IN *Vitro* EVALUATION OF ANTIFUNGAL POTENTIAL OF SELECTED MEDICINAL PLANTS AGAINST *Phomopsisvexans* BRJ2

P. Kushwaha[1] and S.L. Shinde[2]
[1]Department of Botany, Yeshwant Mahavidyalaya, Nanded
[2]Department of Botany, Rajiv Gandhi Mahavidyalaya, Mudkhed
pradeep.envs@gmail.com

ABSTRACT

*The present study was carried out to evaluate the antifungal activity of the ethanol, chloroform and petroleum extracts of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa leaves, bark and fruit against fungal pathogen Viz. Phomopsisvexans BRJ2 (**DDBJ Accession NumberLC601991**)causing fruit rotblight of Brinjal isolated from different localities of Nanded District. Antifungal activity was assessed by agar well diffusion assay. Among the five extracts, ethanol, chloroform and petroleum ether extract exhibited potent antifungal activity against Phomopsisvexans BRJ2. The activity is compared with a standard fungicide Clotrimazol. The present extracts exhibited the growth inhibitory activity in a dose dependent manner. The fruit extracts of all Terminaliaspecies used in the present study showed significant inhibition of the growth of the fungusPhomopsisvexans BRJ2. The best screening results were obtained with ethanol, chloroform and petroleum ether fruit and bark extracts of Terminaliaalata, Terminaliabellirica, Terminaliaarjunaand Terminaliacatappa. So theresults further supported the view that the Terminaliabellirica, Terminaliaarjuna, Terminaliacatappa and Terminaliaalatais promising source of natural useful therapeutic agents.*

Keywords: *Terminalia species, Phomopsisvexans, Brinjal, Antifungal activity, Agar well diffusion*

Introduction

Brinjal crop is subjected to the attack of the many diseases which causes damage altogether growth stages limiting production. The diseases are caused by fungi, bacteria, viruses, nematodes or environmental factors. Among them fungal and bacterial diseases are common in brinjal fields. Widespread fungal diseases of eggplants contains the plant disease caused by (Rhizoctoniasolani, Pythium sp.), Cercospora leaf spot caused by Cercosporamelongenae, Alternaria leaf spot caused by Alternariamelongenae, Phomopsis blight or fruit rot caused by Phomopsisvexans and wilt caused by Fusariumoxysporum f.spmelongenae. Out of them Phomopsis fruit rot caused by Phomopsisvexans and wilt caused by Fusariumoxysporumf.sp. melongenae are the foremost devastating diseases of brinjal growing fields. The main economic a part of the brinjal plant is that the fruit only and therefore the fruit is heavily infected mainly by fungal pathogen i.e. Phomopsisvexans (Das, 1998; Khan, 1999). The cultivators and therefore the retail sellers face heavy economic losses on account of fruit rotting during cultivation. Phomopsis blight is one among the foremost serious fungal disease which attacks all above ground parts of the plants and it amounts to heavy losses under favorable weather for the incidence of the disease.

This disease under suitable weather conditions may cause 12-25% loss in crop yield because of flower drop and fruit rot (Kannanet al., 1998). Phomopsisvexans causes over 50% losses in the production and productivity of brinjal in different parts of the world (Nolla, 1929). In India yield losses due to Phomopsis fruit rot ranged to the extent of 10-20 percent (Panwaret al., 1970). Phomopsisvexans causes fruit rot and leaf blight of brinjal and it reduces yield and marketable value of the crop nearly 20-30 percent (Jain and Bhatnagar, 1980; Kauret al., 1985).

Medicinal plants have forever been considered as a source for the healthy life for people. Therapeutical properties of medicinal plants are very valuable in healing various diseases and the advantages of these medicinal plants are natural. In many parts of the world, medicinal plants have been utilized for its antibacterial, antifungal and antiviral activities for hundreds of years (Subashet al., 2012; Farombi, 2003).

In view of high price and adverse effects of chemical fungicides, different plant

pathologists all over the world are now trying to isolate the eco-friendly antifungal compounds for controlling plant diseases caused by fungal pathogens. Though the fungicides are the most common method to control of plant diseases, however their use is costly as well as environmentally undesirable (Song and Goodman, 2001). Target and beneficial organisms are also killed by pesticide. Biological control of plant diseases does not pose any environmental threat besides being in expansible has gained significant prominence and is considerable as an alternative to chemicals (Fokkema, 1993).

To control the fruit rot blight of brinjal, there are several management practices which have been developed; the present investigation was concentrated on eco-friendly management in promoting the green alternative for the management of fruit rot blight of brinjaland reducing the fungicide usage.

Materials and Methods

1. Isolation of Fungal Pathogens from diseased Brinjal

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

2. Fungal culture and spore collection.

Phomopsisvexans BRJ2 was isolated from the infected fruits of Brinjal plant. 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 Brinjal

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 (Edelet al., 2000; Lievenset 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 2-10^5 spores/ml. Cultures so obtained were stored in the refrigerator at 5^0C, which served as a stock culture for further studies.

8. Antifungal Analysis of the Medicinal Plants under study

Agar Well Diffusion Assay and Antibiogram Analysis

The antifungal potential of medicinal plants Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica and Terminaliacatappa against the fungal pathogen Phomopsisvexans BRJ2was evaluated against fungal strains in ethanol, chloroform and petroleum ether solvent by using agar-well diffusion method as described by (Abdallah, 2014) with some modifications. Before to the experimental phase, all identified fungal isolates were sub-cultured in a tighten bottles containing potato dextrose broth at 25^0C for 48 hours. After incubation, all turbid bottles as a result of growth-were transferred and kept in the refrigerator (4^0C) to keep the microbial growth at the exponential phase until used. Autoclaved Bottles containing 20ml of potato dextrose agar was poured hot on sterile Petri-dishes (90mm in diameter) and left at room temperature until solidified. Working microbial strains were taken from the broth cultures (previously prepared) and adjusted as McFarland standard, then 100µl from each microbial strain was put over potato dextrose agar plates (depending on the type of microorganism) and distributed above the agar using sterile cotton swabs. Wells were punched into the agar with a sterile cork borer (10mm in diameter). Then, 100µl from each extract (500mg/ml) was dropped into the wells, extracts were previously reconstituted in 10% di-methyl-sulphoxide (DMSO) to make a concentration 500mg/ml. 10% DMSO did not show any inhibitory effect on microorganisms. Another well (in the centre) was loaded with 100µl of 10mg/ml clotrimazol for fungi. Plates were incubated at 25^0C for up to 48 hours. The antifungal activities of the tested extracts were determined by measuring the clear zone of inhibition in millimetre (mm).

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. The 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 45-55^0C 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 500mg/ml prior to use.

Results

In the present study, four medicinal plants Viz. Terminaliaalata, Terminaliaarjuna, Terminaliabellirica and Terminaliacatappa were tested against Phomopsisvexans BRJ2 by agar well diffusion technique. In Vitro evaluation of different medicinal plants extract Viz. ethanol, chloroform and petroleum ether was taken for to test the antifungal evaluation.

In this study, the bark, leaves and fruits extracts of the above specified medicinal plants were used. The dried fine powders of these plant parts were extracted in ethanol, chloroform and petroleum ether were used in this screening.

Out of four plants tested for their antifungal activities most of them showed antifungal activity against phytopathogenic fungusPhomopsisvexansBRJ2. This fungal plant pathogen was inhibited by the extracts of leaves, bark, and fruits of medicinal plants used in the present study.

Isolation and identification of fungal pathogen of Vegetable Crops Brinjal

Thefungal pathogen of vegetable crop Brinjal was isolated from infected brinjal 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)**

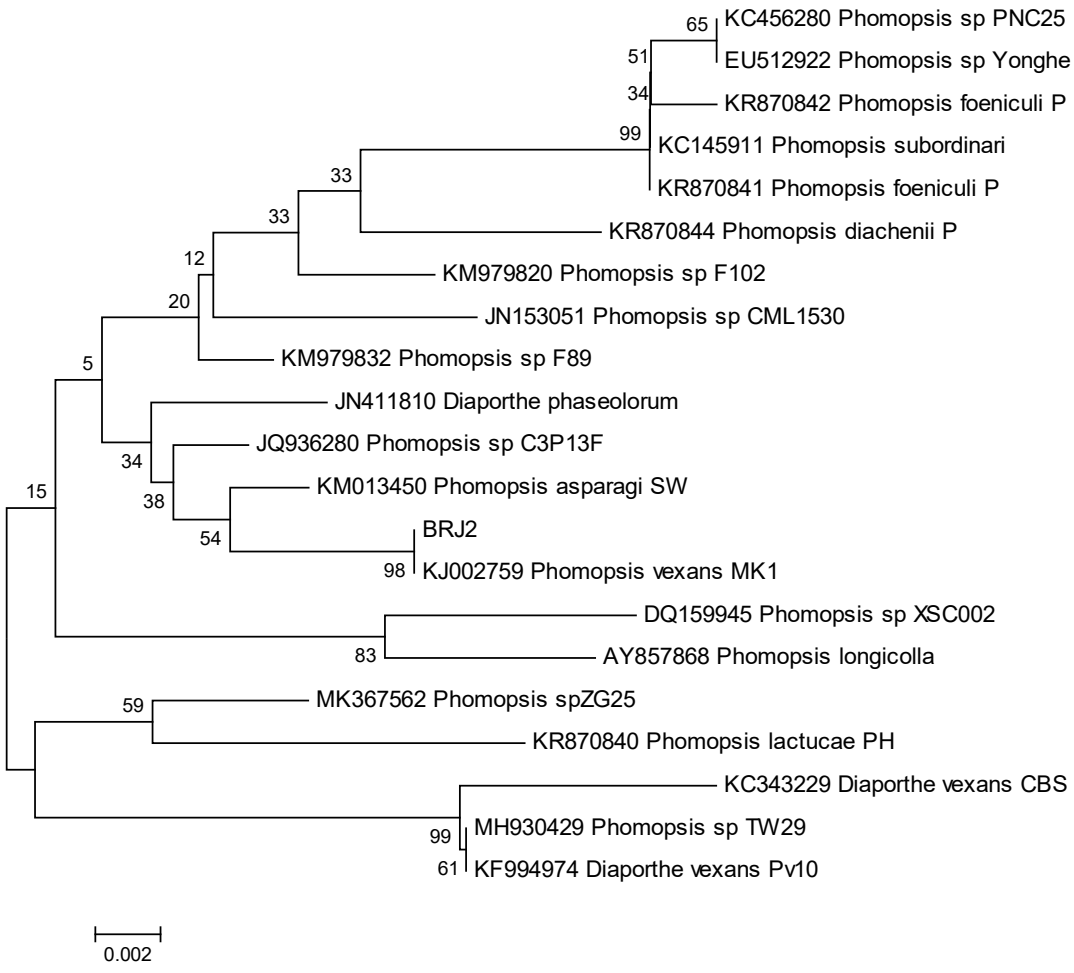


Figure I: Phylogenetic placement of BRJ2 (Accession Number LC601991)

The gene sequences showing relationships among strain BRJ2and the closest type strain species of Phomopsis. 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.

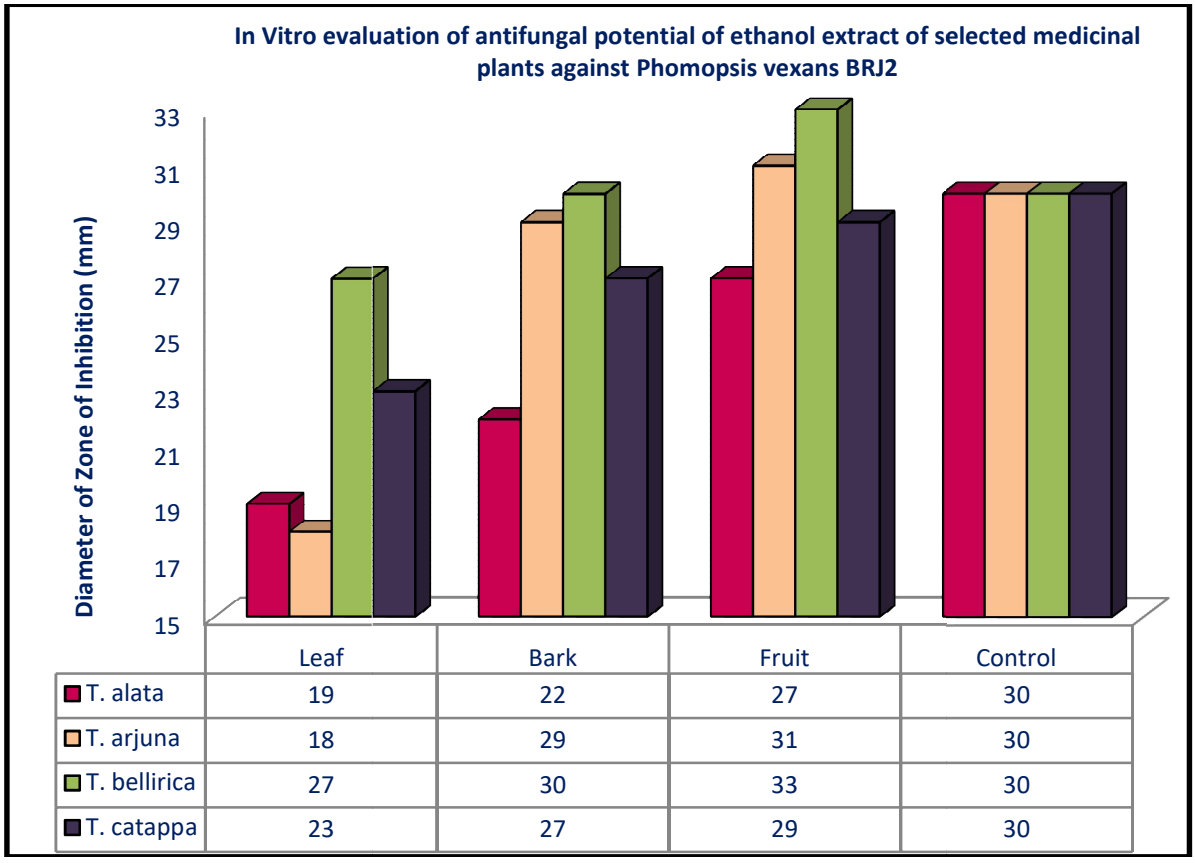
Antifungal Evaluation of Different Medicinal Plant Extracts

In Vitro antifungal potential of ethanolic, chloroform and petroleum ether extracts of medicinal plants *Viz. Terminaliaalata*, *Terminaliabellirica, Terminaliaarjuna*and *Terminaliacatappa*parts like leaves, bark and fruit extracts against *Phomopsisvexans BRJ2* causing fruit rotblight of Brinjalwere determined by Agar well diffusion method on sterile potato dextrose agar (PDA) medium. Among the different medicinal plant extracts used for antifungal activity, ethanol, chloroform and petroleum ether extract of allthe medicinal plants under study were found very significant in inhibiting the maximum growth of the pathogen *BRJ2*. Thediameter of zone of inhibition is depicted in **Table 2. Figure II, Figure III and Figure IV.** Hydroalcoholic extracts of *T.catappa* and *T. mantaly* have been reported to inhibitthe *In Vitro* gr. of *Aspergillusfumigatius*. (Elizabeth K.M. (2005).

Table 2: In Vitro evaluation of antifungal potential of selected medicinal plants against Phomopsisvexans BRJ2

Plants used	Diameter of Zone of growth inhibition (mm)											
	Ethanol				Chloroform				Petroleum Ether			
	Control	Leaf	Bark	Fruit	Control	Leaf	Bark	Fruit	Control	Leaf	Bark	Fruit
Terminaliaalata	30	19	22	27	30	16	22	25	30	17	19	29
Terminaliaarjuna	30	18	29	31	30	25	26	30	30	20	30	33
Terminaliabellirica	30	27	30	33	30	26	29	32	30	24	27	35
Terminaliacatappa	30	23	27	29	30	18	27	27	30	25	28	31

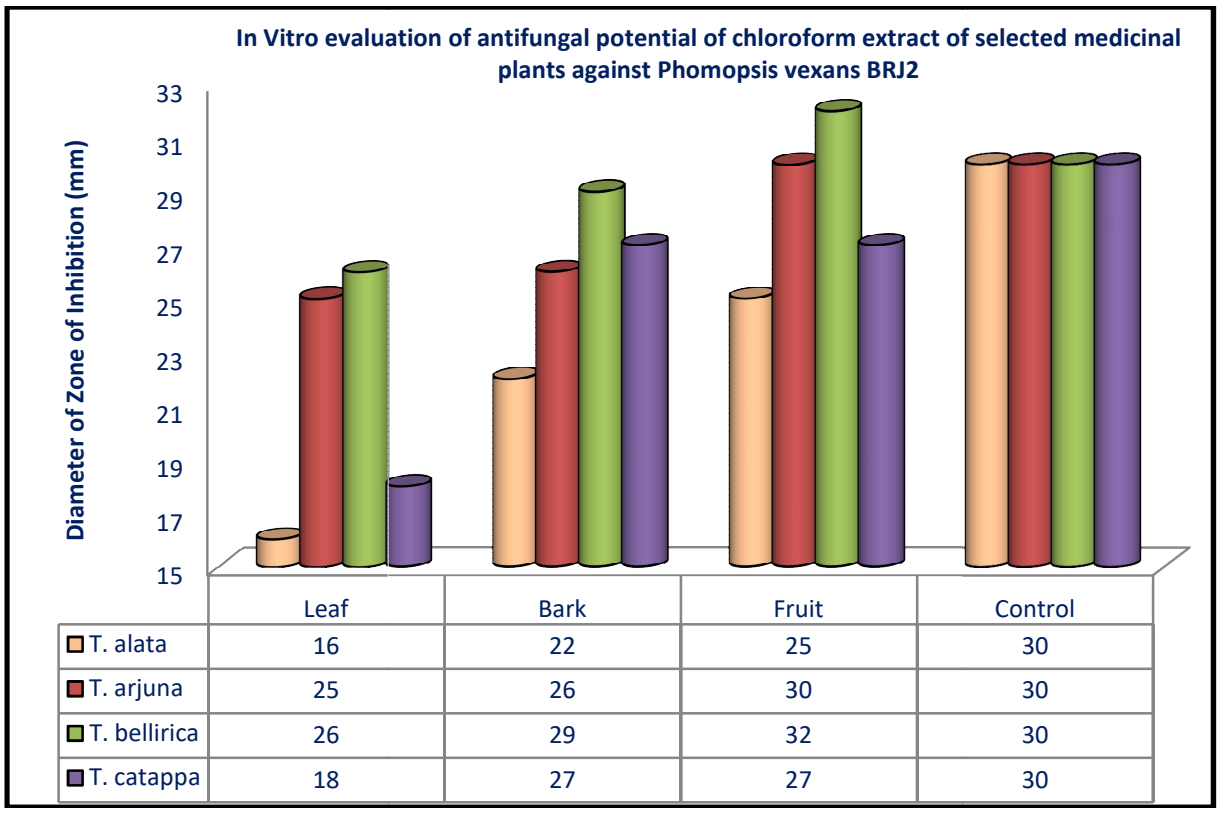
Figure II: In Vitro evaluation of antifungal potential of ethanol extract of selected medicinal plants against Phomopsisvexans BRJ2



As depicted in **Figure II** that the promising fungal isolate BRJ2 shows the largest zone of inhibition to ethanol extract of fruit of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa followed by bark andleaves. As shown in Figure II, the significant zone of inhibition i.e. 27mm, 31mm, 33mm and 29mm observed for fruit of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa respectively. Similarly, the promising fungal isolate BRJ2 shows the effective zone of inhibition to similar extract ofbark which is 22mm, 29mm, 30mm and 27mm respectively for the plants used in the present study. Furthermore, as it is evident from the **Figure II** that, the comparatively less zone of inhibition is observed by the ethanol extract of leaves of medicinal plants used in the study as 19mm, 18mm, 27mm and 23mm respectively. The zone of inhibition observed for control (Clotrimazol) is30mm.

The previous reports on the antifungal activity of the different part of T. chebulaincluding its fruits have primarily focused on activity against C. albicans (Vonshaket al., 2003; Singh et al., 2012). The study conducted by Sakanderet al., (2015) found that the aqueous, ethyl acetate and methanol extract of T. chebula fruit possess antifungal activity with zones of inhibition ranging between 16mm and 47.75mm.

Figure II: In Vitro evaluation of antifungal potential of chloroform extract of selected medicinal plants against Phomopsisvexans BRJ2



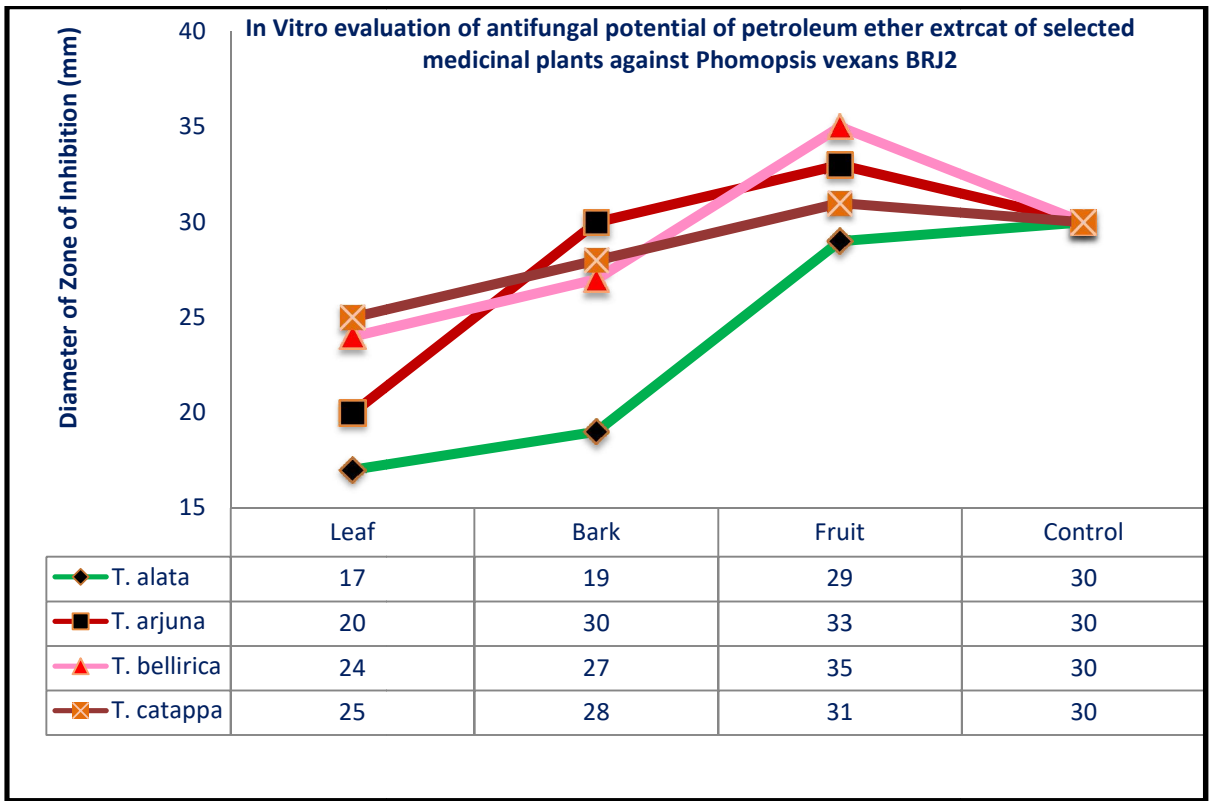
	Leaf	Bark	Fruit	Control
T. alata	16	22	25	30
T. arjuna	25	26	30	30
T. bellirica	26	29	32	30
T. catappa	18	27	27	30

As depicted in **Figure III** that the promising fungal isolate BRJ2 shows the largest zone of inhibition to chloroform extract of fruit of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa followed by bark and leaves. As shown in Figure III, the significant zone of inhibition i.e. 25mm, 30mm, 32mm and 27mm observed for fruit ofTerminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa respectively. Similarly, the promising fungal isolate BRJ2 shows the effective zone of inhibition to similar extract of bark which is 22mm, 26mm, 29mm and 27mm respectively for the plants used in the present study. Furthermore, as it is evident from the **Figure II** that, the comparatively less zone of inhibition is observed by the chloroform extract of leaves

of medicinal plants used in the study as 16mm, 25mm, 26mm and 18mm respectively. The zone of inhibition observed for control (Clotrimazol) is 30mm.

Sakanderet al., (2015) showed in their study that the significant antifungal activity was observed in the aqueous extracts of the fruits of Terminaliachebula (47.75mm) against Microsporumgypseum and the mesocarp of Perseaamericana (40.5mm) against Microsporumcanis. Candida albicans was inhibited by the ethyl acetate (20mm) and aqueous extracts (16mm) of T. chebula fruits and aqueous extract of the seeds of Syzygiumjambos (16mm).

Figure III: In Vitro evaluation of antifungal potential of petroleum ether extract of selected medicinal plants against Phomopsisvexans BRJ2



	Leaf	Bark	Fruit	Control
T. alata	17	19	29	30
T. arjuna	20	30	33	30
T. bellirica	24	27	35	30
T. catappa	25	28	31	30

As depicted in **Figure IV** that the promising fungal isolate BRJ2 shows the largest zone of inhibition to petroleum ether extract of fruit of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa followed by bark and leaves. As shown in Figure IV, the significant zone of inhibition i.e. 29mm, 33mm, 35mm and 31mm observed for fruit ofTerminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa respectively. Similarly, the promising fungal isolate BRJ2 shows the effective zone of inhibition to similar extract ofbark which is 19mm, 30mm, 27mm and 28mm respectively for the plants used in the present study. Furthermore, as it is evident from the **Figure IV** that, the comparatively less zone of inhibition is observed by the petroleum ether extract of leaves of medicinal plants used in the study as 17mm, 20mm, 24mm and 25mm respectively. The zone of inhibition observed for control (Clotrimazol) is 30mm.

Hence the antifungal activity of T. catappa tends to agree with the earlier reports of ManzurAbulet al., 2011; Parekh J. and Chanda S. (2008). The presence of antifungal activity in alcoholic fraction of Terminallia leaf extracts finds agreement with the work of other scientists.

Conclusions

It can be concluded that the antifungal activity of leave, bark and fruit extracts of Terminaliaalata, Terminaliabellirica, Terminaliaarjuna and Terminaliacatappa would

be valuablein treating the fruit rot blight of brinjal and various kinds of plant diseases. These plant extracts could serve as potential sources of new antimicrobials and for green and eco-friendly treatment technology development of different fungal plant pathogens.

Acknowledgement

We would like to offer our sincere thanks to National Center for Cell Sciences, University of Pune Campus for the 18s rRNA sequencing of the isolate. Our sincere thanks to Principal, Yeshwant Mahavidyalaya, Department of Botany, Nanded for providing the Laboratory facilities.

References

1. Abdallah, E. M. (2014). Antimicrobial properties and phytochemical constituents of the methanol extracts of Euphorbia retusa Forssk. and Euphorbia terracina L. from Saudi Arabia. South Asian J. Exp. Biol. 4(2): 48-53.
2. Colak, A., and Bicici, M. (2013). PCR detection of Fusariumoxysporum f. sp. radicislycopersici and races of F. oxysporum f. sp. lycopersici of tomato in protected tomato-growing areas of the eastern Mediterranean region of Turkey. Turkish Journal of Agriculture and Forestry. 37(4): 457-467.
3. Das B.H. (1998). Studies on Phomopsis fruit rot of brinjal. M.sc Thesis, Department of plant pathology, Bangladesh Agricultural University, Mymensingh. 29-64.
4. Edel, V., Steinberg, C., Gautheron, N., and Alabouvette, C. (2000). Ribosomal DNA targeted oligonucleotide probe and PCR assay specific for Fusariumoxysporum. Mycological Research. 104(05): 518-526.
5. Elizabeth K.M. (2005). Antimicrobial activity of Terminaliabellerica. India journal of clinical biochemistry: 20(2): 150-153.
6. Farombi, E.O. (2003). African indigenous plants with chemotherapeutic potentials and biotechnological approach to the production of bioactive prophylactic agents. African Journal of Biotechnology. 2 (2): 662-671.
7. Fokkema N.J. (1993). Opportunities and problems of control of foliar pathogens with Microorganisms. Pesticide Science. 37: 411-416.
8. Jain M.P. and Bhatnagar M.K. (1980). Efficacy of certain chemicals in the control of fruit rot of brinjal. Pesticides. 14: 27-28.
9. Kannan R., Ananthan M. and Balasubramani P. (1998). Anthracnose a menace in chilli cultivation. Spice India. 11: 2-3.
10. Kaur S., Kaur R., Kaur P. and Singh D. (1985). Studies on wilt and fruit rot of brinjal caused by Fusariumsemitectum. Indian Phytopathology. 38: 736-738.
11. Khan N.U. (1999). Studies on epidemiology, seed-borne nature and management of Phomopsis fruit rot of brinjal M.Sc. Thesis, Department of plant pathology Bangladesh Agricultural University, Mymensingh. p. 134.
12. Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A. and Thomma, B. P. H. J. (2006). Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Science.171(1):155–165
13. Manzur A., Raju A. and Rahman S. (2011). Antimicrobial activity of Terminaliacatappa extracts against some pathogenic microbial strains. Pharmacology and pharmacy. 2: 299-305
14. Nolla J.A.B. (1929). The eggplant blight and fruit rot in puerto Rico. Journal of the Department of Agriculture of Puerto Rico. 13:35-57.
15. Panwar N.S., Chand J.N., Singh H. and Paracer C.S. (1970). Phomopsis fruit rot of brinjal (S. melongena L.) in the Punjab. Viability of the fungus and role of seed in disease development. Journal of Research, Punjab Agricultural University, Ludhiana. 7: 641- 643.
16. Parekh J. and Chanda S. (2008). In Vitro antifungal activity of methanol extracts of some Indian medicinal plants against pathogenic yeast and moulds. African journal of Biotechnology. 7(23): 4349-4353

17. Sakander H., Akhilesh B., Koteshwara A.R. (2015). Evaluation of antifungal potential of selected medicinal plants against human pathogenic fungi. Int J Green Pharm. 9:110-117.
18. Singh G. and Kumar P. (2012). Evaluation of antimicrobial activity of alkaloids of Terminaliachebula Retz. Against some multidrug resistant microorganisms. Int J Green Pharm. 6:57-62.
19. Song F. and Goodman R.M. (2001). Molecular biology of disease resistance in rice. Physiological and Molecular Plant Pathology. 59:1-11.
20. Subash, K.R., Bhaarathi, M.G., Jagan Rao, and Cheriyan, B.V. (2012). Phytochemical screening and acute toxicity study of ethanolic extract of Alpinia galangal in rodents. Int.J. Med. Res. Health Sci. 2 (1): 93-100.
21. Tamura K., Nei M. and Kumar S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci. USA, 101:11030-11035.
22. Tamura K., Dudley J., Nei M. and Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) ver. 4.0. MolBiolEvol. 24:1596-1599.
23. Vonshak A., Barazani O., Sathiyamoorthy P., Shalev R., Vardy D., Golan-Goldhirsh A. (2003). Screening South Indian medicinal plants for antifungal activity against cutaneous pathogens. Phytother Res. 17:1123-5.