Septate conidia with a short beak



A



B



D

**Plate 4: Pathogenicity test (Detached leaf method)**

A) Inoculated leaves plus control; B & D) *Alternaria* symptoms visible on inoculated leaves

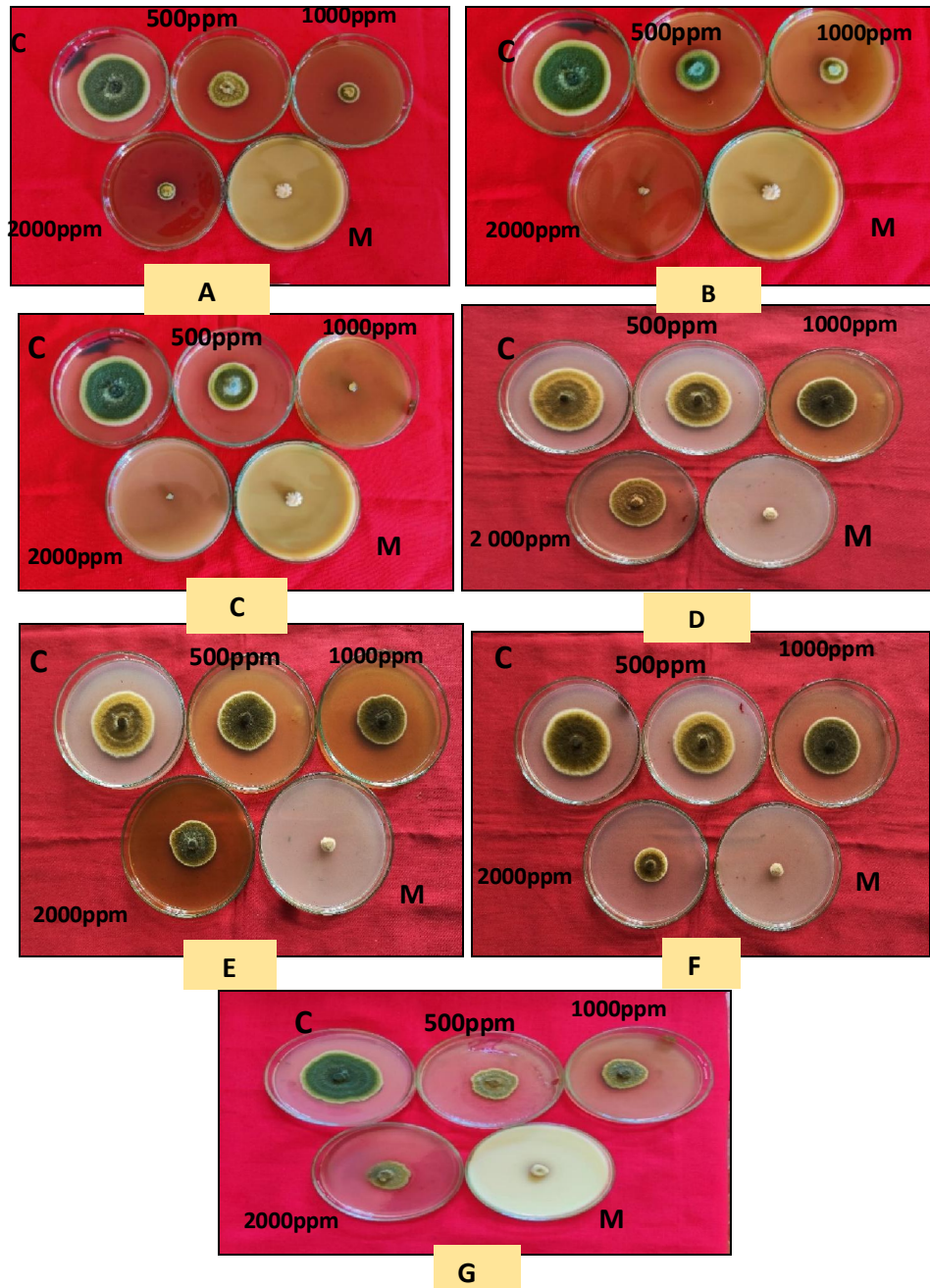
C) Control ; i) Inoculated leaves

*Alternaria mali* by various aqueous plant extracts ranged from 6.2 to 63.9 per cent. Among the plant extracts tested maximum inhibition of mycelial growth (63.9%) over control was observed in case of clove bud extract followed by an inhibition of 41.7 per cent for mint leaf extract, ginger rhizome extract (36.2%), artemisia whole plant extract (18.2%), datura leaf extract (17%). The least inhibition (6.2%) was exhibited by turmeric rhizome extract (Plate-6) followed by garlic (clove) extract (13.7%). It was further observed that the effect of the three concentrations (500ppm,1000ppm and 2000ppm) on mycelial inhibition of *Alternaria mali* varied significantly i-e. with the increase in concentration from 500ppm to 2000ppm there was an increase in the inhibition of mycelial growth of pathogen as well (Fig.1). The mean overall inhibition observed for the three concentrations (500ppm, 1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively (Table 4.1). Further aqueous extract of clove proved significantly most effective at 2000ppm concentration with an inhibition of 73 per cent (Plate-5, Fig.1).This obtained result was almost comparable to the inhibition (73.3%) observed for the fungicide Mancozeb @ 1500ppm used as a check in this study.

#### **4.3.1.2 Effect of the Ethanolic Plant extracts on mycelial growth of *Alternaria mali***

The effect of ethanolic plant extracts on the radial growth of *Alternaria mali* was significant (Table 4.1). Among the seven Ethanolic plant extracts tested, clove extract proved to be most effective with a mycelial inhibition of 69.2 per cent. This was followed by turmeric rhizome extract (49.4%), ginger rhizome extract (32.6%), mint leaf extract (32.3%) and garlic(clove) extract (28.8%) (Plate-6). The least inhibition was observed in case of artemisia whole plant extract (17%) followed by datura leaf extract (18.7%) (Plate-6). The data further reveals that clove ethanol extract proved significantly most effective at 2000ppm concentration with a mycelial inhibition of 100 per cent which was greater than inhibition (73.3%) observed for the fungicide Mancozeb @1500ppm used as a

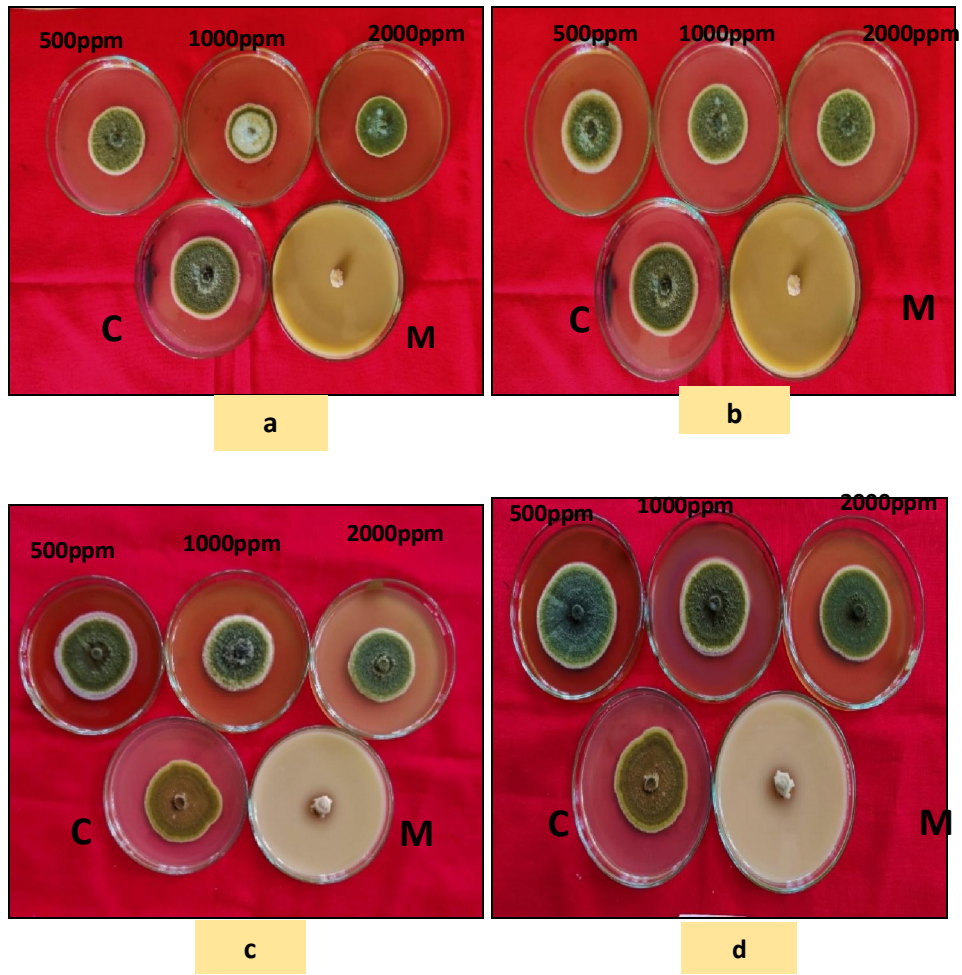
check in this study (Plate-5, Fig.2). Furthermore, the mycelial inhibition showed an increasing trend with the increasing concentration of the test plant extracts (Fig.2). The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively (Table 4.1).



**Plate 5: Response of *Alternaria mali* to various plant extracts**

C: Control M: Mancozeb

A: clove aqueous extract; B: clove ethanol extract; C: clove methanol extract D: garlic aqueous extract; E: garlic methanol extract; F: ginger methanol extract; G: mint methanol extract



**Plate 6: Response of *Alternaria mali* to various plant extracts**

C : Control M : Mancozeb

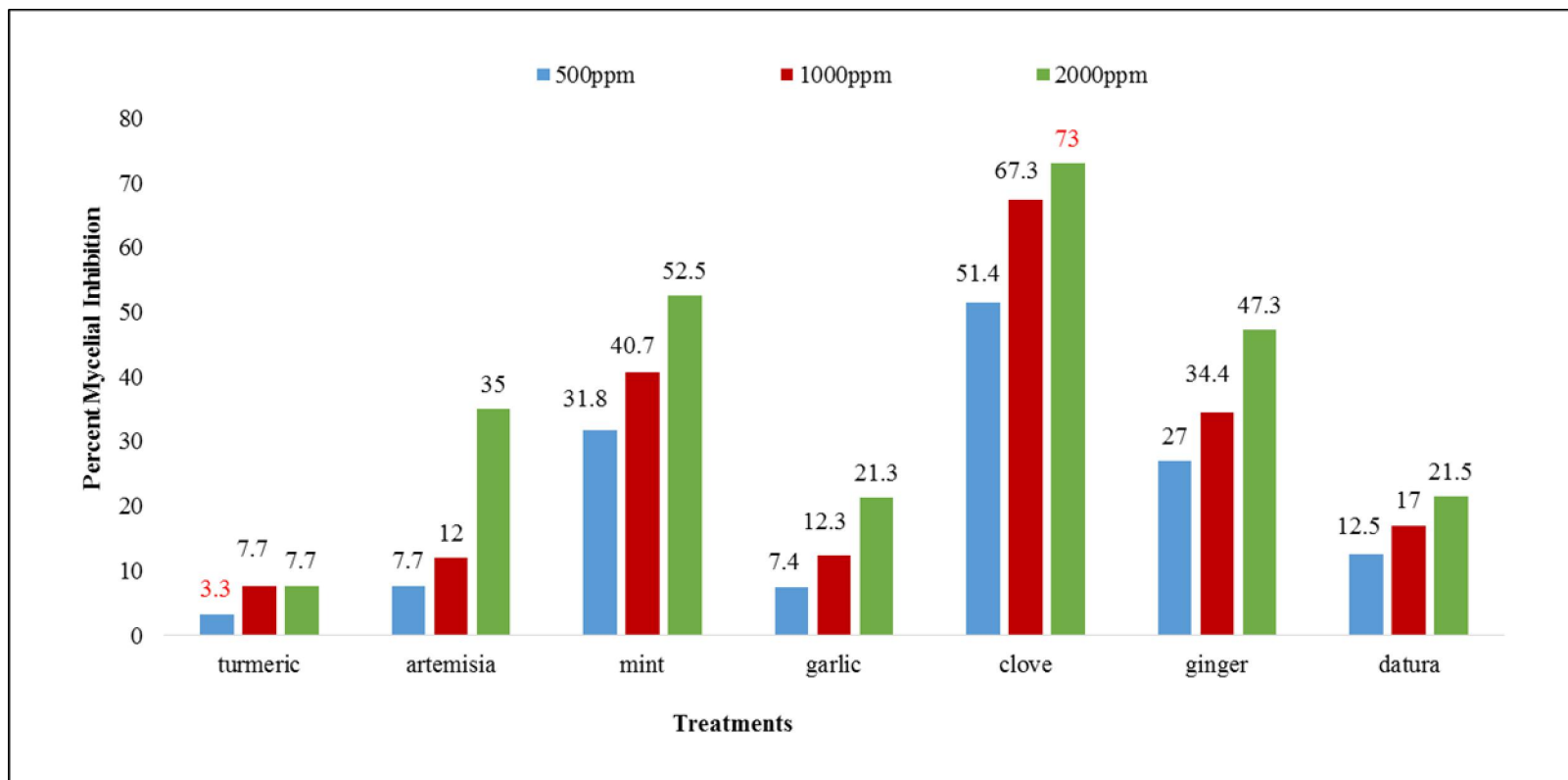
a: garlic ethanol extract; b: artemisia ethanol extract

c: datura ethanol extract; d: turmeric aqueous extract

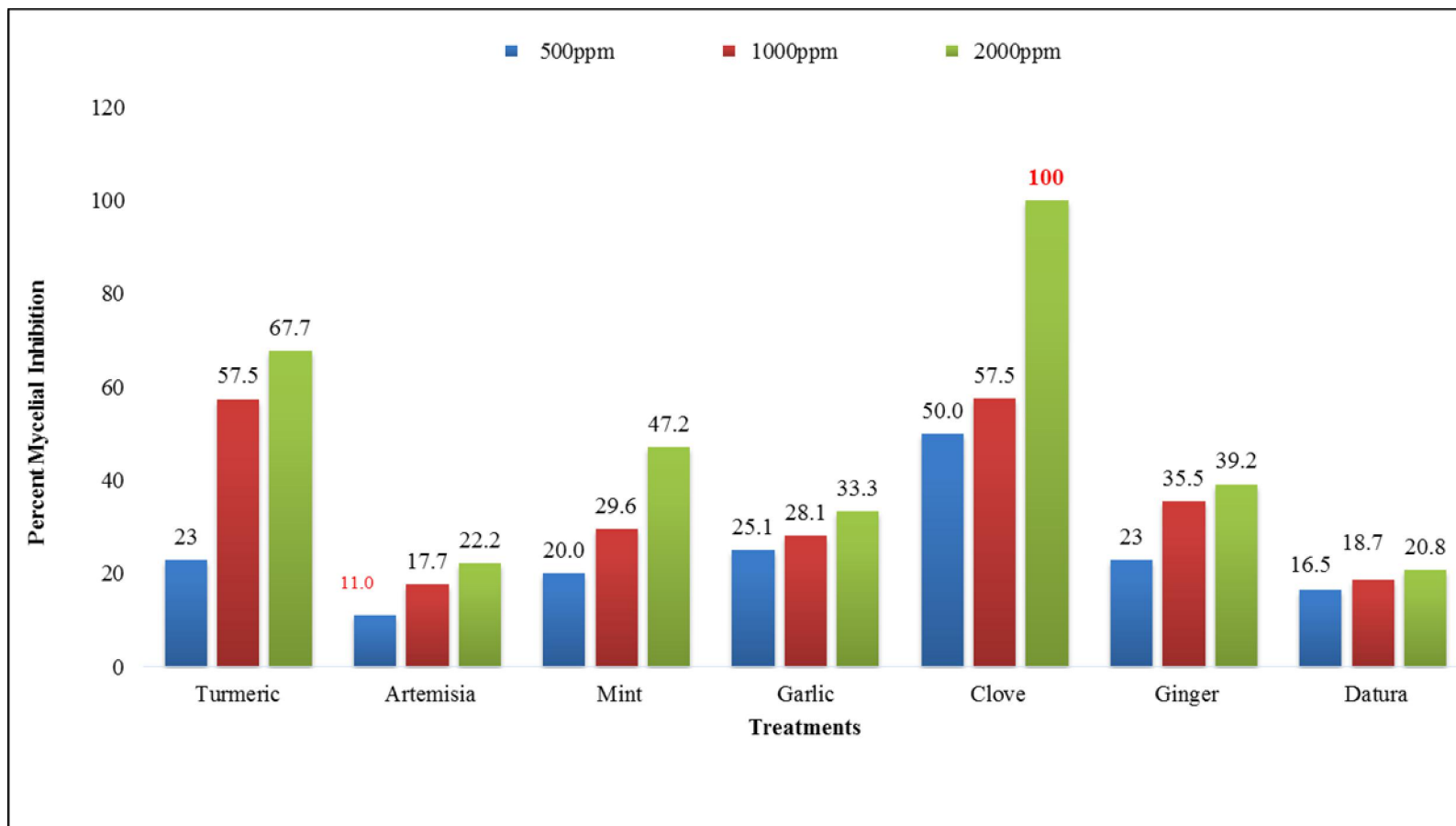
**Table 4.1: Mycelial growth inhibition of *Alternaria mali* by different concentrations of test plant extracts**

Methods Treatments	Aqueous extraction			Sub Mean	Ethanol extraction			Sub Mean	Methanol extraction			Sub Mean	Factor Means	
	500 ppm	1000 ppm	2000 ppm		500 ppm	1000 ppm	2000 ppm		500 ppm	1000 ppm	2000 ppm		Treatment	Conc.
Turmeric	3.3 *(10.5)	7.7 (16.1)	7.7 (16.1)	6.2 (14.2)	23 (28.7)	57.5 (49.3)	67.7 (55.4)	49.4 (44.4)	1.1 (6.0)	26.4 (30.9)	34 (35.7)	20.5 (24.2)	<b>25.4</b> <b>(27.6<sup>d</sup>)</b>	<b>21.3</b> <b>(26<sup>c</sup>)</b>
Artemisia	7.7 (16.1)	12 (20.3)	35 (36.3)	18.2 (24.2)	11.0 (19.1)	17.7 (24.9)	22.2 (28.1)	17.0 (24.0)	19.5 (26.2)	24.4 (29.4)	25.2 (30.0)	23.0 (28.5)	<b>19.4</b> <b>(25.6<sup>c</sup>)</b>	<b>33.2</b> <b>(35.2<sup>b</sup>)</b>
Mint	31.8 (34.3)	40.7 (39.7)	52.5 (46.4)	41.7 (40.1)	20.0 (26.6)	29.6 (32.9)	47.2 (43.4)	32.3 (34.3)	37.8 (37.9)	43.5 (41.3)	47.2 (43.4)	42.8 (40.9)	<b>38.9</b> <b>(38.4<sup>b</sup>)</b>	<b>43.5</b> <b>(42.4<sup>a</sup>)</b>
Garlic	7.4 (15.7)	12.3 (20.5)	21.3 (27.5)	13.7 (21.3)	25.1 (30.1)	28.1 (32.0)	33.3 (35.2)	28.8 (32.4)	10.5 (18.9)	26 (30.7)	33.3 (35.3)	23.3 (28.3)	<b>21.9</b> <b>(27.3<sup>d</sup>)</b>	
Clove	51.4 (45.8)	67.3 (55.1)	73 (58.7)	63.9 (53.2)	50.0 (45.0)	57.5 (49.4)	100.0 (90.0)	69.2 (61.5)	49.5 (44.7)	100 (90)	100 (90)	83.2 (74.9)	<b>72.1</b> <b>(63.2<sup>a</sup>)</b>	
Ginger	27.0 (31.3)	34.4 (35.9)	47.3 (43.4)	36.2 (36.9)	23 (28.7)	35.5 (36.6)	39.2 (38.8)	32.6 (34.7)	8.9 (17.4)	17.0 (24.4)	51 (45.6)	25.6 (29.1)	<b>31.5</b> <b>(33.5<sup>c</sup>)</b>	
Datura	12.5 (20.7)	17.0 (24.4)	21.5 (27.6)	17.0 (24.2)	16.5 (24.0)	18.7 (25.6)	20.8 (27.1)	18.7 (25.6)	10 (18.4)	24.6 (29.7)	33.3 (35.3)	22.6 (27.8)	<b>19.4</b> <b>(25.9<sup>c</sup>)</b>	
Mean	20.2 (24.9)	27.4 (30.3)	36.9 (36.6)	<b>28.1</b> <b>(30.6<sup>b</sup>)</b>	24.1 (28.9)	34.9 (35.8)	47.2 (45.4)	<b>35.4</b> <b>(36.7<sup>a</sup>)</b>	19.6 (24.2)	37.4 (39.5)	46.3 (45.0)	<b>34.4</b> <b>(36.2<sup>a</sup>)</b>		
<b>CD (p&lt;0.05)</b>	Method (M) = 0.61 Concentration (C) = 0.61 Treatment (T) = 0.94 Method*Concentration (M*C) = 1.07 Method*Treatment (M*T) = 1.63 Concentration*Treatment (C*T) = 1.63 Method*Concentration*Treatment (M*C*T) = 2.83													

\*Values in parenthesis are arcsine transformed values and those superscribed with identical letters do not differ significantly.



**Fig. 1:** Effect of different concentrations of Aqueous plant extracts on radial growth of *Alternaria mali*



**Fig. 2: Effect of different concentrations of Ethanolic plant extracts on radial growth of *Alternaria mali***

#### **4.3.1.3 Effect of the Methanolic Plant extracts on mycelial growth of *Alternaria mali***

Perusal of the data revealed that the methanolic plant extracts had significant effect on mycelial growth of *Alternaria mali* (Table 4.1). Among all the plant extracts clove extract exhibited maximum mycelial inhibition of 83.2 per cent over control. This was followed by mint leaf extract (42.8%), ginger rhizome extract (25.6%) (Plate-5), garlic (clove) extract (23.3%) and artemisia whole plant extract (23%). The least inhibition of 20.5 per cent was exhibited by turmeric rhizome extract followed by datura leaf extract (22.6%). The data further reveals an increase in the responsiveness of the pathogen to extracts with an increase in the concentration from 500ppm to 2000ppm (Fig.3). The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively. Furthermore clove methanol extract proved significantly most effective with a mycelial inhibition of 100 per cent observed at both 1000ppm and 2000ppm concentration which was greater than the inhibition (73.3%) observed for fungicide Mancozeb at concentration 1500ppm(Plate-5, Fig.3).

#### **4.3.1.4 Effect of the different solvents on antifungal activity of plant extracts**

Significant differences were observed between Aqueous extracts and Ethanol and Methanol extracts regarding inhibition of mycelial growth. In this study Ethanol and Methanol extracts of the test plants showed better overall results in comparison to the Aqueous extracts. The aqueous extracts exhibited a mean overall inhibition of 28.1 per cent while methanol and ethanol extracts showed comparable results with a mean overall inhibition of 34.4 and 35.4 per cent, respectively (Table 4.1, Fig.4).

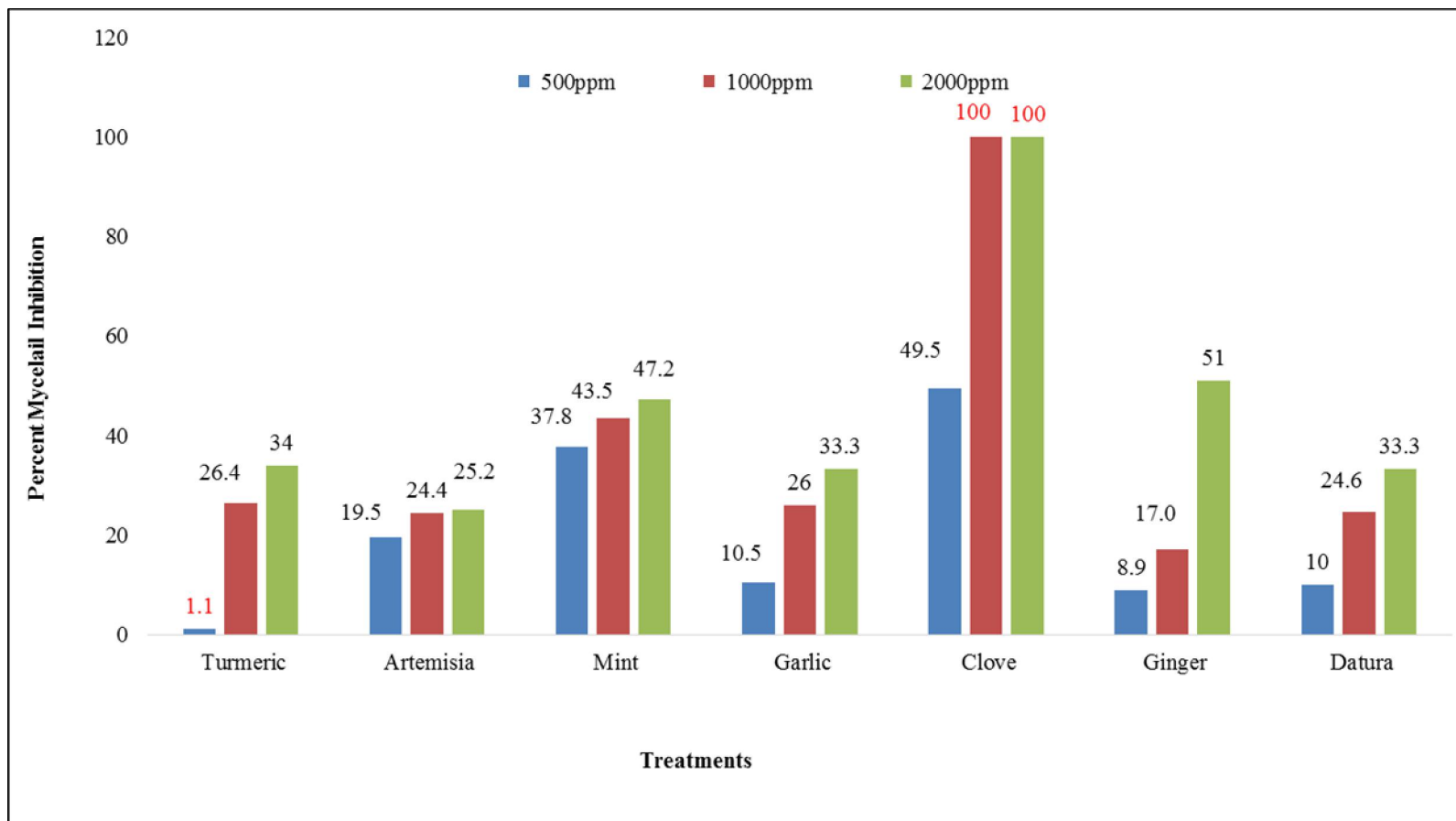
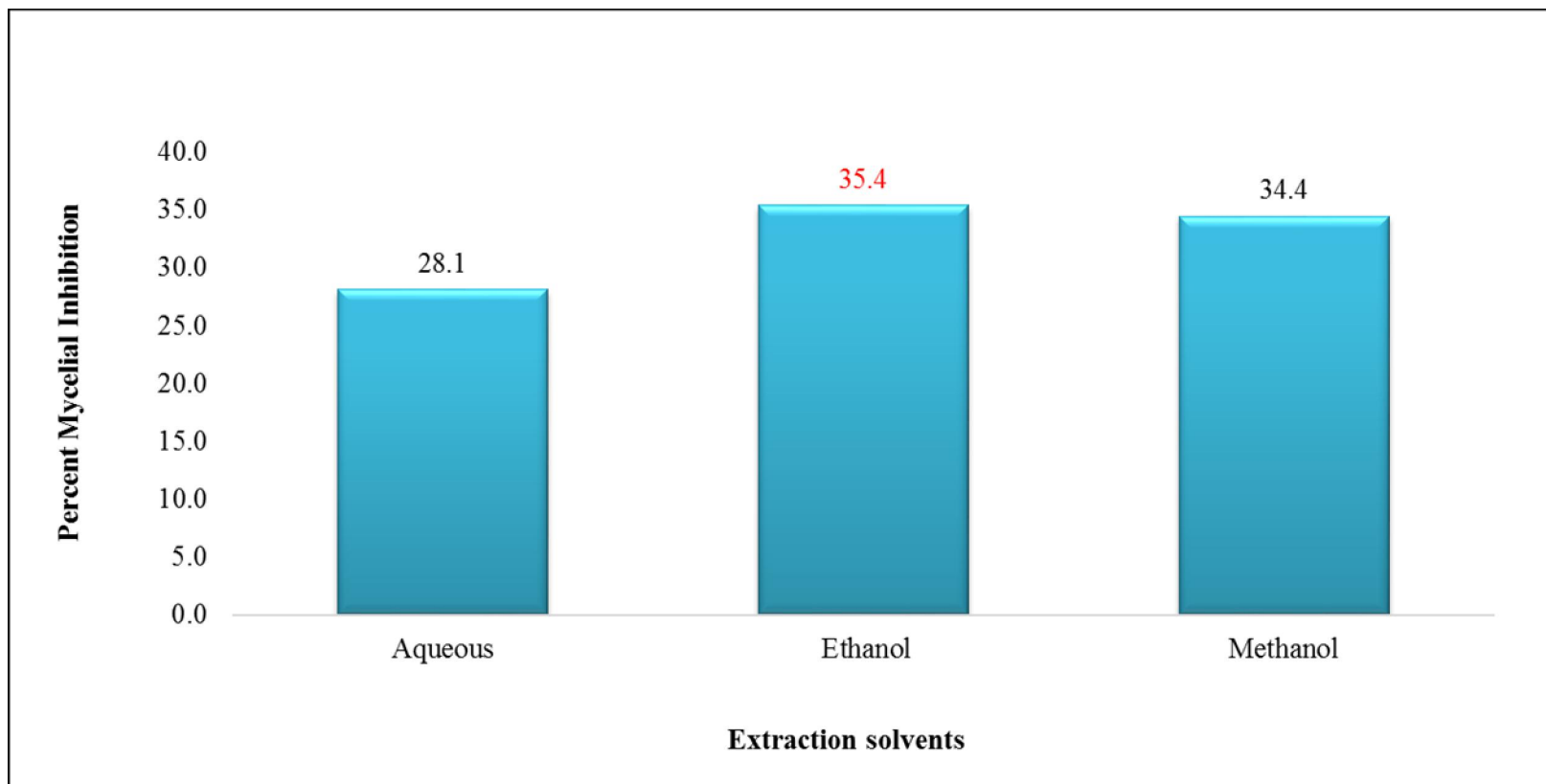


Fig. 3: Effect of different concentrations of Methanolic plant extracts on radial growth of *Alternaria mali*



**Fig. 4:** Effect of different extraction solvents of plant extracts on mycelial growth of *Alternaria mali*

#### **4.3.2 *In vitro* evaluation of the effect of plant extracts on spore germination of *Alternaria mali***

##### **4.3.2.1 Effect of the Aqueous Plant extracts on spore germination of *Alternaria mali***

The aqueous plant extracts showed significant effect on spore germination of *Alternaria mali* (Table 4.2). The results revealed that among the seven aqueous plant extracts tested, maximum conidial germination inhibition of 42.7 per cent was exhibited by ginger rhizome extract followed by clove extract (41.7%), turmeric rhizome extract (33.8%), artemisia whole plant extract (31.5%) and mint leaf extract (31.3%). Garlic clove extract proved to be least effective with an average of only 9 per cent inhibition in spore germination followed by an inhibition of 10.6 per cent in case of datura leaf extract. It was further observed that the effect of the three concentrations (500ppm, 1000ppm and 2000ppm) on conidial germination of *Alternaria mali* varied significantly i-e., with the increase in concentration from 500ppm to 2000ppm there was an increase in the inhibition of germination of pathogen as well (Fig.5). The mean overall inhibition observed for the three concentrations (500ppm, 1000ppm and 2000ppm) was 33.6, 41.5 and 51.1 per cent respectively (Table 4.2).

##### **4.3.2.2 Effect of the Ethanolic Plant extracts on spore germination of *Alternaria mali***

All the ethanolic plant extracts inhibited spore germination in *Alternaria mali* (Table 4.2). Among the seven ethanolic plant extracts tested, highly significant per cent inhibition of spore germination was observed in case of clove bud extract (77.3%). This was followed by ginger rhizome extract (47.6%), garlic clove extract (44.4%), mint leaf extract (35.8%) and artemisia whole plant extract (33.9%). Minimum inhibition of conidial germination was however, provided by turmeric rhizome extract (18.9%). Furthermore, germination inhibition showed an increasing trend with the increasing concentration of the test plant extracts (Fig.6).

The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 33.6, 41.5 and 51.1 per cent respectively (Table 4.2). The data further revealed that clove ethanolic extract proved significantly most effective at 2000ppm concentration with 85 per cent conidial germination inhibition (Fig.6).

#### **4.3.2.3 Effect of the Methanolic Plant extracts on spore germination of *Alternaria mali***

The results of the anti-fungal screening of the methanol extracts of the seven test plants are presented in Table- 4.2. Perusal of the data revealed that of the seven methanol plant extracts maximum conidial germination inhibition was exhibited by ginger rhizome extract (82.2%) followed by clove bud extract and turmeric rhizome extract with 76.3 and 71.5 per cent inhibition, respectively. This was followed by artemisia whole plant extract (54.5%), mint leaf extract (45.4%) and garlic clove extract (41.8%). The minimum inhibition of conidial germination was observed in case of datura leaf extract (30.4%).

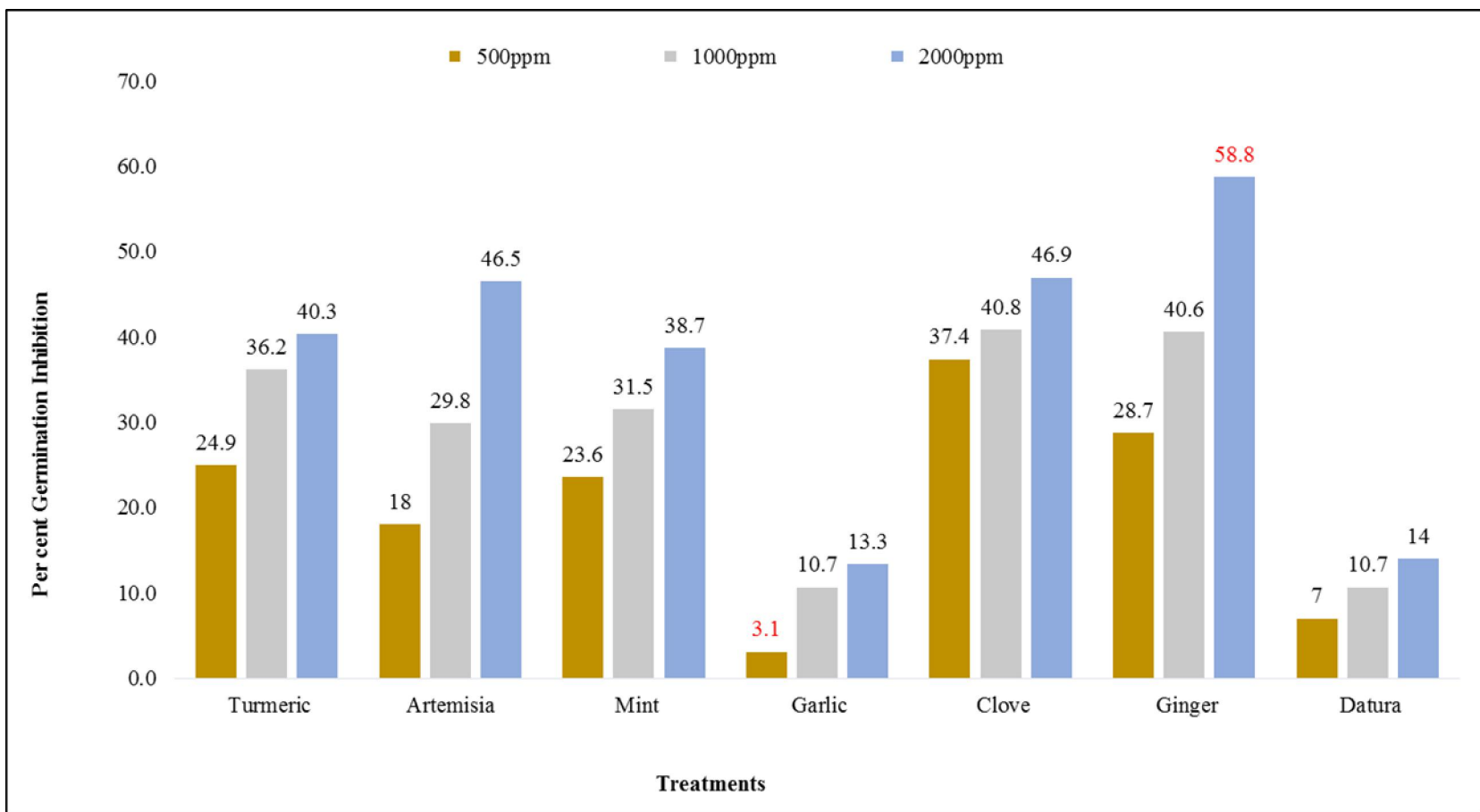
#### **4.3.2.4 Effect of different extraction solvents of plant extracts on spore germination of *Alternaria mali***

Significant differences were observed between aqueous, ethanol and methanol extracts of the test plants regarding inhibition of the conidial germination. The methanolic extracts showed the highest efficacy with a mean overall spore germination inhibition of 57.4 per cent. Also the mean spore germination inhibition in case of the ethanolic extracts was 40.1 per cent while the aqueous extracts exhibited the least effect with an inhibition of 28.6 per cent (Table 4.2, Fig.8).

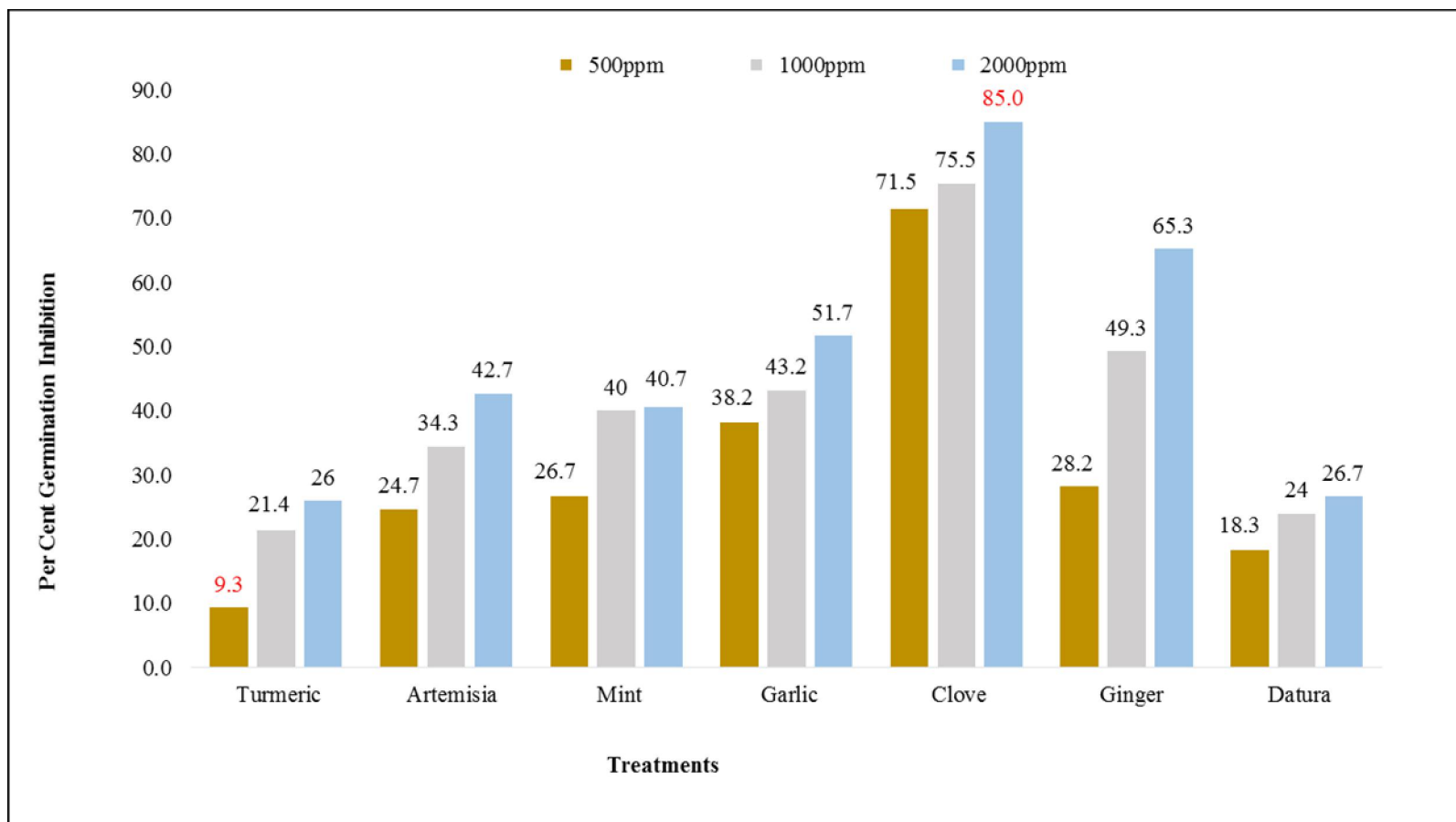
**Table 4.2: Spore germination inhibition of *Alternaria mali* by different concentrations of test plant extracts**

Methods Treatments	Aqueous extraction			Sub Mean	Ethanol extraction			Sub Mean	Methanol extraction			Sub Mean	Factor Means	
	500 ppm	1000 ppm	2000 ppm		500 ppm	1000 ppm	2000 ppm		500 ppm	1000 ppm	2000 ppm		Treatment	Conc.
Turmeric	24.9 *(30.0)	36.2 (37.0)	40.3 (39.4)	33.8 (35.4)	9.3 (17.8)	21.4 (27.6)	26 (30.6)	18.9 (25.3)	61.8 (51.8)	72.3 (58.2)	80.5 (63.8)	71.5 (58.0)	<b>41.4</b> <b>(39.6<sup>c</sup>)</b>	<b>33.6</b> <b>(34.5<sup>c</sup>)</b>
Artemisia	18 (25.1)	29.8 (33.1)	46.5 (43.0)	31.5 (33.7)	24.7 (29.8)	34.3 (35.9)	42.7 (40.8)	33.9 (35.5)	37.2 (37.5)	45.2 (42.2)	81.2 (64.4)	54.5 (48)	<b>40.0</b> <b>(39.1<sup>c</sup>)</b>	<b>41.5</b> <b>(39.8<sup>b</sup>)</b>
Mint	23.6 (29.0)	31.5 (34.2)	38.7 (38.4)	31.3 (33.9)	26.7 (31.1)	40 (39.2)	40.7 (39.6)	35.8 (36.6)	36 (36.9)	44 (41.6)	56.3 (48.6)	45.4 (42.3)	<b>37.5</b> <b>(37.6<sup>d</sup>)</b>	<b>51.1</b> <b>(45.9<sup>a</sup>)</b>
Garlic	3.1 (9.9)	10.7 (19.1)	13.3 (21.3)	9.0 (16.8)	38.2 (38.1)	43.2 (41.1)	51.7 (46.0)	44.4 (41.7)	39.6 (39.0)	40 (39.2)	45.7 (42.6)	41.8 (40.2)	<b>31.7</b> <b>(32.9<sup>c</sup>)</b>	
Clove	37.4 (37.7)	40.8 (39.7)	46.9 (43.2)	41.7 (40.2)	71.5 (57.7)	75.5 (60.3)	85.0 (67.4)	77.3 (61.8)	68.7 (56.0)	70.8 (57.3)	89.3 (70.9)	76.3 (61.4)	<b>65.1</b> <b>(54.5<sup>a</sup>)</b>	
Ginger	28.7 (32.3)	40.6 (39.6)	58.8 (50.1)	42.7 (40.6)	28.2 (32.0)	49.3 (44.6)	65.3 (53.9)	47.6 (43.5)	78.8 (62.6)	80.6 (63.9)	87.1 (69.0)	82.2 (65.2)	<b>57.5</b> <b>(49.8<sup>b</sup>)</b>	
Datura	7 (15.3)	10.7 (19.1)	14 (21.9)	10.6 (18.8)	18.3 (25.3)	24 (29.3)	26.7 (31.1)	23 (28.6)	24 (29.3)	31.3 (34.0)	36 (36.9)	30.4 (33.4)	<b>21.3</b> <b>(26.9<sup>f</sup>)</b>	
Mean	20.4 (25.6)	28.6 (31.7)	36.9 (36.8)	<b>28.6</b> <b>(31.3<sup>c</sup>)</b>	31.0 (33.1)	41.1 (39.7)	48.3 (44.2)	<b>40.1</b> <b>(39.0<sup>b</sup>)</b>	49.4 (44.7)	54.9 (48.1)	68.0 (56.6)	<b>57.4</b> <b>(49.8<sup>a</sup>)</b>		
<b>CD (p&lt;0.05)</b>	Method (M) = 0.74 Concentration (C) = 0.74 Treatment (T) = 1.14 Method*Concentration (M*C) = 1.29 Method*Treatment (M*T) = 1.98 Concentration*Treatment (C*T) = 1.98 Method*Concentration*Treatment (M*C*T) = 3.43													

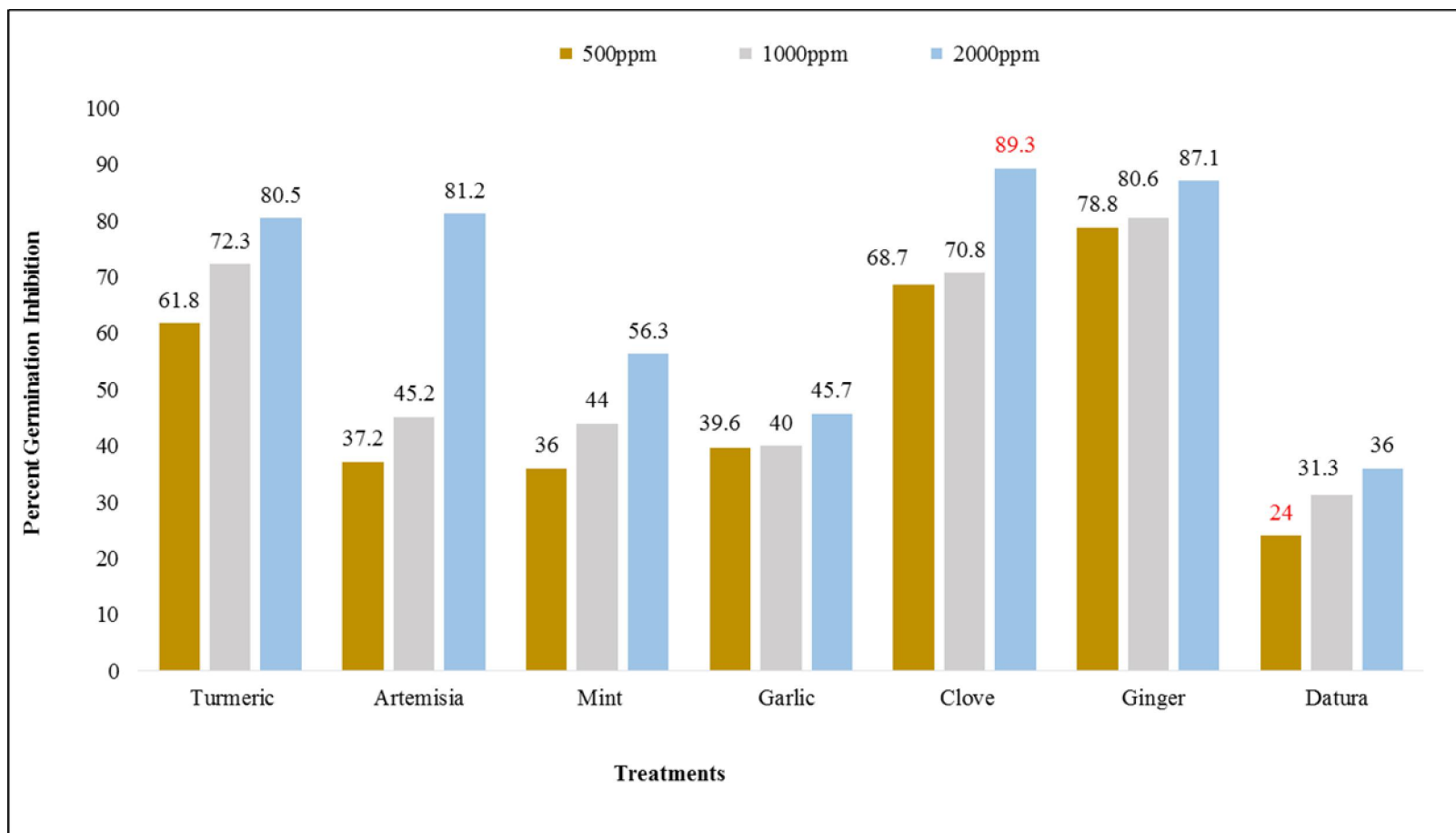
\*Values in parenthesis are arcsine transformed values and those superscribed with identical letters do not differ significantly.



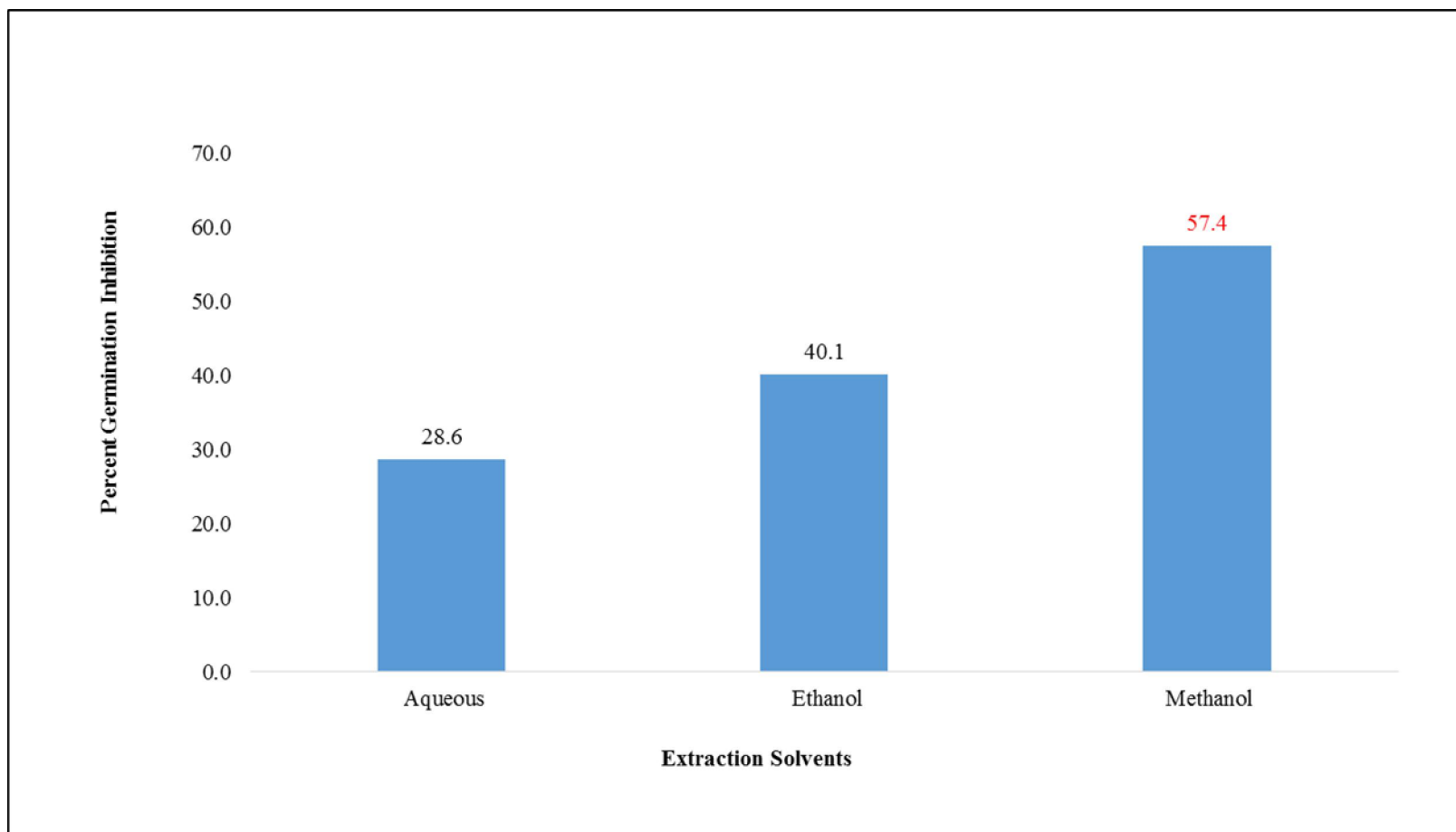
**Fig. 5:** Effect of different concentrations of Aqueous plant extracts on spore germination of *Alternaria mali*



**Fig. 6: Effect of different concentrations of Ethanolic plant extracts on spore germination of *Alternaria mali***



**Fig. 7: Effect of different concentrations of Methanolic plant extracts on spore germination of *Alternaria mali***



**Fig. 8:** Effect of different extraction solvents of plant extracts on spore germination of *Alternaria mali*

#### 4.4 Phyto-chemical profiling of the effective botanical

The results obtained from the anti-fungal bio-assay of the selected plant extracts in this study revealed that methanolic extract of clove proved to be most effective among all for inhibiting both the mycelial growth of *Alternaria mali in vitro* as well as the conidial germination. It exhibited highest efficacy at concentrations 2000ppm and 1000ppm resulting in 100 per cent inhibition of mycelium over control. This was significantly higher than mycelial inhibition of 73.3 per cent exhibited by fungicide Mancozeb @ 1500ppm. Therefore, based on the results of the *in vitro* antifungal assay for clove methanol extract, it clearly suggested the presence of certain phytochemical compounds present in the said extract possessing some anti-microbial properties. For identification of such compounds the extract was subjected to Liquid Chromatography cum Mass Spectroscopy. The experiment was outsourced and conducted at Indian Institute of Technology, Bombay. The phyto-chemical screening of the clove methanol extract revealed the existence of alkaloids, flavonoids, terpenoids, tannins, aldehydes, ketones, glycoside, steroids, carbohydrates and phenolic compounds (Table-4.3). A large number of compounds identified were either phenolic compounds and flavonoids. The phyto-constituents found included poly-phenols such as Eugenol, Gallic acid, Chlorogenic acid certain flavonoids like Quercetin and also compounds with known antifungal properties like Econazole. Also, certain antibiotic compounds like Cefamandole nafate, Demeclocycline were also found (Fig.9).

**Table 4.3 : List of compounds detected in Methanol extract of Clove**

<b>S No.</b>	<b>Name of the Compound</b>	<b>Class</b>
1	Eugenol	Phenolic compound
2	Quercitin	Flavanoid
3	Chlorogenic acid	Phenolic acid
4	Gallic acid	Poly-phenol
5	Cefamandole nafate	$\beta$ - lactam antibiotic
6	Rhein / Cassic acid	Anthraquinone
7	Ursolic acid	Tri- terpene
8	Econazole	Imidazole
9	Aesculin	Coumarin glucoside
10	Sulfabenzamide	Sulfonamide
11	Lecanoric acid	Poly- phenol
12	Rhamnetin	Flavanoid
13	Ellagic acid	Poly- phenol
14	Demeclocycline	Tetracycline antibiotic
15	Luteoline	Flavanoid
16	Diosmin	Flavone glycoside
17	Cefuroxime axetil	$\beta$ - lactam antibiotic
18	Koparin	Flavanoid
19	Geneticin	Aminoglycoside antibiotic
20	Norstictic acid	Depsidone

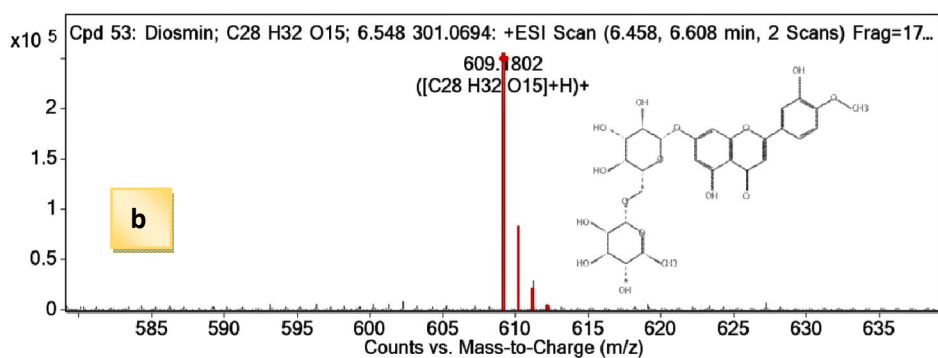
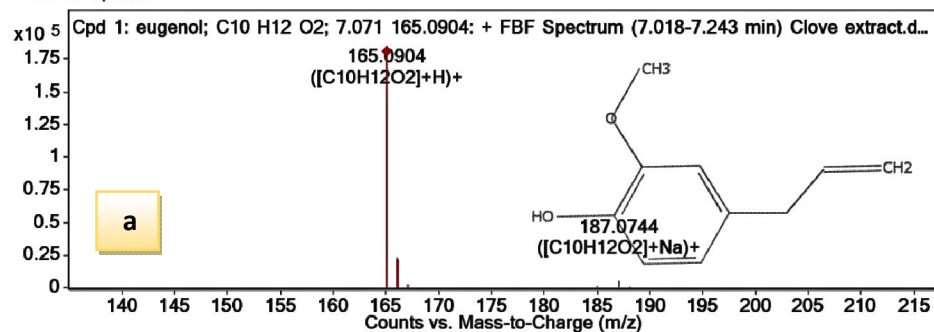
#### **4.5 Binding affinity calculation through CB-Dock**

In order to identify key inhibitor molecules, molecular docking approach was followed as described in chapter 3. Among the 11 molecules (eugenol, quercetin, gallic acid, econazole, ellagic acid, luteolin, demeclocycline, norstictic acid rhein, koparin and diosmin) obtained from LC-MS/MS diosmin, rhein and quercetin proved to be the top 3 in terms of affinity towards the defined enzyme. Diosmin showed the Vina Score (affinity) of -9.6 kcal/mol followed by rhein -9.2 kcal/mol and quercetin -8.7 kcal/mol (Table 4.4). Diosmin is shown in docked state (yellow ball and stick) with lanosterol 14-alpha demethylase (cyan) Fig.9. Co-crystallized ligand is also shown (red ball and stick) in the figure for confirmation of correct determination of active site.

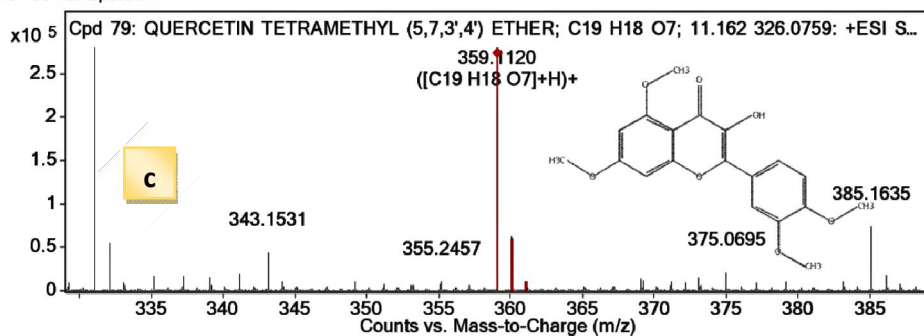
**Table 4.4: Binding affinity values of various molecules against lanosterol 14-alpha demethylase of *Candida albicans***

Name of Ligand	Name of receptor	Binding Affinity (Vina Score) kcal/mol
Eugenol	Lanosterol 14-alpha demethylase	-5.8
Quercetin		-8.7
Gallic acid		-5.6
Econazole		-7.7
Ellagic acid		-7.6
Luteolin		-7.7
Demeclocyclinne		-7.3
Norstictic acid		-8.6
Rhein		-9.2
Koparin		-8.1
Diosmin		-9.6

MS Zoomed Spectrum

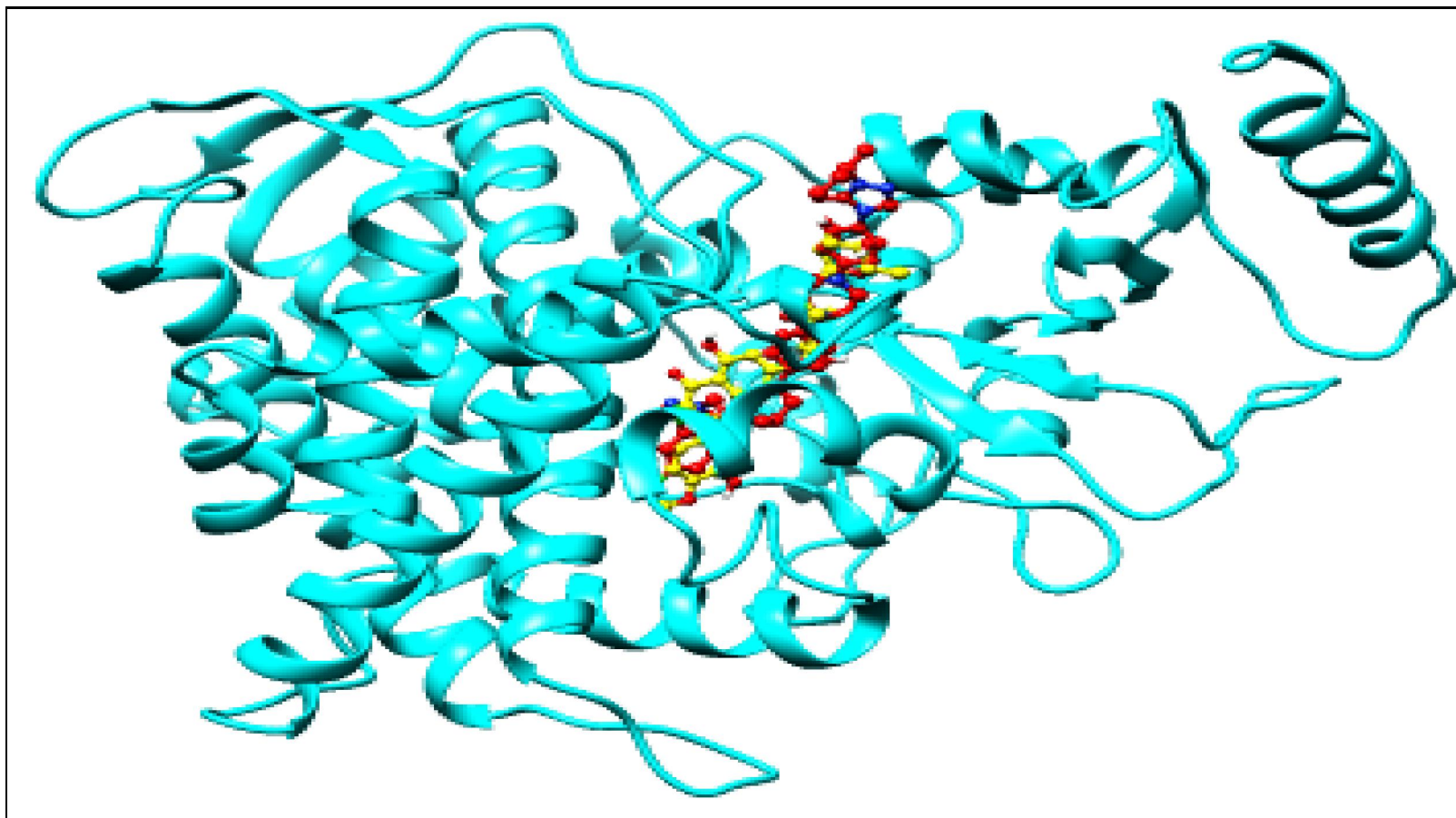


MS Zoomed Spectrum



**Fig. 9: Chromatogram of phytochemical compounds found in clove methanol extract**

a) Eugenol; b) Diosmin; c) Quercetin



**Fig. 10:** Diosmin (yellow) at the active site of lanosterol 14-alpha demethylase (cyan). The co-crystallized ligand (red) has also been shown as reference for correct active site determination

## Chapter-5

### DISCUSSION

*Alternaria* leaf blotch has attained the status of economically important disease affecting the apple crop in many Asian countries including India and Japan (Sawamura *et al.*, 1990). The disease is a threat to the apple industry in Kashmir Valley owing to the severe defoliation resulting from this disease especially in those apple producing regions where susceptible cultivars/strains of Delicious are grown. During the summer of 2013, *Alternaria* leaf blotch severely affected the apple orchards in Kashmir causing severe defoliation and even premature fruit fall resulting into a widespread epidemic affecting almost 70 per cent of the Delicious cultivars (Bhat *et al.*, 2015). Although the disease is managed through chemical fungicides, however, the indiscriminate use could lead to the development of resistance in pathogen. Moreover, in present scenario there has been a shift from the conventional system of disease control involving an excessive use of chemicals to a more organic and sustainable form of disease management. During the last several decades researchers have evaluated plant extracts against various plant pathogens and valuable results have been achieved (Zaker, 2016). Therefore, search for alternatives to chemical fungicides for the management of *Alternaria* leaf blotch of apple has become imperative. The current study was, therefore, conducted to screen out different botanicals for their antifungal efficacy against *Alternaria mali*, causal agent of *Alternaria* leaf blotch of apple, and to figure out the phytochemical compounds in the most effective plant extract. The investigational findings of the present study are briefly discussed as under:

#### 5.1 Isolation and Identification of the pathogen

The cultural characteristics of the fungus recorded on the seventh day of inoculation revealed light to dark olivaceous colony colour with greenish or brownish tinge, velvety in appearance and 45mm in diameter. Morphological

characters studied using microscopy revealed pale brown conidia with obclavate shape having both transverse and longitudinal septation with a short beak. The cultural and morphological characters of isolated fungus are the same as described by Roberts (1924).

## **5.2 Pathogenicity test**

The pathogenicity of *A. mali* (Roberts) was established on wounded detached leaves of apple cv. “Red Delicious”. The characteristic symptoms of disease appeared after 72 hours of the inoculation. Soleimani and Esmailzadeh (2007) also confirmed the pathogenicity of *Alternaria mali* by inoculation with conidial suspension on detached wounded apple leaves.

## **5.3 Antifungal activity assay of the plant extracts**

In our present study seven botanicals were screened *in vitro* for their antifungal efficacy against the pathogen *Alternaria mali*. The investigation involved determination of per cent mycelial inhibition of the fungus exhibited by the plant extract *in vitro* and also the evaluation of inhibition of conidial germination recorded due to each extract.

### **5.3.1 *In vitro* evaluation of the plant extracts on the radial growth of *Alternaria mali***

Extracts of the seven test plants showed varying anti-fungal potential against *Alternaria mali*. The difference in the antimycotic actions of the studied plant extracts could be as a result of solvent used for extraction, the method of extraction, origin and nature of the plants. Further, the efficacy of the plant is also dependent on the responsiveness of the trial phytopathogen and the concentration of the decoctions.

#### **5.3.1.1 Effect of the Aqueous Plant extracts on mycelial growth of *Alternaria mali***

In the present investigation aqueous extract of each of the seven botanicals was tested *in vitro* by Poisoned Food Technique for determination of per cent mycelial inhibition. All the seven botanicals inhibited the mycelial growth of

*Alternaria mali* with per cent inhibition ranging from 6.2 to 63.9 per cent. Among the aqueous extracts tested maximum inhibition of mycelial growth (63.9%) over control was observed in case of Clove bud extract. It proved significantly most effective at 2000ppm concentration with an inhibition of 73 per cent recorded which was almost comparable to the mycelial inhibition (73.3%) exhibited by fungicide Mancozeb, used as a check in the current study. The obtained results for clove bud extract are in agreement with those of Al-Askar and Rashad (2010) who also reported the potent antifungal properties of clove extract against *Rhizoctonia solani* where a 100 per cent inhibition of mycelial growth *in vitro* was recorded at test concentrations of 1, 2,3 and 4 per cent. Reportedly, clove extracts possess antimicrobial and antifungal properties due to the presence of phenolic compounds, such as flavonoids, hydroxybenzoic acids, and hydroxyphenyl propenes (Yassin *et al.*, 2020). The aqueous extract of Mint exhibited an inhibition of 41.7 per cent irrespective of the concentration. Chandel and Sharma (2014) in their study reported inhibition of mycelial growth (31.48%) by aqueous extract of Mint @ 40 per cent against *Rhizoctonia solani*. However, the slight discrepancies may be the variation in the concentrations used and the pathogen tested. Aqueous extract of Ginger rhizome exhibited an inhibition of 36.2 per cent. The results are confirmatory with those reported by Singh *et al.* (2014) stating that the aqueous extract of Ginger caused 30 per cent mycelial growth inhibition against fungus *Fusarium oxysporium*. In the present study, mycelial inhibition (13.7%) was obtained in case of aqueous extract of Garlic clove. Kumar and Singh (2017) in their study reported Aqueous extract of Garlic quite effective against *Alternaria solani*. Aqueous extract of Turmeric rhizome showed least efficacy among the aqueous extracts of different plants tested with an inhibition of 6.2 per cent only. Mamarabadi *et al.* (2018) in their study reported that aqueous extract of turmeric at concentration 16.66mg/ml least effective against fungal pathogen *Geotrichum candidum*, while exhibiting significant inhibition against fungus *Botrytis cinerea* at concentration 25mg/ml. It indicates that the responsiveness towards botanicals varies from one pathogen to another.

With the increase in concentration from 500ppm to 2000ppm, there was an increase in the inhibition of mycelial growth of pathogen as well. The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively. Osman *et al.* (2016) in their study also reported an increased inhibitory effect on mycelial growth of *Alternaria alternata* with the increase in concentration of the test extracts.

#### **5.3.1.2 Effect of the Ethanolic Plant extracts on mycelial growth of *Alternaria mali***

The ethanolic extracts of all the seven test plants exhibited potential antifungal activity against *Alternaria mali*. Among the seven ethanolic plant extracts tested, clove bud extract proved to be most effective with a mycelial inhibition of 69.2 per cent recorded over control. Further, clove ethanol extract at 2000ppm concentration resulted in 100 per cent inhibition of mycelial growth of the fungus. These results concurred with those of Boonpawa *et al.* (2019), demonstrating that clove ethanol extract showed significant antifungal activity against *Aspergillus flavus* inhibiting the mycelial growth by 83 per cent at 10,000ppm concentration. The high antifungal potency of Clove could be attributed to the presence of eugenol (phenolic compound) in clove extract which inhibits the biosynthesis of ergosterol, a component of the microbial cell membranes. This may lead to the disruption of microbial cell membrane permeability causing cell death (Pereira *et al.*, 2013). An inhibition of 28.8 per cent was recorded in case of ethanolic extract of garlic. Kutawa *et al.* (2018) in their study reported that ethanol extract of garlic showed antifungal activity against two fungal pathogens *Fusarium sp.* and *Rhizopus sp.* The antifungal property of garlic extract could be attributed to the presence of compound Allicin which possesses strong antimicrobial and antifungal properties. Further, ethanolic extract of turmeric rhizome exhibited an inhibition of 49.4 per cent in mycelial growth of *A. mali*. These results are confirmatory with those of Kipkogei *et al.* (2019) who reported that ethanolic extract of turmeric demonstrated moderate antifungal activity against *Alternaria alternata*. Turmeric is rich in phenolic

compounds, alkaloids, terpenoids, steroids, flavonoids, tannins, saponins, secondary metabolites such as curcuminoids (curcumin) which could be the active ingredients responsible for the antifungal action (Mamarabadi *et al.*, 2018). The least inhibition (17%) for ethanol extract was observed in case of Artemisia whole plant extract. Rodino *et al.* (2014) in their study found Artemisia extract effective against *Alternaria alternate* inhibiting the mycelial growth of the pathogen. This variation in the results may be attributed to the fact that whole plant extract was used in present study in contrast to leaf extract used in their study.

The mycelial inhibition showed an increasing trend with the increasing concentration of the test plant extracts. The mean overall inhibition observed for the three concentrations (500ppm, 1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively. Clove ethanol extract proved significantly most effective at 2000ppm concentration with a mycelial inhibition of 100 per cent. This was greater than the inhibition of 73.3 per cent observed for the fungicide Mancozeb used as a check in this study. This result is consistent with that of Yassin *et al.* (2020) who found a greater antifungal potency of clove extract against *Candida sp.* in comparison to antifungal medication Terbinafine.

### **5.3.1.3 Effect of the Methanolic Plant extracts on mycelial growth of *Alternaria mali***

In the present investigation, the methanolic extracts of all the test plants exhibited strong antifungal potency against *Alternaria mali*. Methanol extract of Clove bud proved to be most effective with an overall mycelial inhibition of 83.2 per cent observed. Further clove extract at concentrations 1000ppm and 2000ppm resulted in complete inhibition (100%) of mycelial growth of the pathogen. These results are consistent with those of Yassin *et al.* (2020) who observed strong antifungal potency of Clove methanol extract against three *Candida* species (*C. albicans*, *C. glabrata*, and *C. tropicalis*). Least inhibition (20.5%) was observed for methanol extract of turmeric rhizome. Radwan *et al.* (2014), however, in their study reported methanolic extract of turmeric rhizome most effective against

*Colletotrichum sp.* In the present study, methanol extract of datura inhibited the mycelial growth of *Alternaria mali* by 22.6 per cent. Zaker and Mosallanejad (2010) in their study observed no antifungal activity by Methanol and Methanol-water extract of datura against *Alternaria alternate*. However, in another study Kumar and Singh (2017) found datura extract showing good inhibitory effect against *Alternaria solani*. It indicates that antifungal activity of a particular plant extract may vary from one fungal species to another. The responsiveness of the pathogen to extracts increased with an increase in the concentration from 500ppm to 2000ppm. The mean overall inhibition observed for the three concentrations (500ppm, 1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent respectively. Furthermore clove methanol extract proved significantly most effective with a mycelial inhibition of 100 per cent observed at both 1000ppm and 2000ppm concentration which was greater than the inhibition (73.3%) observed for fungicide Mancozeb.

Ethanol and methanol extracts of the test plants showed better overall results in comparison to the aqueous extracts. The aqueous extracts exhibited a mean overall inhibition of 28.1 per cent, while methanol and ethanol extracts showed comparable results with a mean overall inhibition of 34.4 and 35.4 per cent, respectively. Several researchers have also found that the type of extraction solvents have a significant impact on the type and number of bioactive compounds extracted, which results in variation in their activity against different pathogens (Hepsibah and Jothi, 2017; Felhi *et al.*, 2017).

### **5.3.2 *In vitro* evaluation of the plant extracts on spore germination of *Alternaria mali***

The seven botanicals showed varying levels of anti-fungal potential depending on the solvent used for extraction and the concentration at which the *Alternaria mali* was exposed.

### **5.3.2.1 Effect of the Aqueous Plant extracts on spore germination of *Alternaria mali***

In the present investigation aqueous extract of each of the seven botanicals was tested *in vitro* for determination of per cent conidial germination inhibition of *Alternaria mali*. All the seven extracts inhibited the conidial germination of the pathogen, however, exhibited varying antifungal potential. Among the aqueous extracts, maximum inhibition of conidial germination (42.7%) was observed in case of ginger rhizome extract. The obtained results for ginger rhizome extract are in agreement with those of Osman *et al.* (2016) who in their study reported the inhibitory effect of ginger aqueous extract on conidial germination of *Alternaria alternata*. They reported germination inhibition of 69, 77 and 88 per cent at different concentrations of the extract tested. Al-Rahmah *et al.* (2013) attributed the antimicrobial potency of ginger to the presence of phenolic compounds ; gingerol, cendrene, zingiberene, phellandrene, geranial, neral,  $\beta$ -bisabolene, alkaloids, saponins, tannins, flavonoids and terpenoids. Mint leaf extract also exhibited promising results with an inhibition of 31.3 per cent observed for aqueous extract. Taskeen-un-Nisa *et al.* (2011) observed similar results with Mint extract against the fungus *Fusarium oxysporum*. In the present study, aqueous extract of garlic (clove) did not show a good inhibitory effect against *Alternaria mali* with the lowest inhibition of 3.1 per cent observed at concentration of 500ppm among all the extracts evaluated. With the increase in concentration from 500ppm to 2000ppm there was an increase in the inhibition of germination of pathogen as well. The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 33.6, 41.5 and 51.1 per cent respectively. Parveen *et al.* (2017) in their study also observed similar results on inhibition of sporulation of the test fungi (*Dreschslera sp.*, *Aspergillus flavus*, *Aspergillus niger* and *Penicillium expansum*) with an increase in concentration of the test plant extracts.

### **5.3.2.2 Effect of the Ethanolic Plant extracts on spore germination of *Alternaria mali***

All the seven Ethanolic extracts showed inhibitory effect against *Alternaria mali*, inhibiting the conidial germination of the pathogen. Highly significant per cent inhibition of spore germination was observed in case of clove bud extract (77.3%). It proved most effective at concentration 2000ppm with 85 per cent conidial germination inhibition recorded for the same. Our findings for clove bud extract are in tandem with those of Nagerabi *et al.* (2016) who in their study reported clove extract showing significant inhibition of 43 to 96 per cent on spore germination of the fungus *Aspergillus niger*. The antifungal potency of the Clove extract could be attributed to the presence of phenolic compounds, such as flavonoids, hydroxybenzoic acids, and hydroxyphenyl propenes (Yassin *et al.*, 2020). In the present study, for ethanolic extracts, the least inhibition of 9.3 per cent was observed for turmeric rhizome extract at 500ppm concentration. This is the first such study conducted to evaluate the effect of plant extracts against *A.mali*. The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 33.6, 41.5 and 51.1 per cent respectively. The data further revealed that clove ethanolic extract proved significantly most effective at 2000ppm concentration with 85 per cent conidial germination inhibition.

### **5.3.2.3 Effect of the Methanolic Plant extracts on spore germination of *Alternaria mali***

Among methanolic extracts, clove bud extract at concentration 2000ppm exhibited the maximum inhibition (89.3 %) of conidial germination of *Alternaria mali*. Also, ginger rhizome extract exhibited significant inhibition (82.2%) of conidial germination of the pathogen. The obtained results for ginger rhizome are in agreement with those of Prashanth *et al.* (2017) who in their study reported the inhibitory effect of ginger extract on spore germination of *Bipolaris sorokiniana*. Al-Rahmah *et al.* (2013) attributed the antimicrobial potency of ginger to the

presence of phenolic compounds ; gingerol, cendrene, zingiberene, phellandrene, geranial, neral,  $\beta$ -bisabolene, alkaloids, saponins, tannins, flavonoids and terpenoids. Further, methanolic leaf extract of datura showed least inhibitory effect against *Alternaria mali* with an inhibition of 24 per cent at 500ppm concentration observed for the same. Zaker and Mosallanejad (2010) in their study observed no antifungal activity by methanol and methanol-water extract of datura against *Alternaria alternata*. However, Hannan *et al.* (2005) in their study reported datura leaf extract highly effective against *Alternaria alternata*. This discrepancy in the results of different studies may be attributed to variation in extraction methods and concentrations tested. Also Methanol extract of artemisia whole plant exhibited an inhibition of 54.5 per cent on conidial germination of *Alternaria mali*. The antifungal efficacy of artemisia extract in inhibiting the spore germination of pathogenic fungi was also reported by Parveen *et al.* (2017) in their study where they found artemisia extract showing highest inhibition against *Penicillium expansum* followed by *Aspergillus flavus*, *Aspergillus niger* and *Drechslera sp.* at the same concentration.

The methanolic extracts showed the highest efficacy with a mean overall spore germination inhibition of 57.4 per cent. Also the mean spore germination inhibition in case of the ethanolic extracts was 40.1 per cent while the aqueous extracts exhibited the least effect with an inhibition of 28.6 per cent. The reason for greater efficacy of methanolic extracts may be attributed to the fact that methanol when used as an extraction solvent, yields higher polyphenols and flavonoids (Lezoul *et al.*, 2020).

#### **5.4 Phyto-chemical profiling of the effective botanical**

The results of the present investigation effectively demonstrates that clove bud methanolic extract is most effective among all the plant extracts evaluated *in vitro*, for inhibiting both the mycelial growth of *A. mali* as well as the conidial germination. The phyto-chemical screening of clove methanol extract revealed the existence of alkaloids, flavonoids, terpenoids, tannins, aldehydes, ketones,

glycoside, steroids, carbohydrates and phenolic compounds. A large number of compounds identified were either phenolic compounds and flavonoids. The phyto-constituents found included poly-phenols such as eugenol, gallic acid, chlorogenic acid, certain flavonoids like quercetin and also compounds with known antifungal properties like econazole (which is an antifungal drug already available). Our findings are in agreement with Jimoh *et al.* (2017) who in their study on phyto-chemical screening of clove methanol extract using GC-FID (Gas Chromatography with Flame Ionization Detection) found compounds like alkaloids, terpenoids, flavonoids, tannins, aldehydes etc. A major group of compounds, a total of around forty six, identified by them were phenolic compounds like eugenol, luteolin, salicylic acid, gallic acid etc. Similar results were reported by Kumar *et al.* (2010) in their investigation on phyto-chemical screening of dichloromethane extract of clove. The phyto-constituents detected were alkaloids, glycoside, steroids, carbohydrates, terpenoids, tannins and phenolic compounds.

To identify the key inhibitor molecules, the selected phytochemicals (eugenol, quercetin, gallic acid, econazole, ellagic acid, luteolin, demeclocycline, norstictic acid rhein, koparin and diosmin) were docked with lanosterol 14-alpha demethylase enzyme, which is one of the key enzymes in sterol biosynthesis pathway. Diosmin, rhein and quercetin proved to be the top 3 in terms of affinity towards the defined enzyme. The newly identified molecules thus could serve as potential new generation plant-based drugs against *A.mali* after in depth analysis of these molecules. In similar kind of studies, Umesh *et al.* (2020) identified rutin, carpaine, stigmasterol,  $\beta$ -caryophyllene, and  $\alpha$  eudesmol as lead phytochemicals against pathogenic microbes with the approach of molecular docking against trehalose-6-phosphate phosphatase enzyme. However, this study area is in infancy, and requires more in-depth studies to elucidate upon the individual activity, and mechanism of action of the potent inhibitors identified.

## Chapter-6

### SUMMARY AND CONCLUSION

The investigation entitled “Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources” was conducted to screen botanicals against *Alternaria mali*, and to figure out the probable bioactive compounds. The results of the study are summarised as under:

- All the seven botanicals tested in the present investigation showed efficacy against *Alternaria mali* while inhibiting its mycelial growth and spore germination *in vitro*.
- Among the aqueous extracts tested *in vitro*, clove bud extract showed highest efficacy. It proved highly effective at concentration 2000ppm with mycelial inhibition of 73 per cent.
- The methanolic and ethanolic extracts of clove caused significant inhibition of the mycelial growth of *Alternaria mali*. A 100 per cent inhibition was observed for clove ethanol extract at 2000ppm concentration while in case of clove methanol extract a complete inhibition of mycelial growth was observed at both 1000ppm and 2000ppm concentration. This was significantly greater than the inhibition (73.3%) observed for fungicide Mancozeb @ 1500ppm used as a check in this study.
- For mycelial inhibition, among the extraction methods used in the current study, ethanol and methanol extraction showed better results compared to the aqueous extraction. The aqueous extracts exhibited a mean overall inhibition of 28.1 per cent while methanol and ethanol extracts showed comparable results with a mean overall inhibition of 34.4 and 35.4 per cent, respectively.

- In all cases the mycelial inhibition showed an increasing trend with increasing concentration of the test plant extracts. The mean overall inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 21.3, 33.2 and 43.5 per cent, respectively.
- All the seven botanicals used in the current investigation inhibited the conidial germination of *Alternaria mali in vitro*, however, exhibiting varying antifungal potential.
- Among the aqueous extracts, maximum inhibition of conidial germination (42.7%) was observed in case of Ginger rhizome extract.
- For the ethanolic extracts, highly significant per cent inhibition of spore germination was observed in case of Clove bud extract (77.3%). It proved most effective at concentration 2000ppm with 85 per cent conidial germination inhibition. Also, the methanolic extracts of clove at concentration 2000ppm exhibited the maximum inhibition (89.3 %) of conidial germination of *Alternaria mali*.
- Among the extraction methods, the methanolic extraction showed the highest efficacy with a mean overall spore germination inhibition of 57.4 per cent. Also, the mean spore germination inhibition in case of the ethanolic extracts was 40.1 per cent while the aqueous extracts exhibited the least effect with an inhibition of 28.6 per cent.
- Furthermore, germination inhibition showed an increasing trend with the increasing concentration of the test plant extracts. The mean overall spore germination inhibition observed for the three concentrations (500ppm,1000ppm and 2000ppm) was 33.6, 41.5 and 51.1 per cent respectively.
- The phyto-chemical profiling of the highly effective botanical (clove methanol extract) obtained in the current study revealed a number of compounds like Eugenol, Quercetin, Chlorogenic acid, Gallic acid,

Luteoline etc. Majority of the detected compounds were either flavonoids and poly-phenols. Certain compounds like Econazole with known antifungal properties were also found. Antibiotic compounds such as Demeclocycline, Cefamandole nafate etc were also detected.

The conclusions drawn from the present investigation are as follows:

- The results obtained from this study showed that the seven botanicals screened *in vitro* against *Alternaria mali* exhibited antifungal potential against the pathogen with varying efficacy.
- *Syzygium aromaticum* (clove) extracts exhibited a highly antifungal efficiency. In particular, the methanol extract of clove (bud) significantly inhibited both the mycelial growth and spore germination of *Alternaria mali*.
- The results demonstrated a high potency of the clove extract compared with that of fungicide Mancozeb (positive control).
- These preliminary results, obtained from *in vitro* experiments, may be supplemented by other more comprehensive studies *in vivo*, both in controlled greenhouse conditions and in open field to practically evaluate the effectiveness of these botanicals.
- Methanol proved to be an effective solvent in the extraction process producing high yield of clove active constituents.
- The present study conclusively demonstrates that clove is a good source of various phytochemicals like poly-phenols, flavonoids, alkaloids and potent anti-microbial compounds.
- Molecular docking studies revealed that diosmin, rhein and quercetin proved to be the top 3 molecules in terms of affinity towards lanosterol 14-alpha demethylase, a key enzyme in sterol biosynthesis pathway.

- This preliminary finding warrants an in-depth analysis of the usefulness of these compounds against *Alternaria mali* and further research in the direction to elucidate the mechanism of action to develop a plant based drug in future.

## LITERATURE CITED

- Al-Askar, A. A. and Rashad, Y. M. 2010. Efficacy of some plant extracts against *Rhizoctonia Solani* on Pea. *Journal of Plant Protection Research* **50**(3).
- Al-Rahmah, A. N., Mostafa, A. A., Abdel-Megeed, A., Yakout, S. M. and Hussein, S. A. 2013. Fungicidal activities of certain methanolic plant extracts against tomato phytopathogenic fungi. *African Journal of Microbiology Research* **7**(6): 517-524.
- Muthukumaran, P., Akshara, R., Elakiya, C. and Saraswathy, N. 2016. Comparative evaluation of antibacterial, antifungal activity and phytochemical screening of leaf and bark extract of *Solanum trilobatum* L. *Journal of Chemical and Pharmaceutical Research* **8**(7): 198-202.
- Anonymous, 2018a. NHB Statistical Database. <http://www.nhb.gov.in>
- Anonymous, 2018b. *Area and production under major horticulture crops in Jammu and Kashmir*. Directorate of Horticulture, Kashmir. <http://hortikashmir.gov.in/DATA/AREA%20PROD%20NET>.
- Anonymous 2013. Sporious Pesticides, bad weather spoil 60% of apple production. The Daily Kashmir Times. September 22, 2013.
- Ayoub, M and Niazi, A. U. 2001. Control of wheat rust by leaves extract of poisonous phanerogamic plants. *Journal of Biological Science* **8**: 300-305.
- Balamurugan, S. 2014. *In vitro* antifungal activity of *Citrus aurantifolia* Linn plant extracts against phytopathogenic fungi *Macrophomina phaseolina*. *International Letters of Natural Sciences* **13**: 70-74.
- Balunas, M. J. and Kinghorn, A. D. 2005. Drug discovery from medicinal plants. *Life Sciences* **78**: 431-441.

- Bhat, K. A., Peerzada, S. H. and Anwar, A. 2015. Alternaria epidemic of apple in Kashmir. *African Journal of Microbiology Research* **9**: 831-837.
- Boonpawa, R., Sriyoha, F. and Kamnon, G. 2019. Antifungal activity of clove and betel extract against *Aspergillus Flavus* during dried Chili storage. *Asia Pacific Environmental and Occupational Health Journal* **5**(2): 17-22.
- Bulajic, A. and Babovic, M. 1997. *Alternaria mali*, a new pathogen of apple in Yugoslavia. *Jugoslovensko Vocarstvo* **31**: 215-25.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods, a review. *International Journal of Food Microbiology* **94**: 223-253.
- Chimahali, J., Jebamalar, A., Duraikannu, G. and Thirumal, S. 2019. Phytochemical analysis and evaluation of antimicrobial activity in the whole plant extracts of *Gloriosa superba*. *Asian Journal of Pharmaceutical and Clinical Research* **12**(6): 245-249.
- Chandel, S. and Sharma, S. 2014. Botanicals, biofumigants and antagonists application in managing stem rot disease caused by *Rhizoctonia solani* Kuhn in carnation. *Journal of Biopesticides* **7**(1): 3-10.
- Chuang, P. H., Lee, C. W., Chou, J. Y., Murugan, M., Shieh, B. J. and Chen, H. M. 2007. Antifungal activity of crude extracts and essential oil of *Moringa oleifera* Lam. *Bioresource Technology* **98**: 232-236.
- Cortes-Rojas, D. F., Fernandes de Souza, C. R. and Oliveira, W. P. 2014. Clove (*Syzygium aromaticum*): a precious spice. *Asian Pacific Journal of Tropical Biomedicine* **4** (2): 90-96.
- Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* **12**: 564-582.

- Dar, K. B., Bhat, A. H., Amin, S., Zargar, M. A., Masood, A., Malik, A. H. and Ganie, S. A. 2017. Evaluation of antibacterial, antifungal and phytochemical screening of *Solanum nigrum*. *Biochemistry and Analytical Biochemistry* **6**(1).
- Das, K., Tiwari, R. K. and. Shrivastava, D. K. 2010. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research* **4**: 04-111.
- Dev, D., Konda, S., Puneeth<sup>1</sup>, M. E., Tanuja, N., Singh, P. and Narendrappa, T. 2016. *In vitro* evaluation of bioagents and botanicals against *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. causing Anthracnose of pomegranate. *Ecology, Environment and Conservation* **22**(3): 1229-1232.
- Fathoni, A., Saepudin, E., Cahyana, A. H., Rahayu, D. U. C. and Haib, J. 2017. Identification of nonvolatile compounds in clove (*Syzygium aromaticum*) from Manado. **In**: AIP Conference Proceedings.
- Fatimah, A., Alharbi, R. I., Albasher, G., Almeer, R. and Alsaggabi, N. S. 2019. Antifungal potential of aqueous extract of *Boswellia carteri*. *Journal of Pure and Applied Microbiology* **13**(4): 2375-2381.
- Felhi, S., Daoud, A., Hajlaoui, H., Mnafgui, K., Gharsallah, N. and Kadri, A. Solvent extraction effect on phytochemical constituents, profiles, antioxidant and antimicrobial activities and functional group analysis of *Ecballium elatarium* seeds and peels fruits. *Food Science and Technology* **37**: 483-492.
- Filajdic, N. and Sutton, T. B. 1991. Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different varieties of apples to *Alternaria* blotch. *Plant Disease* **75**: 1045-8.

- Gaspar, E. M., Duarte, R. and Santana, J. C. 2018. Volatile composition and antioxidant properties of clove products. *Biomedical Journal of Scientific and Technical Research* **9**: 7270-7276.
- Gatto, A., Ippolito, B., Linsalataa, V., Nicholas, Cascaranoa, A., Nigro, F., Vanadiaa, S. and Venere, D. D. 2011. Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. *Postharvest Biology and Technology* **61**(1): 72-82.
- Grove, G. G., Eastwell, K. C., Jones, A. L. and Sutton, T. B. 2003. Diseases of apple. **In**: Ferree DC, Warrington IJ, eds. Apples: Botany, production and uses. Wallingford, United Kingdom: CABI Publishing 459-88.
- Hannan, A., Mukhtar, I., Riaz, T. and Khan, S. N. 2005. Effect of plant extracts on black point infection of wheat. *Mycopath* pp 53-55.
- Hans, R. and Sharma J. N. 2017. *In Vitro* evaluation of botanicals, bio-agents and fungicides against *Alternaria alternata* causing Mouldy core, Core rot of apple. *International Journal of Agriculture Sciences* **9**(49): 4835-4840.
- Hassanein, N. M., Zeid, M. A., Youssef, I. F. and Mahmoud, D. A. 2008. Efficacy of leaf extracts of neem and chinaberry against early blight and wilt disease of tomato. *Australian Journal of Basic Applied Sciences* **2**: 763: 772.
- Hepsibah, A. H. and Jyothi, G. J. 2017. A comparative study on the effect of solvents on the phytochemical profile and the biological potential of *Ormocarpum cochinchinense* Auct. Non (Lour.). *International Journal of Pharmacy and Pharmaceutical Sciences* **9**: 67-72.
- Hwang, I. C., Kim, J., Kim, H. M., Kim, D. I., Kim, S. G. and Jang, C. 2009. Evaluation of toxicity of plant extract made by neem and matrine against

main pests and natural enemies. *Korean Journal of Applied Entomology* **48**: 87–94.

Jagtap, G. P., Mali, A. K. and Dey, U. 2013. Bioefficacy of fungicides, bio-control agents and botanicals against leaf spot of turmeric incited by *Colletotricum capsici*. *African Journal of Microbiology Research* **7**(18): 1865-1873.

Jimoh, S. O., Arowolo, L. A. and Alabi, K. A. 2017. Phytochemical Screening and Antimicrobial evaluation of *Syzygium aromaticum* extract, essential oil *International Journal of Current Microbiology and Applied Sciences* **6**(7): 4557-4567.

Johnston, A. and Booth, C. 1983. *Plant Pathological Pocket Book*. Commonwealth Mycological Institute, Kew Surrey, England. pp 439.

Keniya, M. V., Sabherwal, M., Wilson, R. K., Woods, M. A., Sagatova, A. A., Tyndall, J. D. A., Monk, B. C., 2018. Crystal Structures of full-length Lanosterol 14 $\alpha$ -Demethylases of prominent fungal pathogens *Candida albicans* and *Candida glabrata* provide tools for antifungal discovery. *Antimicrobial agents and chemotherapy* **62**.

Khalse, K. D., Lal, A. A. and Simon, S. 2017. Efficacy of bio-agents and plant extracts against the *Alternaria* leaf spot of cabbage (*Alternaria brassicae*). *Journal of Pharmacognosy and Phytochemistry* **6**(4): 1980-1982.

Kim M. K., Choi, G. J. and Lee, H. S. 2003. Fungicidal property of *Curcuma longa* L. rhizome-derived curcumin against phytopathogenic fungi in a greenhouse. *Journal of Agricultural and Food Chemistry* **51**(6): 1578–1581.

- Kishore, K. and Sharma, I. M. 2005. Nursery management of leaf blotch disease of apple seedlings. *Plant Disease Research* **20**: 29-31.
- Kipkogei, K. E., Kiptui, K. P. and Ezekiel, K. 2019. Antifungal Potential of *Curcuma longa* (Turmeric) and *Zingiber officinale* (Ginger) against *Alternaria alternata* infecting spinach in Kenya. *World Journal of Agricultural Research* **7**(4): 124-131.
- Koehn, F. E. and Carter, G. T. 2005. The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery* **4**: 206-220.
- Kokab, J., Asad, S. and Zakria, M. 2018. Antifungal evaluation and phytochemical identification of selected botanicals against *Ceratocystis manginecans* causing mango sudden death. *Plant Pathology and Microbiology* **9**(11): 1-12.
- Kumar, P. and Singh, S. 2017. *In Vitro* evaluation of fungicides and plant Extract against *Alternaria solani* (Ellis) causing Early Blight in tomato (*Lycopersicon esculentum* Mill.). *International Journal of Current Microbiology and Applied Science* **6**(9): 820-827.
- Kumar, U., Kumar, B., Bhandari, A. and Kumar, Y. 2010. Phytochemical investigation and comparison of antimicrobial screening of clove and cardamom. *International Journal of Pharmaceutical Sciences and Research* **1**(12): 138-147.
- Kusaba, M. and Tsuge, T. 1994. Nuclear ribosomal DNA variation and pathogenic specialization in *Alternaria* fungi known to produce host specific toxins. *Applied and Environmental Microbiology* **60**: 3055-62.

- Kutawa, A. B., Danladi, M. D. and Haruna, A. 2018. Antifungal activity of garlic (*Allium sativum*) extract on some selected fungi. *Journal of Medicinal Herbs and Ethnomedicine* **4**: 12-14.
- Labbé, C. M., Rey, J., Lagorce, D., Vavruša, M., Becot, J., Sperandio, O., Villoutreix, B. O., Tufféry, P., Miteva, M. A., 2015. MTiOpenScreen: a web server for structure-based virtual screening. *Nucleic acids research* **43**: 448-454.
- Liu, Y., Grimm, M., Dai, W.-t., Hou, M.-c., Xiao, Z.-X., Cao, Y., 2020. CB-Dock: a web server for cavity detection-guided protein–ligand blind docking. *Acta Pharmacologica Sinica* **41**: 138-144.
- Lezoul, N. E. H., Belkadi, M., Habibi, F. and Guillen, F. 2020. Extraction processes with several solvents on total bioactive compounds in different organs of three medicinal plants. *Molecules* **25**: 4672.
- Ma, T., Luo, J., Tian, C., Sun, X. Quan, M. Zheng, C. Kang, L. and Zhan, J. 2015. Influence of technical processing units on chemical composition and antimicrobial activity of carrot (*Daucus carrot* L.) juice essential oil. *Food Chemistry* **1**(170): 394-400.
- Mamarabadi, M., Tanhaeian, A. and Ramezany, Y. 2018. Antifungal activity of recombinant thanatin in comparison with two plant extracts and a chemical mixture to control fungal plant pathogens. **8**: 180-200.
- Mohana, D. C. and Raveesha, K. A. 2007. Anti-fungal evaluation of some plant extracts against some plant pathogenic field and storage fungi. *Journal of Agricultural Technology* **4**(1): 119-137.
- Muthukumar, P., Akshara, R., Elakiya, C. and Saraswathy, N. 2016. Comparative evaluation of antibacterial, antifungal activity and

phytochemical screening of leaf and bark extract of *Solanum trilobatum* L.  
*Journal of Chemical and Pharmaceutical Research* **8**(7): 198-202.

Nagerabi, S. A. F., Ahmed, A. H. M. and Elshafie, A. E. 2016. *In Vitro* evaluation of selected plant extracts as biocontrol agents against Black Mold (*Aspergillus Niger* Van Tieghem) of onion bulbs (*Allium cepa* L.). *International Journal of Scientific and Technology Research* **5**(1): 2277-8616.

Nene, Y. L and Thapliyal, P. N. 2002. *Fungicides in Plant Disease Control*. 3<sup>rd</sup> edition Oxford and IBH Publishing Co. PVT. Ltd., New Delhi 691pp.

Osman, E. H. B., Algam, S. A. E., Osman, M. T., Ali, M. E., Abbo, A. S. H. and Elhassan, S. M. 2016. Antifungal effect of camel urine and ginger water extract against *Alternaria alternata* the causal agent of Early Blight disease of tomato *in vitro*. *International Journal of Agriculture, Forestry and Plantation* **2**: 462-1757.

Parveen, S., Wani, A. H., Bhat, M. Y., Malik, A. R., Koka, J. A. and Ashraf, N. 2017. Antimycotic potential of some phytoextracts on some pathogenic fungi. *Journal of Biopesticides* **10**(1): 60-65.

Patel, P., Patel, N., Patel, D., Desai, S. and Meshram, D. 2014. Phytochemical analysis and antifungal of *Moringa Olefera*. *International Journal of Pharmacy and Pharmaceutical Sciences* **6**(5).

Pereira, F. D. O., Mendes, J. M. and Lima, E. D. O. 2013. Investigation on mechanism of antifungal activity of eugenol against *Trichophyton rubrum*. *Medical Mycology* **51**: 507–513.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., Ferrin, T. E., 2004. UCSF Chimera-- a visualization system

for exploratory research and analysis. *Journal of computational chemistry* **25**: 1605-1612.

Prashanth, J., Vaish, S. S. and Omprakash, S. 2017. *In vitro* studies on the direct effect of Garlic Cloves and different botanical extracts against *Bipolaris sorokiniana* causing Foliar Blight of Wheat. *Biosciences, Biotechnology Research Asia* **14**(3): 1165-1168.

Radwan, M. M., Tabanca, N., Wedge, D. E., Tarawneh, A. H. and Cutler, S. J. 2014. Antifungal compounds from turmeric and nutmeg with activity against plant pathogens. *Fitoterapia* **99**: 341-346.

Ravikumar, M. C., Rajkumar, H. and Garampalli. 2013. Antifungal activity of plants extracts against *Alternaria solani*, the causal agent of early blight of tomato. *Archives of Phytopathology and Plant Protection* **6**: 1-6.

Rijai, L., Rijai, A. J., Kuncoro, H., Badrah, S. and Amir, M. 2017. Secondary metabolites compounds profile by LC-MS/MS and chemical methods from Kolowe Stem Bark (*Chydenanthus excelsus*). *Journal of Pharmaceutical Sciences and Research* **9**(4): 420-428.

Roberts, J. W. 1924. Morphological characters of *Alternaria mali* (Roberts). *Journal of Agricultural Research* **27**: 699-712.

Rodino, S., Butu, M., Petrache, P., Butu, A. and Cornea, C. P. 2014. Antifungal activity of four plants against *Alternaria alternata*. *Scientific Bulletin. Series F. Biotechnologies* **xviii**.

Rout, S. P., Choudary, K. A., Kar, D. M., Das, L. and Jain, A. 2009. Plants in traditional medicinal system-future source of new drugs. *International Journal of Pharmacy and Pharmaceutical Sciences* **1**(1): 1-23

- Salhi, N., Mohammed Saghir, S. A., Terzi, V., Brahmi, I., Ghedairi, N. and Bissati, S. 2017. Antifungal activity of aqueous extracts of some dominant Algerian medicinal plants. *BioMed Research International* pp 1-6.
- Sawamura, K., Jones, A. L. and Aldwinckle, H. S. 1990. *Alternaria blotch*. In: Compendium of Apple and Pear diseases: American Phytopathology Society Press pp 24-5.
- Scheuerell, S. and Mahaffee, W. 2002. Compost tea: Principles and prospects for plant disease control. *Compost Science and Utilization* **10**: 313-338.
- Shahzad A., Bhat, G. N, and Mir, N. A. 2002. *Alternaria mali*-A new pathogen of apple in Kashmir. *SKUAST Journal of Research* **4**: 96-98.
- Sharma, J. N. 2000. Marssonina Blotch: A new disease of apple and its control. *Indian Journal of Plant Protection* **28**: 100-1.
- Sicard, D., Michalakakis, Y., Dron, M. and Neema, C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in three centres of diversity of its host, *Phaseolus vulgaris*. *Phytopathology* **87**: 807-813.
- Singh, P., Mishra, P. and Tripathi, N. N. 2014. Evaluation of plant extract against *Fusarium oxysporum* F. Sp. *Lycopersici*, wilt pathogen of tomato. *International Journal of Food, Agriculture and Veterinary Sciences* **4**(2): 163-167.
- Sofi, T. A., Muzafer, A. B., Dar, G. H., Ahangar F. A. and Hamid, A. 2013. Virulence variation in *Alternaria mali* (Roberts) and evaluation of systemic acquired resistance (SAR) activators for the management of *Alternaria* leaf blotch of apple. *American Journal of Food and Agriculture* **25**(3): 196-204.

- Soleimani, M. J. and Esmailzadeh, M. 2010. First report of *Alternaria mali* causing apple leaf blotch disease in Iran. *Australasian Plant Disease Notes* **2**: 57-58.
- Taskeen-Un-Nisa, Wani, A. H., Bhat, M. Y., Pala, S. A. and Mir, R. A. 2011. *In vitro* inhibitory effect of fungicides and botanicals on mycelial growth and spore germination of *Fusarium oxysporum*. *Journal of Biopesticides* **4**(1): 53-56.
- Trott, O., Olson, A. J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry* **31**: 455-461.
- Umesh, H. R., Ramesh, K. V. and Dearaju, K. S. 2020. Molecular docking studies of phytochemicals against trehalose-6-phosphate phosphatases of pathogenic microbes. *Beni-Suef University Journal of Basic and Applied Sciences* **9**: 1-14
- Vincent, J. M. 1947. Distortion of fungal hyphae in the presence of certain inhibitors. *Nature* pp 15: 850.
- Wink, M. 1993. Production and application of phytochemicals from an agricultural perspective. **In**: T. A. van Beek and H. Breteler (eds), *Proceedings of Phytochemical Society Europe: Phytochemistry and Agriculture*, Clarendon Press, Oxford, pp. 171–213.
- Wijesundara, S. A. D. T. L., Kannangara, B. T. S. D. P. and Abeywickrama, K. 2015. Antifungal activity of *Croton aromaticus* L. *in vitro* against post-harvest fungal pathogens isolated from tropical fruits. *The Journal of Agricultural Science* **11**(2): 105-117.

- Yassin, M. T., Mostafa, A. A. and Al-Askar, A. A. 2020. *In vitro* anticandidal potency of *Syzygium aromaticum* (clove) extracts against vaginal candidiasis. *BMC Complementary Medicine and Therapies* **20**: 1-9.
- Yusoff, S. F., Haron, F. F., Muda Mohamed, M. T., Asib, N., Sakimin, S. Z., Abu Kassim, F. and Izera Ismail, S. 2020. Antifungal activity and phytochemical screening of *Vernonia amygdalina* extract against *Botrytis cinerea* causing Gray Mold disease on tomato Fruits. *Biology* **9**: 286.
- Zaheer, Z., Shafique, S., Shafique, S. and Mehmood, T. 2012. Antifungal potential of *Parthenium hysterophorus* L. plant extracts against *Fusarium solani*. *Scientific Research and Essays* **7**(22): 2049-2054.
- Zaker, M. 2016. Natural Plant Products as Eco-friendly Fungicides for Plant Diseases Control. *A Scientific Journal of Krishi Foundation* **14**(1): 134-141.
- Zaker, M. and Mosallanejad, H. 2010. Antifungal activity of some plant extracts on *Alternaria alternata*, the causal agent of Alternaria leaf spot of potato. *Pakistan Journal of Biological Sciences* **13**(21): 1023-1029.

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## **CERTIFICATE**

Certified that all the corrections/amendments as suggested by External Examiner Dr. Jaspal Kaur, Professor, Department of Plant Pathology, PAU, Ludhiana during Viva-Voce examination held on 09-03-2021 have been incorporated in the manuscript entitled “**Identifying Plant Inhibitors against *Alternaria mali* from Diverse Plant Sources**” submitted by **Ms. Mariya (Regd. No. MSA-2018-1232)**

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