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# Impact of ethanol and chloroform extract of Terminalia chebula seed on early blight pathogen

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*Abstract :* Early blight is a very common and devastating fungal disease of crops that imposes economic loss throughout the world. Spores of Alternaria solani present in the soil for many months to year make it tough to control. When weather suitable, spores germinate to form mycelium affecting plants, initially leaves near the land or soil affected which leads to fruit rot in tomatoes and tuber rot in potatoes. In this study, the antagonistic activity of Terminalia chebula seed was evaluated for its ability to control Alternaria solani and is it really an alternate candidate for synthetic fungicide. Dried fruit seeds of Terminalia chebula extracted with ethanol and chloroform resultant extract were tested for their biological activity by a broth macro dilution method to check spore germination and a well-diffusion method was used to control mycelial growth of Alternaria solani. Ethanol extract ($P<0.010$) and chloroform extract ($P<0.012$) had significant levels of controlling early blight spores. Ethanol extract 20.13mm and chloroform extract 13.9mm zone of mycelial inhibition recorded. Advance studies were carried out on ethanol extract by GCMS and Seed germination to test biological activity. GCMS analysis found 20 active peaks with two of them being new, remaining 18 known compounds identified with their retention time and peak area compared in the NIST library. Structural elucidation was done for 5-Hydroxymethylfurfural, cis-9, cis-12-Octadecadienoic acid, Cis-13-Eicosenic acid and n-Hexadecanoic acid. Seeds of Lycopersicum esculentum treated with ethanol extract increase the germination rate, vigor index, and percentage of germination. The phytochemical compound present in ethanol extract increases protein and protease activity in germinating seeds, a better alternate for synthetic compounds controlling plant pathogenic fungi Alternaria solani at 0.1mg/ml.

*IndexTerms* - Phytochemicals, fungicides, plant pathogen, early blight, Alternaria solani, seed germination, protein and protease activity in the seed..

## I. Introduction

## INTRODUCTION

Early bight is a fungal plant disease affecting tomatoes worldwide. Early blight of tomato (Ramezani, Y., *et al.*, 2019) the symptom visible only after fruiting, initially mature leaves shows discoloured leaf spot at the bottom and spread to other parts of the plant. Early blight of potato (Wolters, P.J., *et al.*, 2019) infecting leaves and stem, if left untreated invade to tubers cause economic loss. *Alternaria solani* reside in soil from a dead infected plant, debris, or contaminated organic manure. The spores of the early blight pathogen are dormant in the soil for many months to years, when the weather is suitable they start to germinate and cause early blight, a devastating disease to tomatoes.

Copper or sulphur-based fungicide (Horsfield, A., *et al.*, 2010) is usually used to control the disease. But tend to accumulate in the soil after prolonged use. Biocontrol agents emerging trend to control this disease but it disturbs the ecological chain. Botanical extract is an alternative to control plant diseases without harming the environment, attracting many researchers and scientists to explore various plants for their antagonistic activity gaining importance as a control measure in organic farming. *Terminalia chebula* dried fruit used in Triphala, Ayurveda medicine to cure many health ailments in India. The phytochemical (Huang, Y., *et al.*, 2016) compounds present in Terminalia chebula dried fruit were well explored by many researchers.

Phytochemicals (Singh, M.K., *et al.*, 2020) in plants help to protect and propagate themselves (Lin, D., *et al.*, 2016). There were many compounds like phenolic compounds, flavonoids, lignans, anthocyanins and xanthones produced by plant demography that determine it (Upadhyay, A., *et al.*, 2014). Phenolic compounds contain benzene rings with a simple hydroxyl group to the high polymer. Phenolic compounds help to pollinate, protect animals, and have antimicrobial effects (Vuolo, M.M., *et al*., 2019). Terpenoids otherwise known as isoprenoids import flavour and fragrance are also known to have antimicrobial activity. This also helps the plant to interact with the environment and tolerance to adverse condition e.g. vitamin A. Alkaloids natural base contain nitrogen, reserve nitrogen, growth stimulator and is mainly involved in plant defence. It tends to be soluble in water under acidic or in neutral, soluble in lipid under basic conditions (Verpoorte, R., 2005). These molecules have a wide range of applications.

Management of these plant diseases is always complicated and there is a need for new compounds to control the growth (Rahman, M., *et al*., 2021). Advanced resistant variety was also used to control the incident but none of them was used to bread (Adhikari, P., *et al.*, 2017). Most of the compounds only prevent the occurrence of disease (Martinko, K., *et al.*, 2021). Control by targeting either mycelium or spore germination without affecting the ecosystem attracts many researchers. This paper is about the ability of *Terminalia chebula* seed extract antagonistic activity on spore germination and mycelial growth inhibition of *Alternaria solani* will reveal a biologically active compound that can be a potential candidate for green fungicide.

The active compound can be applied on seed or foliar to check pathogenic microbial activities depending on the etiology of the pathogen (Dubey, S.C., *et al.*, 2010). Early blight is a soil-born fungus to control it, seed treatment is commonly used with any one of the following methods like seed dressing, seed coating, and seed pelleting (Kaufman, G., 1991). If the coated seed germinates and produces a strong and metabolically active plant, which easily resists plant pathogen (Javed, T., *et al.*, 2018, Javed, T., *et al.*, 2022). Di.Y., *et al.*, 2022 studied the protein and protease involvement in seed germination process.

Before application of active compound in plant or seeds need to support the growth of germination without affecting it. When seeds germinate utilize stored protein by protease enzyme (Müntz, K., *et al.*, 2001) for nitrogen, sulfur, and carbon (Krishnan, H.B. and Coe, E.H., 2001) makes it important criteria hence included in this study.

## NEED OF THE STUDY.

Chloroform and ethanol solvents were used to extract the chemical compounds from the seeds of *Terminalia chebula* dried fruit and tested for their biological activity against wilt fungi. Efficient extract analysed by GCMS and structural elucidation made with NIST library. The same extract was tested for their seed germination and impact on protein and protease content of germinating seed.

## RESEARCH METHODOLOGY

### 3.1 Sample processing

*Terminalia chebula* Retz. Dried fruit sample. Surface washed with sterile distilled water and air dried. These fruits were broken and divided into two parts, with only seeds collected and powdered for further work.

### 3.2 Solvent Extraction

The dried fruit seed extract was obtained using the cold extraction method (Umeh, E.U., *et al.*, 2005). Fifty grams of powdered *Terminalia chebula* seed materials (endo) were weighed into separate sterile conical flasks. The samples were extracted using 250 ml of ethanol and chloroform separately and left for 48 hours at room temperature. The resultant suspensions were filtered into sterile conical flasks. These extracts were air dried to evaporate the organic solvent and the residue was collected, they were labelled appropriately and used for further studies.

### 3.3 Chemical composition

Preliminary qualitative analysis of sugars, amino acids, anthroquinins, coumarins, saponins, tannins, phenolic compounds, flavonoids, terpenoids, and glycosides were made as per method of Gul, R., *et al.*, 2017, to the extent GCMS adopted to elucidate the structure of constituents in the extract.

### 3.4 Biological activity of extracts

Biological activity of ethanol and chloroform extract of seed determined for standard culture *Alternaria solani* ITCC-4632 against its mycelium by well-diffusion and against spore germination by broth dilution method.

### 3.5 Standard fungal culture

*Alternaria solani* ITCC - 4632 obtained from Indian type culture collection (ITCC), New Delhi, India. The strain was well maintained on Potato dextrose agar at 4°C.

### 3.6 Antagonistic activity on mycelium

Seven-day-old cultures of *Alternaria solani* ITCC – 4632 spread on Mueller-Hinton agar. Approximately well (0.65 cm diameter) was made with a cork-borer onto the plates inoculated with test organisms. 50µl of Crude plant extracts of 250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml were respectively added into each well aseptically. Inoculated plates were incubated at room temperature until the fungal growth of the control plates reached the edge of the plate. Three replicates were done for each treatment it measures the antagonistic activity by measuring the zone of clearance of mycelium.

### 3.7 Antagonistic activity on spores:

**Fungal Spore Suspension**

Fifty ml of sterile distilled water was added to the 7-day-old culture of *Alternaria solani* ITCC – 4632, which was grown on potato dextrose agar (PDA) and scraped gently, filtered through a Buchner funnel under sterile conditions. The filtrate was adjusted with half strength Czapek's Dox Broth to $1x10^5$ spores per ml and used for further study.

**Inhibition of spore germination**

100µl of conidial spore suspension with 100µl of *Terminalia chebula* ethanol, chloroform extracts of seed with various concentrations ranging from 1.0, 2.0, and 5.0 mg/ml were added separately and incubated in a shaker at 28℃ with 120 rpm for 10 days. For control, the test tube received 100µl of conidial suspension and 100µl of sterile distilled water. Optical readings were taken at 600 nm every 2-day intervals up to 10 days. All procedures described above were carried out under sterile conditions. Experiments were done in triplicate, mean was calculated, rate of inhibition was calculated from mean by (Ac-At)/AcX100) whereas Ac-Mean value of Negative Control, At-Mean value of test sample.

### 3.8 Phytochemical analysis by GCMS

Ethanol extract analysed by GCMS. The injection quantity of 1µL was used (split ratio 10:1), and the injector temperature was maintained at 250 ℃ (constant). The column oven temperature was set at 50 ℃ for 3 min, raised at 10 ℃ per min up to 280 ℃ and the final temperature was increased to 300 ℃ for 10 min. The contents of phytochemicals present in the test samples were identified based on a comparison of their retention time (min), peak area, peak height and mass spectral patterns with that spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) library.

### 3.9 Seed treatment and germination test

The seeds of *Lycopersicum esculentum* were surface sterilized using sodium hypochlorite (0.1% v/v) for 30 min and followed by a wash with sterile distilled water. Then these seeds were treated with *Terminalia chebula* ethanol extract (0.05, 0.1 and 0.2% w/v), seeds with the volume of extract (20seeds/2ml), and kept in a rocker (with slight agitation) overnight in room temperature. Instead of extracting sterilized distilled water is used as a control. The seeds (50 seeds/Petri plate) were placed in a Petri plate (16cm diameter) lined with moist filter paper and incubated at 28±2ºC under dark conditions for 2 days and further at 12h light/12h dark up to 20 days and the germination % was calculated. The experiment was conducted in a completely randomized design with three replication for treatment. The germination percentage was calculated:

Germination % = (Number of germinated seeds × 100) / Total number of seeds

Seed vigour index = (Germination %) × (Seedling Length)

The germination rate was calculated by the number of seeds newly germinating (Δn) at the time (t) after showing.

Germination rate $=\sum (\Delta n/t)$

### 3.10 Protein Estimation (2)

Germinated plant samples were collected at various germination stages from control and extract-treated plants. They were ground in a pre-chilled mortar and pestle (1g/2ml) with 100 mM phosphate buffer at 4ºC. It was then centrifuged at 4ºC for 10 minutes at 12,000 G in a refrigerated centrifuge. The supernatant contains protein.

To 1 ml of each sample, 5 ml of Bradford's reagent (Coomassie brilliant blue 2mg in 1ml 95% ethanol) was added, mixed thoroughly and absorbance was measured at 595 nm in a Shimadzu (UV-1800) spectrophotometer. Protein contents were determined using Bovine Serum Albumin (BSA) fraction V (Sigma Chem. Co., USA) as a standard.

### 3.11 Protease activity

At various germination stages, protease was extracted from germinating seed tissues by homogenizing in 100 mM phosphate buffer at 4ºC. The enzyme reaction was started by adding casein (1% w/v), a substrate for protease assay (Lowry, O. H., *et al.*). The reaction mixture was incubated at 37º C for 20 min then the enzyme reaction was terminated by adding trichloro acetic acid (5% v/v), causing precipitation of protein. The resulted precipitate was removed by centrifugation at 10000 rpm for 15 min and the supernatant was allowed to react with alkaline copper reagent at room temperature for 10 min. To this reaction mixture, Folin Ciocalteau Reagent (FCR) was added and incubated in dark at room temperature for 30 min. The absorbance was measured at 620 nm after the development of blue colour using a UV-visible spectrophotometer. The enzyme activity was expressed as µ mole/min/g dry weight basis, which corresponded to µ mole of tyrosine equivalent released per minute under the assay conditions

## IV. RESULTS AND DISCUSSION

### 4.1 Chemical composition

Steroid, Reducing sugar, Phenolic compound, Amino acid, Glycoside, Coumarin and anthroquinone were present in ethanol extract of dried fruit seed.

The high amount of tannin and coumarin and the average amount of steroid, terpenoid and saponin were present in the chloroform extract of dried seeds.

### 4.2 Biological activity of extracs

Antagonistic activity on mycelium

Table 1 Mean value of zone of inhibition in mm diameter.

| Solvent Extract | 250 µg/ml | 500 µg/ml | 750 µg/ml | 1000 µg/ml |
|---|---|---|---|---|
| Ethanol | 14.06 ± 0.094 | 15.03 ± 0.047 | 20.13 ± 0.094 | 20.96 ± 0.047 |
| Chloroform | 11.03 ± 0.124 | 14.03 ± 0.205 | 13.9 ± 1.344 | 21 ± 0.006 |

± standard deviation

Positive control Fluconazole inhibits mycelial growth up to 24.94±0.465 mm in diameter. The concentration of 750 µg/ml of phytochemicals in ethanol and chloroform extract diffuse from the well and inhibit *Alternaria solani* ITCC – 4632 mycelial growth up to 20 and 14mm diameter exhibited confirms the presence biologically active molecules in the extract.

### 4.3 Antagonistic activity on spores

Results of ethanol and chloroform extract were expressed in Fig 1 and 2 using prism version 3.0. Spore germination is effectively controlled by ethanol and chloroform extract. Compared it with positive control Amphotericin. 91% of initial spore growth was inhibited later 88% of growth was controlled by ethanol extract. 77% of initial spore growth and later 69% inhibited by chloroform extract.

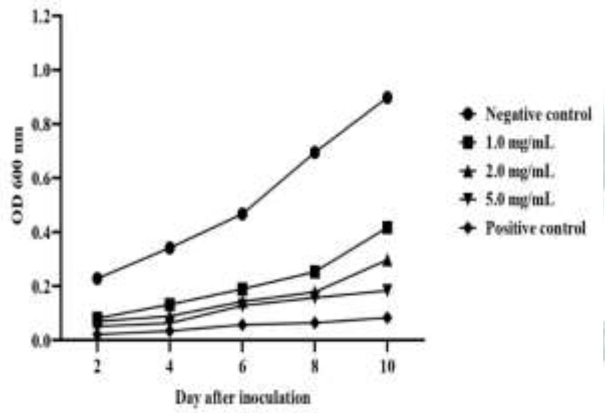



Fig. 1: Effect of Terminalia chebula Ethanol extract against spores of Alternaria solani ITCC - 4632

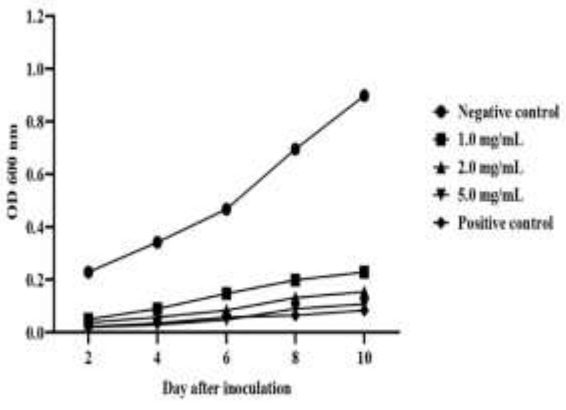



Fig. 2: Effect of Terminalia chebula chloroform extract against spore of Alternaria solani ITCC - 4632

### 4.4 Statistical analysis of Efficiency of solvent extract

To test the significance of ethanol and chloroform extract against *Alternaria solani* ITCC – 4632, statistical analysis was performed with excel using a two-tail T-test analysis of p-value calculated for each extract (2mg/ml) inhibit spore germination 0.0120, 0.0108 respectively. Accepting significant biological activity of both the extract as p less than 0.05. Further compounds in ethanol extract were analysed by GCMS.

**4.5 GCMS**

X-axis retention time and Y-axis mass value of the specific compound area of peak at the point of retention revealed the presence of active constituents; these are tabulated with their retention time, molecular formula, molecular weight, and peak area. GC-MS analysis of ethanol extracts *Terminalia chebula* confirms the presence of a total of 20 compounds in the chromatogram (Graph 1, Table 2). With the help of the NIST library structure of 5-Hydroxymethylfurfural, cis-9, cis-12-Octadecadienoic acid, Cis-13-Eicosenic acid, n-Hexadecanoic acid and other compounds elucidated (Fig 3). The biological activity of ethanol extract was tested on *Lycopersicum esculentum* seed germination, impact on protein and protease activity in germinating seeds was also monitored.

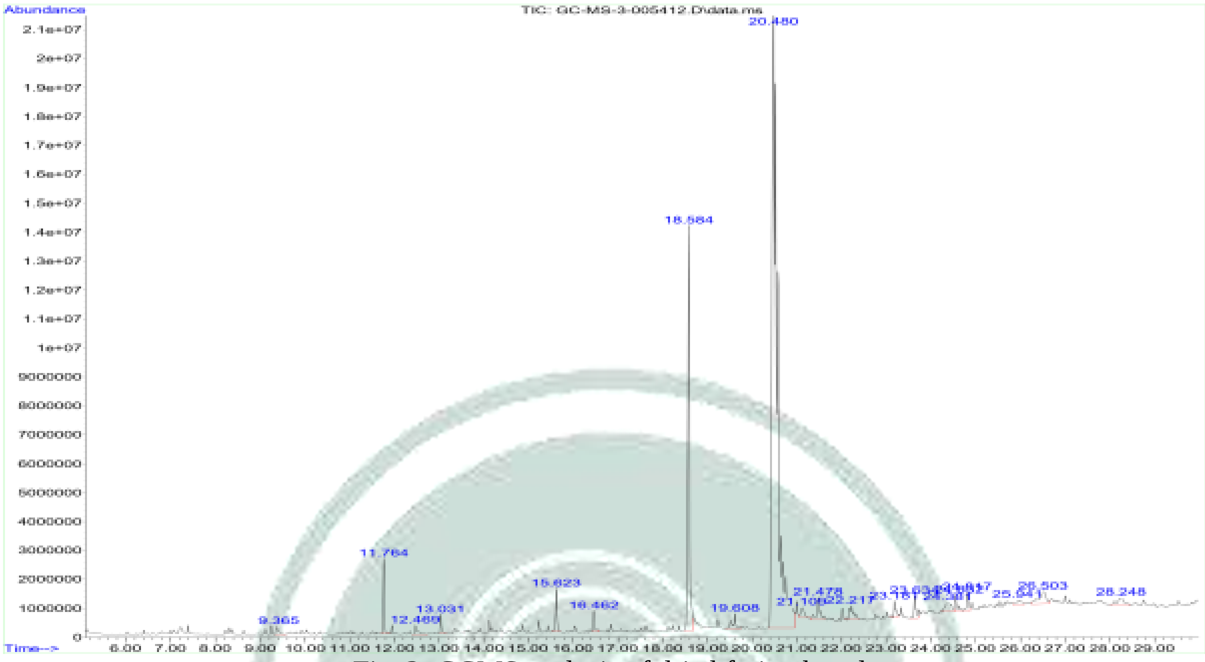



Fig. 3: GCMS analysis of dried fruit ethanol extract

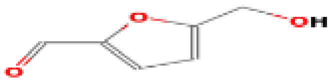
Table 2. The GC-MS of ethanol extract of Terminalia Chebula

| RT | Compound name | Molecular formula | Molecular weight | Peak area |
|---|---|---|---|---|
| 9.365 | 2,3-Dihydrooxazole, 2-t-butyl-4-(1-hydroxy-1-methylethyl)-3-methoxycarbonyl-5-methyl- | C6H10O | 98.14 | 0.48 |
| 11.764 | 5-Hydroxymethylfurfural | C6H6O3 | 126.1100 | 1.93 |
| 12.469 | 3,5-Dimethyl-1-dimethylphenylsilyloxybenzene | C16H20OSi | 256.4149 | 0.51 |
| 13.031 | Cyclododecane | C12H24 | 168.3190 | 0.61 |
| 15.623 | D-Allose | C6H12O6 | 180.16 | 1.40 |
| 16.462 | Tetradecanoic acid | C14H28O2 | 228.3709 | 0.65 |
| 18.584 | n-Hexadecanoic acid | C16H32O2 | 256.4241 | 15.27 |
| 19.608 | Heptadecafluorononanoic acid, tridecyl ester | C22H27F17O2 | 646.4214 | 0.92 |
| 20.480 | 9-Octadecenoic acid, (E)- | C18H34O2 | 282.4614 | 67.22 |
| 21.109 | 9,12-Octadecadienoic acid (Z,Z)- | C18H32O2 | 280.4455 | 0.75 |
| 21.478 | 9,12-Octadecadienoic acid (Z,Z)- | C18H32O2 | 280.4455 | 1.52 |
| 22.217 | cis-13-Eicosenoic acid | C20H38O2 | 310.5145 | 1.13 |
| 23.181 | 9,17-Octadecadienal, (Z)- | C18H32O | 264.4461 | 0.70 |

| | | | | |
|---|---|---|---|---|
| 23.634 | cis-9-Hexadecenal | C16H30O | 238.4088 | 0.84 |
| 24.381 | Zinc, bis[[5,5'-methylenebis[3,4-dihydro-4,4-dimethyl-2H-pyrrol-2-onato]](1-)-N1,N1']-, (T-4)- | C9H10N2 | 146.19 | 1.19 |
| 24.632 | 1,4-Methanonaphthalen-9-ol, 1,2,3,4-tetrahydro- | C11H12O | 160.2124 | 0.65 |
| 24.817 | 9,17-Octadecadienal, (Z)- | C18H32O | 264.4461 | 0.75 |
| 25.941 | 2-Methyl-Z,Z-3,13-octadecadienol | C19H36O | 280.5 | 0.50 |
| 26.503 | - | - | - | 1.87 |
| 28.248 | - | - | - | 1.10 |

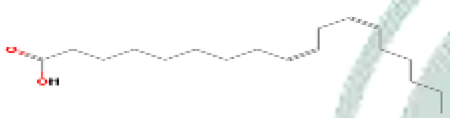
- New compound, no match in NIST library, RT retention time

5-Hydroxymethylfurfural



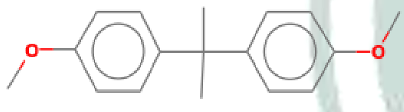
Cis-13-Eicosenic acid

cis-9, cis-12-Octadecadienoic acid



n-Hexadecanoic acid

2,2-Bis(4'-methoxyphenyl)propane



n-Tridec-1-ene

Hexadecyl bromoacetate

trans-$\delta^9$-Octadecenoic acid

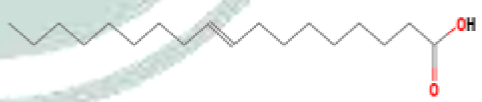


Fig. 4: Structure of biologically active compound in the extract from NIST library.

### 4.6 Seed treatment and germination

Seeds treated with ethanol extract at the various concentration (0.05, 0.1, 0.2mg) along with control seeds are allowed to germinate for up to 20 days. The results expressed in table 3 reveal 44, 91, 38 per cent increase in the percentage of seed germination than the control seed with respect to 0.05, 0.1 and 0.2mg/ml of ethanol extract treated seeds. Vigour index and germination rate were highest in 0.1mg/ml treated seeds.

Table: 3 Seed treatment and germination

| **Experiment** | **Control** | **Treatments** | | |
|---|---|---|---|---|
| | | **0.05** | **0.1** | **0.2** |
| Percentage of seed germination* | 18 | 35 | 45 | 30 |
| | 43 | 54 | 76 | 47 |
| | 69 | 87 | 94 | 81 |

| | | | | |
|---|---|---|---|---|
| | 87 | 98 | 100 | 93 |
| Vigour Index | 956.1 | 1614.1 | 1827 | 1416.4 |
| Germination Rate | 1.21 | 1.36 | 1.38 | 1.29 |

*12, 24, 48 and 72-hour day old plant.

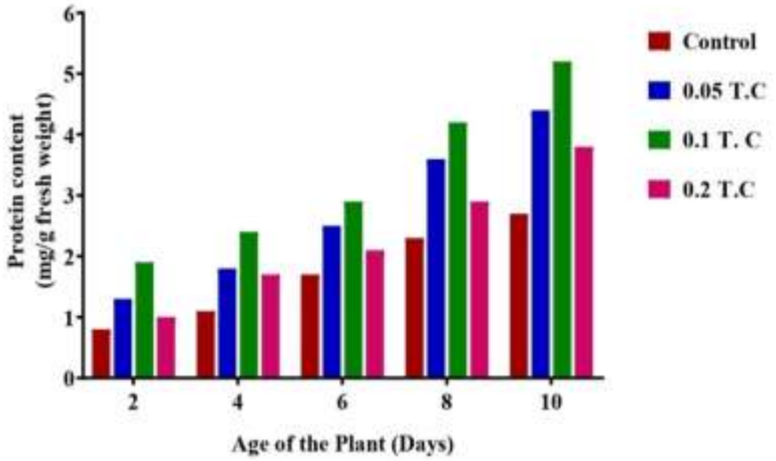



Fig. 5: Protein content in germinating seeds

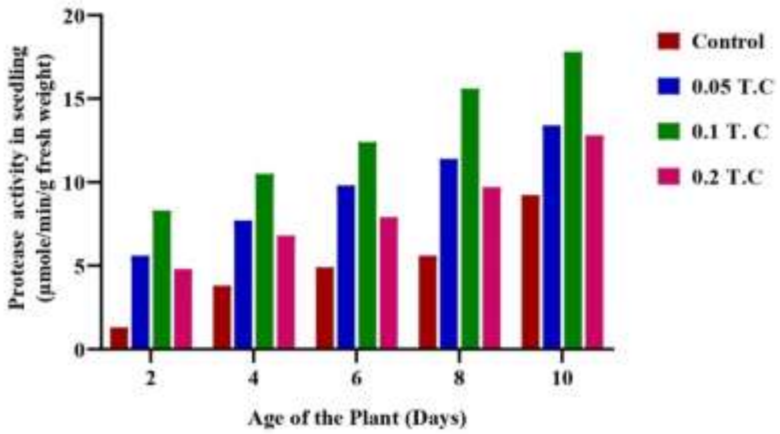



Fig. 6: Protease activity in germinating seeds

Treated seeds with various concentrations (0.05, 0.1, 0.2mg/ml) of ethanol extract show a better response and produce a strong plant than the control seeds of tomato treated with sterile distilled water, the protein and protease activity increased by 92 and 91 per cent respectively than the control (Fig 4 and 5) at the 10$^{th}$ day of germination treated with 0.1mg/ml of ethanol extract. Ethanol extract positively influences the germination process it was observed that 0.05mg/ml and 0.2mg/ml is less active than the concentration of 0.1mg/ml, this may be of effectiveness of the extract and the seeds reactivity.

The fight against plant pathogenic fungi is one of the difficulties of concern to scientists. As a result, researchers are exploring new natural control strategies against fungi using medicinal plants (Dubey, S.C. *et al.*, 2010, Rahman, M. and Borah, M., 2021). Precautions were taken into consideration to avoid loss or destruction of extracted phytochemicals so the choice of solvent was based on target polar and nonpolar compounds, hence ethanol and chloroform were used in this study. The cold extraction method was employed to extract the organic heat-sensitive compound from the studied dried seed. The basic experiments with GCMS done here confirm the presence of a biologically active natural antifungal compounds, biological activity of 0.1mg/ml of extract outstanding activity than any other concentration against spores and mycelium of *Alternaria solani* ITCC – 4632, then it is considered as agent for early blight of tomato at mentioned concentration. *Lycopersicum esculentum* (tomato) seeds were chosen as a model plant as it is easily grown in the greenhouse (Gupta, M.K., *et al.*, 2012). The results revealed 0.1mg/ml concentration of ethanol extract supports the significant growth of seeds into a young tomato plant. This mid-range concentration yields the best results, is often seen when the extract containing growth stimulating phytochemical that can become toxic or inhibitory at higher doses, here both biological activity and seed treatment were revealed 0.1mg/ml extract was the one looking for, protein and protease activity also added value to it.

**Conclusion**

This preliminary study is a key to the development of plant-based natural fungicide to control the plant pathogenic fungi *Alternaria solani*. The results confirm the presence of phytochemicals in *Terminalia chebula* seed as a potential alternate for controlling early blight in invitro and the ethanol extract does not negatively affect the protein and enzyme protease of germinating tomato seeds. Ethanol extract-treated seeds of tomato (invivo) have a higher germination rate and vigour index than the control, which reveals the application of ethanol extract can't affect the seed germination process. In other words, ethanol extract is safe and can be used as a seed coat to confer protection from *Alternaria solani*. These invivo studies were confined to the greenhouse, pathogen inoculation was avoided as it is a quarantine pathogen that cannot be applied to plants (Destructive insects and pests Act 2 of 1914) in field conditions. The seeds of *Terminalia chebula* was found to cause adverse effect but there is no strong proof to confirm it. It need to evaluate its safety but this study focused on plant fungi.

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Competing Interest - The author declare that they have no competing interests
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