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The Effect of Some Medicinal Plant Extracts on *Fusarium oxysporum* f.sp. *lycopersici* Causal Agent of Tomato Wilts Disease in Laboratory and Greenhouse Conditions

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ABSTRACT

Fusarium oxysporum f.sp. *lycopersici* is an important disease agent of tomato which causes wilt and seedling. The present study was performed to evaluate the antifungal effect of *Achillea millefolium, Salvia verticillata and Ziziphora clinopodioides* extracts and their abilities to inhibit the fungus. For this, methanol extracts of reference plants was extracted and tested in concentrations ranging from 1 , 1.5 and 2 mg/ml on mycelial growth of *Fusarium oxysporum*. The same extracts were then tested for antifungal activity *in vivo* in the greenhouse on inoculated tomato plants. *Z. clinopodioides* demonstrated highest antifungal activity against mycelial growth of *F. oxysporum* strain that recorded 77.1%, 62.03% and 61.99% at 2, 1.5 and 1 (mg/ml), respectively. the MIC value for of *Z. clinopodioides* against *F. oxysporum* was 3.125 mg/ml followed *A. millefolium* and *S. verticillata* extract having 6.25 mg/ml. The MFC of extracts was found to be 6.25 mg/ml in *Z. clinopodioides* and 12.5 mg/ml for *A. millefolium* and *S. verticillate.* In greenhouse experiment employing methanol extracts of three plant species showed an increase in the mean plant height and also fresh and dry weight of root and shoot with the consequent reduction in the disease symptoms of the tomato seedlings. Overall, the results showed significant growth inhibition activity of *Z. clinopodioides* methanol extract against *F. oxysporum* in both *in vitro* and greenhouse condition. Although the extracts of *A. millefolium* and *S. verticillata* which had no effect *in vitro* assays, in greenhouse conditions, these plants showed considerable antifungal activity.

1. Introduction

Fusarium wilts disease of tomato caused by (*Fusarium oxysporum* f.sp. *lycopersici*) (f.sp; form special), is one of the most prevalent and destructive disease, causing infection and losses to crop growers (Reis et al., 2005; Sudhamoy et al., 2009). It is now a major concern not only in Iran, but also in other region of the world (Amini, 2009).

The use of synthetic fungicides is mainly practiced for management of wilt disease (El-Sheekh et al., 2020). This measure is not an eco-friendly approach and may cause adverse effects on the environment and human health (Poussio et al., 2018). The increased awareness of the environmental problems associated with fungicides has led to the search for alternative methods to control fungal disease by using

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compounds derived from plant sources (Tegegne et al., 2008). The antimicrobial activity of medicinal plant has been studied for many years. Medicinal plants appear to have a rich source of metabolite and are known to have minimal environmental impact and danger in contrast to synthetic fungicide (Poussio et al., 2018). Among forty plants of different families which were tested against *Fusarium oxysporum* f.sp. *cicero*, *Chenopodium ambrosioides* showed the highest inhibition (Minz et al., 2012). Satish et al., (2009) reported that among 46 aqueous extracts of various medecinal plants against *Fusarium* spp. only 12 plants have showed significant antifungal activity. *Allium ursinum* flower extract inhibited mycelial growth of some pathogenic plant including *Fusarium oxysporum* (Pârvu et al., 2011). Hence, medicinal plants extract can be one of the promising ecofriendly alternative methods for controlling of plant diseases for human consumption (Rongai et al., 2015).

In view of these, in the present investigation, methanol extracts of three medicinal plants including*; Achillea millefolium*, *Salvia verticillata* and *Ziziphora clinopodioides* were studied for evaluation their antifungal activity and identify the reliable concentration of plant extract that have fungicidal properties. In addition, to study the effect of these natural extracts on greenhouse condition in tomato seedlings subjected to fusarium wilt.

2. Materials and Methods

2.1. Preparation of extracts

Leaf samples of *A. millefolium*, *S. verticillata* and *Z. clinopodioides* were collected from natural sites located in Espiran, Tanriz province, Iran, during April and June 2018. The samples were thoroughly washed in running water and dried under laboratory conditions. Subsequently, dry materials made into powdered by using pistol and mortar (Zaker and Mosallanejad, 2010).

2.2. Methanol extract

Forty gram of dry powder materials of each plant were added to 400 ml of pure methanol (Germany, Merck) and homogenized for 24 hours at room temperature;150 rpm. After 24 hours mixtures kept away from direct sunlight under laboratory conditions for 48 h. Subsequently, the mixture was passed through Whatman filter paper (No: 0.22 micron) and then shaken at 160 rpm at 40°C to obtain clear extracts. The methanol was completely removed from clear solutions using a rotary evaporator (IKA Germany, moder RC10) (Zaker and Mosallanejad, 2010). Three extract concentrations (1, 1.5 and 2mg/ml) were selected by pre-test for pathogen prepared in methanol solvent.

2.3. Isolation of the pathogen

Fusarium oxysporum isolate used in this study was collected from a farm in southwestern of Tabriz in East Azerbaijan province of Iran, during summer 2018. For identification, the purified isolates of fungus was identified according to their cultural and morphological characteristics as described by (Leslie and Summerell, 2006). The isolates were grown on potato dextrose agar (PDA, Merck) medium to determine their growth rate and colony pigmentation and the cultures were incubated at 26ºC and 30ºC for 7–10 days in the dark condition. To investigate the presence and shape of the macroconidia, microconidia and chlamydospores, isolates were also placed on CLA (carnation leaf agar (natural medium)) and SNA (Synthetic nutrient-poor agar (Nirenberg 1976)) plates and then incubated for 14 days under fluorescent and near-ultraviolet lights conditions (Joshi et al., 2016).

2.4. Antifungal screening

2.4.1. Antifungal activity in vitro

In order to evaluate the effect of different extracts on mycelial growth of *F. oxysporum*, poisoned food technique was used (Singh et al., 2008). PDA medium was autoclaved at 121°C for 20 minutes and kept under sterilized hood to cool up to 40°C. The extracts were mixed with sterile molten PDA to obtain final concentrations of 1, 1.5 and 2 (mg/ml). 15-20 ml of each media was separately poured into petri dishes, allowed to cool and solidify. After complete solidification of the medium, 5mm disc of seven days old culture of the *F. oxysporum* was inoculated in to Petri dishes. The plates were incubated at 25± 1°C. The Petri dishes containing media devoid of the extract

but with same amount of distilled water served as a negative control. Experiments were carried out in a completely randomized design. The measurements of the mycelial growth dynamic of the fungus were recorded when with 72, 144 and 216 hours after inoculation. Four replicates were used per treatment. The percent inhibition of fungal growth was estimated by using following formula (Mohana and Raveesh 2007):
Percentage inhibition= (C-T)/C×100

Where C; average diameter of the fungal colony of the control plate and T; average diameter (cm) of the fungal colony treated with the treatment plate.

2.4.2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

In order to determine MIC and MFC microtiter plate method (Pfaller *et al*., 2004) was used. For this, a broth dilution was applied. MIC method was performed in sterile, flat-bottomed 96-well microplate. Dry weight of the extracts was determined (Derwich et al., 2010). Potato Dextrose Broth (PDB (Merck, Germany)) was used for the antifungal study and All the extract dissolved in PDB were first diluted to the highest concentration (50 mg/ml) to be tested and then two-fold serial dilution (1/2,1/4,1/8,1/16,1/32, 1/64, 1/128) was made in the concentration range from 0.39 to 50 mg/ml. In each well, 100 µl of each extract dilution was mixed with 100 µl of the PDB. The sample was subjected to an eight-fold dilution series in order to give final amounts of the original suspension. For broth dilution, 50 µl of 10^6 CFU/ml suspension of pathogen strain separately was added to each well containing various extracts at concentrations of 0 (control), 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 mg/ml in broth medium. The two wells were considered positive and negative by adding 100 µl of PDB culture medium, one containing fungal suspension and the other lacking fungal suspension. The microplates were incubated at 25°C and observed for visible growth after 48 h. 10 µl of each well was poured onto the slide and examined exactly by microscope to the determination of germination or non-germination of spores. The lowest concentration of extracts that failed to show any visible growth was considered as the MIC.

MFC was determined by sub-culturing the negative wells on potato dextrose agar (PDA) medium. For determined of MFC, 30 µl of contents of each well that showed complete inhibition was sub-cultured on to PDA plates. Subsequently, the plates were incubated at 25°C in the dark until growth was seen in the growth control subculture. The lowest concentration of extract with no visible growth after 48 h was defined as the MFC (Abdolmaleki *et al*., 2008).

2.4.3. Greenhouse assay

Seeds of tomato (super strain cultivar) were disinfected using a 1% solution of sodium hypochlorite for 15 min and sown in an autoclaved sandy soil. After almost three weeks, plants were transplanting at 2-4 leaf stage into pots containing sterilized soil. Immediately prior to transplanting roots soaked in fungal spore suspension (10^8 spores/ml^1) of *F. oxysporum*. Then 1 mg/ml, of plant extracts were added to each pot at the same time. Each plot contain one plants. Water was used in the inoculated and non inoculated control plants. Plants were grown under 25+- 2 C° (day/ night temperature) , 65 =-5% relative humidity and natural photoperiod.

Each treatment was replicated four times and treatments were arranged in a randomized complete design. Water was applied daily in order to maintain soil moisture at field capacity. Fresh and dry weights of shoots, roots, and plant height were measured 40 days after treatments (El-Sheekh et al., 2020)

2.5. Statistical analysis

The experimental design was completely randomized with four repetitions for each treatment. Statistical analysis of the data obtained in the present study was analyzed by analysis of variance by using SPSS software (ver. 22) and grouping of the treatments was done by Duncan's multiple range test ($p<0.05$) where the comparison of means of different treatment was performed using factorial design.

3. Results

3.1. Mycelial growth inhibition assays

According to Table 1, the methanol extract of *Z. clinopodioides* at different concentration demonstrated highest antifungal activity against

mycelial growth of *F. oxysporum* strain that recorded 77.1%, 62.03% and 61.99% at 2, 1.5 and 1 (mg/ml), respectively. In contrast, the methanol extracts of *A. millefolium* and *S. verticillata* at different concentrations had lowest effect on the growth of *F. oxysporum* (Table 1). At 1 (mg/ml) concentration, inhibitory growth of 16.08 and 13.6 % were recorded for *A. millefolium* and *S. verticillata*, respevtively. Followed by 20.96 and 13.06% for *A. millefolium* and *S. verticillata* at 1.5 (mg/ml) concentration, respevtively (Table 1). The methanol extract of *A. millefolium* showed inhibition of 22.11% followed by 14.13 in *S. verticillata* at 2 (mg/ml) concentration (Table 1).

3.2. MIC and MFC

The MIC values of different extracts with respect of different plant extracts were determined using the broth dilution method. The range of MIC of different extracts recorded was 3.125 to 6.25 mg/ml. In the present study, the MIC value for methanolic extract of *Z. clinopodioides* against *F. oxysporum* was 3.125 mg/ml followed *A. millefolium* and *S. verticillata* extract having 6.25 mg/ml. The results of MIC values determination are shown in Table 2.

The MFC of methanolic extract which caused total inhibition of *F. oxysporum* was found to be 6.25 mg/ml in *Z. clinopodioides* and 12.5 mg/ml MFC for methanolic extracts of *A. millefolium* and *S. verticillata* (Table3).

3.3. Greenhouse assay

The efficacy of *Z. clinopodioides*, *A. millefolium* and *S. verticillata* extracts at 1 mg/ml concentration in suppressing disease was analyzed under greenhouse condition. According to the results given in Table 4, the plant extracts exhibited an *in vivo* antifungal effect against tomato wilt caused by *F. oxysporum*. A significant ($p \leq 0.05$) increase in plant height, fresh weight and dry weight were observed in plants grown from *Z. clinopodioides*, *A. millefolium* and *S. verticillata* inoculated when compared with the plants grown from the respective pathogen-inoculated seedlings (Table 4). Results revealed the significant effect of the treatments.

Plant heights of tomato increased depending on the plant extract inoculated, in comparison to negative controls. Shoot fresh weight of tomato seedling were stimulated by *Z. clinopodioides*, *A. millefolium* and *S. verticillata* by 4.99, 4.23 and 2.86 g. All plants, inoculated with the above extracts had higher shoot dry weight in comparison to the control plants which inoculated with *F. oxysporum*. The increase was highest in plants inoculated with *Z. clinopodioides* (0.29 g) followed by *A. millefolium* (0.26 g) and *S. verticillata* (0.16 g). Root fresh and dry weights of inoculated plants were higher in comparison to negative controls. *Z. clinopodioides*, *A. millefolium* and *S. verticillata* increased root fresh weight by 0.54, 0.43 and 0.25 g and root dry weight by 0.18, 0.15 and 0.10 g (Table 4). Shoot and root fresh and dry weight of tomato seedling was most affected by *Z. clinopodioides* in comparison to controls (Table 4).

4. Discussion

In order to some synthetic fungicides are known to be effective in pathogenic disease control and their prolonged usage could cause some health problems, there is an increasing interest in finding alternative, safe and natural methods to develop new antifungal agents (Al-Samarrai et al., 2012; Lopez-Reyes et al., 2013; Alkooranee et al. (2020). Plant extracts especially medicinal plants products are the most interested possible natural substitutes for conventional synthetic fungicides (Ogbo and Oyibo 2008; Li et al., (2017). Less side effects, lack of pathogenic resistance, low production costs, soil decomposition and lack of contamination are the reasons for the preference of plant extracts over chemicals (Choudhury et al., 2017).

In this study, three medicinal plants including *Z. clinopodioides, A. millefolium and S. verticillata* were screened for their antifungal properties at three different concentrations (1, 1.5 and 2 mg/ml). The study demonstrated the plant extract such as *Z. clinopodioides* had considerable effect on the growth rate of *F. oxysporum* in laboratory and greenhouse condition. Although *A. millefolium* and *S. verticillata* showed less inhibitory effect in laboratory, reference plants showed considerable antifungal activity in greenhouse condition.

Table 1. The effect of various plant extract at different concentration.

Plant species	Mycelial growth (cm)			Inhibition (%)		
	1	1.5	2	1	1.5	2
Z. linopodioides	2.09	1.93	1.25	61.99^{b}	62.03^{b}	77.10^{a}
A. millefolium	4.53	4.27	4.21	16.08^{d}	20.96^{c}	22.11^{c}
S. verticillata	4.8	4.78	4.6	13.6^{e}	13.6^{e}	14.13^{d}

Numbers within a column followed by the same letter are not significantly different at ($p< 0.05$); different letters mean significantly different

Table 2. MIC of plant extracts against *F. oxysporum*

	MIC in mg/ml							
	0.39	0.78	1.56	3.125	6.25	12.5	25	50
Z. clinopodioides	+	+	+	-	-	-	-	-
A. millefolium	+	+	+	+	-	-	-	-
S. verticillata	+	+	+	+	-	-	-	-

Table 3. MFC of plant extracts against *F. oxysporum*

	MFC in mg/ml							
	0.39	0.78	1.56	3.125	6.25	12.5	25	50
Z. clinopodioides	+	+	+	+	-	-	-	-
A. millefolium	+	+	+	+	+	-	-	-
S. verticillata	+	+	+	+	+	-	-	-

Table 4. Effect of plant extract treatments on shoot and root fresh and dry weight of tomato seedlins in pots inoculated with *Fusarium oxysporum.*

Treatments	Mean plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Control (non-inoculated)	33.75^{a}	7.14^{a}	0.46^{a}	0.63^{a}	0.21^{a}
Control (inoculated)	10.75^{e}	1.06^{d}	0.06^{d}	0.13^{c}	0.04^{c}
Z. clinopodioides	26.25^{b}	4.99^{b}	0.29^{b}	0.54^{a}	0.18^{bc}
A. millefolium	22.25^{c}	$4.23b^{c}$	0.26^{bc}	0.43^{ab}	0.15^{bc}
S. verticillata	18.25^{d}	2.86^{c}	0.16^{c}	0.25^{bc}	0.10^{b}

Numbers within a column followed by the same letter are not significantly different at ($p< 0.05$); different letters mean significantly different

Ziziphora clinopodioides L., a perennial plant belongs to the Lamiaceae family is a well-known traditional medicinal herb which is the most common species has been reported from Iran (Salehi et al., 2005). There are various reports about antimicrobial (Ji et al., 2012), antifungal (Behravan et al., 2007) and antioxidative (Tian et al., 2011) properties of *Z. clinopodioides*. Previous investigations revealed presence of pulegone and isomenthone compounds, piperitenone, menthone, phenolic constituents, flavonoids, polysaccharides, fatty acids and sterols in this plant (Ozturk and Ercisli 2007; Yu et al., 2012; Tian et al., 2012). Amiri (2009) showed that pulegone and thymol are the main components in *Z. clinopodioides* of Razan region of Iran.

In our research, the methanol extract of *Z. clinopodioides* at 2 mg/ml concentration indicated considerable antifungal activity against

F. oxysporum, while 1.500 and 1 mg/ml showed moderate inhibition. The growth inhibition percentages of the methanolic extracts of reference plant showed highest inhibition efficiency ranging from 61.99 to 77.1%. In general, growth inhibition percentages of *Z. clinopodioides* increased with increasing the concentration of extract. *Z. clinopodioides* at 2 mg/ml concentration demonstrated highest antifungal activity against mycelial growth of *F. oxysporum* strain that recorded 77.1%. The present study also revealed that *Z. clinopodioides* extracts has both fungistatic and fungicidal activities and showed 3.125 mg/ml of MIC and 6.25 mg/ml of MFC. Similar studies have been carried out by different researcher on antifungal activity of plant extracts on the mycelial growth of *Aspergillus flavus* and *A. fumigatus* (Haghighi and Khosravi 2010), *Rhizoctonia solani* (Foroughi et al., 2013), *Sclerotinia sclerotiorum* (Ma et al., 2016). Thus, *Z. clinopodioides* is more likely to be developed into a novel fungicide against phytopathogenic fungi. Based on our knowledge, in comparison to many other pharmaceutical-industrial plants, there is a very little data about chemical and antifungal composition of *Z. clinopodioides* in Iran. Hence, it will be necessary to further research on investigation antifungal compounds which result in inhibitory effect on pathogenic fungi.

Achillea L. is a large genus belonging to the family Asteraceae and its species known as medicinal plants (Benedek and Kopp 2007). These species are used in cosmetics, fragrances and also agriculture (Aydin and Sevindik 2018). Some reports indicated that in order to *A. millefolium* includes variety of flavonoid; it can be used as a natural antifungal agent for the treatment of several infectious diseases affecting fruits, vegetables and humans (Trumbeckaite et al., 2011; Candan et al., 2003). Candan et al., (2003) showed that water-insoluble parts of the methanolic extracts were found to have moderate activity against *Clostridium perfringens* and the yeasts. Sevindik et al (2016) also revealed that the essential oils obtained from *A. millefolium* had an inhibition effect on *Staphylococcus aureus*, *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus cereus*. . They found that *A. millefolium* extract had antimicrobial effects on both bacteria and fungi, they also observed no antimicrobial activity against tested organisms for n-hexane, Chloroform and methanol extracts of *A. millefolium* subsp. *pannonica*. In another study, promising water-insoluble fractions of the methanol extract of this plant showed moderate activity against *Clostridium perfringens* and *C. albicans*. The hexane–ether–methanol extract of *A. millefolium* was found to be mildly active against *E. coli*, *P. aeruginosa*, *S. aureus*, *Salmonella enteridis*, *Aspergillus niger*, and *C. albicans* (Stojanovic et al., 2005).

Salvia L. is one of the largest genera of the family Lamiaceae and is widely distributed all over the world. *Salvia* species have been used based on their well-characterized antioxidant, aromatic and also antimicrobial properties (Vallejo et al., 2006). Although the oils of *S. verticillata* showed high antibacterial activity, the oils of reference plant exhibited no or slight antifungal property (Yousefzadi et al., 2007). The essential oil of *S. verticillata* were tested against *Candida albicans* and were found to be very effective (Altun et al., 2007). Although some reports have revealed the antimicrobial activity of essential oils of *Achilia*, the information on the antimicrobial activity of reference plant extracts is limited (Candan et al 2003; Sökmen et al., 2004; Karamenderes et al., 2007; Karaalp et al., 2009).

In present study, *A. millefolium* and *S. verticillata* was not found to be effective and showed little ability to inhibit *F. oxysporum* strain *in vitro*. In our research, the lowest growth inhibition was recorded by *S. verticillata*, ranged between 13.6-14.13%. *A. millefolium* showd also low activity against *F. oxysporum* and the growth inhibition was recorded ranging from 16.08 to 22.11%. According to the results, MIC and MFC values of *A. millefolium and S. verticillata* are identical ranged between 6.25 and 12.5 mg/ml against pathogen isolate, respectively. However, in previous studies of references plants showed considerable antifungal activities. In the current study, we observed low activity of *A. millefolium* and *S. verticillata* extracts at tested concentrations against *F. oxysporum*, this discrepancy might reflects the differences in plant subspecies, antimicrobial assay, extraction methods and microbial strains (Karaalp et al., 2009). Most research on inhibitory effects of plant extracts is limited to laboratory conditions (Stojanovic et al., 2005; Rongai et al., 2015; Aydin and

Sevindik, 2018) and their effects have been less studied in greenhouse conditions. Due to the need to study the inhibitory effect of plant extracts in greenhouse conditions, the extracts were also tested in greenhouse conditions and it was observed that extract of all three plants had significant effects on improving plant growth traits. Although in the present study *A. millefolium* and *S. verticillata* showed low antifungal activities *in vitro*, reference plants revealed considerable antifungal activity in greenhouse condition. The antifungal effects of Achillea have been shown in field conditions (Baka et al., 2016). In addition to the antifungal properties of these plant extracts, their effect on plant growth and general well-being of plants might be another effective process. Therefore, further studies are needed to determine the cause.

Conclusion

According to this study, the extract of *Z. clinopodioides* had inhibitory effect on the mycelia growth of *F. oxysporum*. In vivo results under greenhouse conditions confirmed that this plant extract can be used as a viable and safe alternative for controlling of *F. oxysporum*. Thus, it can be suggested for use against the fusarium wilt of tomato. There is a tremendous need for novel antimicrobial agents from various sources. Since these plants are wild and available, they could be used as an economic and eco-friendly agent in disease management. Screening of plant extracts is a primary source of discovery and identifying potentially useful molecules against infectious diseases. It can be recommended further studies to understand the interference of *Z. clinopodioides* on other phytopathogenic fungi and also studies on the chemical constituents and further antimicrobial activity.

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Refereces

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