IN VITRO MANAGEMENT OF FOUR TOMATO FUNGAL PATHOGENS USING PLANT EXTRACTS AND FERMENTED PRODUCTS

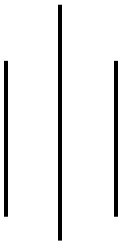
A Dissertation Submitted To:

CENTRAL DEPARTMENT OF BOTANY

TRIBHUVAN UNIVERSITY

For the partial fulfillment of the requirements for

Master of Science in Botany



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CENTRAL DEPARTMENT OF BOTANY

TRIBHUVAN UNIVERSITY

KIRTIPUR, KATHMANDU, NEPAL

May, 2015

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RECOMMENDATION

This is to certify that Miss Radha Shrestha has completed this dissertation work entitled "IN VITRO MANAGEMENT OF FOUR TOMATO FUNGAL PATHOGENS USING PLANT EXTRACTS AND FERMENTED PRODUCTS" as a partial fulfillment of Masters of Science Degree in Botany under my supervision with special paper "Plant Pathology and Applied Mycology". This is her original research work and has been carried out under my supervision. To the best of our knowledge, this thesis work has not been submitted for any other degree in any institution.

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LETTER OF APPROVAL

This is to certify that the dissertation work entitled "IN VITRO MANAGEMENT OF FOUR TOMATO FUNGAL PATHOGENS USING PLANT EXTRACTS AND FERMENTED PRODUCTS" submitted by Radha Shrestha has been accepted for the partial fulfillment of requirement for Master of Science on Botany.

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ACKNOWLEDGEMENTS

At first, I want to acknowledge my supervisor Dr. Ganesh Prasad Neupane for his sincere supervision and direction for the completion of this thesis.

I am very grateful to Head of Department (HOD) Prof. Dr. Pramod Kumar Jha for his encouragement and support in administrative work. Thanks to Prof. Dr. Usha Budathoki, Dr. Ram Deo Tiwari, Dr. Chandra Prasad Pokhrel and Dr. Sanjaya Jha for their direct and indirect encouragements. Also thanks to all the teachers of Central Department of Botany for their encouragement.

I want to express my sincere gratitude to Lab Assistance sir Bishnu Khatri and Sailendra Kumar Singh, guard Narayan Prashad Ghimire and Sambhu Bista for their help during Lab work. Also thanks to Bhakta Pradhan, Min Bahadur KC and Sitaram Upadhya for preparing letter for NARC.

Thanks to Chetana Manandhar of Plant Pathology Division, National Institute of Research Centre (NARC) for providing articles.

Thanks to my class mates Narendra Raj Adhikari, Bina Bhattarai, Gopal Sharma, Ghanashyam Chaudhary, Bhuwaneshwori Joshi, Anil Joshi and Chandra Mohan Gurumachan. Also thanks to my senior Miss. Suprava Shrestha and junior Sujan Balami for providing technical idea to complete my lab work. And thanks to my all friends, all seniors and juniors.

Finally, thanks to my late mother for giving birth to me and thanks to my father for educating me up to this level and all my brothers and sisters for their inspiration and financial support.

Radha Shrestha

ABSTRACTS

Tomato, a popular vegetable crop, used in various dishes contains most of the essential nutritional requirements. It is the major commercial vegetable that can be grown throughout the country. *Alternaria* sp., *Colletotrichum* sp., *Stemphylium* sp. and *Verticillium* sp. are the important infectious causal agents that had threatened tomato. Regular use of chemical fungicides creates a hazardous effect to the environment and human health too. Fresh plant extracts and fermented products are easily available, easy to prepare, non hazardous to environment and human too. The organic compounds and secondary metabolites present in them are lethal to mycelia growth of these pathogens.

Three wild plants (*Agave cantula, Ageratina adenophora* and *Melia azeradach*) are used for experimentation to control the mycelia growth of four tomato test pathogens in both form i.e. fresh extract and fermented form. Mancozeb (0.2mg/ml) used as positive control showed the reduction in mycelia growth of *Alternaria* sp., *Colletotrichum* sp., *Stemphylium* sp. & *Verticillium* sp. by 63.30%, 100%, 74.02% and 58.33% respectively. In the experiments, among three tested samples, both form of *Agave cantula* showed significant inhibitory effect to mycelia growth of three tomato pathogens v.i.z. *Colletotrichum* sp. (100% inhibition at 50% conc. of fresh extract and 100% inhibition at 60% conc. of ferment), *Stemphylium* sp. (83.33% inhibition at 60% conc. of fresh extract and 80.9% inhibition at 60% conc. of ferment) and *Verticillium* sp. (88.11% inhibition at 60% conc. of fresh extract and 88% inhibition at 60% conc. of ferment). Fresh extract and fermented product of *A. cantula* showed less inhibitory effect to the mycelia growth of *Alternaria* sp. (42.58% inhibition at 60% conc. of fresh extract and 44.29% at 60% conc. of ferment). It may be effective to *A. solani* at higher concentration.

Key words: Tomato, *Alternaria* sp., *Stemphyllum* sp., *Verticillium* sp., *Colletotrichum* sp., Fresh plant extract, Fermented plant extract, *Agave cantula*, *Ageratina adenophora and Melia azeradach.*

ACRONYMS

%:	Percentage
℃:	Degree Celsius
µl:	Micro liter
µm:	Micro meter
cm:	Centimeter
gm:	Gram
g:	Gravity
L:	Liter
mg:	Milli gram
MIC:	Minimum Inhibition Concentration
ml:	Milli liter
PDA:	Potato Dextrose Agar
SDW:	Sterilized Distilled Water
W/V:	Weight/Volume
WA:	Water Agar
No.:	Number
NC:	Negative control

PC:	Positive control
DW:	Distilled water
Conc.:	Concentration
Ha:	Hectare
t:	ton
t Ha^{-1}:	Ton per hectare
yr:	Year
sp.:	Species
lbs:	Pounds
Fig:	Figure

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CHAPTER I

1. INTRODUCTION

1.1 BACKGROUND

1.1.1 Tomato and its nutritional value

Tomato (*Lycopersicon esculentum* Mill.) is the edible, often red fruit/berry of night shade family Solanaceae. The species originated in the South American Andes (Encyclopedia of Life, 2014) and its use as a food originated in Mexico, and spread throughout the world following the Spanish colonization of the Americas. Its many varieties are now widely grown, sometimes in greenhouses in cooler climates. Hydroponic tomatoes are also available, and the technique is often used in hostile growing environments, as well as high-density plantings.

Tomato plants typically grow to 1–3 meters (3–10 ft) in height and have a weak stem that often sprawls over the ground and vines over other plants. It is a perennial in its native habitat, although often grown outdoors in temperate climates as an annual. An average common tomato approximately weighs 100 grams (Rijkzwaan.nl, 2010 & Enzazaden.nl, 2009).

Tomato is consumed in diverse ways, including raw, as an ingredient in many dishes, sauces, salads, and drinks. Tomato is a good source of energy. Besides, carbohydrates, fats, proteins, vitamins, trace elements like magnesium, potassium, phosphorus, etc, and other constituents like water and lycopene are also present (Table 1). Tomato varieties are available with double the normal vitamin C (Double rich), 40 times normal vitamin A, high level of anthocyanin (resulting in blue tomatoes), and two to four times the normal amount of lycopene (numerous available cultivars with the high crimson gene) (USDA, 2009).

The red pigment contained in tomatoes is called lycopene. This compound appears to act as a natural antioxidant that neutralizes free radicals that can

damage cells in the body. Lycopene has also been shown to improve the skin's ability to protect against harmful UV rays (BBC News retrieved, 2010). The lycopene from tomatoes has no effect on the risk of developing diabetes, but may help relieve the oxidative stress of people who already have diabetes (Valero *et.al,* 2011). Two powerful compounds found in tomatoes i.e. coumaric acid and chlorogenic acid are thought to block the effects of nitrosamines. Tomatoes also contain other protective mechanisms, such as antithrombotic and anti-inflammatory functions. Research has additionally found a relationship between eating tomatoes and a lower risk of certain cancers (American Cancer Society, retrieved 2014) as well as other conditions, including cardiovascular disease, osteoporosis, ultraviolet light-induced skin damage, and cognitive dysfunction.

Table 1: Nutritional contents of Tomato (red tomatoes, raw)

Contents	**Per 100 g of *Lycopercicon escluenta***
Energy	74KJ(18KCal)
Carbohydrates	3.9g
Sugar	2.6g
Dietary fiber	1.2g
Fats	0.2g
Proteins	0.9g
Vitamins	
Vitamin A equivalent	5% (42µg)
Beta carotene	4% (449 µg)
Lutein zeaxanthin	123 µg
Thiamine(B1)	3% (0.037mg)
Niacin(B3)	4% (0.594mg)
Vitamin B6	6% (0.08mg)
Vitamin C	17% (14mg)
Vitamin E	4% (0.54mg)
Vitamin K	8% (7.9 µg)
Trace metals	
Magnesium	3% (11mg)
Manganese	4% (0.114mg)
Phosphorus	3% (24mg)
Potassium	5% (237mg)
Other constituents	
Water	94.5 g
Lycopene	2573mg

Source*:* *USDA Nutrient Database, 2009*

1.1.2 Tomato production

Tomato is a major horticultural crop with an estimated global production of over 120 million metric tons (F.A.O. 2007). About 161.8 million tons of tomatoes were produced in the world in 2012. China is the largest producer, accounted for about one quarter of the global output, followed by India and the United States. For one variety, plum or processing tomatoes, California accounts for 90% of U.S. production and 35% of world production (FAOSTAT, 2012). There are around 7,500 tomato varieties grown for various purposes.

Tomato is also one of the major commercial vegetable crops in Nepal (Ghimire *et. al*., 2000/ 2001). They grow best in terai, low and mid hills, and its demand is increasing in mid hills for cash generation (Pandey & Chaudhary, 2004). Total area and production of this crop in Nepal is 10,530 ha and 72,657 t respectively with an average productivity of 6.9 t ha-1 (Shrestha and Ghimire, 1996), which is very low as compared to the experimental yield of tomato in the country. The crop is grown in winter in the terai and inner-terai and can be grown in two seasons, spring and rainy in the low and mid hills of Nepal (Ghimire *et. al*., 2000/2001). Plastic house technology is one of the viable alternatives to produce quality tomato in the high hills (Chapagain *et. al*., 2010).

1.1.3 Tomato fungal diseases

Fungi are an important group of microorganisms responsible for various diseases of plants and cause a considerable loss in yield (Kharde *et. al*., 2010). Some species of fungus produce mycotoxins that are very toxic to humans. For e.g. Sphinganine-analog mycotoxins(SAM's) produced by *Fusarium moniliforme* of tomato inhibit *de novo* sphingo lipid (ceramide) biosynthesis *in vitro*, which leads to a variety of cellular responses, including accumulation of sphingoid bases in animal cells(Merrill *et.al*., 1997). Tomato diseases are caused by fungi, bacteria, virus and nematodes. Tomato diseases caused by fungi includes leaf blights, leaf spots, mildews, rots of (root, stem, fruit), wilt diseases, etc, and cause severe damage to crops. Different groups of fungi like *Alternaria, Septoria,*

Phytophthora, etc, are responsible to cause leaf disease. Disease on leaf causes degradation of photosynthetic area and loss of crop production. Species of *Fusarium* and *Verticillium* cause wilting disease. *Colletotrichum*, *Stemphylium*, etc. causes fruit rot diseases.

1.1.4 Tomato diseases in Nepal

Since 2010 more than 40 tomato diseases has been studied in Nepal. Report shows that disease in tomato is caused by both infectious and non infectious agents. It also reports that majority of disease causing agent is fungal pathogens infecting different plant parts. Here are the lists of fungal diseases of tomato in Nepal reported by NARC (Plant Pathology Division) from 2010 to 2013 (Table 2).

Table 2: Fungal diseases of tomato in Nepal

S.N	Date of isolation(yr)	Disease	Fungal Pathogen
1.	2010/11	Flower bud drying	*Cladosporium* sp.
		Blossom blight	*Cladosporium* sp.
		Root rot	*Rhizoctonia* sp.
		Root rot	*Rhizoctonia solani*
		Early blight	*Alternaria solani*
		Fruit rot	*Cladosporium* sp.
		Late blight	*Phytophthora infestans*
		Seedling disease	*Rhizoctonia* sp.
2.	2011/12	Wilt	*Fusarium solani*
		Leaf blight	*Rhizoctonia solani*
		Leaf spot	*Septoria lycopersici*
		Root rot	*Fusarium* sp.

3.	2012/13	Root rot	*Pythium* sp.
		Powdery mildew	*Leveilulla taurica*
		Root rot	*Rhizoctonia* sp.
		Leaf spot	*Septoria* sp.
		Root rot	*Fusarium solani*
		Late blight	*Phytopthora infestans*
		Leaf spot	*Cladosporium* sp,
			Colletotrichum sp.
		Root rot	*Rhizoctonia solani*
		Leaf blight	*Alternaria* sp.
		Bleaching/ necrosis	*Fusarium* sp.

Source: *NARC (Plant Pathology Division), Annual Report (2010- 2013)*

1.1.5 Test Pathogens

Four fungal pathogens were isolated from tomato leaf and were used for antifungal assay (Table 3).

a. *Alternaria sp. (*Nees.)

The study on the morphological characteristics of *Alternaria* sp. indicated the septate mycelium. Conidiophores of the fungus were formed singly or in groups, straight or flexuous, olivaceous brown. The conidia were solitary, straight or slightly flexuous, muriform and ellipsoidal tapering to a beak, pale or olivaceous brown, (157.08-171.36) μm in length and (71.4-85.68) μm width with 2 -7

transverse septa and 1-4 longitudinal septa. Colony color was greenish green; parasitic on tomato leaf causing leaf blight disease.

b. *Colletotrichum sp.* (Corda.)

Morphological characteristics of *Colletotrichum* sp. (Corda.) shows septate mycelium, acervuli disc shaped/cushion shaped (214.2-242.76 x 171.36-214.2) µm, waxy, sub epidermal, dark, spines or setae at the edges or among the conidiophores; conidiophores simple, elongate; conidia hyaline, 1-celled (114.2-128.52x85.67-99.96) µm ,oblong; parasitic on tomato leaf, associated with *Leveilulla taurica* causing powdery mildew of tomato. Fast growing mycelium (covers the PDA plate having diameter 9cm within 4 days. White mycelium at early stage of growth but darkens later with dark spots of acervuli on maturity.

c. *Stemphylium sp.* (Wallr.)

Mycelium of *Stemphylium* (Wallr.) is septate; conidiophores dark, simple, short, bearing (3-5) terminal conidia; conidia dark with cross and longitudinal septa, ovoid , smooth; parasitic on tomato leaf associated with *Leveilulla taurica* causing powdery mildew of tomato. Color of colony was light grey at the center with wooly colorless aerial mycelium at the early stage of growth but darkens into dark leathery mass at maturity.

d. *Verticillium sp.* (Nees.)

Fast growing mycelium, covers the PDA plate within 4 days, mycelium septate and branched. Conidiophores slender, branched, some branches verticilliate (in whorls); conidia ovoid to ellipsoid (42.84-57.12 x 28.56-42.84) µm, hyaline, 1-celled, borne in small cluster (generally 4 in number) apically, vascular parasites causing wilts of higher plants also parasitic on other fungi. Color green at centre surrounded by white fluffy mycelia at the early stage of mycelia growth.

Table 3: Characteristics of test pathogens

Name of fungus	Isolation date	Plant part used for isolation	Colony color	Mycelium character	Conidial character
Alternaria sp.	2071/1/25	Leaf	Greenish green	Septate, branched, hyaline	Conidia in acropetal chain or single, olive brown, cross and longitudinal septa, obclavate to ovoid, apical beak
Colletotrichum sp.	2071/5/12	Leaf	Faint brown with scattered black acervuli	Septate, branched	Conidia inside acervuli,1-celled, hyaline, oblong
Stemphyllum sp.	2071/4/28	Leaf	Ash color at early stage but darkens later	Septate, branched	Terminal3-5 dark conidia, cross and longitudinal septa, ovoid, smooth.
Verticillium sp.	2071/4/12	Leaf	Green mycelia surrounded by white fluffy mycelia	Septate branched	Terminal 4-5(in cluster) conidia on verticillate conidiophores, oval to ellipsoid, hyaline, 1 celled.

Source: *Barnett, 1960*

1.1.6 Fresh plant extracts

Higher plants are a treasure house of phytchemicals which serve as valuable drugs that has controlled several fetal disease worldwide (Umamekeshwari *et al.*, 2008). Many angiosperm plants are store houses of effective chemotherapeutants and biological screening of these plants has proved that these can be used for treating diseases (Tewari *et al.*, 1988).

Natural plant products are important sources of new agrochemicals for the control of plant diseases (Kagale *et al.,* 2004). Furthermore, biocides of plant origin are non-phytotoxic, systemic and easily biodegradable (Qasem & Abu-Blan, 1966). It is now known that various natural plant products can reduce populations of foliar pathogens and control the disease development. Additionally, these plant extracts have considered as potential and environmentally safe alternatives, and plays a vital role in integrated pest management programs (Bowers & Locke, 2004).

A number of plant species have been reported to possess natural substances that are toxic to several plant pathogenic fungi (Goussous *et al.,* 2010). Several higher plants and their constituents have shown success in plant disease control mechanisms, and are proved to be harmless and non-phytotoxic unlike chemical fungicides (Singh *et.al.,* 1983; Alam *et.al.,* 2002). Evidence suggests that plant extracts can be used against microbes causing diseases in plants. So, remarkable antifungal effect of plant extracts on the germination (Dubey, 1991), and extracts of plant parts for controlling the disease (Pandy *et.al.,* 1983; Chary *et.al.,* 1984; Singh and Dwivedi, 1990) have been reported earlier.

A large number of plants have been reported to possess fungi toxic properties against plant pathogens which could be exploited commercially with practically no residual or toxic effect on ecosystem (Kumar *et al.,* 2008).

1.1.7 Fermented plant product

Fermented products are the secondary metabolites produced by living organism (plants, animals, and microorganisms). Louis pasture was first to study the activity of microorganism causing fermentation process. These fermented products are natural antimicrobial products. Microorganisms that have been used in fermentation produce different antimicrobial metabolites including organic acids, hydrogen peroxide, ethanol, and diacetyl in addition to bacteriocins. Not only that, fermented products make a house for those anaerobic microbes or those already present in phylosphere that are fetal to plant pathogenic organisms.

Secondary metabolites with antimicrobial activity can be found in most organisms (Koehn and Carter, 2005) including: plants such as fruits, vegetables, seeds, herb, and spices; animal sources such as milk, eggs, and tissues (Bhatnagar and Kim, 2010) and microorganisms such as bacteria and fungi (Gyawali and Ibrahim, 2012). Recently, plants especially herbs and spices are given more attention in natural antimicrobial research (Hayek *et. al*., 2013). Secondary metabolites formed from fermentation of some plant parts also have inhibitory effect on causal agents of plant disease. Application of these fermented products as foliar spray and seed treatment may either reduce the infections or severity of diseases. Preparation and application of fermented plant products is an eco-friendly method, less costly and may be more effective than fresh plant extract. This is because fermented product contains extra secondary metabolites apart from the constituents of plant itself, also it consists the mass of other organisms that may be lethal to plant pathogens.

Here are the lists of three wild plants used to test the antifungal activity against four tomato test pathogens. These plants are used in both fresh and fermented form (Table 4).

Table 4: List of plants used in antifungal activity

Scientific Name	**Local Name**	**Common Name**	**Family**
Agave cantula L.	Kettuke	Century plant	Agavaceae
Ageratina adenophora (Spreng.) king & H. Rob.	Kalimunte	Eupatory, sticky snake root	Asteraceae
Melia azedarach L.	Bakaino	China cherry	Meliaceae

Source*:* *Keshab Shrestha, 1998*

1.1.8 Use of fungicides and their effects

Fungi are an important group of microorganisms responsible for various diseases of plants and cause a considerable loss in yield (Kharde *et. al*., 2010). A number of chemical fungicides are available in the market for the crop protection. Some of them are excellent in terms of efficacy and cost benefit. However their indiscriminate use has created the problems of air, soil and water pollution, development of resistance in target organisms and serious health hazards due to the toxicity of their residues. The use of synthetic chemicals as antimicrobial, for the management of plant diseases has undoubtly increased crop protection but with considerable deterioration of environmental quality and human health (Cutler and Cutler, 1999).

A large number of plants have been reported to possess fungi toxic properties against plant pathogens which could be exploited commercially with practically no residual or toxic effect on ecosystem (Kumar *et al*., 2008). There is a growing concern in recent years both in developed and developing countries, about the use of hazardous fungicides for controlling plant disease (Kadar *et. al*., 2014).

1.2 Objectives

General Objectives

- To test fungicidal effect of fresh plant extracts and fermented plant product against four fungal pathogens of tomato.

Specific Objectives

- To compare fungicidal effect of fresh plant extracts and fermented plant product with chemical fungicide mancozeb (0.2mg/ml) against four tomato pathogens.

- To compare fungicidal effect of plant extracts with fermented plant product against four tomato pathogens.

1.2 Justification of the study

Tomato is very nutritious vegetable which contains major essential nutrients required for human consumption. The nutrition present in it is available to consumer more when cooked then raw. Red pigment (lycopene) present in tomato act as natural antioxidant that prevents from harmful UV rays (BBC News retrieved, 2010), neutralizes free radicals, relieve the oxidative effect from diabetes (Valero *et.al,* 2011). Coumaric acid and chlorogenic acid present in tomato block the effect of nitrosamines. It also contains antithrombotic and anti-inflammatory properties Further, regular use of tomato in dish controls different diseases like cancer, cardiovascular disease, osteoporosis, ultraviolet light-induced skin damage, and cognitive dysfunction (Vegparadise.com. 2009, retrieved).

Tomato is the second largest vegetable crop produced after potato throughout the world. Total area and production of tomato in Nepal is 10,530 ha and 72,657 t respectively with an average productivity of 6.9 t ha-1 (Shrestha and Ghimire, 1996). Tomato can be grown in winter, spring and rainy seasons (Ghimire *et. al*., 2000/2001). The crop is grown in winter in the terai and inner-terai and can be grown in two seasons, spring and rainy in the low and middle hills of Nepal. Though tomato is best suited to the Terai, in low and mid hills, it is becoming increasingly attractive for cash generation in the high hills also (Pandey & Chaudhary 2004). From few decades ago, plastic house technology has encouraged the growers to cultivate tomato because it is one of the viable alternative means for quality tomato production in the high hills (Chapagain *et. al*., 2010). Plastic house tomato production technology is getting popularity among the farmers day by day as a new frontier in the eastern high hills of Nepal (Chapagain *et. al*., 2010).

Fungal pathogen causes maximum crop loss in tomato plant. These pathogens either infect leaves causing degradation of photosynthetic area or wilting the plant or causing the rot of fruit, stem or root, which later reduces the crop production. Among the test pathogens, *Alternaria* sp. causes leaf blight, *Colletotrichum* sp. and *Stemphylium* sp. causes fruit rot and *Vetticillium* sp. causes wilt disease in tomato. Early blight and fruit rot caused by *Alternaria solani* is one of the most catastrophic diseases incurring loss both at pre- and post-harvest stages in tomato (Naik *et. al*., 2010).

Excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide (Sharma and Parihar, 2010). Further, the cost of these fungicides is comparatively high and their application results in development of resistance in the pathogens against these fungicides (Nigam *et.al*., 1994). Apart from plant extracts and fermented products there are numerous alternative methods generated to control fungal pathogens instead of chemical compounds. E.g. Essential oils, biological control agents like bacteria, fungi, actinomycetes, etc.

Fresh plant extracts contains several organic compounds that are inhibitory to the wide range of plant pathogens. Fermented plant products contain secondary metabolites and also include mass of diverse microbes that may be lethal to the plant pathogens. These extracts and fermented products of plant either control the mycelia growth or spore germination later reducing disease intensity or severity. These plant products are locally available and can be prepared traditionally. And their application does not require complicated preventive measure as that of chemical fungicides.

Three wild and locally available plants viz. *Agave cantula*, *Ageratina adenophora* and *Melia azedarach* are used for experimentation. These plants are being mentioned by IPM (Integrated Pest Management) for the preparation of extract to control pathogens of different vegetables like Cauliflower, Cabbage, Chilly,

Brinjal, etc. And their preparation is traditional process in Sanga village of Kavre district.

1.4 Limitation of the study

- Pathogenecity test was not done because three of the test pathogens were found associated with other diseases. *Verticillium* sp. was found associated with *Septoria* sp. causing septoria leaf spot. Similarly, *Colletotrichum* sp. and *Stemphylium* sp. were found associated with *Leveilulla taurica* causing powdery mildew in tomato.
- MIC (Minimum Inhibition Concentration) was not performed.
- Conidial measurement of *Stemphylium* sp. was not performed.

CHAPTER II

2. LITERATURE REVIEW

Hadian, (2012) found Neem as the most efficient extract against two tomato pathogens i.e. *Fusarium oxysporum f.sp lycopercici* (98% inhibition) and *Rhizoctonia solani* (96% inhibition).

Strange and Scott, (2005) studied the comparative impact of plant pathogens on causing disease and crop production losses. Among the plant pathogens, fungi cause the greatest impact with regard to diseases and crop production losses. The losses include considerable foliage and post harvest losses of vegetables and fruits which are brought about by decay due to fungal plant pathogens.

Iannou, (2000) proved that *Fusarium oxysporum f.sp lycopercici* (Sacc.) Snyder and H.N. Hasen, is economically important wilting pathogen of tomato whereas Honda *et. al*., (1999) proved *Rhizoctonia solani* Kuhn, is one of the most soil borne plant pathogenic fungi in the world.

Osman and Rehiayam, (2003) found that inappropriate use of agrochemicals especially fungicides possesses adverse effect on ecosystem and a possible carcinogenic risk than insecticides and herbicides together.

Studies of Zhonghau and Michalidies, (2005) showed that resistance by pathogens to fungicides has rendered certain fungicide ineffective.

Singh *et. al*., (1983); Alam *et. al*., (2002) tested antifungal activities (In-Vitro) of some plant extracts and smoke on four fungal pathogens of different host which showed that several plant extracts and their constituents are successful in controlling plant diseases and are proved to be harmless and non-phytotoxic unlike chemical fungicides.

Rai, (2002) studied occurrence and in-vitro management of some seed borne pathogens of Maize and Sorghum. It showed neem extract was most potent among tested plant extract against *Fusarium moniliform* in sorghum.

Agbenin and Marley, (2006) analyzed in-vitro assay of some plant extract against *Fusarium oxysporum f.sp.Lycopercici* causal agent of tomato wilt that showed dry neem seed extract gave 100% inhibition of mycelia growth.

Study of Wilson *et. al.*, (1997) on evaluation of plant extract and essential oil for antifungal activity against *Botrytis cinerea* demonstrated that garlic extract act as good antifungal activity and it could be useful as post harvest treatment.

Singh and Singh, (2005) studied the management of mushroom pathogens through botanicals. From the study it was supposed that inhibition observed due to *Allium sativum* may be due to the presence of sulphur compounds and allicin.

Lee *et. al*., (2003) tested fungicidal activity of *ar*-turmerone identified in *Curcuma longa* against six phytopathogenic fungi and found *Curcuma* rhizome contains sesquiterpene ketone *ar*-turmerone that act as biological fungicides and confirms its usefulness as good disease control agents.

Carpinella *et. al*., (2003) tested antifungal effects of different organic extracts from *Melia azedarach* L. on phytopathogenic fungi and their isolated active compounds. The result from the study showed that extracts from different parts of *Melia azedarach* L. act as potential antifungal agents for selected phytopathogenic fungi and extracts from seed kernel were highly effective on *Rhizoctonia solani* and *Fusarium oxysporum*.

Abada *et. al*., (2008) studied the effect of some chemical salts on suppressing the infection by early blight disease of tomato on which study showed *Alternaria* fungus can cause disease on all the parts of plant(leaf blight, stem collar rot and fruit lesions) and resulting on severe damage during all the stages of plant development.

Kagale *et al.,* (2004) studied the antimicrobial activity and induction of systemic resistance in rice by leaf extract of *Datura metel* against *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae*. From the study it was analyzed that natural plant products are important sources of new agrochemicals for the control of plant diseases.

Study of Qasem & Abu-Blan, (1966) on fungicidal activity of some weed extract against different plant pathogens, furthermore emphasized that biocides of plant origin are non-phytotoxic, systemic and easily biodegradable.

Bowers & Locke, (2004) studied effect of formulated plant extracts and essential oils on population density of *Phytophthora nicotianae* in soil and Phytophthora blight in greenhouse. Study reports, various natural plant products can reduce populations of foliar pathogens and control the disease development, and then these plant extracts have a potential as environmentally safe alternatives and as components in Integrated Pest Management (IPM) programs.

Dushyent and Bohra, (1997) studied the effect of 11 different plant extracts on the mycelia growth of *A. solani* and found that leaf extracts of some plants, i.e. *Tamarix aphylla* and *Salsola baryosma*, totally inhibited the growth of the pathogen *in vivo*.

Wszelaki and Miller, (2005) determined the efficacy of disease management products in organically produced tomatoes and reported that garlic extracts significantly reduced the early blight disease on tomato.

Vijayan, (1989) from the study on early blight of tomato caused by *Alternaria solani* reported that the bulb extract of *Allium sativum*, leaf extract of *Aegle marmelos* and flower extract of *Catharanthus roseus* inhibited the spore germination and mycelia growth of *A. solani*.

Investigations on the mechanisms of disease suppression by plant products, studied by(Amadioha, 2000) suggested that the active principles present in plant extracts may either act on the pathogen directly or induce systemic resistance in host plants resulting in a reduction of the disease development (Kagale *et al.*, 2004).

Study of Nashwa and Abo-Elyousr, (2012) on evaluation of various plant extract against the early blight disease of tomato plants under greenhouse and field conditions indicates that the foliar sprays of tomato plants with plant extracts resulted in a significant reduction in early blight infection. It also demonstrated that many plant extracts, e.g. from *Ocimum basilicum*, *Azedarach indica*, *E. chamadulonsis*, *D. stramonium*, *N. oleander*, and *A. sativum*, can be used for the biocontrol of early blight disease.

Several authors including Curtis *et al.*, (2004); Krebs *et al.*, (2006) and Latha *et al.*, (2009) reported that plant extracts from 20 non-host plant species caused a reduction of the early blight disease and suppressed the mycelia growth of *Alternaria solani.* All treatments with tested plant extracts, improved the yield of tomato plants compared to the infected control.

Study of Dimoglo *et al.*, (1985) on structure activity correlations for the antioxidant and antifungal properties of steroid glycosides revealed that steroidal saponins present in *Agave americana* L. have antifungal activity against agricultural pathogens.

Data collected by USDA, (2009) on vegparadize.com reports lists of all the essential nutrients present in tomato.

BBC News, (retrieved 2010) reported that lycopene (red pigment) present in tomato fruit, improves the skin's ability to protect against harmful UV rays on regular consumption.

Valero *et.al,* (2011) studied the Meta-analysis on the role of lycopene in type 2 diabetes mellitus and reported that lycopene from tomatoes has no effect on the risk of developing diabetes, but may help relieve the oxidative stress of people who already have.

American Cancer Society, (retrieved 2014) studied the lycopene of tomato and found the relationship between eating tomatoes and a lower risk of certain cancers.

Food and Agriculture Organization, (F.A.O. 2007) estimated tomato as the major horticultural crop with an estimated global production of over 120 million metric tons.

Food and Agriculture Organization of United Nations, (2014) estimates about 161.8 million tonnes of tomatoes were produced in the world in 2012. China, the largest producer, accounted for about one quarter of the global output, followed by India and the United States. For one variety, plum or processing tomatoes, California accounts for 90% of U.S. production and 35% of world production.

Ghimire *et.al.,* (2000/2001) studied status of tomato yellow leaf curl virus in tomato in western hills of Nepal and found tomato as one of the most important vegetable crops grown from subsistence to commercial scale in Nepal. It also reports that the crop is grown in winter in the terai and inner-terai and can be grown in two seasons, spring and rainy in the low and middle hills of Nepal.

Study of Shrestha and Ghimire, (1996) explored total area and production of tomato in Nepal is 10,530 ha and 72,657 t, respectively with an average productivity of 6.9 t ha-1 which is very low as compared to the experimental yield of tomato in the country.

Pandey and Chaudhary, (2004) studied evaluation of tomato varieties and their planting dates for commercial production under Jumla agro-ecological condition. Their study reports that though tomato is best suited to the terai, in low and midhills, it is becoming increasingly attractive for cash generation in the high hills also.

Chapagain *e.t al*., (2010) studied up-scaling of polyhouse tomato production technology in mid and high hills of eastern Nepal. Their study reports plastic house tomato production technology is getting popularity among the farmers day by day as a new frontier in the eastern high hills of Nepal harmful side effects of chemical fungicides on human and environment.

Khadre *et. al.*, (2010) studied effect of plant extracts on fungal pathogens causing leaf blight of tomato in in vitro and reported fungi are an important group of microorganisms responsible for various diseases of plants and cause a considerable loss in yield.

Study of Bauman, (2007) reports that fungus *Claviceps purpurea* causes the ergot poisoning. An individual infected with the mycotoxin present in ergot experiences hallucination, gangrene, and blood flow restrictions in his/her limbs. Humans usually get infected with the fungus after eating cereal grains contaminated with *C. purpurea.*

Nepal Agriculture Research Council (NARC), Plant Pathology Division (2010/11), studied total 20 tomato diseases caused by different causal agents.

Among 20 tomato diseases, 11 of them were found fungal diseases. On ascending year (2011/12), total 14 tomato diseases were studied and 6 fungal diseases was reported. Similarly, on 2012/13, 10 tomato diseases were diagnosed from different regions of the country which reported 6 fungal diseases.

Kagale *et al.,* (2004) their report on antimicrobial activity and induction of systemic resistance in rice by leaf extract of *Datura metel* against *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* concluded, natural plant products are important sources of new agrochemicals for the control of plant diseases.

Fungicidal activity of some common weed extracts against different plant pathogenic fungi studied by Qasem J. R. and Abu-Blan H. A., (1996) resulted biocides of plant origin are nonphytotoxic, systemic and easily biodegradable.

Study of Bowers J.H and Locke J. C., (2004) on effect of formulated plant extracts and oils on population density of *Phytophthora nicotianae* in soil and control of Phytophthora blight in the greenhouse reported that various natural plant products can reduce populations of foliar pathogens and control the disease development, so that these plant extracts have a potential as environmentally safe alternatives and as components in integrated pest management programs.

Goussous *et. al.,* (2010) evaluated antifungal activity of several medicinal plants extracts against the early blight pathogen (*Alternaria solani*) and concluded that a number of plant species possess natural substances that are toxic to several plant pathogenic fungi.

Kumar *et. al*., (2008) studied on assessment of *Thymus vulgaris L*. essential oil as a safe botanical preservative against post harvest fungi infestation of food commodities and concluded that large number of plants possess fungi toxic

properties against plant pathogens which could be exploited commercially with practically no residual or toxic effect on ecosystem.

Singh *et. al*., (1983) studied on isolation and properties of fungi toxic principle from *Zingiber officinale* and Alam *et. al., (*2002) studied antifungal activities of some plant extracts and smoke on four fungal pathogens of different hosts and concluded that several higher plants and their constituents are successful in plant disease control and are harmless and non phytotoxic unlike chemical fungicides.

Dubey, (1991) evaluated fungicidal effect of essential oils of three higher plants on sclerotia of *Macrophomia phaseolina* and reported plant extracts shows remarkable antifungal effects on the germination of fungal spores.

Pandy *et. al.*, (1983) studied control of pestalotia fruit rot of guava by leaf extracts of two medicinal plants; Chary *et.al*. 1984 screened indigenous plants for their antifungal principles; Singh and Dwivedi 1990 evaluated economic efficacy of different fungicides for the control of leaf spot of cauliflower and come up with common conclusion that extracts of plant parts can effectively control plant diseases.

Study of koehn and Carter, (2005) on the evolving role of plant products on drugs discovery reports that natural products are chemical compounds or substance produced by a living organism or found in nature that possesses pharmacological or biological activity. Also they reported secondary metabolites with antimicrobial activity can be found in most organisms which include plants such as fruits, vegetables, seeds, herb, and spices.

Bhatnagar and Kim, (2010) from their study on immense essence of excellence: marine microbial bioactive compounds found that secondary metabolites with

antimicrobial activity can also be found in animal sources such as milk, eggs, and tissues.

Similarly, Gyawali and Ibrahim, (2012) studied impact of plant derivatives on the growth of food borne pathogens and the functionality of probiotics and reported secondary metabolites with antimicrobial can also be gained from microorganisms such as bacteria and fungi.

Study of Hayek *et. al.,* (2013) on antimicrobial products reports that at recently, plants especially herbs and spices are been given more attention in natural antimicrobial research.

Cutler and Cutler, (1999) after study on their biological active natural product concluded that use of synthetic chemicals as antimicrobial for the management of plant diseases undoubtly increases crop protection but on the other hand considerably deteriorate environmental quality and human health.

Kadar *et. al*., (2014) experimented in vitro effect of two fungicides on pathogenic fungi causing root rot of tomato in Algeria and reported that there is a growing concern in recent years both in developed and developing countries, about the use of hazardous fungicides for controlling plant disease.

Antifungal activity of extracts obtained from actinomycetes studied by Sharma and Parihar, (2010) concluded that excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide.

Study of Naik *et. al*., (2010) on morphological ,physiological, pathogenic and molecular variability among isolates of *Alternaria solani* from tomato reports early blight and fruit rot caused by *Alternaria solani* is one of the most catastrophic diseases incurring loss both at pre- and post-harvest stages in tomato.

Nigam *et. al.,* (1994) reported that the cost of chemical fungicides and antibiotics are comparatively high and their constant application results in development of resistance in pathogens against these fungicides and antibiotics.

Barnett, (1960) illustrated the key to identify the genera of imperfect fungi on book illustrated genera of imperfect fungi.

Shekawat and Prashad, (1971) gave the method to prepare fresh plant extracts for antimicrobial assay. In this method, fresh plant parts to be used for anti microbial test are weighed, cleaned, ground, filtered through whatmann No.1 filter paper, diluted and freshly used for antimicrobial assay.

Pundir Ram Kumar *et. al*., (2013) gave the method to prepare fermented plant product for antimicrobial assay. In this method, plant parts are cleaned, weighed, mixed with non-ionized NaCl, kept in air tight jar and incubated for 28 days at room temperature.

Grover and Moore, (1962) gave the formula to calculate the percent inhibition in mycelia growth was determined with the help of mean colony diameter.

Rao and Srivastava, (1994) introduced the poisoned food technique to perform antifungal assay.

CHAPTER III

3. MATERIALS AND METHOD

3.1 Materials

Three wild plants were used for the preparation of fresh extract and fermented product. All the glass wares, different equipments and chemicals used during experiments are mentioned in Appendix-I.

3.2 Methods

3.2.1 Collection of diseased plant parts

Diseased plant parts were collected from Sanga village of Kavre district. Diseased plants parts i.e. leaves were collected for isolation of pathogens. The infected plant parts were collected in different seasons i.e. from 2014-4-23 to 2014-7-14.

3.2.2 Preparation of PDA media

For preparing PDA media 100 gm of peeled potato was cut into small pieces and boiled for sometime in 500ml distilled water. The cooked potato pieces were filtered by muslin cloth, 20 gm dextrose was dissolved thoroughly and the volume of filtrate was maintained to 1000 ml by adding more water. Twenty gram of agar was added and stirred gently to get thoroughly mixed. The mouth of flask was covered with aluminium foil and tied with sterilized rubber. And the media was autoclaved for 30 minutes in 121℃ with 15 lbs pressure.

Similarly, Water agar (WA) was prepared by mixing 20 gm agar with distilled water and final volume of mixture was made 1000 ml. These contents after mixing was sterilized in autoclave with 15 lbs pressure at 121℃ for 15 minutes. Antibiotic was added after the media has cooled to 45-50℃.

3.2.3 Isolation of fungal pathogens

For the isolation of fungal pathogens, diseased plant parts with infection was cut and inoculated in water agar media after surface sterilization first with tap water

and then with 70% ethanol for 2 minutes. Final surface sterilization of infected leaf was done with sterilized distilled water. Inoculated WA media was incubated at room temperature for 4-5 days till the growth of mycelia and appearance of reproductive structures. After the growth of mycelia and conidia of selected fungal pathogens on WA media, mycelia with media were cut and re inoculated in PDA for pure culture. Subculture was done 3 times till pure culture of selected fungal pathogen was obtained. Isolation of fungal pathogens was done in different seasons i.e. from 2014-5-6 to 2014- 7-20.

3.2.4 Conidial measurement

Size of the conidia of test pathogens was measured using ocular micrometer. For the measurement of conidial size, ocular micrometer was first standardized with stage micrometer to obtain calibration factor. Size of the test fungus was calculated by multiplying calibration factor with the size gained from ocular micrometer. Calibration factor was calculated by using following formula given below:

$$\text{One ocular division (in } \mu\text{m)} = \frac{\text{No. of divisions on stage micrometer}}{\text{No. of divisions on ocular micrometer}} \times 100$$

Measurement of different conidia was carried out for 10 times to find the particular range of conidia. Calculation of conidial measurement of four test pathogens is mentioned in Appendix II.

3.2.5 Identification of test fungus

Four tests fungi were identified with the help of literature (Barnett, 1960 and Ellis, 1971).

3.2.6 Maintenance of pure culture

The pure culture of test fungi was preserved by sub culturing them in sterilized petriplates containing PDA, every week.

3.2.7 Preparation of one week old culture

One week old culture is required to test antifungal activity tests. It was prepared by transferring pure culture of fungus on sterilized petriplate containing PDA media and incubated at 25±2℃ for a week. Three petriplates of pure culture for each pathogen was maintained as stock culture. The stock culture was kept in refrigerator maintaining temperature 10℃. Inoculum disc from stock culture was used for further antifungal assays. The inoculums discs used for mycelia growth inhibition was from fourth batch culture.

3.2.8 Preparation of fresh plant extract

Fresh leaves of *Agave cantula*, *Ageratina adenophora* and *Melia azeradach* were used for antifungal assays. Extract of fresh leaves was prepared by little modification with the method of Shekawat and Prashad, (1971). The freshly collected leaves materials were washed separately with tap water and finally with repeated changes for three times with sterile distilled water. They were separately ground in 15% ethanol at the rate of 1mg/ml of leaf tissue in a sterilized mortar and pestle. The extract was passed through two layered muslin cloth. Then it was centrifuged at 5000g for 30 minutes and finally filtered sterilized through Whatman No. 1 filter paper. This formed the standard plant extract solution (100%). The extract obtained was not filtered through Seitz filter to eliminate contamination due to bacteria, instead PDA during each test was amended with antibiotic (Nemox).

3.2.9 Dilution of fresh plant extract

The extract was further diluted to make different concentrations i.e. 30%, 40%, 50% and 60% by adding distilled water. For 30% extract solution, 1.5ml stock solution was added with 3.5ml distilled water to make total 5ml solution. Similarly for 40%, 50% and 60% extract solution 2ml, 2.5ml and 3ml stock solution was added with 3ml, 2.5ml and 2ml distilled water was added respectively.

3.2.10 Preparation of fermented plant product

Ferment of selected three plant species was prepared by the method as described by Kumar *et al*. (2013). Washed healthy leaves were cut into pieces with the help of scissors. Leaf pieces were weighed 50 g in two layer followed by sprinkling of NaCl (non-iodized). The leaf pieces and salt were placed in alternating layer in a wide mouth jar. Heavy weight of the mixture was placed in the jar and pressed gently to squeeze out the juice. The jar was covered with sterile lid and incubated at room temperature for 28 days for completion of fermentation. The liquid at the bottom of jar forms the standard solution (100%).

3.2.11 Dilution of fermented plant product

The ferment was further diluted to make different concentrations i.e. 30%, 40%, 50% and 60% by adding distilled water same as for dilution of plant extract, i.e. for 30% extract solution, 1.5ml stock solution was added with 3.5ml distilled water to make total 5ml solution. Similarly for 40%, 50% and 60% extract solution 2ml, 2.5ml and 3ml stock solution was added with, 3ml, 2.5ml and 2ml distilled water respectively.

3.2.12 Antifungal assay

Determination of percent mycelia growth inhibition was performed by poisoned food technique method given by (Grover and Moore, 1962). Three ml (10%) of each concentration of fresh plant extract was poured into sterilized 50ml conical flask followed by adding 27ml melted PDA. The conical flask was swirled gently to allow thorough mixing of content. In control set of fresh extract (15%) ethanol extract was amended. Then the mixture was poured in 3 petriplates, 10 ml each with the help of sterilized measuring cylinder. After the solidification of media one inoculum disc of 4mm diameter (prepared with the help of sterilized cork borer) of the test fungus was aseptically inoculated upside down of each petriplates and incubated at temperature 25± 2℃ for 7 days in case of *Alternaria* sp. and *Stemphylium* sp. Similarly, 4 days of incubation for *Colletotrichum* sp. and *Verticillium* sp. For each control and treatment, 3 replicates were maintained for each test. Average diameter of fungal colony was measured on 7^{th} day of incubation for *Alternaria* sp. and *Stemphylium* sp. and 4^{th} day for *Colletotrichum* sp. and *Verticillium* sp. respectively. And the percentage of mycelia growth inhibition was calculated separately.

Similar process was applied for antifungal assay in case of fermented plant product except that control was maintained without addition of ferment on PDA. For positive control 0.2mg/ml Mancozeb was amended with PDA during antifungal assay.

3.2.13 Calculation of Mycelia Growth Inhibition

Percentage of inhibition in mycelia growth was determined with the help of mean colony diameter, and was calculated by using following formula given by (Rao and Srivastava, 1994):

$$\text{Percent Inhibition} = \frac{gc - gt}{gc} \times 100$$

gc= Growth of mycelium colony after incubation in control set

gt= Growth of mycelium colony after incubation in treatment set

3.2.14 Data analysis by t-test

Data was analyzed statistically using t-test from Excel 2007. Means of three replicates was calculated and compared. The inhibitory effect of plant extracts and fermented products were compared with that of positive control (0.2mg/ml Mancozeb). P value was observed <0.005. Average of mycelia growth of test pathogens was plotted against different concentration of plant extracts and fermented product in bar graph along with error bar**.**

CHAPTER IV

4. RESULTS

4.1 Isolation of test pathogens

Three fungal isolates were obtained from naturally occurring tomato leaves showing leaf blight (in case of *Alternaria* sp.) and leaf spot (in case of *Colletotrichum* sp., *Stemphylium* sp. and *Verticillium* sp.) symptoms. *Verticillium* sp. was found associated with Septoria leaf blight disease while *Colletotrichum sp*. and *Stemphylium sp*. was found associated with powdery mildews caused by *Leveilulla taurica.* The pathogens were identified based on the morphological characteristics (Barnett, 1960).

4.2 Antifungal activity of three wild plants against mycelia growth of test pathogens.

Three wild plant species (Table 4) were selected and evaluated for antifungal test against four tomato pathogens (Table 3). Selected plants were tested against pathogens in both forms i.e. as fresh plant extract (Shekawat and Prashad, 1971) and fermented (Pundir Ram Kumar *et al*., 2013) form. The result was compared with Mancozeb (0.2gm/ml). All the result shows increase in percent of inhibition of mycelia growth as increased in concentration of both plant extracts and ferment.

4.2.1 Inhibition of mycelia growth by fresh plant extract

Among the extracts of three plant species, *Agave cantula* showed effective inhibition of mycelia growth of test pathogens. It effectively inhibited the mycelia growth of three associated pathogens (*Colletotrichum* sp. *Stemphylium* sp. and *Verticillium* sp.) rather than *Alternaria* sp. in selected concentration (30%, 40%, 50% & 60%). Mancozeb (0.2mg/ml) inhibited the mycelia growth of *Alternaria* sp., *Colletotrichum* sp., *Stemphylium* sp. & *Verticillium* sp. by 63.30%, 100%, 74.02% and 58.33% respectively.

Table 5: Antifungal activity of ethanol extract from *Agave cantula* at different concentrations against test pathogens.

	% inhibition mycelia growth					
			Concentration of *Agave* extract			
Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	25.16*	28.12*	36.77*	42.58*
Colletotrichum sp.	0	100	100*	100*	100*	100*
Stemphylium sp.	0	74.02	81.95*	85.56*	87.45*	88.83*
Verticillium sp.	0	58.33	84.66*	85.56*	87.44*	88.11*

= p-value<0.05; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

Table 5 indicates that ethanol extract (15%) of *Agave cantula* was effective in inhibiting the mycelia growth of three associated tomato pathogens i.e. 100% inhibition at 30% concentration for *Colletotrichum* sp., 88.83% inhibition at 60% concentration for *Stemphylium* sp. and 88.11% inhibition at 60% concentration for *Verticillium* sp. *Agave cantula* was not effective in suppressing the mycelia growth of *Alternaria* sp. as compared to other test pathogens. It inhibited the growth of mycelia of *Alternaria* sp. by 42.58% at 60% concentration. But it is promising that it can control *Alternaria* sp. at higher concentration. A significant inhibitory effects of *A. cantula* on mycelia growth in four tomato test pathogens was evident ($P<0.05$).

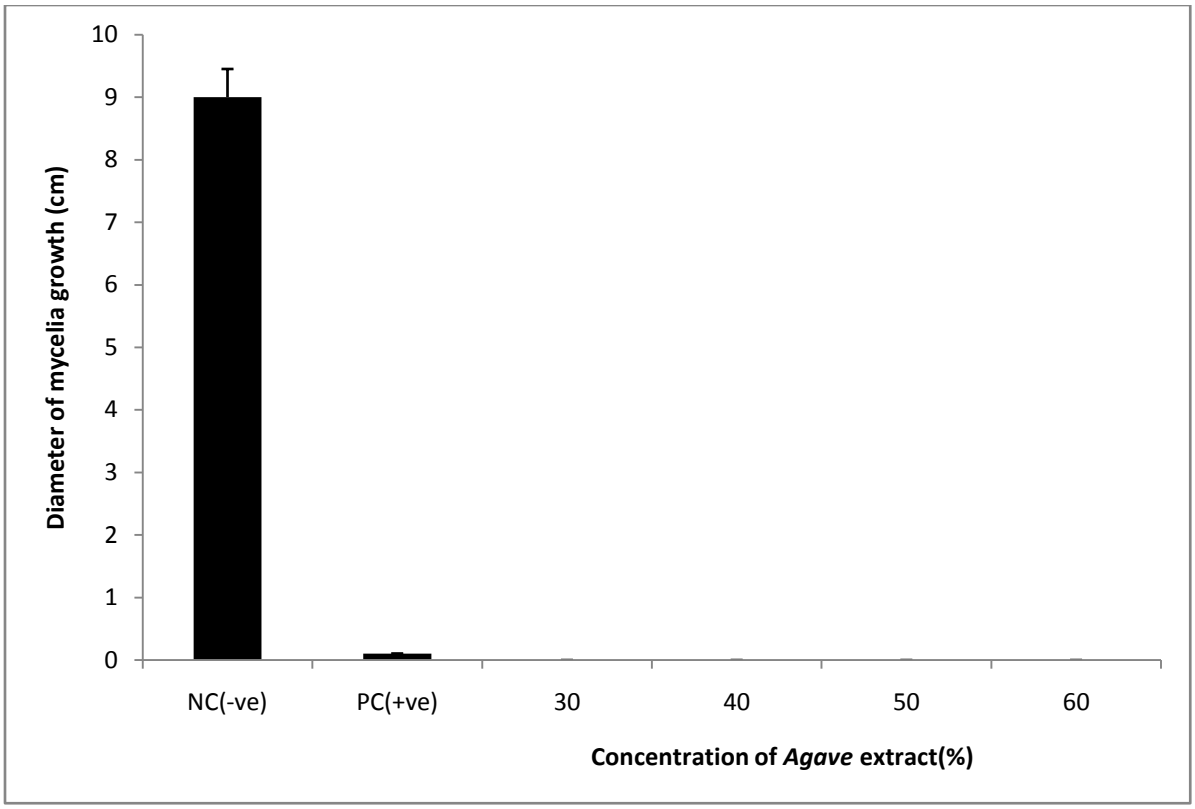


Fig1: Diameter of mycelia growth of *Colletotrichum* sp. on different concentration of *Agave* extract.

Figure 1 shows the growth of *Colletotrichum* mycelia on negative control 15% ethanol extract i.e. 9cm and that on positive control (0.2mg/ml) mancozeb less than 0.5 cm. The graph shows no growth of mycelia on given concentrations of *Agave* extract.

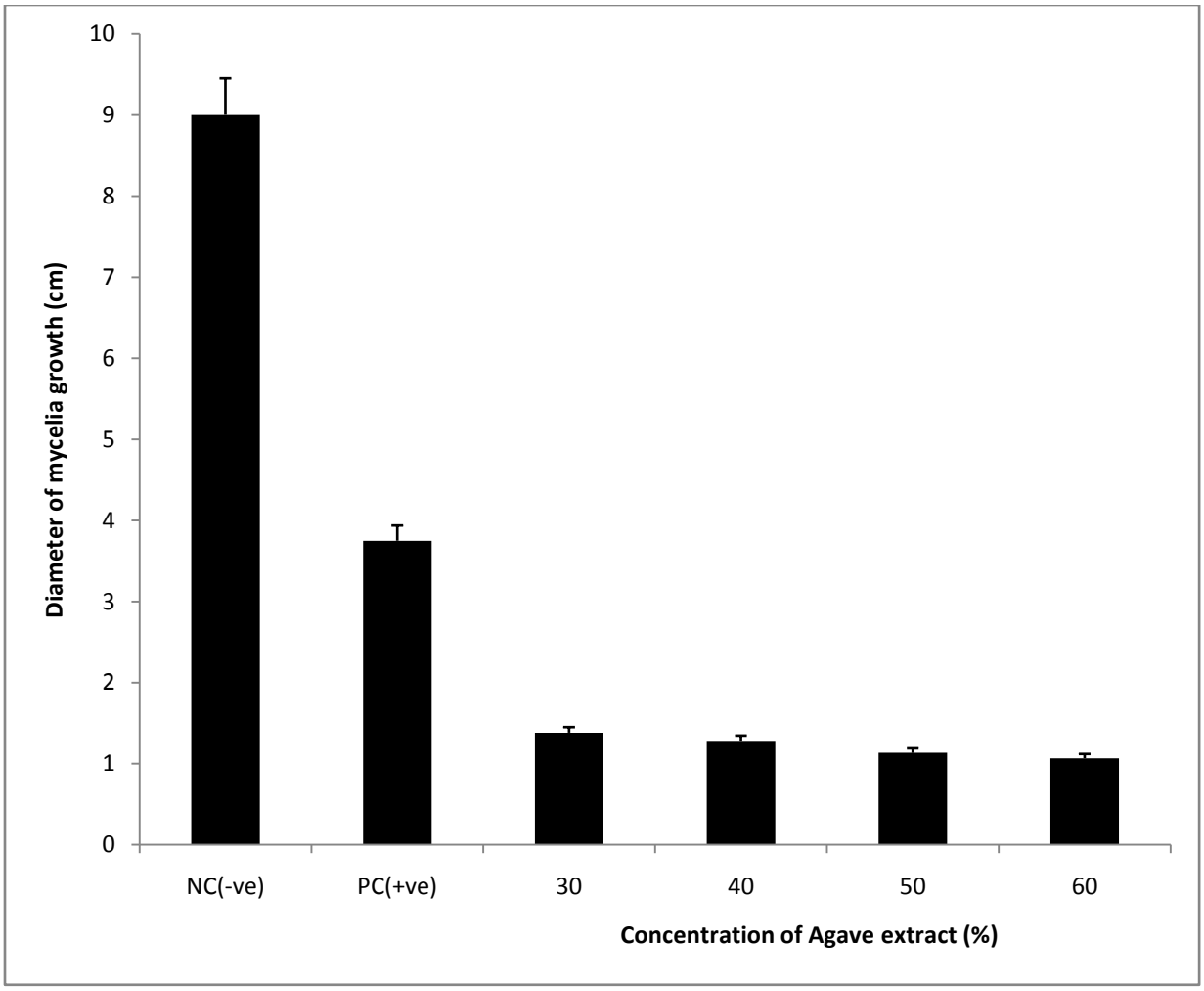


Fig 2: Diameter of mycelia growth of *Verticillium* sp. on different concentration of *Agave* extract

Graphical representation on figure 2 shows the growth of *Verticillium* mycelia on negative control, positive control (mancozeb 0.2mg/ml) and different concentration of *Agave* extract (30-40) %. Diameter of mycelia has reduced as increase in concentration of extract. **Error bar indicates statistically significant inhibitory effect to mycelia growth of *Verticillium* by all the concentrations of *Agave* extract.**

Table 6: Antifungal activity of ethanol extract from *Ageratina adenophora* at different concentrations against test pathogens

	% inhibition mycelia growth					
			Concentration of *Ageratina* extract			
Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	3.63*	5.09*	7.87*	9.69*
Colletotrichum sp.	0	100	0*	0*	0*	0*
Stemphylium sp.	0	74.02	12.33*	12.98*	14.02*	15.58*
Verticillium sp.	0	58.33	0*	0*	0*	0*

= p-value<0.05; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

In Table 6, *Ageratina adenophora* (15% ethanol extract) showed no inhibitory effect to the *Colletotrichum* sp. and *Verticillium* sp. at the selected concentrations. Least inhibitory effect was shown to *Alternaria* sp. i.e. 3.63%, 5.09%, 7.87% & 9.69% inhibition at concentrations 30%, 40%, 50% & 60% respectively. Similarly to *Stemphylium* sp., inhibition percent of mycelia growth was 12.33%, 12.98%, 14.02% & 15.58% at concentrations 30%, 40%, 50% & 60% respectively. T-test on the mycelia inhibition data showed significant effect of the plant extract of *A. adenophora* on test pathogens i.e. ($P<0.05$).

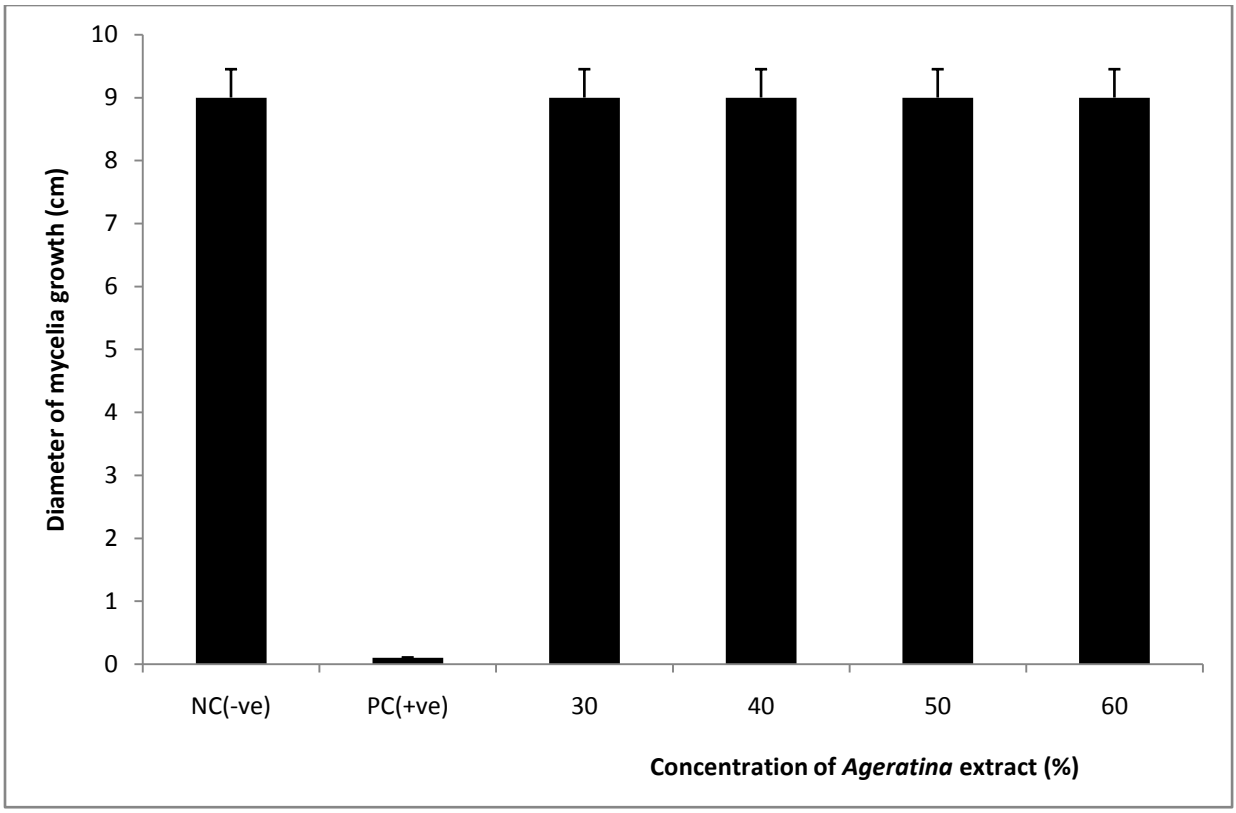


Fig 3: Diameter of mycelia growth of *Colletotrichum* sp. on different concentration of *Ageratina* extract

Bar graph on figure 3 represents effective inhibition of mycelia growth of *Colletotrichum* sp. by positive control mancozeb 0.2 mg/ml. It also represents no inhibitory effect to the mycelia growth of *Colletotrichum* sp. by *Ageratina* extract at any given concentrations.

Table7: Antifungal activity of ethanol extract from *Melia azedarach* L. at different concentrations against test pathogens

	% inhibition mycelia growth					
			Concentration of *Melia* extract			
Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	27.43*	28.37*	29.32*	31.08*
Colletotrichum sp.	0	100	0*	0*	0*	0*
Stemphylium sp.	0	74.02	16*	18.07*	19.55*	26.96*
Verticillium sp.	0	58.33	0*	0*	0*	0*

= p-value<0.005; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

As *Ageratina adenophora*, *Melia azedarach* also showed no inhibitory effect to the mycelia growth of *Colletotrichum* sp. and *Verticillium* sp. at selected concentrations (Table 7). Efficacy of *Melia* extract on suppressing the mycelia growth of *Alternaria* sp. was comparatively greater than that to the mycelia of *Stemphylium sp.* But the effectiveness is less than 50%.i.e mycelia of *Alternaria* was inhibited by 27.43%, 28.37%, 29.32% and 31.08% at concentrations 30%, 40%, 50% & 60% respectively. Similarly, mycelia growth of *Stemphylium* sp. was inhibited by 16%, 18.07%, 19.55% and 26.96% at concentrations 30%, 40%, 50% and 60% respectively. In both cases, inhibition percent was increased as increased concentration of ethanol extract. Statistical analysis indicates significant inhibitory effect of extract of *M. azedarach* on mycelia growth of test pathogens ($P<0.05$).

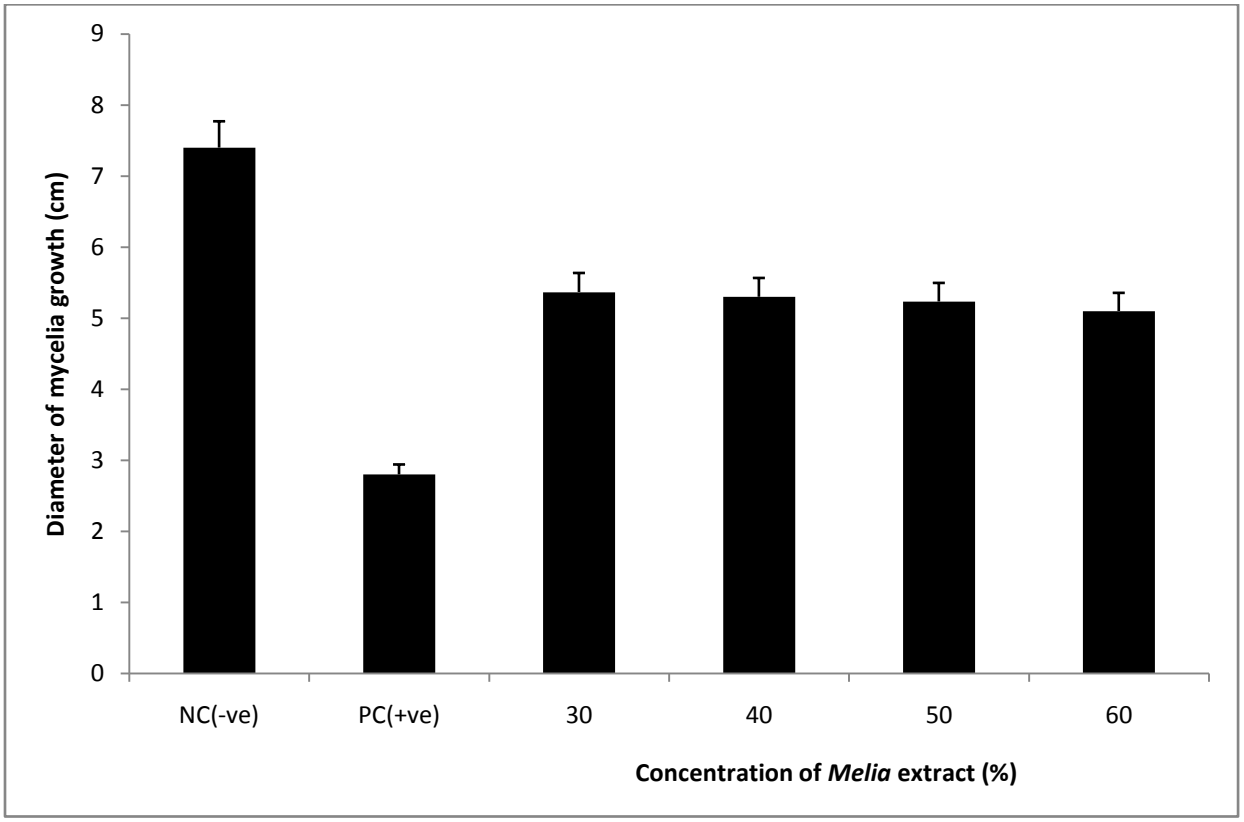


Fig 4: Diameter of mycelia growth of *Alternaria* sp. on different concentration of *Melia* extract.

Graphs on figure 3 indicates the mycelia growth of *Alternaria* sp. on negative control, positive control and different concentration of *Melia* extract. Less inhibitory effect to the growth of *Alternaria* was shown by *Melia* extract in comparision to positive control (0.2 mg/ml) mancozeb. The reduction in diameter of mycelia growth has increased as increased in concentration of extract but the difference is comparatively less between 10^{th} % concentration.

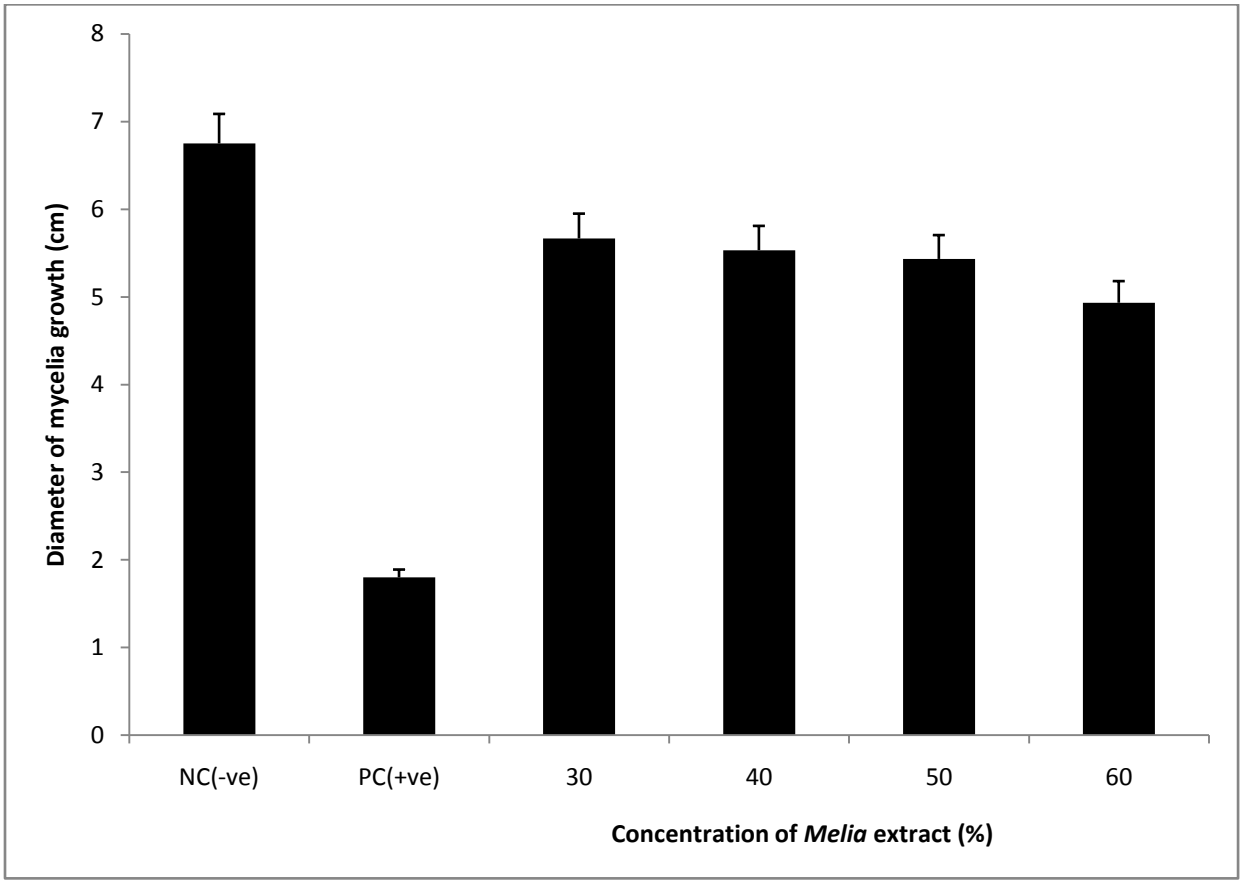


Fig 5: Diameter of mycelia growth of *Stemphylium* sp. on different concentration of *Melia* extract.

Figure 4 indicates less reduction in mycelia growth of *Stemphylium* sp. by *Melia* extract in comparison to positive control mancozeb (0.2mg/ml). But there is increased in growth reduction as increase in concentration of *Melia* extract.

4.2.2 Inhibition of mycelia growth by fermented plant product

In case of fermented product, ferment of *Agave cantula* showed maximum effective for the inhibition of mycelia growth of test pathogens followed by ferment of *Melia azedarach*. *A. cantula* suppressed the mycelia growth of *Colletotrichum* sp., *Stemphylium* sp. and *Verticillium* sp. effectively. Control for that of mycelia growth of *A. solani* was also effective but less than 50% inhibition.

Table 8: Antifungal activity of fermented product from *Agave cantula* at different concentrations against tomato leaf pathogenic fungi

	% inhibition mycelia growth					
			Concentration of *Agave* ferment			
Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	30.53*	32.76*	41.41*	44.29*
Colletotrichum sp.	0	100	88.11*	89.44*	91.44*	100*
Stemphylium sp.	0	74.02	52.21*	72.21*	77.86*	80.91*
Verticillium sp.	0	58.33	80*	82.44*	84.44*	88*

= p-value<0.005; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

Ferment of *Agave cantula* (Table 8) showed greater inhibitory effect on the mycelia growth of *Colletotrichum* sp. followed by *Verticillium* sp. i.e. it inhibited mycelia growth of *Colletotrichum* sp. by 88.11%, 89.44%, 91.44% and 100% at concentrations 30%, 40%, 50% and 60% respectively. Similarly, *Agave* ferment suppressed growth of mycelia of *Verticillium* sp. by 80%, 82.44%, 84.44% and 88% at concentrations 30%, 40%, 50% and 60% respectively.

Inhibition of mycelia growth of *Stemphylium* sp. was found maximum at 60% i.e. 80.91% inhibition. Similarly at 30%, 40% & 50% concentrations the inhibition was 52.21%, 72.21% & 77.86% respectively.

Lesser effect of *Agave* ferment was shown to the mycelia growth of *A. solani* i.e. at 30%, 40%, 50% and 60% concentration inhibition was 30.53%, 32.76%, 41.41% & 44.29% respectively. Ferment of *A. cantul*a showed significant inhibitory effect on the mycelia growth of test pathogens (P<0.05).

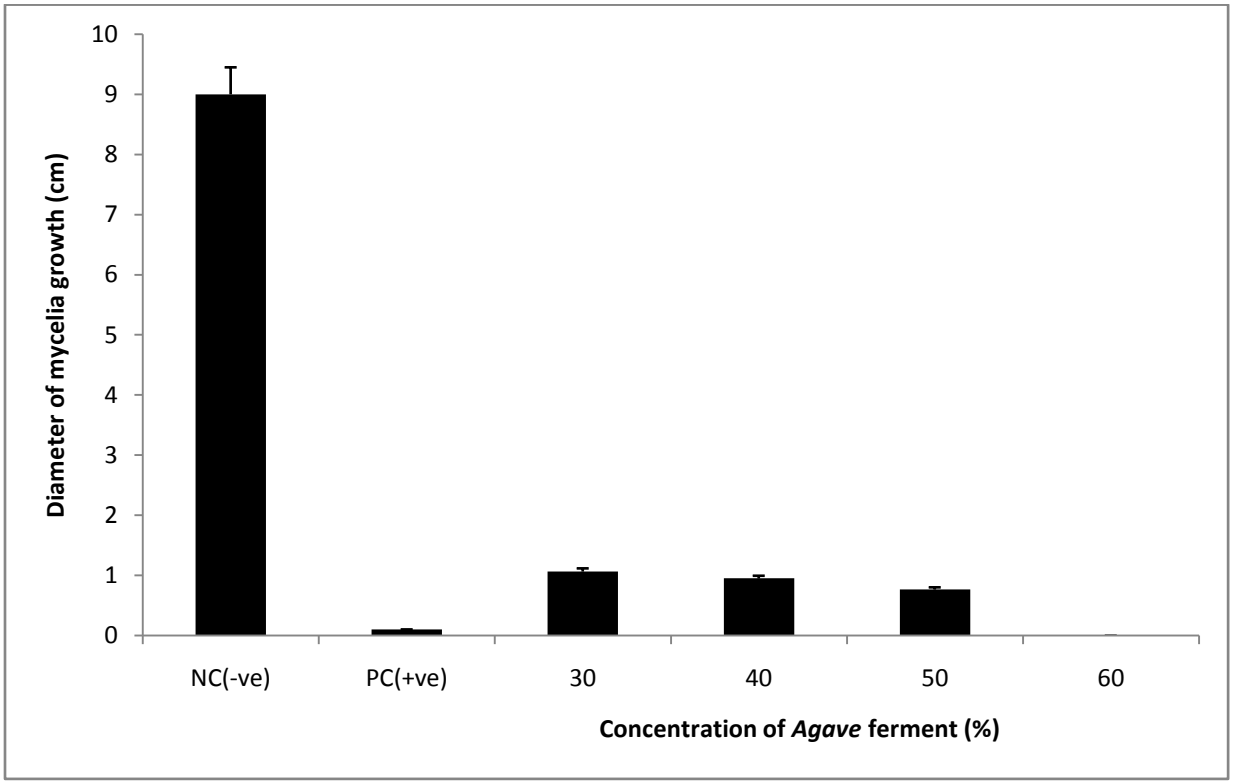


Fig 6: Diameter of mycelia growth of *Colletotrichum* sp. on different concentration of *Agave* ferment

Figure 5 indicates that mycelia growth of *Colletotrichum* on positive control was less than 0.5 cm. Among the different concentration of *Agave* ferment, it has significant inhibitory effect on mycelia growth of *Colletotrichum* at 30% concentration. Diameter of mycelia has decreased from 30 to 50% as increase in cocentration of *Agave* ferment and no growth at 60% concentration.

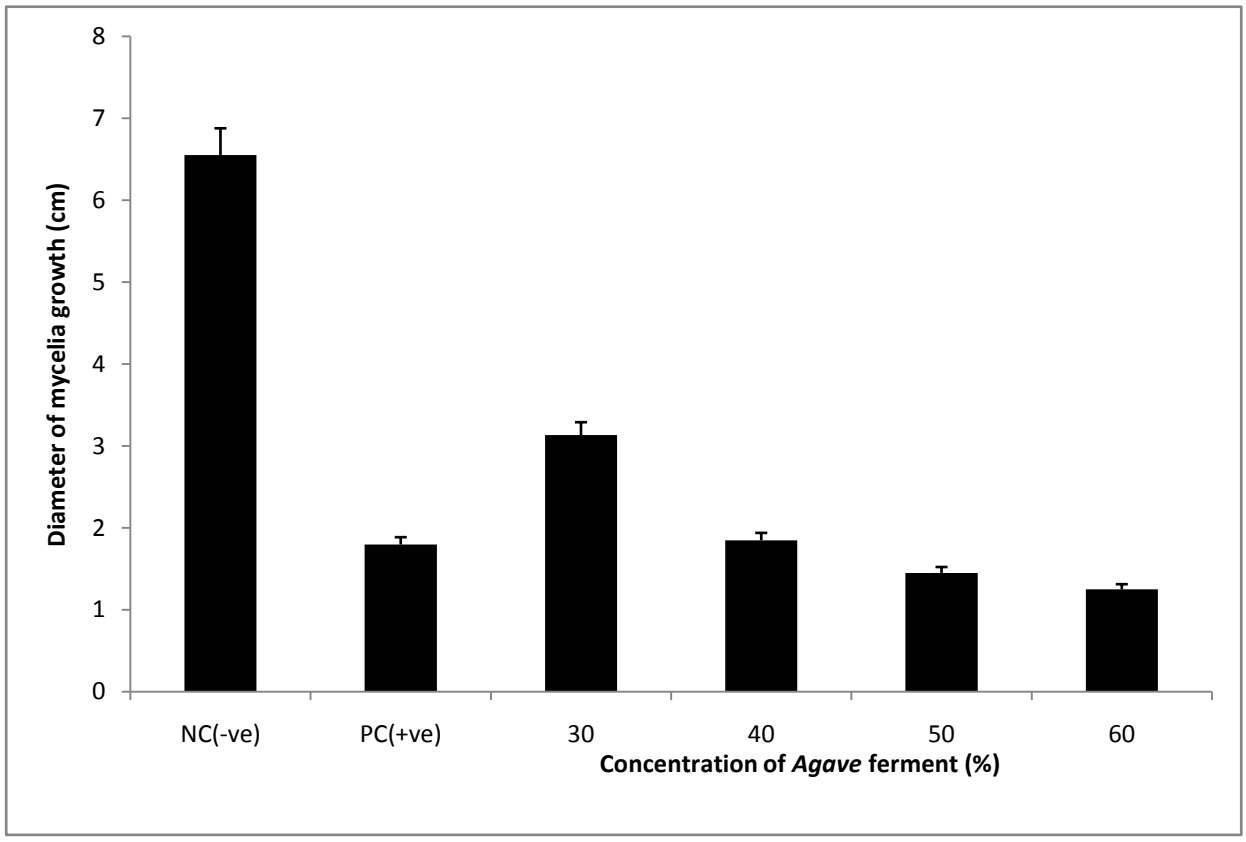


Fig 7: Diameter of mycelia growth of *Stemphylium* sp. on different concentration of *Agave* ferment

In figure 6, there is significant inhibitory effect shown by 40%, 50% and 60% concentration of *Agave* ferment on mycelia growth of *Stemphylium* than by 30% concentration. There is increase in inhibitory effect on mycelia growth of *Stemphylium* as increase in concentration of *Agave* ferment.

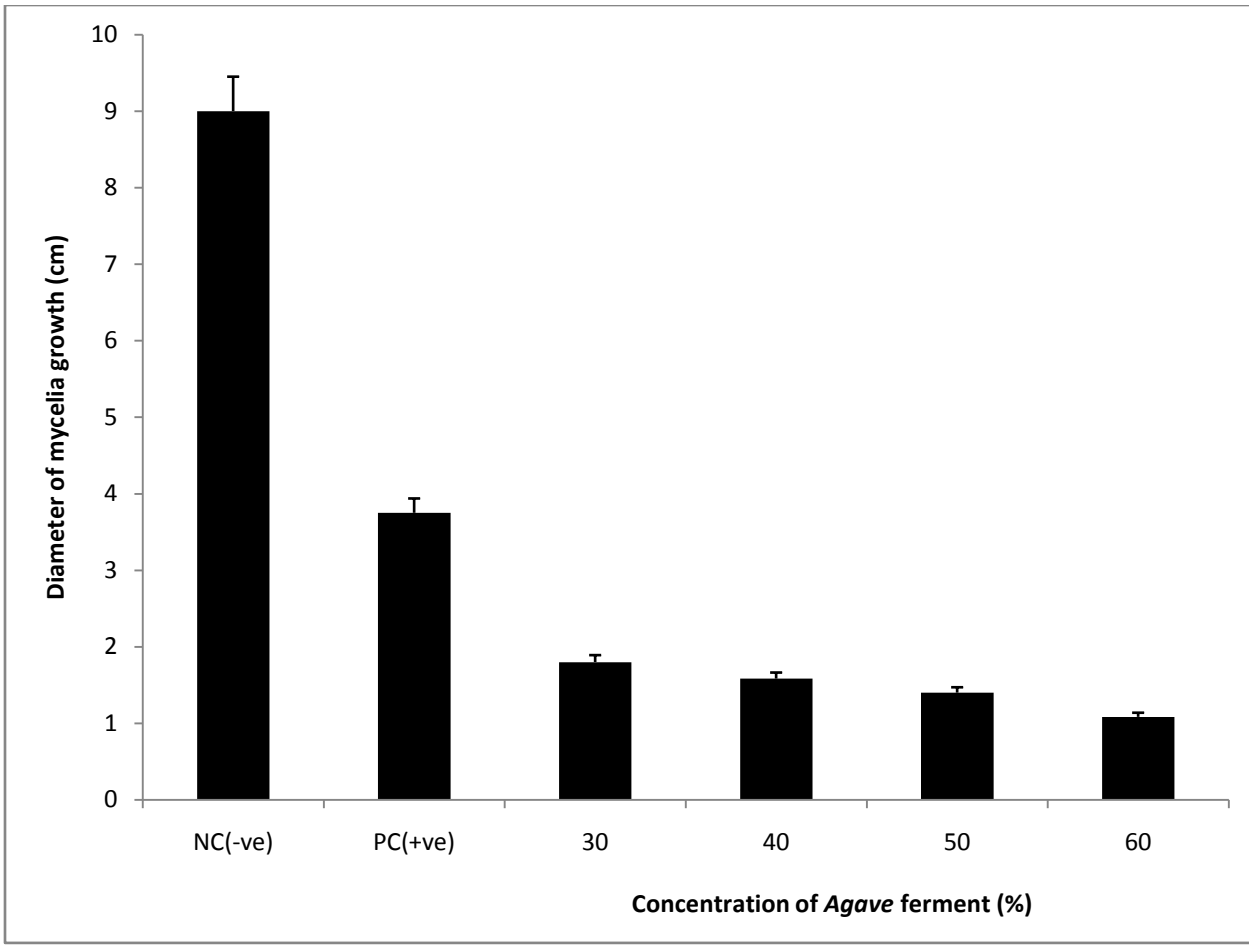


Fig 8: Diameter of mycelia growth of *Verticilllium* sp. on different concentration of *Agave* ferment

Figure 7 indicates significant inhibitory effect to mycelia growth of *Verticillium* by *Agave* ferment. There is increase in inhibition of mycelia growth as increase in concentration of *Agave* ferment. Error bar at 30% indicates significant inhibitory effect to the mycelia growth of *Verticillium* sp.

Table 9: Antifungal activity of fermented leaf extract from *Ageratina adenophora* at different concentrations against tomato leaf pathogenic fungi

	% inhibition mycelia growth					
			Concentration of *Ageratina* ferment			
Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	1.69*	2.22*	3.52*	3.66*
Colletotrichum sp.	0	100	0*	0*	0*	0*
Stemphylium sp.	0	74.02	28.04*	29.02*	31.48*	31.98*
Verticillium sp.	0	58.33	0*	0*	0*	0*

= p-value<0.005; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

Ageratina ferment (Table 9) showed no effectiveness in reduction of mycelia growth of *Colletotrichum* sp. and *Verticillium* sp. Full growth of mycelia was observed over PDA media. It suppressed the growth of mycelia of *Stemphylium* sp. by 28.04%, 29.02%, 31.48% and 31.98% at concentrations 30%, 40%, 50% and 60% respectively. Comparatively lesser effect was shown to the mycelia growth of *Alternaria* sp. i.e. 1.69%, 2.22%, 3.52% & 3.66% inhibition at 30%, 40%, 50% and 60% respectively. The observation suggests that the fungicidal effect of *Ageratina* ferment have been found promising against tomato pathogens like *Alternaria* and *Stemphylium*. And the effect can be increased further by using the ferment at higher concentrations. Statistical analysis showed significant effect of *A. adenophora* ferment on mycelia growth of test pathogens ($P<0.05$).

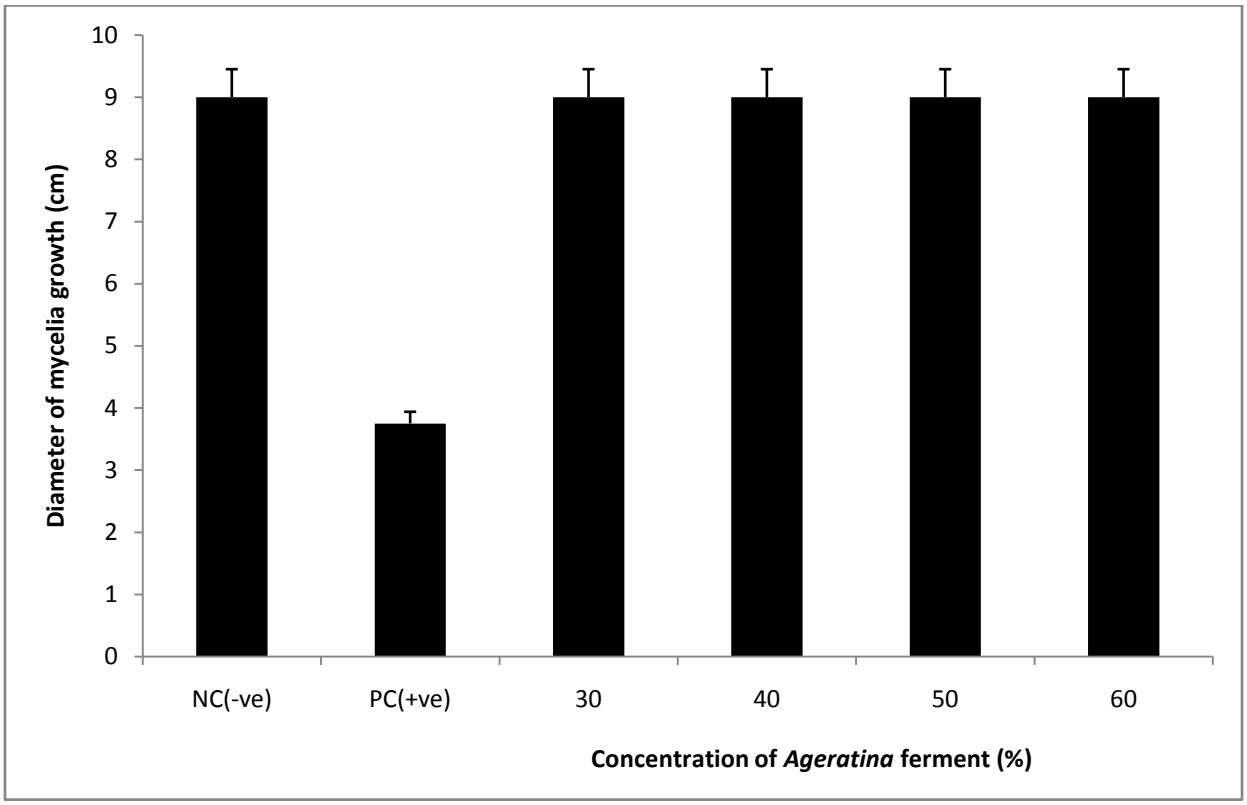


Fig 9: Diameter of mycelia growth of *Verticilllium* sp. on different concentration of *Ageratina* ferment

Figure 9 represents the antifungal activity of Ageratina ferment against mycelia growth of Verticillium sp. Bar graph shows comparatively greater inhibitory effect of positive control 0.2 mg/ml mancozeb rather than Ageratina ferment (0% inhibition).

Table 10: Antifungal activity of fermented leaf extract from *Melia azeradach* at different concentrations against tomato leaf pathogenic fungi

	% inhibition mycelia growth
	Concentration of *Melia* ferment

Fungal strains	NC (Ethanol)	PC (DW)	30	40	50	60
Alternaria sp.	0	63.3	6.25*	7.75*	11*	11.87*
Colletotrichum sp.	0	100	17.44*	18.33*	20.22*	23.33*
Stemphylium sp.	0	74.02	24.59*	28.19*	37.7*	42.29*
Verticillium sp.	0	58.33	0*	0*	0*	0*

= p-value<0.005; NC= negative control; PC= Positive control

Each treatment was replicated three times. Means followed by same letter within the row do not differ significantly at p<0.05 according to t-test Excel 2007.

Melia ferment in Table 10 results no effective for the inhibition of mycelia growth of *Verticillium* sp. i.e. 0% inhibition at all concentrations. It showed comparatively greater efficacy in reducing mycelia growth of *Stemphylium* sp. followed by *Colletotrichum* sp. but less than 50% inhibition. In *Stemphylium* sp., 24.59%, 28.19%, 37.70% and 42.29% was inhibited at concentrations 30%, 40%, 50% & 60% respectively. Similarly in *Colletotrichum* sp. 17.44%, 18.33%, 20.22% and 23.33% was inhibited at 30%, 40%, 50% and 60% concentrations. *Melia* ferment showed comparatively lesser effect in controlling mycelia growth of *Alternaria* sp. i.e. 6.25%, 7.75%, 11% & 11.87% inhibition at 30%, 40%, 50% and 60% concentrations. Ferment of *M. azedarach* can be effective against *Stemphylium*, *Colletotrichum* and *Alternaria* on higher concentration. There is significant effect of *M. azedarach* on test pathogens ($P<0.05$).

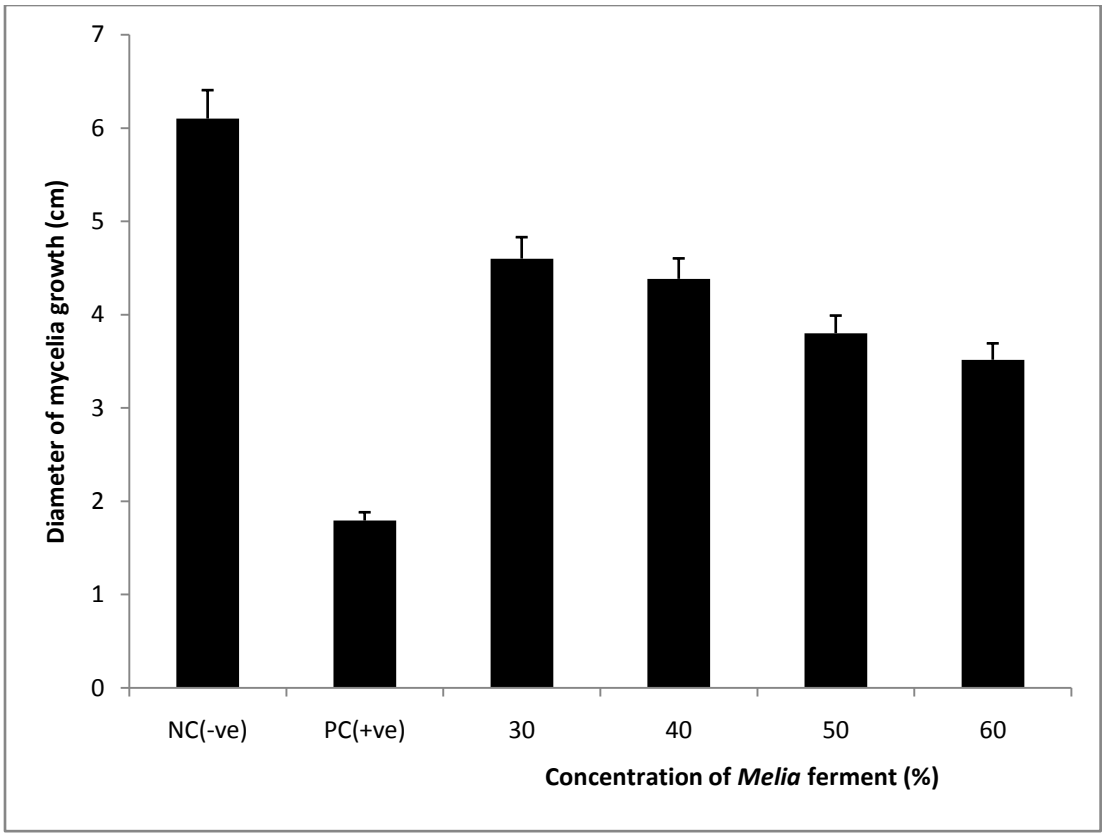


Fig 10: Diameter of mycelia growth of *Stemphylium* sp. on different concentration of *Melia* ferment.

Figure 8 shows less inhibitory effect to mycelia growth of *Stemphylium* by different concentration of *Melia* ferment. But there is increase in inhibition of mycelia growth of *Stemphylium* by *Melia* ferment.

CHAPTER V

5. DISCUSSION

Among the isolated fungal test pathogens, only the *Alternaria* sp. was isolated from the infected leaf that shows characteristic symptoms caused by alternaria leaf blight. Remaining three fungal pathogens was found associated with other tomato fungal pathogens. From the experimentation, *Verticillium* sp. was found associated with Septoria leaf blight disease while *Colletotrichum* sp. and *Stemphylium* sp. was found associated with powdery mildews caused by *Leveilulla taurica.* These associated pathogens do have the indirect effect on increasing disease severity on the crops.

Although Pathogenecity test (Koch's Postulates) is one of the methods to identify certain pathogenic fungi. Pathogenecity test of *Alternaria* sp. causing alternaria leaf blight was only able to perform and not the rest three associated pathogens (*Colletotrichum* sp., *Stemphylium* sp. and *Verticillium* sp.)

Agave cantula, Melia azeradach, Ageratina adenophora with other wild plant species like *Artemisia sp.* and *Azeradach indica* in the form of mixture either as fresh extract or in the form of ferment mixed with cow urine is traditionally applied in sanga village of Kavre district to control vegetables like cauliflower, cabbage, chilly, potato (small scale field), etc after the influence of IPM program. The process to prepare both forms either fresh or ferment does not need any sophisticated precautions because they are natural plant products, locally available to farmers and can be easily prepared.

Mancozeb is commonly and excessively used fungicide used at Sanga village and other neighbor villages to control leaf spot, leaf blight and fruit rot diseases in tomato and also potato. Mancozeb 0.2mg/ml is standard concentration recommended by manufacturing company. Mancozeb (0.2mg/ml) inhibited the mycelia growth of *Alternaria* sp., *Colletotrichum* sp., *Stemphylium* sp. & *Verticillium* sp. by 63.30%, 100%, 74.02% and 58.33% respectively. The

fungicide seems effective in controlling fruit rot diseases caused by *Colletotrichum* sp. and *Stemphylium* sp. rather that leaf spot disease caused by *Alternaria* sp. and wilt disease caused by *Verticillium* sp.

Even if, it is better to find MIC (Minimum Inhibitory Concentration) value for valuable results and recommendations but due to limitation of time and laboratory facilities, it was unable to perform.

It is the better method to eliminate bacterial and fungal contamination and reduce the degradation of extract and fermented product. From the experimentation it was observed that membrane filter was unable to filter extracts from water solvents. It filtered the solvent extracts of higher concentration but not with less concentration i.e.15% ethanol. During filtration, viscous layer blocked the membrane filter for further filtration. So, as an alternative, antibiotic Nemox was used to make the media bacteria free.

Little modification to the method given by Sekwat and Prashad (1971) was done during preparation of fresh plant extract. Ethanol with 15% was used instead of 100% water so as to dissolve organic compounds present in plant parts. On the other hand, only 15% ethanol was used because 30% ethanol completely inhibited the mycelia growth of fungal test pathogens.

Ageratina adenophora contains a novel quinic acid derivative; 5-0-trans-0-coumarolquinic acid methyl ester (1), together with three known ones, chlorogenic acid methyl ester (2), macranthoin F (3) and macranthoin G(4). These organic compounds were isolated from aerial parts of A. adenophora. Compound (1) was found to act as antifungal activity against spore germination of Magnoporthe grisea with IC (Inhibition Concentration) value 542.3µm. These four compounds were also tested for their antioxidant activity against DPPH (1, 1, diphenyl-2-picrylhydrazyl) radical (Zang et. al., 2013). Six compounds namely β-sitosterol(1), β-amyrin(2), Ursolic acid(3), Benzoic acid(4), 3,5-dimethoxybenzoic acid (5) and Maesol(6) were isolated from the chloroform

fraction through column chromatography from leaves extract of *Melia azeradach*. All compounds showed their highest antifungal activity after 24 hours of incubation against *Ascochyta rabiei* that cause chickpea blight disease.

Both forms of *Agave cantula* i.e. ferment and its extract form are effective in inhibiting the mycelia growth of three fungal test pathogens (*Colletotrichum* sp., *Stemphyilum* sp. and *Verticillium* sp.) comparing to *Melia* and *Ageratina*. It may be effective to *Alternaria* sp. at higher concentrations. Chemical analysis of *Agave* spp. reveals an important richness of phenolic compounds, including flavonoids, homoisoflavonoids, and phenolic acids which are effective on broad diversity of biological activities associated to those compounds, as antioxidant, antibacterial, antifungal, antinematod, and immuno-modulatory capabilities. The phenols of *Agave* also have a major potential as specific chemotaxonomic markers (Almaraz- Abarca *et. al*., 2013). Antioxidant and antifungal properties of steroid glycosides revealed that steroidal saponins present in *Agave americana* L. have antifungal activity against agricultural pathogens (Dimoglo *et al.*, 1985).

Alternaria sp. causing the leaf blight in tomato plant causes great crop loss. It is considered as the major tomato fungal pathogens. Early blight and fruit rot caused by *Alternaria solani* is one of the most catastrophic diseases incurring loss both at pre- and post-harvest stages in tomato (Naik *et. al*., 2010). Among the plants used for antifungal assay against *Alternaria* sp., both forms of *Agave cantula* inhibited the mycelia growth of *Alternaria* (60% extract inhibited by 42.58% and 60% ferment inhibited by 44.29%) followed by extract of *Melia azeradach* at 60% inhibited mycelia growth by 31.08%. Organic compounds and antifungal properties present in *Melia* plant species remains less effective in controlling mycelia growth of *Alternaria* sp. So, mancozeb 0.2 mg/ml remains greater effective in controlling mycelia growth of *Alternaria* sp. (63.30% inhibition) compared to extract and ferment of selected plant species.

Colletotrichum sp. causes fruit rot disease in tomato. Although it causes fruit rot disease, it was isolated from infected tomato leaf associated with powdery mildews caused by *Leveilulla taurica.* Mycelia growth of *Colletotrichum* sp. was completely inhibited by extract and ferment of *Agave cantula.* Ferment of *Melia azeradach* showed less effectiveness in reducing mycelia growth of *Colletotrichum* sp. Extract of *Melia & Ageratina*, similarly ferment of *Ageratina* was not effective in controlling mycelia growth of *Colletotrichum* sp. Extract and ferment of *Agave* showed similar inhibitory effect to the mycelia growth (100% inhibition) as that by 0.2 mg/ml Mancozeb.

Stemphylium sp. also causes fruit rot disease in tomato. It was also found associated with powdery mildews caused by *Leveilulla taurica* from infected leaf. Extract and ferment of *Agave cantula* inhibited mycelia growth of *Stemphylium* by 88.83% and 80.91% at 60% concentration respectively. Extract of *Agave* had greater effectiveness in suppressing mycelia growth of *Stemphylium* as compare to its ferment form. Comparing extract and ferment of *Ageratina* and *Melia*, ferment form of both plant species had greater inhibitory effect to the mycelia growth of *Stemphylium* sp. At 60% concentration, extract and ferment of *Ageratina* suppressed mycelia growth of *Stemphylium* sp. by 15.58% & 31.98% respectively. Similarly, that of *Melia* suppressed it by 26.96% & 42.29% respectively. Both extract and ferment of *Agave cantula* showed greater controlling effect to the mycelia growth of *Stemphylium* than 0.2 mg/ml Mancozeb (74.02% inhibition).

Verticillium sp. is one of the important wilt causing fungus. Extract and ferment of *Ageratina* and *Melia* had no inhibitory effect on mycelia growth of *Verticillium* sp. while that of *Agave* inhibited by 88.11% at 60% extract solution and 88% at 60% ferment solution. Ferment of *Agave* in case of *Verticillium*, showed greater efficacy in controlling mycelia growth than extract solution. But both forms of *Agave* had greater inhibitory effect to the mycelia growth of *Verticillium* sp. as compared to 0.02 mg/ml Mancozeb (58.33% inhibition).

CHAPTER VI

6. CONCLUSION

Chemical fungicides are toxic to non target organism, environment polluters, harmful to human and other animal if not used scientifically. Their regular use develops resistance in pathogens. They are costly for farmers with low economic condition. Locally available plant extracts and fermented products are eco friendly and does not have side effect on human and environment.

Fresh extract and ferment of *Agave cantula* inhibited mycelia growth of test pathogens (*Colletotrichum, Stemphylium* and *Verticillium* spp.) significantly. It may be effective to *Alternaria* sp. at higher concentration. So, the extract and ferment of *A. cantula* were promising compared to *Ageratina adenophora* and *Melia azeradach*.

In case of *Ageratina*, 15% ethanol extract had no inhibitory effect to the mycelia growth of *Colletotrichum* sp. and *Verticillium* sp. It has less effect on reducing the growth of *Alternaria* sp. and *Stemphylium* sp. Its ferment form also has no inhibitory effect to mycelia growth of *Colletotrichum* sp. and *Verticillium* sp. but less effect to that for *Alternaria* sp. Its ferment form at higher concentration can effectively reduce the mycelia growth of *Stemphylium* sp.

As *Ageratina*, 15 % ethanol extract of *Melia* also has no inhibitory effect to the mycelia growth inhibition of *Colletotrichum* sp. and *Verticillium* sp. while less effect to the mycelia growth of *Alternaria* sp. and *Stemphylium* sp. Ferment of *Melia* has no inhibitory effect to the mycelia growth of *Verticillium* sp. while less to the mycelia of *Alternaria* sp. But higher concentration of *Melia* ferment can effectively reduce the mycelia growth of *Colletotrichum* sp. and *Stemphylium* sp.

So, *Agave* ferment and its extract form are effective in inhibiting the mycelia growth of three fungal test pathogens (*Colletotrichum* sp., *Stemphyilum* sp. and *Verticillium* sp.) comparing to *Melia* and *Ageratina*. It may be effective to *Alternaria* sp. at higher concentrations followed by *Melia* extracts.

CHAPTER VII

7. RECOMMENDATIONS

The extract and ferment of *Agave cantula* can be experimented in field as foliar spray in higher concentration to control tomato pathogens.

The solution of this plant source can also be used to test *in vitro* to control spore germination and also seed pathogens of tomato by seed treatment.

Agave cantula contains some allergic substances which lead to appearance of reddish rashes over the skin if its extract remains on skin. So, it would be better to use gloves during cutting its leaves into pieces to prevent skin form allergy. Or skin should be massage with mustard oil after allergic reaction with its extract.

REFERENCES

Alam S, Akther N, Begum F, Banu M S, Islam M R, Chowdhury A N and Alam M S. 2002. Antifungal activities (*in vitro*) of some plant extracts and smoke on four fungal pathogens of different hosts. *Pakistan J. Bio. Sci*. **5(3)**: 307-309.

Almaraz-Abarca N, Delgado-Alvaroda E A, Reyes J A, Uribe- Soto J N & Gonzalez- Valdez L S.2013. The phenols of the genus Agave (Agavaceae). Journal of Biomaterials and Biotechnology. **4**.Pp:9-16.

Almaraz-Abarca N, Gonzalez E, Campos M G, Avila-Sevilla Z E, Delgado-Alvaroda E A & Avila-Reyes J A.2013. Variability of the foliar phenol profile of the *Agave victoriae-reginae* (Agavaceae). Botanical Science.in press.

American Cancer Society. 2010(Retrieved June 2014). Lycopene.

Barnett H L.1965 (3rd printing). *Illustrated Genera of Imperfect Fungi*. Burgees Publishing Company. Pp: 55, 127 and 195.

Bauman R. 2007. *Microbiology: With diseases by taxonomy*. San Francisco, Calif.: Pearson/Benjamin Cummings.

BBC News. 2008(retrieved).*Tomato dishes 'may protect skin.*

Bhatnagar I and Kim S K. 2010. Immense essence of excellence: marine microbial bioactive compounds . *Marine Drugs*. **8**: 2673.

Bowers J H and Locke J C.2004: Effect of formulated plant extracts and oils on population density of *Phytophthora nicotianae* in soil and control of Phytophthora blight in the greenhouse. *Plant Disease*. **88**: 11–16.

Chapagain T R, Piya S, Mandal J L and Chaudhary BP.2010. Up-scaling of polyhouse tomato production technology in mid and high hills of eastern Nepal. *Proceedings of ninth national outreach research workshop*. Kathmandu (Eds. Paudel M N, Barakoti T P and Ghimire Y N). Outreach Research Division, Nepal Agriculture Research Council, Kathmandu. Pp 116-120.

Chary M P, Reddy E A and Reddy S M.1984.Scernning of indigenous plants for their antifungal principles. *Pesticides.* **18**: 17-18.

Cutler H G and Cutler S J. 1999.Biological active natural product: *Agrochemical CRS Press, Boca Raton: USA.*Pp:299.

Dubey R C. 1991. Fungicidal effect of essential oils of three higher plants on sclerotia of *Macrophomia phaseolina*. *Indian Phytopath*. **44**:241-243.

Ellis M B. 1971. Dematicious Hypomycetes. Common wealth mycological institute,Kew.Pp:608.

Encyclopedia of Life.2014 (retrieved). *Solanum lycopersicum- Tomato*.

Enzazaden.nl. 2009. Het gemiddelde vruchtgewicht van Ingar ligt tussen 100–110 gram. *Enza Zaden – Teeltnieuws*.

Food and Agriculture Organization of the United Nations .2014.FAOSTAT: Production-Crops, 2012 data.

Ghimire S R, Subedi P P and Sylvia P G. 2000/2001. Status of tomato yellow leaf curl virus in tomato in the western hills of Nepal. *Nepal Agric. Res.* J. **4&5**.

Goussous S J, Abu-El-Samen F M and Tahhan R A. 2010. Antifungal activity of several medicinal plants extracts against the early blight pathogen (*Alternaria solani*). *Archives of Phytopathology and Plant Protection*. **43**: 1746–1758.

Grover R K and Moore J D. 1962. Toximetric studies of fungicides against brown rot organism. *Sclerotina fruticola*. *Phytopathology.* **52:**876-880.

Gyawali R and Ibrahim S A.2012. Impact of plant derivatives on the growth of food borne pathogens and functionality of probiotics. *Applied Microbiology and Biotechnology*.**1**

Hayek S A, Gyawali R and Ibrahim S A. 2013. Antimicrobial natural products. *A. Mendez-Vilas, Ed.*

Kadar N H, Benaouili H, Benichou S, Kihal M , Benali S and Henni J E. 2014. Invitro effect of fungicides on two pathogenic fungi causing root rot on tomato in Algeria. *Africal Journal of Agriculture Research*.**9(33)**:2584-2587.

Kagale S, Marimuthu T, Thayumanavan B, Nandakumar R and Samiyappan R. 2004. Antimicrobial activity and induction of systemic resistance in rice by leaf extract of *Datura metel* against *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae*. *Physiological and Molecular Plant Pathology*. **65**: 91–100.

Kharde M N, Wabale A S, Adhav R M, Jadhav B D, Wabale A M and Pandey M. 2010. Effects of plant extracts on fungal pathogens causing leaf blight of tomato in vitro. *Asian J. Exp. Biol. Sci. Spl*. Pp: 121-123.

Koehn F E and Carter G T.2005. The evolving role of natural products in drug history. *Nature Reviews Drug Discovery*. **4**:206.

Kumar A, Shukla R, Singh P, Prasad C S and Dubey N K. 2008. Assessment of *Thymus vulgaris L*. essential oil as a safe botanical preservative against post harvest fungi infestation of food commodities. *Innovat. Food Sci. Emerg. Technol*. **9**:575–580.

Kumar P R, Amandeep K, Neha K and Pranaya J. 2013. Fermented sauerkraut juice as antimicrobial agent:In vitro study. *International Research Journal Of Pharmacy*.**4(12)**

Merrill A H, Jr. Schmelz E M, Dillebay D L, Spiege S,Shayman J A, Schroeder J J, Riley R T, Voss K A & Wang E. 1997. Toxicol.Appl.Pharmacol. 142.Pp: 208-225.

Naik M K, Prasad Y, Bhat K V and Devika Rani G S.2010. Morphological, physiological pathological and molecular variability among isolates of Alternaria solani from tomato. *Indian Phytopath*.**6(33)**:168-173.

Pandey Y R and Chaudhary B N. 2004. Evaluation of tomato varieties and their planting dates for commercial production under Jumla agro-ecological condition. In**:** *Proceedings of the Fourth National Horticultural Research Workshop* (March 2-4, 2004), Kathmandu (Eds. Khatri B.B, Sharma B.P., Khatiwada PP, Paudyal KP, Khadge BR and Regmi HN). Horticulture Research Division, Nepal Agriculture Research Council, Kathmandu, pp. 380- 385.

Pandy R S, Bhargava S N, Shukla D N and Dwivedi D K. 1983. Control of pestalotia fruit rot of guava by leaf extracts of two medicinal plants. *Rev.Maxicana de Fitopatologia.* **2**: 15-16.

Plant Pathology Division (NARC). 2011/12. *Annual Report*. National Agricultural Research Council (NARC), Khumaltar, Lalitpur. Pp; 69.

Plant Pathology Division (NARC).2010/11. *Annual Report*. National Agricultural Research Council (NARC), Khumaltar, Lalitpur.

Plant Pathology Division (NARC).2012/13. *Annual Report*. National Agricultural Research Council (NARC), Khumaltar, Lalitpur.

Qasem J R and Abu-Blan H A. 1996: Fungicidal activity of some common weed extracts against different plant pathogenic fungi. *Journal of Phytopathology*. **144**: 157–161.

Rao G P and Srivastava A K. 1994. Toxicity of essential oils of higher plants against fungal pathogens of sugarcane. *Current Trend in Sugarcane Pathology*. (eds.) Rao GP, Gillasple A G, Upadhaya P P, Bergamin A, Agnihotri V P and Chen C T. International Books and Perodicals Supply Service, Pitampura, Delhi, Pp. 347-365.

Rijkzwaan.n. (2010). Het gemiddeld vruchtgewicht ligt tussen de 102 en 105 gram en de kwaliteit is goed. *Tomaat september 2010, RZ Seeds & Services*.

Shah P K, Amatya J and Dahal P R. 2006(revised edition). *Practical Microbiology*. Delta offset press. Pp: 176.

Sharma H and Parihar L. 2010. Antifungal activity of extracts obtained from actinomycetes. *Journal of Yeast and Fungal Research*. **1(10)**: 197-200.

Shekhawat P S and Prasad R. 1971.Antifungal properties of some plant extracts, in inhibition of spore germination. *Indian Phytopath*.**24**: 800-802.

Shrestha K,1998. Dictonary of Nepalese plant names. *Mandala Book Point*. Kathmandu, Nepal.

Shrestha T N and Ghimire N P. 1996. Fresh vegetable production in Nepal. Paper presented at the National Seminar on Vegetable Development, 11- 12 June 1996.

Singh R K and Dwivedi R S.1990. Fungicidal properties of neem and babul gum against Sclerotium rolfsii. *Acta. Bot. Indica.* **18**:260-262.

Singh Y, Tripathi N N and Dixit S N. 1983. The isolation and properties of fungi toxic principle from *Zingiber officinale*. *Indian J. Plant. Pathol*. **1**:89-96.

Tewari S N, Sukla H S,Biswal M K,and Nayak. **1988**. *Nati.Acad.Sci.lett.* **11**: 369-373.

Umamekeshwari A, Shreevidya R and Nuni A. **2008**. *Advances in biological Research*. **2(1-2)**:1-5.

Valero M A, Vidal A, Burgos R, *et al.* (2011). Meta-analysis on the role of lycopene in type 2 diabetes mellitus. *Nutr Hosp (Meta-analysis) (in Spanish; Castilian)* **26 (6)**: 1236–41.

Vegparadise.com.2009(retrieved). Vegetarians in Paradise/Tomato History, Tomato Nutrition, Tomato Recipe.

Vicario R. 2014(retrieved).*Slow Food® Upstate*.

Zhang W, Liu W X, Zhena M F, Ql X Y, Wan F H, Wang J, Lei T, Zhou Z & Tan J W. 2013. Bioactive quinic acid derivative from *Ageratina adenophora. Molecules:***18(1).**Pp:14096-104.

APPENDIX-I

Apparatus and Equipments

Apparatus: Petri plates, Funnel, Micro pipette, Glass pipette, Beakers, Whatman No.1 filter paper, Conical flasks, Mortar and pestle, Cellophane tape, Scale, Pencil, Notebook, Knife, Inoculating loop, Needle, Marker, Camera, Slides, Cover slips, Newspaper, Alumimiun foil, Tying rubber, Plastic jar, Cork borer, Measuring cylinder.

Equipments: Laminar air flow hood, Growth chamber, Incubator, Autoclave, Heater, Burner, Refrigerator, Microscope, Ocular and stage micrometer**.**

Chemicals: Potato extract, Agar, Dextrose, Ethanol (70%), Distilled water, Cotton blue, Lacto phenol, Antibiotic (Nemox), Non- ionized salt.

APPENDIX-II

Micrometry

Calculation of calibration factor for one ocular division (in 40X)

$$\text{One ocular division } (\mu m) = \frac{\text{No. of division on stage micrometer}}{\text{No. of divisions on ocular micrometer}} \times 100$$

$$= 10/7 \times 10(\mu m)$$

$$= 14.28\mu m$$

So, the value of calibration factor is 14.28µm.

a) Size of conidia of *Alternaria* sp.

From ocular measurement

Length=11 to 12

= 11x 14.28µm; 12x14.28µm

=157.08µm; 171.36µm

Breadth= 5 to 6

=5x14.28µm; 6x14.28µm

=71.4µm; 85.68µm

Therefore, Size of conidia of *Alternaria* sp. was (157.08µm-171.36 µm) x (71.4µm-85.68µm).

b) Size of acervuli of *Colletotrichum* sp.

From ocular measurement

Length=15 to 17

= 15x 14.28µm; 17x14.28µm

=214.2µm; 242.76µm

Breadth= 12 to 15

=12x14.28µm; 15x14.28µm

=171.36µm; 214.2µm

Therefore, Size of acervuli of *Colletotrichum* sp. was (214.2µm-242.76µm) x (171.36µm-214.2µm).

Similarly, Size of conidia of *Colletotrichum* sp.

From ocular measurement

Length=8 to 9

= 8x 14.28µm; 9x14.28µm

=114.24µm; 128.52µm

Breadth= 6 to 7

=6x14.28µm; 7x14.28µm

=85.67µm; 99.96µm

Therefore, Size of conidia of *Colletotrichum* sp. was (114.2µm-128.52µm) x (85.67µm-99.96µm).

c) Size of conidia of *Verticillium* sp.

From ocular measurement

Length=3 to 4

= 2x 14.28µm; 3x14.28µm

=42.84µm; 57.12µm

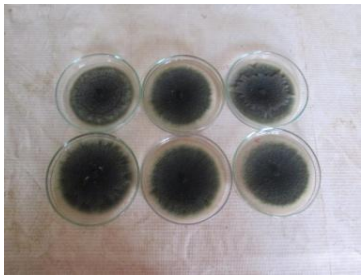
Breadth= 2 to 3

=12x14.28µm; 15x14.28µm

=28.56µm; 42.84µm

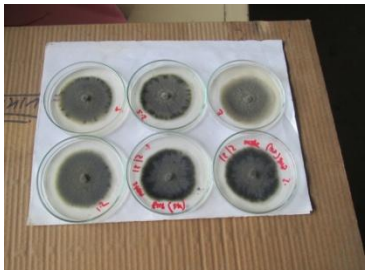
Therefore, Size of conidia of *Colletotrichum* sp. was (42.84µm-57.12µm) x (28.56µm-42.84µm).

PHOTOPLATE I

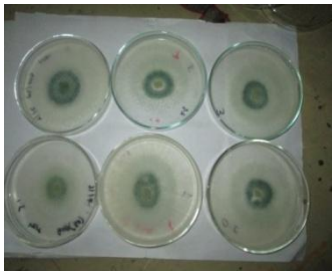


Colony of *Alternaria* sp. on PDA

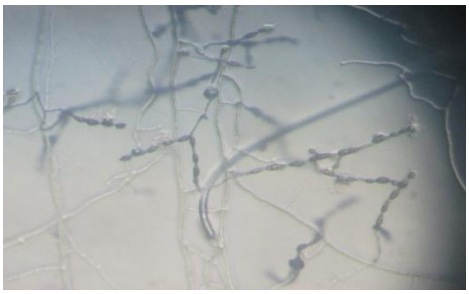
Colony of *Colletotrichum* sp. on PDA



Colony of *Stemphylium* sp. on PDA



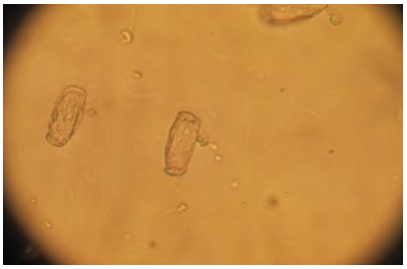
Colony of *Verticillium* sp. on PDA



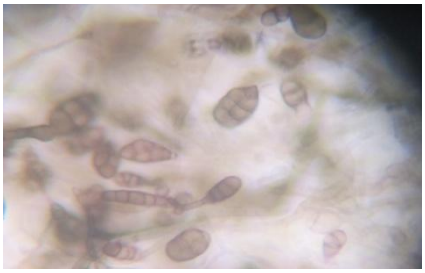
Alternaria sp. on water agar

Powdery mildew by *Leveilulla taurica*

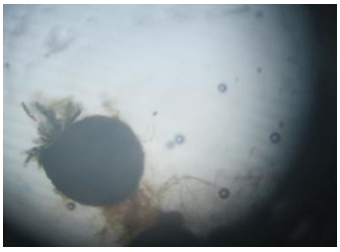
PHOTOPLATE II



Leveilulla taurica conidia



Alternaria sp. on PDA



Acervilus with conidia of *Colletotrichum* sp.

Verticillium sp. on PDA

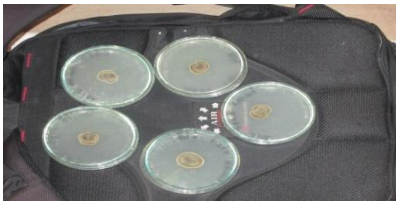
Blight symptoms by *Alternaria* sp.

Blight symptoms by *Alternaria* sp.

Helminthosporium sp. associated with *Alternaria* infected leaf

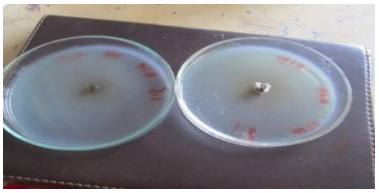
Alternaria sp. from

PHOTOPLATE III

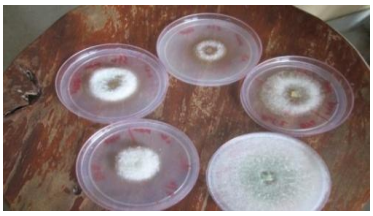


Alternaria on 0.2% Mancozeb extract

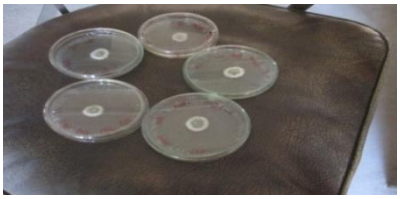
Colletotrichum on *Ageratina*



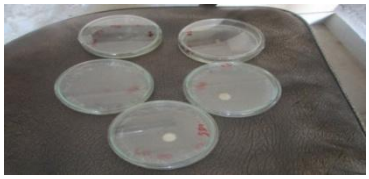
Colletotrichum on 30% *Agave* extract (0.05 -0.4%)



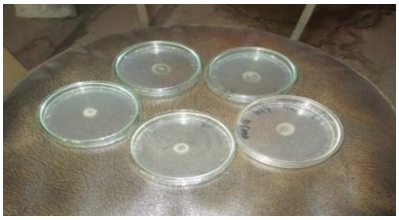
Verticillium on Mancozeb



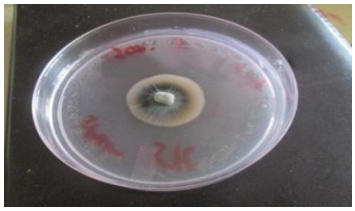
Verticillium on 30% *Agave* ferment Mancozeb(0.05 -0.4%)



Colletotrichum on



Colletotrichum on 30% Agave ferment Mancozeb



Stemphylium on 0.05%