



Antifungal activity of aqueous extract of *Trifolium resupinatum* on phytopathogenic fungi

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Abstract

This study aims to evaluate the antifungal activity of the aqueous extract of *Trifolium resupinatum* against nine phytopathogenic fungi (*Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Macrophomina phaseolina*, *Curvularia austriaca*, *Drechslera halodes*, *Bipolaris* sp., *Alternaria alternata*). The study was conducted using three concentrations *i.e.*, 5%, 10% and 20% of the *T. resupinatum* aqueous extracts. Scanning Electron Microscopy (SEM) was employed to examine ultrastructural alterations of treated and untreated mycelia of *C. austriaca*. Fourier Transform Infrared (FTIR) spectrometry was used to detect significant functional groups present in *T. resupinatum* aqueous extracts. *T. resupinatum* aqueous plant extracts showed effective activity against all selected phytopathogenic fungi, based on concentration dependents. SEM of untreated fungal cells did not exhibit structural changes, while treated cells exhibited deformation of the cellular structure, distortion and folding of the outer surfaces, and compacted conidia. FTIR spectrum demonstrated that *T. resupinatum* aqueous plant extracts exhibited various reactive functional groups including alcohols, carboxylic acids and aromatic substances. This study concluded that the aqueous extract of *T. resupinatum* might be used as a natural antifungal agent, which control infections caused by phytopathogenic fungi in the agricultural fields.

Keywords: *Trifolium resupinatum*, Antifungal activity, phytopathogenic fungi, aqueous extract

Introduction

Despite the widespread use of antifungals, scientists estimate that 10 to 23% of the world's crops are lost to fungal diseases each year, with an additional 10 to 20% post-harvest lost. As the effects of global warming, fungal diseases are slowly spreading toward the Polar Regions, producing an increase in the number of nations where these infections are a problem because they harm both agriculture production and harvests. The stem rust fungal diseases, which typically occur in the tropics, thus previously it was observed only in European countries. Furthermore, the researchers caution that fungi's resistance to higher temperatures may raise the likelihood that opportunistic pathogens could develop the ability to infect plants (agriculture crops), mammals (including humans).

As fungal infections become more frequent in European countries due to global warming and as they continue to become resistant to antifungals. This troubling trend may only get worse for emerging nations and also Western countries will be severely impacted by this problem. Particularly, the five important crops (rice, wheat, maize, soya beans and potatoes), fungal infections currently lead to losses equal to food provide between 600 million and 400 million populations for each year.

Traditionally, farmers were used organic and natural fungicidal agents in the early days of agricultural cultivations. In the later 19 century, growth of global population and urbanization, demands more food grains and agricultural practices. Consequently, there was the need of increased the agricultural productivity and also use of upgraded controlling methods to control the losses of food grains due to microbial pathogens. Eventually, there was increasing applications synthetic fungicides agents to control plant diseases caused by microbial pathogens

without knowing impact of environmental concern. After a period of time environmentalist and other research scientist reported the impact of synthetic fungicide in the agricultural field and surrounding the water bodies ^[1]. The WHO and other research communities focused on toxic impact of synthetic fungicides on human and animal health ^[2]. Three active fungicides chemicals such as tebuconazole, epoxiconazole and prochloraz were more toxic and it damage mitochondrial, membrane and caspases 3/7 activities ^[3]. Chemical pesticide exposure in humans is associated with chronic diseases such as cancer, cardiac, respiratory and neurological disorders. The study also reported almost 55 % of applied fungicides are found in air, fresh water and soil ^[4]. Additionally, fungi are extremely resistant, remaining viable in soil for four decades, and airborne spores it will travel among continents, the agriculture production are projected to surge in the near future ^[5]. In this concern, to protect the agriculture production of crop from fungal infections and its diseases, a more unique eco-friendly approach needed. One among the approach, use of medicinal and aromatic plant extracts to control fungal infection and its diseases globally.

Amongst various medicinal and aromatic plants, *Trifolium resupinatum*, (also known as Persian clove) belongs to *Leguminosae* family, (*Fabaceae*) genera which comprises about 240 species ^[6]. It has been used in herbal medicine due to its variety of health benefits ^[7, 8]. The *Trifolium* species is commonly planted in subtropical and Mediterranean regions which have approximately 103 species. It is fitted to a wide range of soil types and it has been used as forage plants in different countries ^[9].

T. resupinatum and other types of clovers have been used as a traditional medicine in different countries for different diseases. It's been used as a remedy for eczema and

psoriasis. In Turkish culture, it has been employed in tonic mixtures, expectorants, analgesics, and sedatives. While in Pakistan, it was shown to be effective in treating fever, sore throat, pneumonia and meningitis [6]. *T. resupinatum* contains flavonoids, isoflavonoids, tannins, and other antioxidants. The presence of these precious complexes, makes it perfect as a useful agent for analgesic, antiseptic, anti-inflammatory, anticancer, treat hypertension, antifungal, antibacterial, anti-*Helicobacter pylori*, insecticidal, antihepatotoxic, antidiabetic and in angiogenesis [7, 8, 10].

The earlier study reported antimicrobial effectiveness of *Trifolium* species (for example *T. pratense*, *T. resupinatum*, *T. alexandrinum* and *T. incarnatum*) [11-14]. Additionally, *Trifolium* sp., plant extracts shown effective activity against a variety of both human and plant pathogenic fungi such as *Candida albicans* [11], *Aspergillus niger* [14], *Microdochium nivale* [15], *Botrytis cinerea* and *Sclerotinia trifoliorum* [16].

There are many fungi that cause necrotic fungal diseases of plants, which economically affect agricultural crops, such as *Fusarium* and its species (*F. solani*, *F. oxysporum*, *F. proliferatum*, *F. verticillodes*). *Macrophomina phaseolina* is also a common soil-borne fungus found worldwide. It leads to many infections including charcoal rot, stem and root rot, and seedling blight and it cause significant losses in crop yield. The genus *Curvularia* contains about 80 species, its common pathogen of many plant species. *Drechslera halodes* is a typical plant pathogenic fungus that causes leaf and crown spot disease and root rot of many plants. *Bipolaris* is a filamentous, deciduous fungus, grown in soil and on plant waste, and known to cause plant ailments. *Alternaria alternata* is a necrotic fungal pathogen in many commercially important crops [18-22].

Thus, numerous studies have evaluated the antimicrobial activities of plant extracts against a variety of pathogens [22-25], the antifungal activity of *T. resupinatum* against plant phytopathogenic fungi remains elusive. Therefore, the current study aims to evaluate *in vitro* antifungal activity of *T. resupinatum* aqueous extract against nine phytopathogenic fungi such as *Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium verticillodes*, *Macrophomina phaseolina*, *Curvularia austriaca*, *Drechslera halodes*, *Bipolaris* sp., and *Alternaria alternata*. In addition, to identify the chemical composition of aqueous extracts by Fourier Transform infrared (FTIR) spectrometry and to characterize the ultra-structural deterioration of fungi with *T. resupinatum* aqueous extract with aid of Scanning Electron Microscopy (SEM).

Materials and Methods

1. Plants Collection

T. resupinatum plant was collected from one of the wild parks at east Riyadh, Saudi Arabia, during the period of April 2022. The plant parts were washed with running tap water followed by distilled water and dried in shade for two weeks. Using a milling machine, dried samples were milled into a fine powder. To avoid contamination, the samples were labeled and stored in air-sealed glass containers at 4°C for extraction and later investigations [25].

2. Preparation of Aqueous *Trifolium resupinatum* Extract

The 40 g of dried *T. resupinatum* powder was mixed in a glass conical flask containing 400 ml of sterilized distilled water. The flask was covered with foil well and then subjected to vortexing on the rotary shaker (250 rpm/ 45 °C) for 24 h. The next day, the crude aqueous plant extract was filtered through Whatman No. 1 filter paper. Later, the extract was centrifuged at 10,000 rpm/ 30 min to eliminate any suspended residues. Then, the extract was concentrated and dried in a rotary evaporator at 40°C under decreased pressure. Then, purified plant extract was stored in sterile bottles at 4°C for future use [23].

3. Phytopathogenic Fungal strains

The phytopathogenic fungal strains (*Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium verticillodes*, *Macrophomina phaseolina*, *Curvularia austriaca*, *Drechslera halodes*, *Bipolaris* sp., *Alternaria alternata*) used in this research were provided from the Department of Plant Protection, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia. All the fungi strains were maintained on Potato Dextrose agar (PDA) at 18° C by subsequent subculture.

4. Evaluation of Fungicidal activity of Ridomil Standard

The standard fungicide Ridomil was used as a standard control and stock solution was prepared at 1.04 mg/ml. From the stock solution prepared four different concentrations as follows; 0.025, 0.125, 0.52, and 1.05 mg/ml, respectively. Then each concentration was poured in to 5 PDA plates for each tested fungi, by mycelium disks (6 mm in diameter). The inhibition activity of the fungicide on colony growth was determined by measuring the diameter (in mm) of developing colonies after 10 days of incubation at 25°C [27].

5. *In vitro* Antifungal Activity

The agar dilution method described by Fatimah [18] and Alotibi *et al.* [24], was used for determining the inhibition of mycelial growth of the tested fungi by the plant extract. The aqueous extract was diluted in distilled water to give the required concentration and then sterilized by passing through 0.45 µm sterile bacteriological filters. A known concentrations of aqueous extract was mixed aseptically with equal volume of molted cooled (40-45°C) PDA to give the required final concentrations of 5, 10 and 20 mg/ml), based on the dry weight of dried plant powder. The control was kept without plant extract. After pouring the medium into sterilized petri dishes, the plates were left a while for solidification. Subsequently, for the bioassay, a 6 mm disc was taken from the edge of the activity growing colonies of the tested fungal strains and placed in the center of PDA plates that contains specific concentration of plant extract (as treated) and positive control was Ridomil. All plates were incubated at 28±2 °C for 5 days. Each assay was replicated three times. Radical mycelial growth was assessed by computing the average orthogonal colony diagonals for each replication. The mycelia growth inhibition determination, colonies diameter was measured after 5 days. The data were computed as a percentage of mycelial growth inhibition and given in millimeters diameter according to the following equation;

$$\text{Growth Inhibition (\%)} = \frac{\text{DC} - \text{DT}}{\text{DC}} \times 100$$

Where: DC= colony diameter of control (untreated), and DT = colony diameter of treated ones [26].

6. Scanning Electron Microscopy (SEM) examination

SEM was used to examine the cellular structure of mycelia that were treated with *T. resupinatum* aqueous extract, and compared with the untreated control mycelia. The first step of the SEM method is an initial fixation by buffering Glutaraldehyde (2.5%) night-long in a refrigerator. The second step is washing with phosphate buffer (pH 7.2) to fix it later by buffering with Osmium Tetroxide 1%. The third step is dehydration of the sample by ethanol at serial concentrations. Then the sample was freeze-dried by using a critical point dryer. After that, samples were mounted on gold plated stubs. The final step is to examine the samples under a JEOL-SEM (Model JSM- 6060 LV, Japan).

7. Infrared Spectrometry Analysis

The functional group of chemical components of *T. resupinatum* aqueous extracts was determined by using Fourier Transform Infrared (FTIR) spectrophotometer. The functional groups were separated based on their peak ratio. The FTIR spectra (mid region of 400-4000 cm^{-1}) were produced using a sophisticated computer-controlled spectrophotometer (Nicolet 6700, Thermo Scientific, USA) outfitted with a beam splitter, DTGS detector, and OMNIC software.

8. Statistical Analysis

All data procured from the experiments were means of 3 replicates. Data were recorded as the mean with standard deviations (SD). One-way analysis of variance (ANOVA) was used to analyze data. The differences were considered significant when the probability value is $p < 0.05$. Statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 18.

Results

1. Antifungal Activity

The antifungal activity of *T. resupinatum* aqueous extracts was demonstrated at concentrations of 5%, 10% and 20% are summarized in Table 1. An interesting finding was that *T. resupinatum* aqueous extract showed significant activity against tested nine phytopathogenic fungi. Table 1 showed remarkable controlling activity nine among seven of the tested fungi with 100% growth of inhibition at 20%

concentration, followed by 97.93% (*Alternaria alternata*) and 79.93% (*Curvularia austriaca*). The additional concentrations (5% and 10%) similarly showed a significant antifungal effect against all selected fungi in comparison to the control group (Table 1, Figures 1, 2 and 3).

2. In vitro evaluation of the antifungal activity of Ridomil

Evaluate the antifungal activity of Ridomil as standard fungicidal agent. The result showed that *F. solani*, *Bipolaris* sp., and *A. alternata* were presented high growth of inhibition (87%, 95.86% and 96.86%) at 1.04 mg/ml respectively, whereas the other fungi were completely inhibited (100%) at 1.04 mg/ml. Finally, the most susceptible fungi to Ridomil were *F. oxysporum* then, *F. proliferatum* and the least susceptible fungi to Ridomil was *C. austriaca* (Table 1 and Figure 3).

3. Scanning Electron Microscopy Examination

The morphological alterations observation by SEM of *Curvularia austriaca* untreated with the aqueous extract of *T. resupinatum* were selected and compared with 20% treated plant. The biomass of the untreated *Curvularia austriaca* control group had normal tubular filaments, intact fungal and conidial growth, and no structural modifications were observed (Figure 4 A). SEM images of *Curvularia austriaca* treated with *T. resupinatum* aqueous extract of displayed a distortion of the cellular structure, presence of shrunken curly filaments of different sizes, as well as the images revealed distorted and rucked outer surfaces, and compact conidia (Figure 4B).

4. Fourier Transform Infrared Spectroscopy analysis

The FTIR Figure 5 presents the spectra of the *T. resupinatum* aqueous plant extract with the prominent absorption band at 3411 cm^{-1} was assigned to O-H stretching vibrations in phenolic acids and saponins, as well as, to some extent, N-H stretching vibrations in amines in the protein. The asymmetric and symmetric stretching bands of -CH shown at 2932 cm^{-1} and 2851 cm^{-1} for plant extract, while the peak at 1635 cm^{-1} obviously shows C=O stretching in carboxyl, ketones or C-N bending in the amide group as well as for C=C. On the other hand, the aliphatic and aromatic C-H plane deformation vibrations of the methyl, methylene, and methoxy groups were evident in the vibration band at 1407 cm^{-1} for *T. resupinatum* extract, the peak was found at 1073 cm^{-1} plant extract, for the stretching vibration bands of C-O and C-O-C, whereas the C-H bending was seen at 608 cm^{-1} for *T. resupinatum* extract.

Table 1: Effect of different concentrations of aqueous extracts of *Trifolium resupinatum* on growth diameter (mm) and percentage of inhibition of growth against selected phytopathogenic fungi on PDA media after 7 days of incubation at 25° C.

Plant pathogens	Growth diameter (mm)				Inhibition of growth %			Ridomil (Standard) (0.125 mg/ml)
	Control	5.0 %	10.0 %	20.0 %	5.0 %	10.0 %	20.0 %	
<i>F. solani</i>	80.0±0.00	15.83±8.70*	15.0±8.67*	15.0±8.66*	62.50±0.0*	80.23±1.0*	100.0±0.0*	50.83±0.83*
<i>F. oxysporum</i>	80.00±0.00	25.83±0.83*	0.00±0.00*	0.00±0.00*	67.73±1.0*	100.0±0.0*	100.0±0.0*	18.33±0.83*
<i>F. proliferatum</i>	80.00±0.00	13.3±0.83*	1.67±1.67*	0.00±0.00*	83.36±1.0*	97.93±2.0*	100.0±0.0*	23.33±0.83*
<i>F. verticillioides</i>	80.00±0.00	25.0±0.00*	0.00±0.00*	0.00±0.00*	68.80±0.0*	100.0±0.0*	100.0±0.0*	34.16±0.83*
<i>M. phaseolina</i>	80.00±0.00	10.00±0.00*	0.00±0.00*	0.00±0.00*	87.50±0.0*	100.0±0.0*	100.0±0.0*	40.00±0.00*
<i>C. austriaca</i>	80.00±0.00	38.33±2.20*	29.17±0.83*	21.7±0.83*	52.10±2.7*	63.53±1.0*	72.93±1.03*	59.16±0.83*
<i>D. halodes</i>	80.00±0.00	29.17±0.83*	0.0±0.0*	0.00±0.00*	63.53±1.0*	100.0±0.0*	100.0±0.0*	36.66±0.83*
<i>Bipolaris</i> sp.	80.00±0.00	14.17±0.83*	10.0±0.00*	0.00±0.00*	82.33±1.0*	87.50±0.0*	100.0±0.0*	40.0±1.44*
<i>A. alternata</i>	80.00±0.00	33.3±0.83*	20.83±0.83*	1.67±1.7*	58.36±1.0*	73.97±1.0*	97.93±2.06*	66.66±0.83*

Data represented as mean ± standard error of triplicates. *P-values < 0.05 were considered significant.

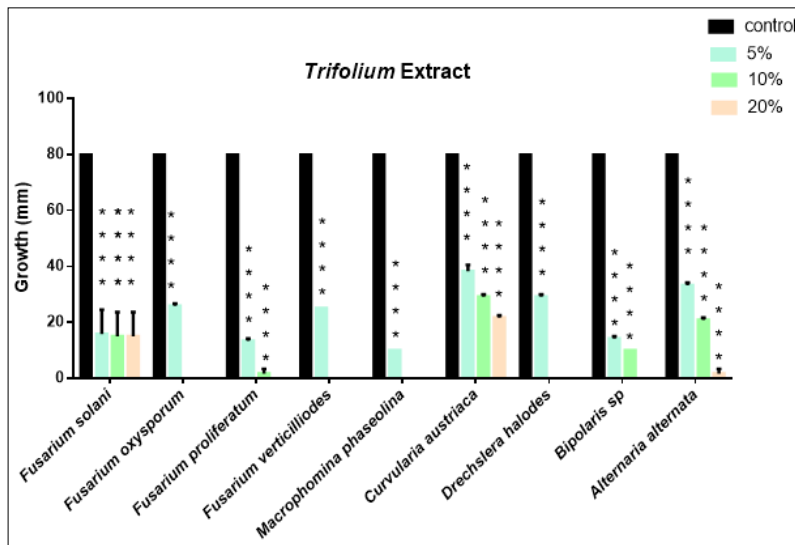


Fig 1: Influence of *Trifolium resupinatum* plant extracts at various concentrations on growth rate (mm) on selected phytopathogenic fungi. (Data represented as mean ± standard error of triplicates. * P-values < 0.05 were considered significant)

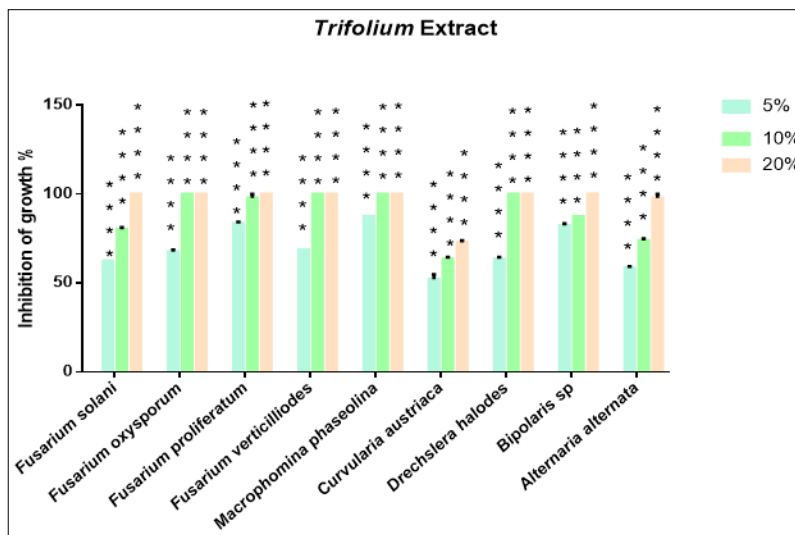


Fig 2: Influence of *Trifolium resupinatum* plant extracts at various concentrations on inhibition of growth percentage of selected phytopathogenic fungi. (Data represented as mean ± standard error of triplicates. *P-values < 0.05 were considered significant).

Tested plant fungal pathogens	Control (without any agents)	Concentration of plant extracts			Ridomil (Fungicide) 0.125(mg/ml)
		5.0%	10%	20%	
<i>F. solani</i>					
<i>F. oxysporum</i>					
<i>F. proliferatum</i>					

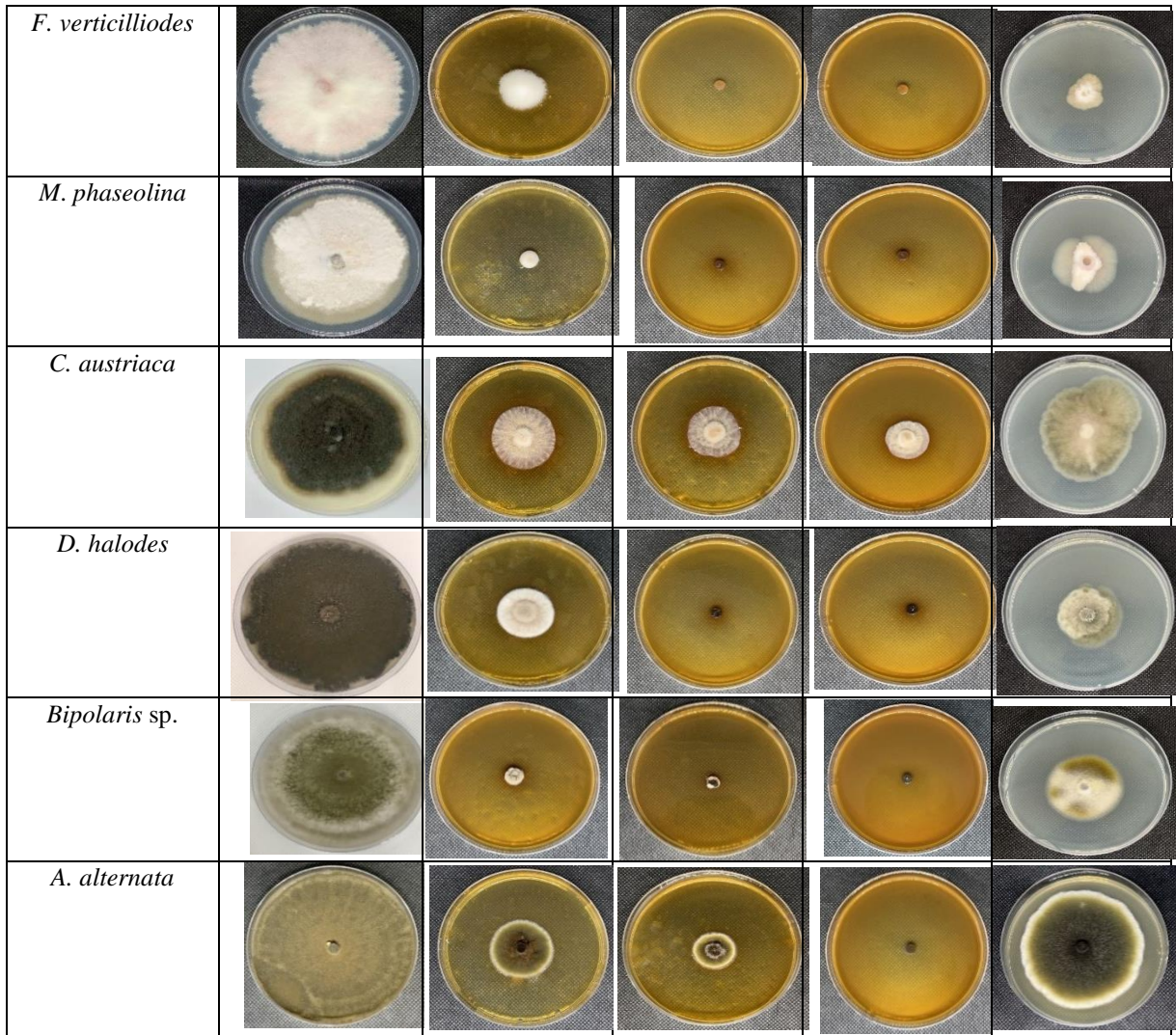


Fig 3: *In vitro* activity of aqueous extract of *Trifolium resupinatum* on nine phytopathogenic fungi

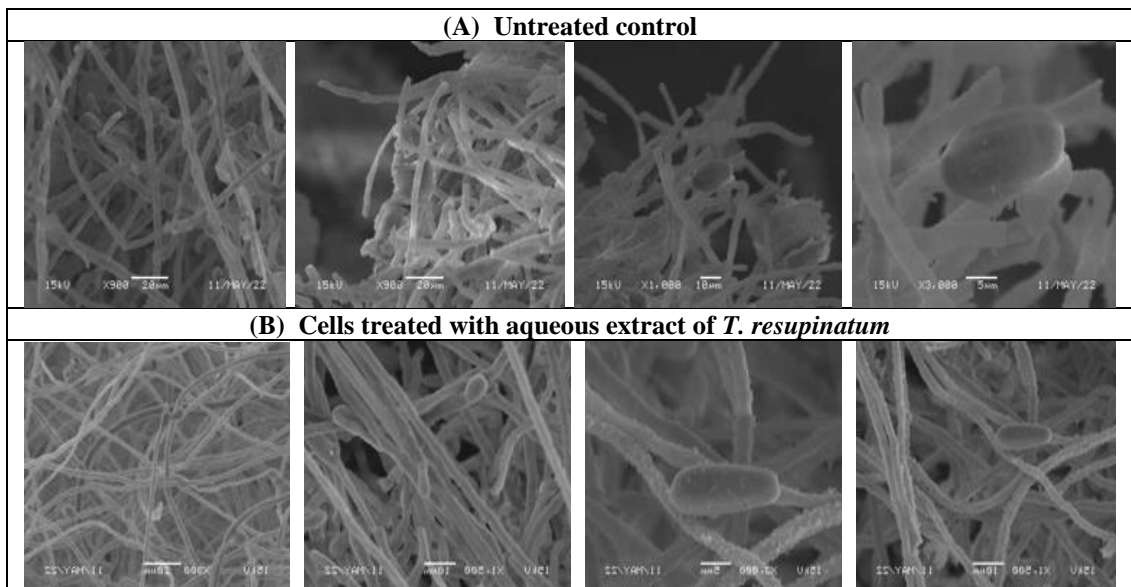


Fig 4: SEM micrographs of *Curvularia austriaca* (A) control; (B) treated by *T. resupinatum* 20% concentration.

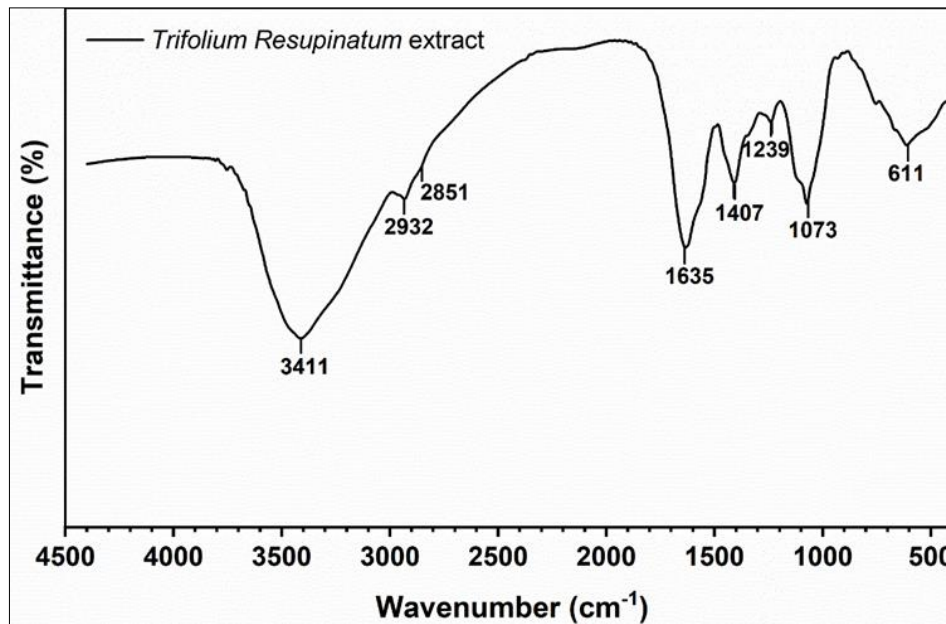


Fig 5: FTIR spectra of the *Trifolium resupinatum* extract.

Discussion

Plant diseases caused by fungi cause severe damage to many economic crops around the world. Although synthetic commercial anti-fungal agents and fungicides are the first-line of defense against fungal infections in crops, these fungicides are toxic and result in deleterious effects on the agricultural crops, soil, other living organisms and surrounding environments [27].

The current study was to investigate the antifungal activity of *T. resupinatum*'s aqueous extract and Ridomil as a positive control against selected nine phytopathogenic fungi such as *Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Macrophomina phaseolina*, *Curvularia austriaca*, *Drechslera halodes*, *Bipolaris* sp., and *Alternaria alternata*. The study outcomes publicized that aqueous extracts of *T. resupinatum* exhibited significant activity against seven fungi i.e., *Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium verticillioides*, *Macrophomina phaseolina*, *Drechslera halodes*, *Bipolaris* sp. followed by *Alternaria alternata* and *Curvularia austriaca*. Particularly, the extracts inhibited 100 % mycelial growth of seven fungi out of nine tested. The current results were significantly exhibited effective antifungal activity by the *T. resupinatum*'s aqueous extract then standard Ridomil. The early report of Cohen [28], investigate antifungal activity of Ridomil against *Phytophthora infestans* on tomato plants. It was control fungi at a concentration of 0.25 mg/ml. About the mycelial growth showed effective controlled against six fungi with 0 %, growth diameter, whereas *Curvularia austriaca* showed 29.17±0.83 % of growth diameter. Subsequently the results evidenced by FTIR spectrum and SEM micrographs. The FTIR spectrum presented the existence significant functional groups of *T. resupinatum* aqueous extract. Thus, the functional groups play a vital role for the antifungal activity. The SEM micrographs also clearly demonstrated that effective antifungal activity against *Curvularia austriaca* at 20 % concentrations. The earlier reports described about flavonols and Isoflavonoids from *Trifolium* sp., exhibited antifungal activity against both human and plant fungal pathogens [8]. Hence, the plant

Trifolium species contains various vital constituents like alkaloids, chalcones, flavonoids, flavones, catechins, tannins, and polyphenols which makes it more potent medicinal properties and also used as a resource plant for therapeutic agents in both agriculture and medical sectors [27]. In this current examination results also showed that *T. resupinatum* aqueous extracts at concentrations (5%, 10%, and 20%) exhibited antifungal activity against the tested nine phytopathogens. The results were astounding given that seven of the examined fungi were totally inhibited (100% growth of inhibition) at 20% concentration, and 97.93% and 79.93% against *Alternaria alternata* and *Curvularia austriaca* at 20% concentration, respectively. Similar to the control group, the rest of the concentrations (5% and 10%) showed marked antifungal activity against all chosen fungi. These results support previous studies that noted the importance of *Trifolium* species as antimicrobial activity [11, 26].

The current outcomes observed was significantly outstanding than the earlier study of Esmaeili *et al* [11] they found that an ethanol-methanol extract of *T. pratense* inhibited both fungal and bacterial pathogens with an effect diameter range of inhibition control. Thus, the current findings were exhibited significant with fungal growth inhibition by *Trifolium* species aqueous extract. Also, *T. resupinatum* extracts was more potent antifungal efficacy than the other species they used by other earlier reports [29]. Regarding, FTIR analyses completely revealed that phytochemical functional groups from the *T. resupinatum* extracts. The functional groups including phenolic acids, saponins, aliphatic and aromatic, methyl, methylene, and methoxy. The current results supported with earlier report of Khurram *et al* [30] and Athar [31], with effective antifungals activities against fungal pathogens. Khatami *et al* [12] was found that *T. resupinatum* seed exudate exhibits antifungal activity against *Neofusicoccum parvum* and *Rhizoctonia solani*, due to the presence of important functional groups revealed by FTIR spectrum. Similar antifungal activity was demonstrated by Al-Otibi [25] which studied the effect of aqueous extracts from *Acacia tortilis* and *Acacia senegal*, which was explained by the presence of polyphenols,

flavonoids, and several vital plant components, which were detected by the FTIR spectrum. The SEM micrographs examination was carried out for ultrastructural alterations in mycelial growth inhibition study. The SEM micrographs of *Curvularia austriaca* treated with *T. resupinatum* extract revealed alteration and deformation on the hyphae's structure and conidial's surface and size. Overall, the study found that *T. resupinatum* plant aqueous extracts exhibited significantly potent antifungal activity against tested nine phytopathogenic fungi, particularly more effective against *Fusarium* species. Furthermore, *in vitro* and *in vivo* studies are needed to evaluate and authenticate the outcomes of this current investigation. Thus, the high percentage of active extract in *T. resupinatum* plant aqueous extracts validates the usage of these plant species as natural antifungal agent formulated as an effective product for sustainable eco-friendly agriculture production [32, 33].

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