THYMUS SERPYLLUM – A NOVEL BIOCONTROL AGENT AGAINST LEAF SPOT PATHOGENS OF *SOLANUM MELONGENA*

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ABSTRACT

Solanum melongena L. production is facing a lot of threats in Pakistan which are responsible for its low productivity. Like many other diseases; leaf spot is very important due to its significant yield losses. Therefore, control of this disease is obligatory to reduce the causal agent lower than commercial thresh hold level. Biological control provides safe fungal management program. Presently, the research was under taken to ascertain the antitoxic effect of *Thymus serpyllum* against eggplant leaf spot pathogens i.e., *Exserohilum rostratum, Cladosporium cladosporioides* and *Curvularia clavata*. For this, 10 concentrations of methanolic extract of plant (0.5% to 5%) were employed against the pathogens. Data analysis depicted that development of all the pathogens was greatly inhibited by all the concentrations while 5% concentration found to be the utmost operative in subduing the growth of all the pathogens. *Curvularia clavata* was found to be the most susceptible among all the pathogens. In pot trials, protective assays proved more pronounced in controlling the disease. The work concludes that organic extract of *T. serpyllum* has stable compounds which have the ability to inhibit the damaging properties of pathogens. This fact guides towards biocontrol using such plants against the phytopathogens in a vast range. The study can be extended to isolate and purify the compounds and its production on commercial scale to manage these pathogens in field conditions.

Key words: Biocontrol, Eggplant, Leaf spot disease, Methanolic plant extract, *Thymus serpyllum.*

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1. INTRODUCTION

The vegetable crop eggplant (known as brinjal in Pakistan) is widely cultivated for its edible fruit in sub-tropics and tropics particularly in U.S.A. India, Pakistan, China, Philippines, Bangladesh, Egypt, France, Italy, Middle East and Far East (Anonymous 2010). It comprises of more than 2000 species distributed in 75 genera. Eggplants have a unique range of health benefits and have a great content of body building factors, dietary fibers, minerals, vitamins, antioxidants, nutrients and proteins (Matsubara et al 2005; Obho et al 2005). It covers 9,044 ha region with annual productivity of 88,148 tons in Pakistan (FAO 2017). Among various vegetables, eggplant production is also facing a lot of threats in Pakistan. Numerous aspects are accountable for reduced productions that include abiotic factors and biotic influences, for example, pests and pathogens (Borkakati et al 2019). The common diseases include bacterial wilt, fungal wilt, Phomopsis blight, mosaic and damping off. Common fungal diseases affecting eggplant are damping off and leaf spots which cause severe damages in the nursery as well as in fields (Crop Protection Compendium 2010). Thus to manage eggplant fungal diseases, some preventive measures can be taken by using resistant crops plant; sanitizing all utensils; crop rotation using non-vulnerable crops; soil disinfection and solarization. Application of fungicides to seeds to eradicate fungal pathogen; superficial sowing of seeds or interruption in planting have also shown effectiveness against certain pathogens that cause different eggplant diseases but the most effective method is suggested to be biological control. Phytochemicals as well as their essential oils possess antimycotic potential against a huge number of mycotic disorders. The extensive range of antimicrobial secondary metabolites like tannins, terpenoids, alkaloids, flavonoids, and glycosides etc. is reported in plants (Dahanukar et al 2000; Shafique et al 2005; Bajwa et al 2007; Shafique et al 2015; Shafique et al 2019).

Thymus serpyllum; native to Europe and North Africa; is an evergreen shrub commonly known as Breckland thyme, wild thyme or creeping thyme of family Lamiaceae (Sathianarayanan et al 2011) and is commonly grown in old stony, wild fields and dry grasslands (Snezana et al 2015). In Pakistan wild thyme yields about 29g/kg. It is aromatic, antiseptic, stimulant, antispasmodic, diuretic and emmenagogue (Grondahl & Bodil 2008). It is the best medicine for flu, fever, headache, constipation, and indigestion, diseases related to bones, depression, fatigue, anxiety, sleeplessness, urinary tract infections, gout, and removing contagions of the reproductive organs (Blumenthal et al 2000). Thyme encompasses plenty of anti-inflammatory composites, comprising luteolin and rosmarinic acid and numerous phytochemicals (ursolic acid, rosmarinic acid and luteolin) which are concomitant to anti-cancer activity in lab experiments (Rasooli & Mirmostafa 2002). Thymus has fungicidal and disinfectant properties. An antimicrobial analysis publicized suppressive activity of ethanol and aqueous extracts of *T. serpyllum* against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa*. Generally, *T. serpyllum* oil suppresses fungal biomass production more efficiently in comparison to any other antimycotic remedy (Jari´c et al 2014). Accordingly the present research was conducted to observe the effectiveness of *T. serpyllum* against the pathogens (*Exserohilum rostratum, Cladosporium cladosporoides,* and *Curvularia clavata*) isolated from Eggplant. The isolation and identification of these fungi as the leaf spot pathogens of eggplant was reported by a number of workers and their pathogenic potential was endorsed by implementation of Koch's postulates (Shafique et al 2019; Shafique et al 2020).

2. MATERIALS AND METHODS

2.1 Selection and Assortment of Allelopathic Plant Species

Thymus serpyllum was experimented as a biological control plant. The plant is fabricated by numerous natural constituents possessing medicinal and antagonistic properties against a number of microorganisms (Ouedrhiri et al 2017). Therefore, wild thyme was opted in this study. The plant sample was procured from the mountainous area Gilgit Baltistan, Pakistan. Wild thyme is a small shrub therefore, it is collected as whole plant including stem, leaves and flowers and sun-dried for several days. The dried material (almost 250 g) was then grinded to make fine powder.

2.2 Preparation of Methanolic Plant Extract

Hundred grams (100 g) dried powdered thyme material was soaked in 1000 mL methanol for about 30 days. Afterwards, material was sieved first with the muslin cloth and then through the Whatman filter paper No. 1. The filtrate was vaporized under vacuum in a rotary evaporator or kept at 45 °C in an oven.

2.3 Antifungal bioassays

2.3.1 Preparation of Fungal Growth Medium

The organic solvent extract bioassays were preceded in 2% Malt extract broth medium to grow fungi in 250 mL conical flasks. Methanolic plant extract was prepared with the method of Shafique et al (2019). To prepare the stock solution of methanolic residues, 5 ml of Dimethyl sulfoxide (DMSO) was added in 16.2 gm of crude followed by the addition of sterilized water to make 27 ml of the final concentration of 10% stock solution. For the evaluation of bio efficacy of extract, ten concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0%) were prepared while control received only DMSO+water. One mL of conidial suspension (5.0×10^5 conidia/mL) of *Exserohilum rostratum*, *Cladosporium cladiosporoides* and *Curvularia clavata* was inoculated to all sets separately in four replicates and incubated at 28 ± 2 ° C for almost 10 days.

2.3.2 Fungal Biomass Determination

After 10 days of inoculation, fungal mass from each treatment was filtered on pre-weighed filter papers to determine dry weight yield according to practice of Bajwa et al (2007). Dehydrated fungal mass was used to find out the percentage of fungal biomass increase or decrease to estimate the comparative effect of employed concentrations of methanolic extracts of *T. serpyllum* on *E. rostratum*, *C. cladiosporoides* and *C. clavata.* Percentage reduction in fungal biomass due to various concentrations of the extracts over control was calculated by applying the following formula:

Growth inhibition (%) =

$$\frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

2.4 Pot Trials

The effect of selected allelopathic plant; capable of high antifungal potential against the pathogens *E. rostratum, C. cladosporoides,* and *C. clavata*; was evaluated. For this; protective and curative assays were employed in pot trials in triplicate containing 4 plants in each replicate.

2.4.1 Protective Potential of *T. serpyllum*

Brinjal plants were developed in pots under greenhouse conditions and at the stage of two weeks growth; they were sprayed with 5 mL of different concentrations i.e. 1, 3 and 5% of methanolic extracts of *T. serpyllum*. After 3 days, 1 mL of fungal inoculum was sprayed to similar set. The pots were enclosed in polythene bags individually and watered regularly. After 15 days of incubation at 24 – 30 °C disease indications, % age disease index and % age control was verified. Disease index was calculated with the following formula to determine disease control:

Disease Index (D.I) =

$$\frac{\text{Number of diseased plants}}{\text{Total Number of sampled Plant}} \times 100$$

Percentage control =

100 – Percentage disease index

2.4.2 Curative Potential of *T. serpyllum*

Brinjal plants were sprayed with 1 mL of fungal inoculum under the green house conditions. Pathogens were allowed to establish for 3 days prior application of 5 mL of methanolic extract of *T. serpyllum* using different concentrations i.e. 1, 3, and 5%. The plants were shielded with polythene bags and soil was moistened regularly. Then, after fourteen days of spray % age disease index and percentage control was calculated for each concentration using above formulae.

3. RESULTS

The methanol extract of *Thymus serpyllum* was used to test the antifungal activity against all the pathogens using 10 concentrations (0-5%). All the different applications of plant extract displayed variable but significant results against all three pathogens *in vitro* conditions.

3.1 Effect of *Thymus Serpyllum* on the Growth of Test Fungi

The *in vitro* antifungal efficacy of *T. serpyllum* extract was recorded against *E. rostratum* and the upshots are summarized in Fig. 1. The data depicted a significant inhibition when exposed to different concentrations of extract with respect to control. The lower concentrations ranging from 0.5 to 3.5% ascertained less lethal to the target pathogen and instigated about 22–45% arrest in fungal growth, while the maximum reduction of approximately 80% in fungal growth was demonstrated by 5% concentration.

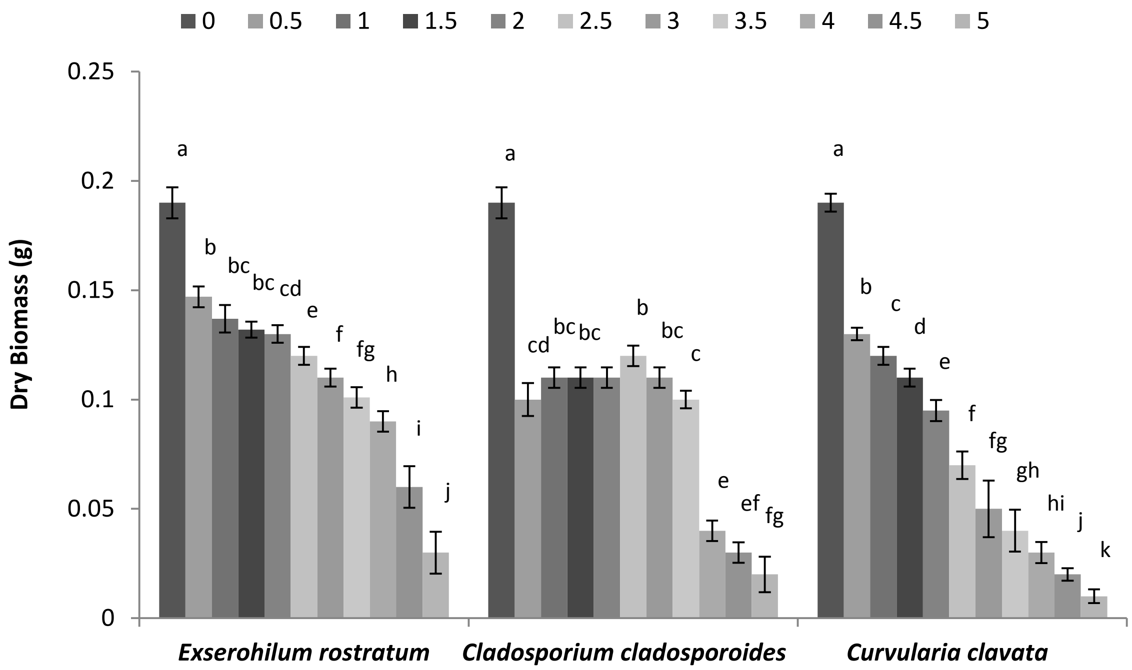

Fig. 1: Effect of different concentrations of methanolic extract of *Thymus serpyllum* on the growth of *Exserohilum rostratum, Cladosporium cladosporoides* and *Curvularia clavata*.

The vertical bars indicate standard error of means of four replicates. Values with different letters show significant difference (p≤0.05) as determined by Duncan's multiple range (DMR) test.

The data obtained from antifungal activities of methanolic extracts of *T. serpyllum* exhibited the variable antifungal activity against *C. cladosporioides* at different concentrations (Fig. 1). All the concentrations showed auspicious outcomes in subduing the fungal growth as the lowest concentration (0.5%) induced about 47% arrest in fungal growth. Almost same level of reduction in biomass production was noticed by 1 to 3.5% concentrations with insignificant differences. The higher concentrations i.e., 4–5% were found to be the most effective in biomass reduction as these concentrations induced up to 79 to 89% suppression in growth, respectively.

Antifungal effect of *T. serpyllum* against *C. clavata* growth depicted a dosage dependent response as a steady and significant arrest in growth was noticed by increasing the concentrations. The maximal fungitoxic stress was indicated by 5% concentration that induce a drop of approximately 95% in dry biomass production of *C. clavata* while the other concentrations (0.5–4.5%) tempted about 31 to 89% biomass reduction, respectively (Fig. 1).

3.2 Pot Trials

In pot trials, the evaluation of protective and curative potential of methanolic extract of *T. serpyllum* presented healthy results with a significant protection of brinjal plants from fungal pathogens at all the employed concentrations (Fig. 2-4).

3.2.1 Effect on Percentage Disease Control of *Exserohilum rostratum*

The results of protective analysis revealed that 5% concentration of plant extract provided plants with the maximum disease control of about 89% and it was found to be decreased with decreasing concentrations. Protective ability of 3% concentration was found to be second in its effect among all the treatments used with a significant disease control of about 84%. In case of curative bioassays, again ascending pattern of disease control was attained against *E. rostratum* with maximum curative activity at 5% concentration exhibiting approximately 73% disease control (Fig. 2).

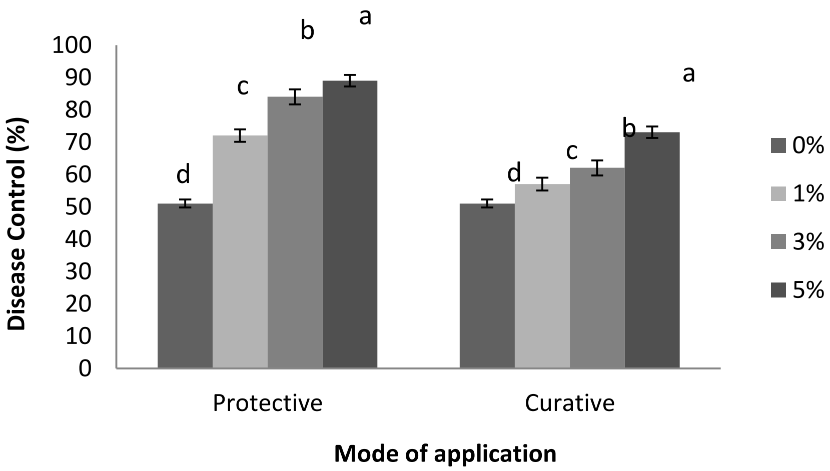

Fig. 2: Effect of protective and curative treatment of methanolic extract of *Thymus serpyllum* on percentage disease control of *Exserohilum rostratum*.

Vertical bars indicate standard error of means of four replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

3.2.2 Effect on Percentage Disease Control of *Cladosporium cladosporioides*

The data regarding the efficacy of methanolic extract of *T. serpyllum* in protective and curative trials against *C. cladosporioides* revealed that the extract exhibited significant but comparatively less inhibition in disease control of brinjal in protective as well as curative bioassays as compared to *E. rostratum*. About 76-86% reduction in disease control was noticed due to the extract of *T. serpyllum* in protective assays. The curative assay displayed 54-68% disease control in brinjal plant (Fig. 3).

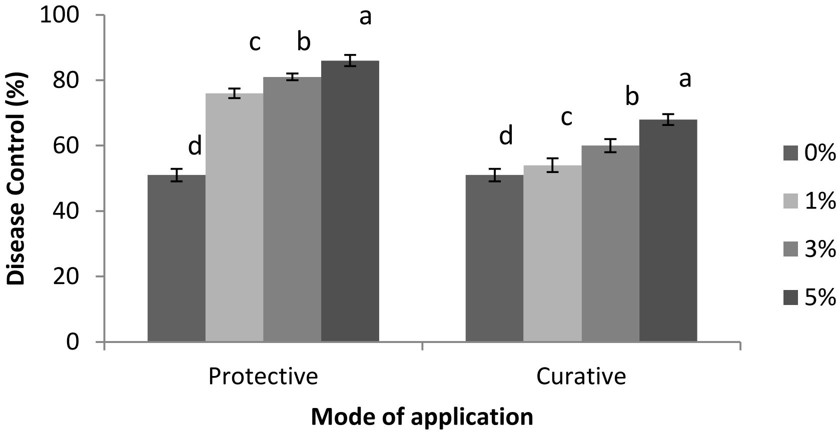

Fig. 3: Effect of protective and curative treatment of methanolic extract of *Thymus serpyllum* on percentage disease control of *Cladosporium cladosporioides*.

Vertical bars indicate standard error of means of four replicates. Values with different letters show significant difference (p≤0.05) as determined by Duncan's multiple range (DMR) test.

3.2.3 Effect on Percentage Disease Control of *Curvularia clavata*

The data regarding disease control induced by different concentrations of methanolic extract of *T. serpyllum* prior and after the pathogenic attack is portrayed in Fig. 4. The disease was found to be effectively controlled by all the concentrations of allelopathic spray and dosage effect was clearly evident. The protective application of extract presented more favorable results in defeating the pathogenic spell as it exhibited about 74-89% disease control by 1-5% extract concentrations. While in curative treatment, 53-72% disease control was observed (Fig. 4).

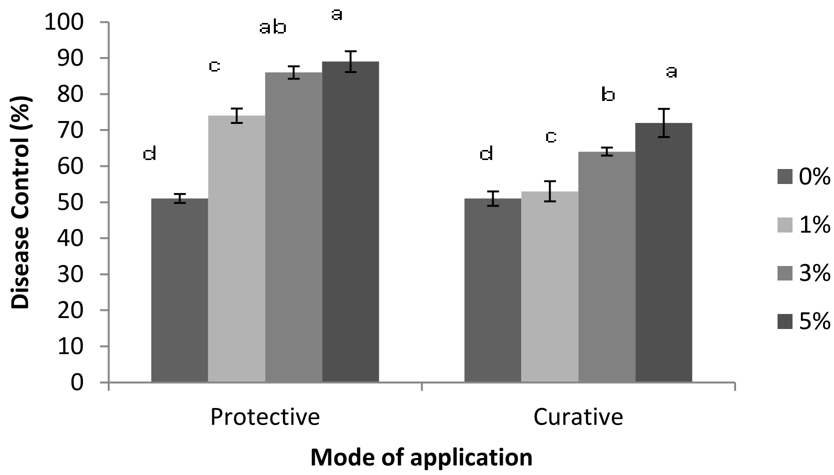

Fig. 4: Effect of protective and curative treatment of methanolic extract of *Thymus serpyllum* on percentage disease control of *Curvularia clavata*.

Vertical bars indicate standard error of means of four replicates. Values with different letters show significant difference (p≤0.05) as determined by Duncan's multiple range (DMR) test.

The complete comparative analysis of protective and curative activity revealed that application of extract prior to pathogenic infection was the best strategy to control the disease.

4. DISCUSSION

Solanum melongena is grown as economically important edible fruit. Leaf spot on brinjal is its destructive disease in Pakistan. There are numerous techniques aimed at the execution of this disease, with varying effects; and still researchers are keen about finding out the best (Jetiyanon et al 2003; Thippeswamy et al 2006). To manage eggplant diseases, some preventive measures can be taken by using resistant varieties; adopting different cultural practices, and utility of chemicals (Dasgupta & Maiti 2008). However, to abate the reliance on fungicides and elude side effects of chemicals, the biological control seems to be effective choice for fungal management. Numerous plant extracts e.g., lemon, citronella, clove, mint, thyme, and oregano oils, have been employed as biocontrol measure to replace the conventional synthetic pesticides (Bajwa et al 2007; Masoko et al 2007; Shafique et al 2015). The action of plant extracts is due to their bioactive compounds against fungi through preventing their growth (Al-Samarrai et al 2012; Banaras et al 2015; Shafique et al 2019).

Presently, biological control potential of methanolic extract of *T. serpyllum* was tested against all the pathogenic fungi. Previously, only a few scientists have ascribed the antimicrobial activity of different species of Thymus genus (Jari´c et al 2014). It is reported to contain the high concentrations of carvacrol in its essential oil (Rahman & Gul 2003). Fungitoxic potential of *T. serpyllum* oil on asexual replication of 5 *Aspergillus* species viz., *A. awamori, A. niger, A. flavus, A. foetidus* and *A. oryzae* were scrutinized (Rahman & Gul 2003). The oil not only possessed the antifungal assets but subdued spore formation and establishment and mycelial growth as well. The potency of suppressive influence was found to be variable with species and the concentration of oil used (Farrukh et al 2012). Generally, *T. serpyllum* oil perused more arrest in fungal biomass production as compared to other antimycotic remedy (Jari´c et al 2014). However, the present study provides a novel implementation of methanolic extract of *T. serpyllum* against the pathogenic fungi causing leaf spot of brinjal in Pakistan.

Currently, ten concentrations (0-5%) of plant extract demonstrated distinctly capricious antagonistic responses against the pathogens *in vitro* settings. A number of evidences also report analogous effects of different plant extracts against known pathogenic fungi (Khan et al 1998; Bajwa et al 2007; Baraka et al 2011; Farrukh et al 2012; Javaid & Munir 2012; Shafique et al 2015), which endorse the possession of antifungal substitutes in the test species. In a study, biological control of *Alternaria arborescens* and *Phyllosticta aristolochiicola* was conducted by Shafique et al (2018) using different concentrations of methanol extract of *Cinnamomum verum*. In case of *A. arborescens,* 0.5% concentration induced approximately 90% suppression in fungal growth. Contrastingly *P. aristolochiicola* was least affected (4–10% arrest) at lower concentrations of extract however 2.0% or more of the extract was evidenced the most potent as fungal pathogens failed to grow at these concentrations.

In current research, the higher concentrations (4-5%) supported the greatest depression in mycelial growth of all the targeted pathogens. This decline in fungal biomass in the presence of phytochemicals could probably be attributed to the reduced rate of mitosis (Bajwa et al 2004) and enhanced cellular respiration (Singh et al 1995). These results are supported by the previous investigation in which fungistatic bioassays were designed to assess the antimycotic effect of ten concentrations of methanolic extracts of fruit, bark and leaves of *Eucalyptus citriodora* against *Fusarium oxysporum*.

Amongst every treatment, the supreme upshot was depicted at the highest concentrations i.e., 4.5-5% (Shafique et al 2015).

The data obtained from the current study revealed that *E. rostratum, C. cladosporioides* and *C. clavata* showed approximately 80, 89 and 95% growth inhibition respectively at the highest concentration of 5% extract. Whereas at the lowest concentration (0.5%); the growth inhibition was 22, 47 and 31% by *E. rostratum, C. cladosporioides* and *C. clavata,* respectively. However; overall, the plant extract of *T. serpyllum* proved supremely operative in subduing the progression of *C. clavata* at the most. This intangible study notifies that *T. serpyllum* possess innate capability to bring antagonistic effects on fungal pathogens. The antimicrobial activities of Thymus can be attributed to the presence of high concentrations of carvacrol and thymol, which are known to occur at very high concentrations in members of the *Lamiaceae* family, such as *Thymus, Coridothymus, Satureja* and *Origanum* (Chorianopoulos et al 2004; Bounatirou et al 2007). The relative intensity of this effect however varied with the species involved, as well as the particular concentrations of the extract employed. Variability in antifungal response against different pathogens might be linked to the differences in concentration and amount recovered based on several factors, including species of plant used, method of extraction, solvents, and extraction time, which in turn may differ in their antifungal potency (Labib & Aldawsari 2015). Differences can also be attributed to raw materials used (dried or fresh), types of soils used for cultivation, the harvesting time in the year, or differences in oil extraction techniques (Kalemba & Kunicka 2003).

The present study was extended for evaluating the biocontrol potential of *T. serpyllum* extract prior and after the pathogen inoculum using pots trials. It was revealed from the study that application of extract prior to pathogenic infection provided the maximum disease control up to 86-89% by all the pathogens. In a contemporary study Shafique et al (2019) also concentrated upon evaluation of antifungal potential of methanolic leaf extract of *Euclaptus citriodora* to control Fusarium Wilt caused by *F. oxysporum* in *Capsicum annuum*. They testified that the spray of extracts prior to infection provided protection from fungal pathogen with the maximum disease control i.e., 85%.

Furthermore, isolation and purification of antifungal compounds from natural plant extracts is prerequisite that play a significant role in suppressing the growth of fungal pathogen. It is further recommended that effective fungitoxic constituents should be commercially analyzed in field or greenhouse applications to check the chemical nature of these constituents.

5. CONCLUSION

The current work reports the novelty of *T. serpyllum* which contains the plant metabolites that are the precious benediction of nature for disease management against the most devastating pathogens by acting as defense materials against them. The growth of all the pathogens was greatly inhibited by 5% concentration of methanolic extract of *T. serpyllum*.

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