ANTIFUNGAL EFFICACY OF *Terminalia species* AGAINST *ColletotrichumfructicolaTMT2* CAUSING ANTHRACNOSE FRUIT ROT BLIGHT IN TOMATO- A GREEN APPROACH

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ABSTRACT

*The present study was executed to evaluate the antifungal efficacy of the aqueous, ethanol, chloroform, petroleum ether and hexane extracts of Terminaliaalata, Terminaliaarjuna, Terminaliabellirica and Terminaliacatappa leaves, bark and fruit against Colletotrichumfructicola TMT2 (**DDBJ Accession NumberLC604629**) fungal species causing Anthracnose fruit rot Blight in Tomato. Antifungal activity was assessed by food poison technique. Among the five extracts, ethanol, chloroform and petroleum ether extract exhibited potent antifungal activity against ColletotrichumfructicolaTMT2. The activity is compared with a standard fungicide Carbendazim.*

Keywords: Terminalia species, Solanumlycopersicum, *Anthracnose,* Colletotrichumfructicola, *antifungal activity.*

Introduction

Crop production faces numerous issues among which fungal diseases are chief cause of yield loss throughout the world which results in an excessive economic burdens. Fungi are familiar with the infection to the plants during the developmental stages as well at post-harvest period. The degradation of fruits, vegetables and grains caused by the pathogenic fungi may lead to the loss of entire product. Synthetic chemical agents are heavily employed to control the phytopathogenic fungi, resulting in the resistance development in pathogens and the accumulation of these chemicals in the environment. So there is necessitating for botanicals for ecofriendly applications to control the crop damage caused by fungi, nematodes, bacteria and other organisms. Many medicinal plants have fungicidal properties against different fungal species including phytopathogenic fungi.

Tomato (*Lycopersiconesculentum* Mill) belonging to Solanaceae family and genus *Lycopersicon*is considered to be the most important vegetable and popular horticultural crop grown worldwide (Nonnecke, 1989). Tomato appropriately called as 'Super food' is one of the primeval crop popular since the mid 19th century because of its variegated climatic adaptability and appreciative nutritive significance.Red tomatoes may contribute protection to human health as it consists of an antioxidant and a carcinogenic substance (Zhang *et al.* 2015). They are composed of important constituents for bone health, neurological diseases and anticancer benefits.Numerous microorganisms *Viz*. bacteria, viruses, fungi, nematodes, abiotic factors and inadequate fertilization have been determined to reduce the quality and yield of tomato crop. Tomato crop has been desired model plant to assess plant pathogen interactions (Arie*et al.,* 2007). The tomato plant is damaged by distinct diseases induced by nematodes, bacteria, fungi and viruses (Ramezani, 2014).Anthracnose on tomatoes is caused by the fungus *Colletotrichumspp.* which is primarily a pathogen of tomato fruit. Since the fruits are ripening, the symptoms first become evident as small and circular indented areas, which then later develop darkened centers. The diseased spots continue to grow bigger with time as each infection site besides spreads deeper into the fruit. With warm, humid and moist weather from the rainfall or over-head irrigation the fungus produces salmon-colored spores which are exuded from black fungal material in the center of the spots. These spores are further spread by splashing water.

For the management of plant diseases, including fungal disease, various chemical fungicides are used. The use of chemicals to suppressthe fungal growth resulted into newer races of pathogens that becameresistant to these chemicals. Sometimes these pathogens

are muchaggressive in their action in comparison with their ancestral counterparts. Moreover, such chemicals are incorporated into food web and foodchain, giving rise to the newer concepts like 'Biomagnification' and 'Bioaccumulation'. Now-a-days, there is general awaking in world community onenvironmental issues. However, everybody thinks and act as per hiscomfort demands.The consumers want cheap food without health risks. The concept of biocontrol has gained much importance in recentyears. This includes use of microbes that inhibits growth of pathogens.The use of plant extracts is also well acknowledged since great antiquity.For eco-friendly management of fungal diseases, recent trend isusing plant based formulations.

In the present investigation, effect ofvarious plant part extracts like leaves, bark and fruit of *Terminaliaalata, Terminaliaarjuna, Terminaliabellirica* and *Terminaliacatappa* were tested for their inhibitory activity againstthe fruit rot blight pathogen *Colletotrichumfructicola TMT2*of tomato by % mycelial growth inhibition method. The observations were compared with theactivity of fungicide. The results obtained are discussed in contestof recent work on these aspects.

Materials and Methods

1. Isolation of Fungal Pathogens from diseased Tomato

Field survey was carried out to record the disease incidence by symptomological study on infected Tomato growing regions located at Sonkhed, Ta: Loha, District: Nanded, Maharashtra state, India. Infected Tomato fruitswere collected to isolate the causal fungal organism. All the samples were brought to the laboratory for further study.

2. Fungal culture and spore collection.

Colletotrichumfructicola TMT2 was isolated from the infected Tomato fruits.The fungal culture was grown in Potato dextrose agar (PDA) medium for 7-10 days and spore suspension was filtered with sterile muslin cloths; conidia spores were collected and spore suspension was adjusted to 2-105 spores/ml.

3. Isolation and identification of fungal pathogen of Vegetable Crops Tomato

Infected fruits (1cm) were placed on to wet blotter disc following the Standard Blotter Method (SBM) (ISTA, 2003). The plates were incubating for 7 days at 25^0C. After incubation, fungi developed on each samples were examined under compound microscope and identified based on colony morphological characters. Selected fungal pathogen was isolated from colonies showing the suitable characters and sub cultured on Potato Dextrose Agar (PDA) plates. Discrete fungal colonies separated on the basis of morphology were then grown on fresh PDA plates in order to obtained pure cultures. All fungal cultures were maintained routinely on PDA slants and stored at 4^0C until use and served as stock cultures. Subcultures were routinely made after every month.

The fungi were conventionally identified and characterized based on their morphological characters and microscopic analysis by using taxonomic guides and standard procedures.

4. Molecular Characterization of Fungal Isolates by 18s rRNA Sequencing

Molecular characterization of fungal pathogens by 18s rRNA Sequencing was carried out for the identification of fungal pathogens (Edel*et al*., 2000; Lievens*et al*., 2006; Colak and Bicici, 2013). The 18S rRNA and ITS region were sequenced at National Center for Cell Sciences, University of Pune Campus, Pune.

5. Molecular identification of fungal isolates:

The 18S rRNA and ITS region were sequenced at National Center for Cell Sciences, University of Pune Campus, Pune.

6. Phylogenetic Analysis:

The generated sequences were analyzed at the National Center for Biotechnology Information Bethesda, MD. www.nbi.nlm.nih.gov/BLAST for closed homology using BLASTn algorithm. The related sequences for the isolates were downloaded from the NCBI database were aligned by using CLUSTAL X2 multiple sequence alignment tool, the Phylogenetic

evolutionary history was inferred using the Neighbor Joining Method analysis (Tamura *et al*., 2004). Phylogenetic analyses were conducted in MEGA 4.0. Phylogenetic tree building was performed using MEGA 4.0 (Tamura *et al*., 2007).

7. Purification and Maintenance of Pure Culture

All the fungal cultures were grown in Potato dextrose agar medium for 7-10 days and spore suspension was filtered with sterile muslin cloths; conidia spores were collected and spore suspension was adjusted to $2\text{-}10^5$ spores/ml. Cultures so obtained were stored in the refrigerator at 5^0C, which served as a stock culture for further studies.

8. Evaluation of Antifungal Efficacy of Plant Extracts on Mycelial Growth of Fungal Pathogen through Poisoned Food Technique

To evaluate the efficacy of plant extracts *Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica* and *Terminaliacatappa* against the fungal pathogen *Viz. Colletotrichumfructicola TMT2*, poisoned food technique was carried out (Singh and Tripathi, 1999; Schmitz., 1930). Potato dextrose agar (PDA) medium was mixed with plant extracts prepared in water, ethanol, chloroform, hexane and petroleum ether and poured in to petri plates. Five mm disc of 7 day old culture of the test fungus was placed at the center of the petri plates and incubated at 25± 2^0C for 7 days. After incubation the colony diameter was measured in millimeter (mm). Three replicates were maintained for each treatment. PDA medium without the plant extract prepared in different solvents was served as control. Synthetic fungicides, clotrimazol were used as standard for comparison.

The per cent inhibition of fungal growth was determined by using the formula given by (Vincent, 1927; Singh and Tripathi, 1999; Schmitz., 1930).

Percentage of Inhibition (%)

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

Where, I = Per cent inhibition
C = Colony diameter in control
T = Colony diameter in treatment

9. Plant Material and Extract Preparation

9.1 Collection of Plant Material

Four widely growing native medicinal plants (botanicals) *Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica* and *Terminaliacatappa* were collected from in and around Nanded region to evaluate their antifungal efficacy. Plants were selected based on criteria such as the presence of antimicrobial properties according to literature or traditional knowledge, easy availability in bulk and having very less commercial value.

The identity of medicinal plants was verified from the herbarium of Science College Nanded. Leaves, bark and fruits were collected from in and around Nanded region. All healthy plant samples were stored in plastic bags and brought back to laboratory. Botanicals were thoroughly washed in running tap water; both fresh healthy materials and shade dried materials were used for further work.

9.2 Surface Sterilization of Plant Material

The collected plant material was thoroughly washed under the running tap water followed by surface sterilization with 1% H_2O_2 and then washed with autoclaved distilled water. The surface sterilized plant material were then dried in oven at 40^0 C for 5 days or until they were dried completely.

9.3 Preparation of Plant Extracts

The plant materials were shade-dried at room temperature and powdered using electric blender. The powdered plant materials (100g) were sequentially extracted with water, ethanol, chloroform, hexane and petroleum ether. After 72 hours of soaking of the plant material in each solvent, the extract was filtered through Whatmann's filter paper using vacuum. Solvent in the extract was removed using rotary vacuum evaporator at 45^0C -55^0C and the dried extract was stored at -20^0C in the refrigerator for further bioassay. Antifungal assay was performed by dissolving the dried extract in five different solvents *Viz.* water, ethanol, chloroform, hexane and petroleum

ether to a final concentration of 1mg/ml prior to use.

Results

Three medicinal plants *Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica and Terminaliacatappa* were tested against *Colletotrichumfructicola TMT2*bypoisoned food technique. *In Vitro* evaluation of different medicinal plants extract *Viz*. aqueous, ethanolic, chloroform, hexane and petroleum ether was taken for to test the antifungal evaluation.

Isolation and identification of fungal pathogen of Vegetable Crops Tomato

Th efungal pathogen of vegetable crop Tomato was isolated from infected Tomatohaving whitish cottony growth and then identified by using 18s rRNA analysis.

Phylogenic Analysis of BRJ2:

The phylogenetic tree was constructed by using Neighbour joining method by Kimura – 2 parameter with 1000 replicates in MEGA 4.0. According to the sequencing similarities anmultiple alignments, the present isolate was identified. The sequence obtained of the present isolate have been deposited in DNA Databank of Japan (DDBJ) and accession number obtained (**Figure I)**.

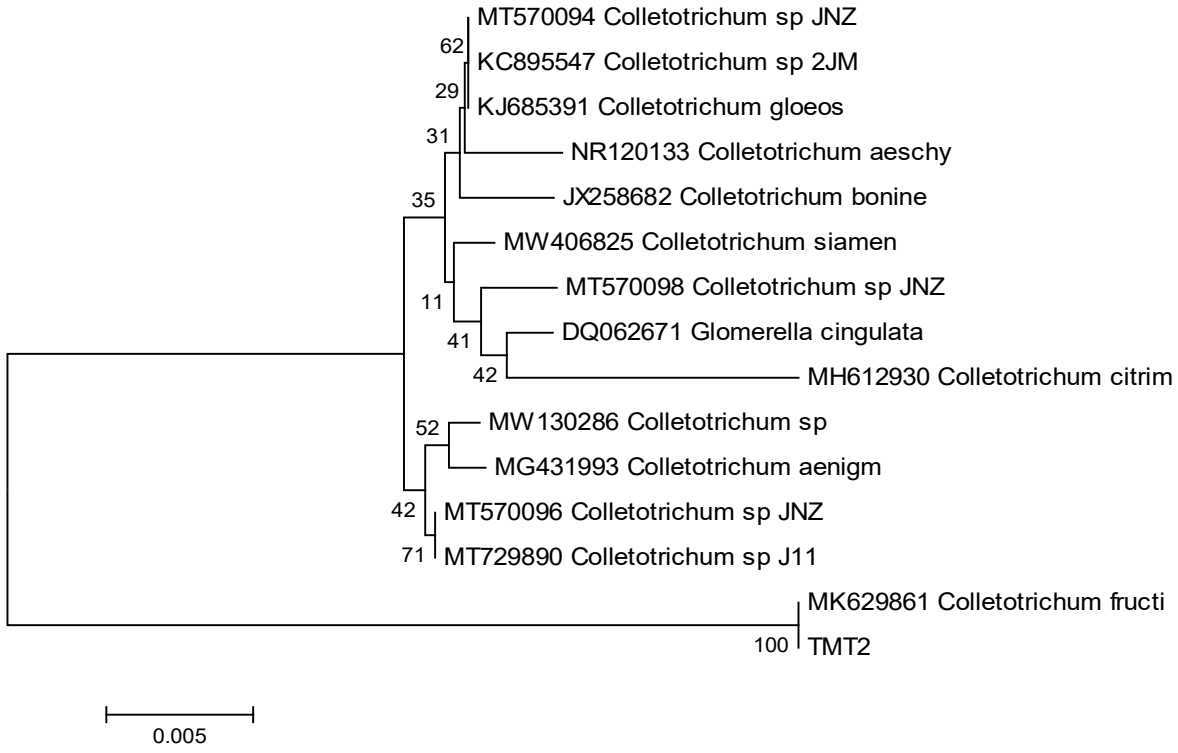


Figure I: Phylogenetic placement of TMT2 (Accession Number LC604629)

The gene sequences showing relationships among strain TMT2and the closest type strain species of Colletotrichum. Numbers at nodes indicate percentage of bootstrap support based on a Neighbor-joining analysis of 1,000 resampled datasets. Bar 0.002substitutions per nucleotide position.

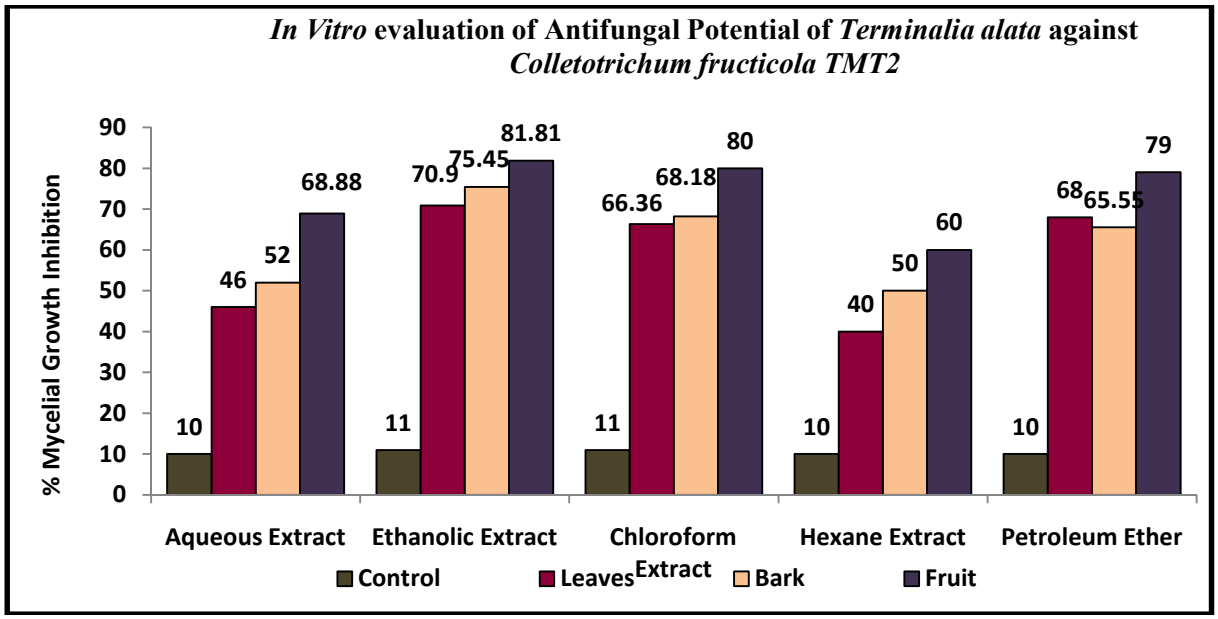
Evaluation of Antifungal Efficacy of Plant Extracts on Mycelial Growth of Fungal Pathogen through Poisoned Food Technique

To evaluate the efficacy of plant extracts *Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica* and *Terminaliacatappa* against the fungal pathogens *Viz. Colletotrichumfructicola TMT2*, poisoned food technique was carried out (Singh and Tripathi, 1999; Schmitz., 1930).

Table 1: *In Vitro* Evaluation of Antifungal Potential of *Terminaliaalata* against *Colletotrichumfructicola TMT2*

Plant used	Leaves			Bark			Fruit		
	Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition
Aqueous Extract									
T. alata	10.00	3.2	46.00	9.00	3.1	52.00	9.00	2.8	68.88
Ethanolic Extract									
T. alata	11.00	3.2	70.90	11.00	2.7	75.45	11.00	2.00	81.81
Chloroform Extract									
T. alata	11.00	3.7	66.36	11.00	3.5	68.18	11.00	2.2	80.00
Hexane Extract									
T. alata	10.00	6.0	40.00	10.00	5.0	50.00	10.00	4.0	60.00
Petroleum Ether									
T. alata	10.00	5.4	68.00	10.00	4.8	65.55	10.00	2.1	79.00

FigureII: *In Vitro*Evaluation of Antifungal Potential of*Terminaliaalata*against *Colletotrichumfructicola TMT2*



In Vitro* evaluation of Antifungal Potential of *Terminalia species* against *Colletotrichumfructicola TMT2

All the aqueous, ethanolic, chloroform, hexane and petroleum ether plant extract of four medicinal plants *Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica*and *Terminaliacatappa*showed antifungal activity against the test fungal pathogen *Colletotrichumfructicola TMT2*with some showing an outstanding antifungal activities than others. Whereas the control Carbendazimwere also taken for antifungal test evaluationagainst *Colletotrichumfructicola TMT2*.

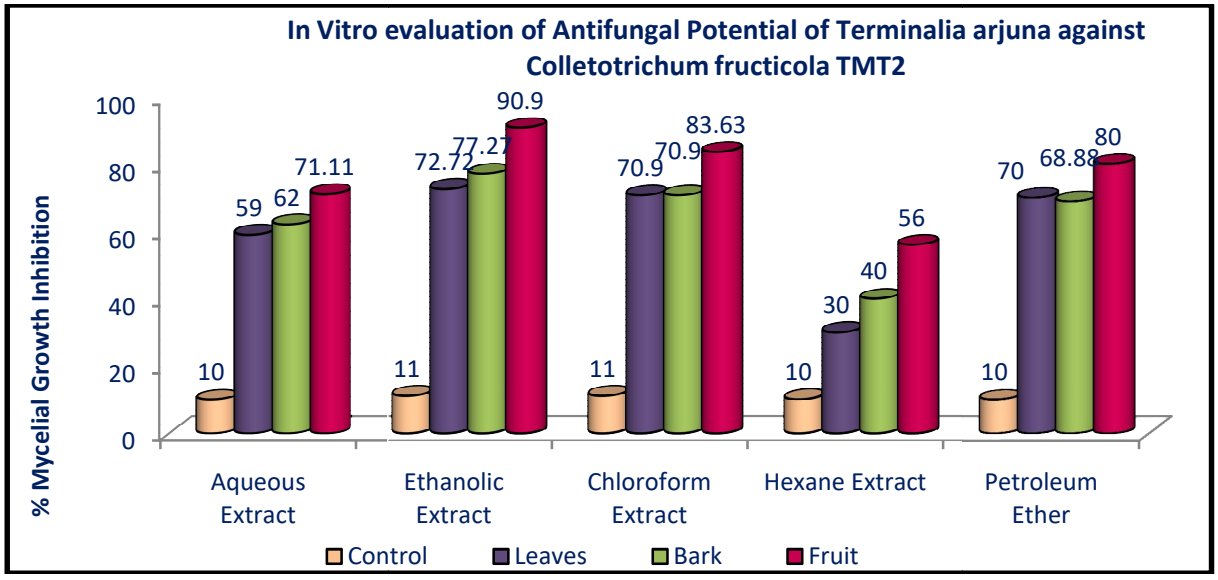
It is found from the **Figure II** that the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 81.81% to ethanolic fruit extract of *Terminaliaalata* followed by bark extract andleaves extract which is 75.45%, 70.90% respectively.Similarly, the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 80.00% to chloroform fruit extract of *Terminaliaalata* followed by bark extract andleaves extract which is 68.18% and 66.36% respectively. Similarly, the promising fungal isolate *TMT2* shows the percent inhibition of mycelial growth i.e. 79.00% to petroleum ether fruit extract of *Terminaliaalata* followed by bark

extract and leaves extract which is 65.55% and 68.00% respectively. Furthermore, as it is evident from the **Figure II** that, the comparatively less percent inhibition of mycelial growth is observed in aqueous and hexane extract of *Terminaliaalata*. The percent inhibition of mycelial growth observed for control of five extract aqueous, ethanol, chloroform, petroleum ether and hexane of *Terminaliaalata*is10%, 11%, 11%, 10% and 10%respectively.

Table 2: *In Vitro* Evaluation of Antifungal Potential of *Terminaliaarjuna* against *Colletotrichumfructicola TMT2*

Aqueous Extract									
Plant used	**Leaves**			**Bark**			**Fruit**		
	Control	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**
T. arjuna	10.00	3.0	59.00	9.00	2.8	62.00	10.00	2.0	71.11
Ethanolic Extract									
T. arjuna	11.00	3.00	72.72	11.00	2.5	77.27	11.00	1.00	90.90
Chloroform Extract									
T. arjuna	11.00	3.2	70.90	11.00	3.2	70.90	11.00	1.8	83.63
Hexane Extract									
T. arjuna	10.00	7.0	30.00	10.00	6.0	40.00	10.00	4.4	56.00
Petroleum Ether									
T. arjuna	10.00	4.1	70.00	10.00	3.8	68.88	9.00	2.6	80.00

Figure3: *In Vitro* Evaluation of Antifungal Potential of *Terminaliaarjuna* against *Colletotrichumfructicola TMT2*



It is found from the **Figure II** that the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 90.90% to ethanolic fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 77.27%, 72.72% respectively.Similarly, the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 83.63% to chloroform fruit extract of *Terminaliaalata* followed by bark extract and leaves extract which is 70.90% and 70.90% respectively. Similarly, the promising fungal isolate *TMT2* shows the percent inhibition of mycelial growth i.e. 80.00% to petroleum ether fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 68.88% and 70.00% respectively. Furthermore, as it is evident from the **Figure II** that, the comparatively less percent inhibition of mycelial growth is observed in aqueous and hexane extract of *Terminaliaalata*. The percent

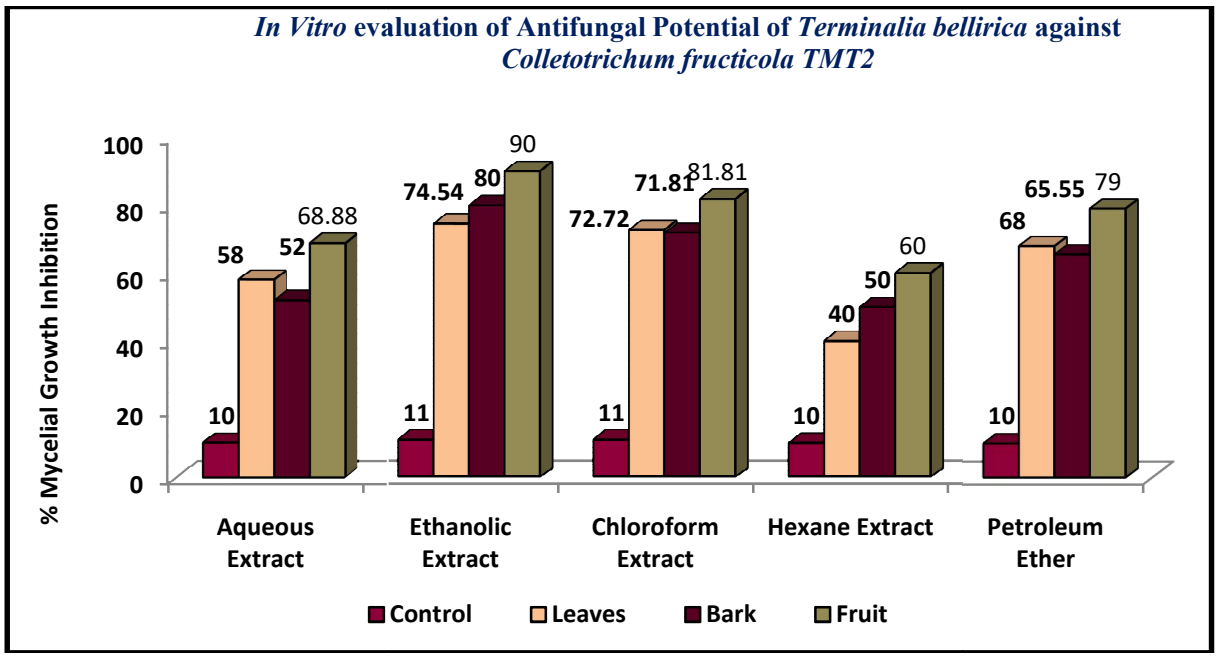
inhibition of mycelial growth observed for control of five extract aqueous, ethanol, chloroform, petroleum ether and hexane of *Terminaliaalata* is 10%, 11%, 11%, 10% and 10%respectively.

The ethyl acetate extracts of the *Pestalotiopsis species*endophytes of *Terminaliaarjuna* and *Terminaliachebula* showed greater antifungal activity against *Alternariacarthami*, *Macrophominaphaseolina*, *Fusariumverticilloides*, *Fusariumoxysporum*, *Phomasorghina* and *Sclerotiniasclerotiorum*, withthe zone of the inhibition diameter ranging from 4mm to 25mm (Tejesvi*et al*., 2007)

Table 3: *In Vitro*Evaluation of Antifungal Potential of*Terminaliabellirica*against *Colletotrichumfructicola TMT2*

Aqueous Extract									
Plant used	**Leaves**			**Bark**			**Fruit**		
	Control	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**
T. bellirica	10.00	3.2	58.00	9.0	3.1	52.00	10.00	2.1	68.88
Ethanolic Extract									
T. bellirica	11.00	2.8	74.54	11.0	2.2	80.00	11.00	1.1	90.00
Chloroform Extract									
T. bellirica	11.00	3.00	72.72	11.0	3.1	71.81	11.00	2.0	81.81
Hexane Extract									
T. bellirica	10.00	6.0	40.00	10.0	5.0	50.00	10.00	4.0	60.00
Petroleum Ether									
T. bellirica	10.00	4.2	68.00	10.0	4.0	65.55	9.00	2.8	79.00

Figure 4: *In Vitro* Evaluation of Antifungal Potential of *Terminaliabellirica*against *Colletotrichumfructicola TMT2*



It is found from the **Figure II** that the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 90.00% to ethanolic fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 80.00%, 74.54% respectively.Similarly, the promising fungal isolate *TMT2* shows the largest percent inhibition of mycelial growth i.e. 81.81% to chloroform fruit extract of *Terminaliaalata* followed by bark extract and leaves extract which is 71.81% and 72.72% respectively.

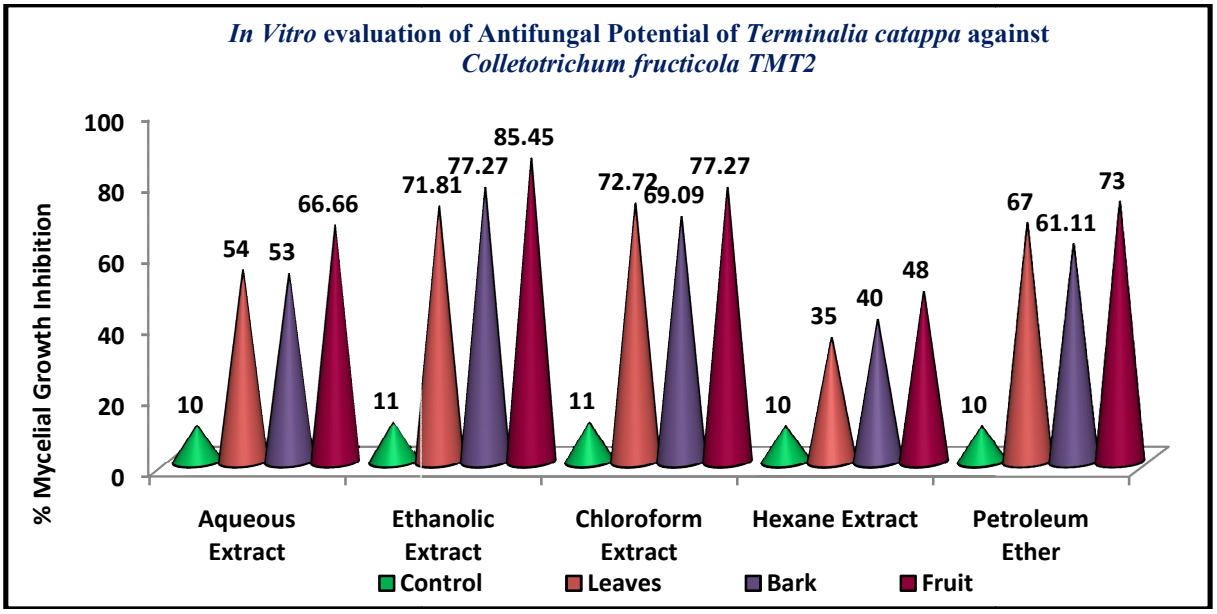
Similarly, the promising fungal isolate *TMT2* shows the percent inhibition of mycelial growth i.e. 79.00% to petroleum ether fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 65.55% and 68.00% respectively. Furthermore, as it is evident from the **Figure II** that, the comparatively less percent inhibition of mycelial growth is observed in aqueous and hexane extract of *Terminaliaalata*. The percent inhibition of mycelial growth observed for control of five extract aqueous, ethanol, chloroform, petroleum ether and hexane of *Terminaliaalata* is 10%, 11%, 11%, 10% and 10% respectively.

Prathyusha*et al.,* (2015) studied plant pathogenic *Fusarium*and *Colletotrichum.* These cultureswere alsoinhibited by the crude extracts of *A. sclerotigenum*. The inhibition against *Fusariumoxysporum*was more at highconcentration while *C. dematium*was inhibited even at low concentrations.

Table 4:*In Vitro* Evaluation of Antifungal Potential of *Terminaliacatappa* against *Colletotrichumfructicola TMT2*

Aqueous Extract									
Plant used	**Leaves**			**Bark**			**Fruit**		
	Control	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**	**Control**	**Treated**	**% Inhibition**
T. catappa	10.00	3.3	54.00	9.0	3.5	53.00	10.00	2.7	66.66
Ethanolic Extract									
T. catappa	11.00	3.1	71.81	11.0	2.5	77.27	11.00	1.6	85.45
Chloroform Extract									
T.catappa	11.00	3.00	72.72	11.0	3.4	69.09	11.00	2.5	77.27
Hexane Extract									
T. catappa	10.00	6.5	35	10.0	6.0	40	10.00	5.2	48.00
Petroleum Ether									
T. catappa	10.00	4.6	67.00	10.0	4.7	61.11	9.00	3.0	73.00

Figure 5:*In Vitro* Evaluation of Antifungal Potential of *Terminaliacatappa* against *Colletotrichumfructicola TMT2*



It is found from the **Figure II** that the promising fungal isolate TMT2 shows the largest percent inhibition of mycelial growth i.e. 85.45% to ethanolic fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 77.27%, 71.81% respectively.Similarly, the promising fungal isolate TMT2 shows the largest percent

inhibition of mycelial growth i.e. 77.27% to chloroform fruit extract of *Terminaliaalata* followed by bark extract and leaves extract which is72.72% and 69.09% respectively. Similarly, the promising fungal isolate TMT2 shows the percent inhibition of mycelial growth i.e. 73.00% to petroleum ether fruit extract of *Terminaliaarjuna* followed by bark extract and leaves extract which is 67.00% and 61.11% respectively. Furthermore, as it is evident from the **Figure II** that, the comparatively less percent inhibition of mycelial growth is observed in aqueous and hexane extract of *Terminaliaalata*. The percent inhibition of mycelial growth observed for control of five extract aqueous, ethanol, chloroform, petroleum ether and hexane of *Terminaliaalata* is 10%, 11%, 11%, 10% and 10%respectively.

Shikha Mandloi (2013) studied that *Curvalarialunata* was found to be the most susceptible to ethanol fractions of *Terminaliacatappa*. An inhibition of 74% growth was observed. Next to this *Aspergillusniger* was 57.33% inhibited. *A. alternate* was least susceptible. However, methanol extract of both plants tested against all four fungi showed significant antifungal activity. The percent of inhibition of methanol extract was more than 50% against *Curvularialunata*and *Trychophytontonsurans Aspergillusniger*. Results of these studies have shown that methanol extract of *T. catappa* has good antifungal activity.

Conclusion

Antifungal activity of leaves, bark and fruit extracts of *Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa*has revealed its medicinalpotential and represents it as one of the most important medicinal plant.That's why;development of modern antifungal drugfrom this plant can be emphasized for control of fruit rot blight in Tomato.This would lead to the development of costeffective herbal drugs. It is concluded that the potential antifungal activity shown by the ethanolic fruits extract of all the plants used in the present study and itsactive constituents would be helpful in interacting various kinds of plant diseases.

Secondary metabolites of plant origins have noticeable impressions on numerous research fields and furthermore added benefit of producing inexpensively. Finding of more such products is a boon for eco-friendly management of vegetable crop, in turn they revolutionizethe redundancy of the synthetic chemicals.

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