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RESEARCH ARTICLE

IN VITRO, CONTROL OF *FUSARIUM OXYSPORUM FS LYCOPERSICI* BY USING AQUESOUS EXTRACTS OF *UMBELLIFERAE* FAMILY MEMBERS IN GAZA STRIP

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ABSTRACT

Fusarium oxysporum fs lycopersici (FOFL) is considered as one of the most important plant pathogen causes fusarium wilt specifically in tomato. In the present study, the antimicrobial activity of Umbelliferae members carrot, Parsley, Celery, Coriander, Fennel, Caraway, Anise, Dill and Cumin aqueous extracts on phytopathogenic fungus (FOFL). These extracts used at different concentration (1,2,4 and 8%) and proved their bioactivity as antifungal agents for the inhibition of *Fusarium oxysporum* radial growth, fungal biomass and sporulation. Carrot and Anise were showed more than inhibition of radial growth than chemical fungicides, but all plant aqueous extracts were showed the inhibition of fungal spores less than chemical fungicides (Bavistin at 10 ppm).Our results in this study revealed that significant antagonistic effects of Umbelliferae members against the *(FOFL)* and therefore could be used a viable option for natural pesticides.

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INTRODUCTION

Tomato (*Lycopersicon esculntum* Mill) belongs to the family Solanaceae and it is considered one of the world's most important and popular vegetables (Pritech *et al.,* 2011). It is one of the most important vegetable crops grown throughout the world under field and greenhouse conditions (Kaloo, 1983). In term of human health, tomato is a major component is the daily diet in many countries, and constitute an important source of minerals, vitamins, and antioxidants (Grierson and Kadar, 1986). It is the most important tropical vegetable crop widely used throughout the world (Hadian et *al.,* 2011). Plant diseases have caused serious losses to human including starvation, direct economic loss and reduce the aesthetic values of landscape plants and home gardens (El kichaoi *et al.,* 2016). Disease development is a dynamic process and can alter over time. A low disease loss in your fields in the recent past does not ensure disease losses will remain low. (*South Carolina Pest Management Handbook for Field Crops, 2015*). There are many plant pathogens which have profound effects on mankind such insect pests and plant pathogen that can destroy more than 40% of all food production each year

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(El kichaoi *et al.,* 2016). The most common diseases on tomato includes early blight, anthracnose, bacterial wilt, bacterial canker, tomato spotted wilt, *Verticillium* wilt and *Fusarium* wilt (Winaned *et al.,* 1999). The wilt diseases are caused by bacteria (*Pseudomonas spp*) and fungi (*Fusarium* and *Verticillium* spp) (Mardi *et al.,* 2002). Tomato *Fusarium* wilt is considered as one of the most important diseases of tomato both in fields and greenhouse- grown tomatoes worldwide (Abdel Monaim, 2012). *Fusarium oxysporum fs lycopersici* is economically important wilting pathogen of tomato (Hadian *et al.,* 2011). *Fusarium oxysporum fs lycopersici* is a soil inhabitant and attacks plant through the roots. Over the years, farmers have over depend on chemicals for both pests and disease control and these have damaging effects on the natural environment, the agro-ecosystem and to human being. Use of chemicals has led to build-up of phytotoxicity in the soil. In Gaza strip tomato producers grow transgenic cultivars in their fields especially Sun red cultivar. The tomato seeds were imported due to there resistant to soil borne diseases such as *Fusarium, Verticillum and* soil *Nematodes*. According to farmers observations, some cultivars have suscbtability to vascular wilt disease. For this reason we use a transgenic tomato as a case study. In this study, during the fusarium pathogenicity test, most tomato strains were resistant to the pathogen except Sun red cultivar which was suscbtible to

disease. In this study *Fusarium oxysporum f.sp. lycopersici* was isolated and identified based on the information of morphological and microscopical charecteristics provided by Booth 1971. The present study aims to assess the impact of aqueous extracts of nine Umbillefera family members (Anise, Coriander, Celery, Fennel, Cumin, Dill, Parsley, Carrot and Caraway) on phytopathogenic fungus *Fusarium oxysporum fs lycopersici* in vitro and determined the effect of these extracts with different concentration (1,2,4 and 8%) on radial growth, sporulation and fungal biomass of *Fusarium oxysporum fs lycopersici*.

MATERIAS AND METHODES

Collection of samples

A total of 10 diseased tomato plants were collected by simple random technique from different agricultural areas of Gaza strip. Field diagnosis of diseased plant samples were done by critically observing the vascular wilt symptoms.

Culture medium

The culture medium for fungal isolation was potato dextrose agar (Dhingra and Sinclair 1985).The pH of the medium was adjusted with hydrochloric acid to 4.6 to strict bacterial growth, appropriate growth media are slightly acid for most fungi, in contrast in the requirements for bacteria, which are generally intolerant acid conditions. Alternatively chloramphenicol (0.19 g/L) was added to the medium and its pH adjusted to 6.5 (Pitt and Hocking 1991).

Isolation of *Fusarium oxysporum* from diseased plants

F. oxysporum was isolated from diseased tomato plants collected from an infested field in Gaza strip. Parts of plants with symptoms of *F. oxysporum* infection were surface sterilized by immersion in 0.3% sodium hypochlorite (NaOCl) for 10 min, rinsed in sterile distilled water, transferred to potato dextrose agar (PDA) medium in 90 x 15 mm Petri dishes and incubated in dark at 28° C for 7 days. Fungal isolate was tested for pathogenicity on tomato seedlings **(Reis and Boiteux, 2007)** and found to be pathogenic. Spores were collected in sterile distilled water after 7 days growth in PDA medium, adjusted 10^5 to spores ml^{-1} using a hematocytometer and used as inoculum for further studies.

Maintenance and storage of *Fusarium oxysporum*

Fungal isolates were cultured on PDA. Isolates were subcultured by transferring a small block of agar from the edge of a colony onto a fresh plates. Plates were incubated at 28° C in the dark. For the production of a fungal spore inoculum flask cultures with PDA medium were incubated at 28° C with constant shaking at 200 rpm in the dark. Conidia were harvested by filtering the culture through sterile micro cloth then suspended in 50% (v/v) glycerol and stored at -80° C or as dry pellets preserved on sterile filter paper stored at -80° C (Fang, 1998).

Identification of *Fusarium oxysporum*

Purified colonies were subcultured to PDA for 7-10 days before any attempts were made to identify them. The plate containing the PDA culture was examined microscopically for the features of the fungus (Summerel *et al.,* 2003). If microconidia and chlamydospores were present, their shape, size and the manner in which they were formed noted; examination was also carried out on the hyphae, on the surface of the agar, or embedded in the agar. Microscopic slide preparations were made for detailed examinations of macroconidia, microconidia and the conidiogenous cells producing the macroconidia (Booth, 1984).

Preparation of aqueous extracts of seeds and shoots of Umbelliferae plants

Fresh specimens of each plant were washed thoroughly 2-3 times with running tap water, followed with sterile distilled water, and then air- dried on a sterile blotter. An aqueous extract was prepared by blending 20g of the selected plant part in 100ml sterile distilled water in a waring blender (waring international, new itartford,CT, USA) for 10 minutes. The macerate was filtered through for layers of cheesecloth, and then through whatman No.1 filter paper. The solution was sterilized by filtration through a bacteriological filter (0.22µm pore size). The extracts were preserved aseptically in bottles at -18 ºC until needed (Hughes, 2002).

Preparation of different dilutions of plant extract

In the current study, different aqueous concentrations (1%, 2%, 4% and 8 %) were prepared from nine plant extracts of Cumin, Coriander, Anise, Caraway, Carrot, Parsley, Dill, Fennel and Celery.

Effect of different concentrations of aqueous extract on radial growth of *Fusarium oxysporum fs lycopersici*

The plant extract at various concentrations (1%, 2%, 4% and 8%, w/v) of effective plant were amended with PDA. The amended medium was dispensed into sterile Petri plates and allowed to solidify. Two perpendicular lines were drawn at the bottom of each plate to cross each other at the center of the plate. Each plate was inoculated with the fungus. A 4mm mycelia disc of each of the test organism was inoculated on each amended agar plate. Inoculated plates were incubated at 25 ºC and growth measured along the perpendicular lines. Daily radial growth of each test organism in any of the test extracts was recorded for 7 days. Each treatment was replicated three times. The media amended with DMSO and recommended fungicide (Bavistin) for respective fungal strain were consider as negative and positive control respectively. The diameter of fungal colony was measured daily. The inhibitory activity to the radial growth (IR) was determined according to the following formula (Pinto *et al*., 1998):

IR (%) = dc – dt/Dc x100
where: IR = inhibitory activity to the radial growth
dc = average increase in mycelia growth in control plates
dt = average increase in mycelia growth in treated plates.

Effect of Extracts on Sporulation

Spores were harvested in sterile distilled water from a culture maintained in slant PDA. The suspension was passed through a filter paper (Whatman No.2) to separate the spore and mycelia or hypae. A 200 µL spore suspension (2 x 10^5 spores/ml^{-1}) was added into 10 ml potato dextrose broth in a test tubes containing various concentrations of plant extract, 1%, 2% , 4% and 8% (w/v) separately . The culture tubes were incubated in the dark under room temperature 25 ºC for five days. The number of spores were counted using haemocytometer under light microscope. The inhibitory activity to the spore formation (IS) was calculated according to the following formula (Pinto *et al.,* 1998):

IS (%) = dc – dt x 100/dc

where:
IS = inhibitory activity to the sporulation
dc = spore's density on control (without extract treatment)
dt = spore's density with extract treatment.

Effect of Extracts on Fungal Biomass

The effect of plant extract on fungal biomass was carried out according to the method described by (Pinto *et al.,* 1998). 100 ml of potato-dextrose broth (PDB) medium was placed in a 200 ml Erlenmeyer flask. The plant extract was added into the flask at concentration varied from 1%, 2%, 4% and 8% (w/v). The medium was then inoculated with 1 ml of spore suspension (the spore density was 2 x 10^5 spores/ml). The final volume of the culture was 100 ml with five flasks for each concentration. The cultures were incubated in the dark for 8 days under room temperature. The biomass was harvested through centrifugation at 5000 rpm for 5 minutes. The pellet (biomass) was taken and placed on glass filter paper and dried in an oven at 60^0 C until constant weight. The inhibitory activity against the fungal biomass (IB) was calculated according to the formula (Pinto *et al.,* 1998):

IB (%) = Wc – Wt x 100 / WCc
Where: IB = inhibitory activity to the fungal biomass
Wc = dry weight of biomass on control (without extract treatment)
Wt = dry weight of biomass with extract treatment.

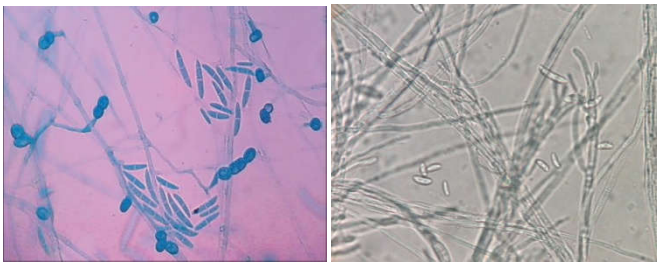
Data Collection and Statisitcial Analysis

The effect of aquesous plant extract on *F. oxysporium fs lycopersici* was examined by Excel soft ware 2007.

RESULTS

Characterization and identification of *Fusarium oxysporum f.sp.lycopersci*

Based on the information of morphological characteristic on the isolates provided by Booth 1971 was identified. *Fusarium oxysporum* produced abundant, single-celled, oval microconidia (figure 1). Short and long, four to five septate, slightly falcate microconidia were the most important morphological characteristics in identifying *Fusarium oxysporum*. Chlamydospores were usually terminal and one or tow celled.



(a) **(b)**

A: chlamydospores and macroconidia of *Fusarium oxysporum*
B: abundant, single celled, oval or oblong microconidia and short and unbranched microconidiophores of *Fusarium oxysporum*

Figure 1. Microconidia, macroconidia and chlamydospores of Fusarium oxysporum

Figure 2. Illustrate the aerial mycelium of *Fusarium oxysporum f.sp lycopersici* ranged from white to dark purple color on PDA

Figure 3. Colonies of *Fusarium oxysporum f.sp lycopersici* on Czapek agar

Figure 4b. Symptoms of fusarium wilt on tomato plants in open field

Figure 4a. Illustrate the browning of the vascular system on stem

Figure 5. Reaction of resistant and susceptible tomato cultivars to *Fusarium oxysporum f.sp lycopersici* A. Resistant plant no yellow or wilt B. Infected plant yellow and wilt leaves

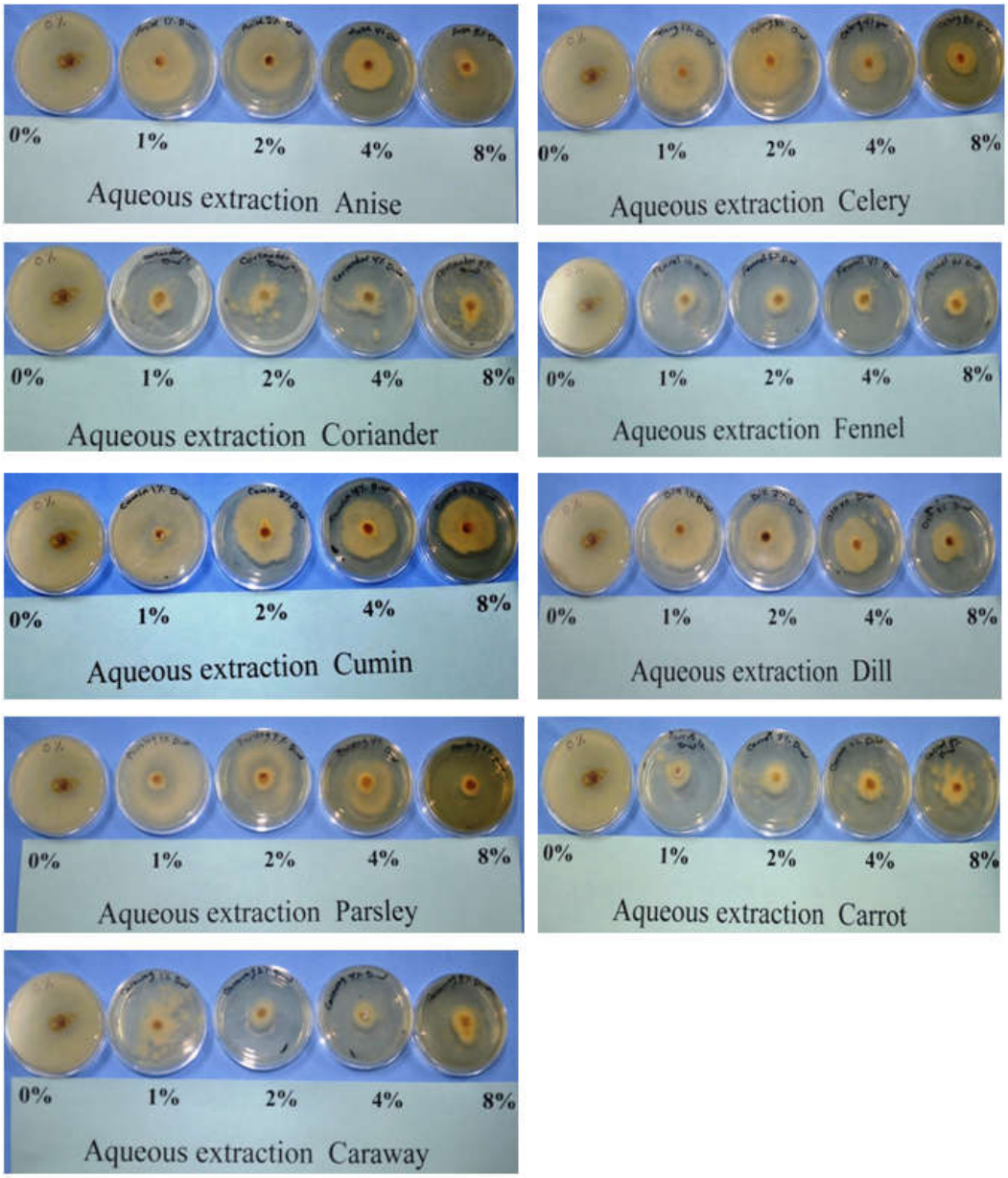
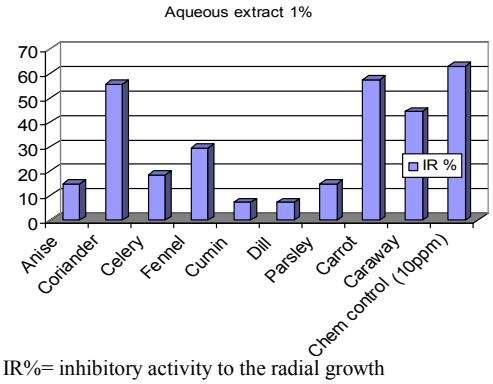


Figure 6. Illustrate the Effect of aqueous extracts of the used Umbillefera plants (Cumin, Coriander, Anise, Caraway, Carrot, Parsley, Dill, Fennel and Celery) at different concentration on the radial growth of *Fusarium oxysporum f.sp lycopersici*

The isolates had different colony characteristics and pigmentation. The aerial mycelium of *Fusarium oxysporum* isolates ranged from white to dark purple on Potato Dextrose Agar (Figure2). Most typical symptoms of the disease appeared on tomato plants in field production (Figure 4.b). The symptoms appeared on older plants during mid-growing season under warm weather conditions. One of the typical leaves wilted and dried up. In many cases one side of the plant was affected first. Infection usually occurred on plants in the form of chlorosis, leaf wilting and browning of the vascular system (Figure 2a) cross-section of the stem revealed necrosis of the vessels.

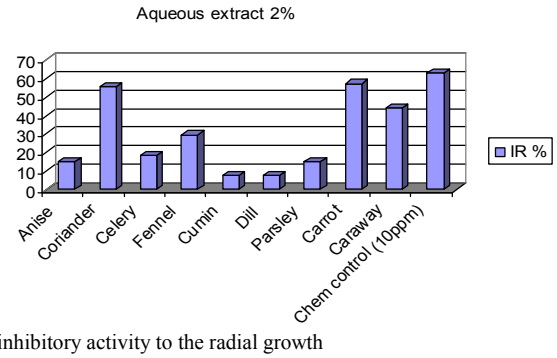
Pathogenicity and disease incidence testing

After 10 days of inoculation of 10 ml 10^7 spores/ml suspension of *Fusarium oxysporum* spores. According to the degree of resistant to the disease , the cultivar sun red was classified susceptible 91.66% (number 6, 81 - 100 % of leaves yellow and wilted) and the tomato 593 cultivar was highly resistant (number 1 no yellow leaves and wilted). The pathogenicity test showed that the earliest symptom of wilt disease on tomato plants is the yellowing of the older leaves. The yellowing gradually affects most of the foliage and is accompanied by wilting of the plant. The wilting becomes more extensive for day to day until the plant collapse and dies. The observation on plant growth characters and reduction of plant after the inoculation of pathogen are presented in figure 3.



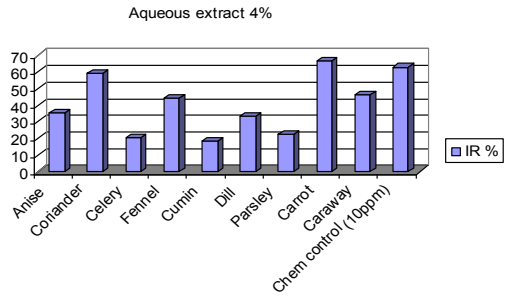
IR%= inhibitory activity to the radial growth

Figure 6. Effect of aqueous extracts of the used Umbillefera plants at concentration 1% on the radial growth of *Fusarium oxysporum f.sp lycopersici*



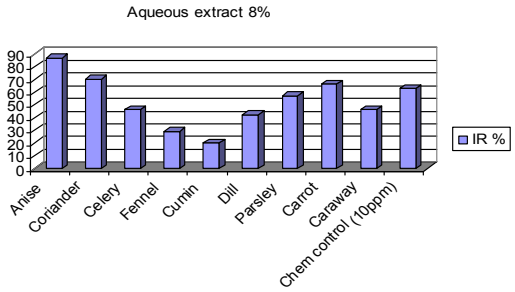
IR%= inhibitory activity to the radial growth

Figure 7. Effect of aqueous extracts of the used Umbillefera plants at concentration 2% on the radial growth of *Fusarium oxysporum f.sp lycopersici*



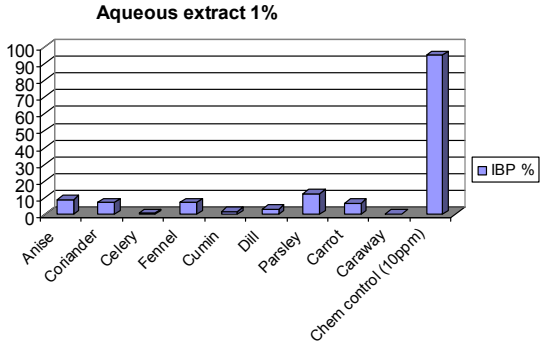
IR%= inhibitory activity to the radial growth.

Figure 8. Effect of aqueous extracts of the used Umbillefera plants at concentration 4% on the radial growth of *Fusarium oxysporum f.sp lycopersici.*



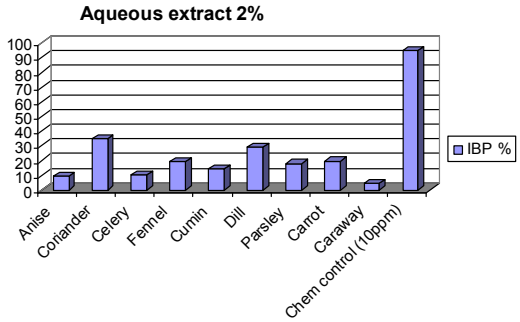
IR%= inhibitory activity to the radial growth.
IR%= inhibitory activity to the radial growth.

Figure 9. Effect of aqueous extracts of the used Umbillefera plants at concentration 8% on the radial growth of *Fusarium oxysporum f.sp lycopersici*



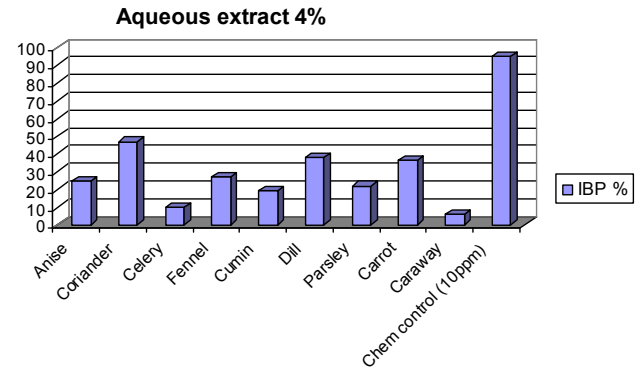
IB%= inhibitory activity to the fungal biomass production

Figure 10. Effect of aqueous extracts of the used Umbillefera plants at concentration 1% on the dry weight of *Fusarium oxysporum f.sp lycopersici*



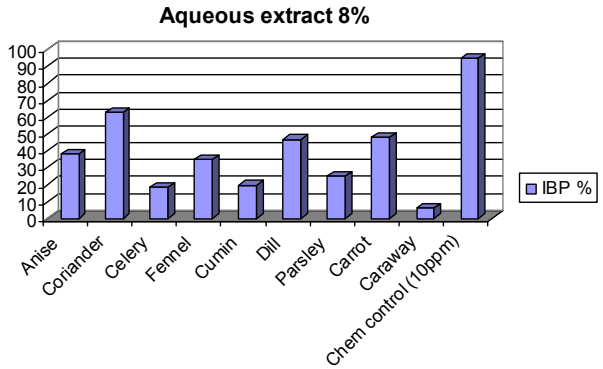
IB%= inhibitory activity to the fungal biomass production

Figure 11. effect of aqueous extracts of the used Umbillefera plants at concentration 2% on the dry weight of *Fusarium oxysporum f.sp lycopersici*



IB%= inhibitory activity to the fungal biomass production

Figure 12. effect of aqueous extracts of the used Umbillefera plants at concentration 4% on the dry weight of *Fusarium oxysporum f.sp lycopersici*



IB%= inhibitory activity to the fungal biomass production

Figure 13. Effect of aqueous extracts of the used Umbillefera plants at concentration 8% on the dry weight of *Fusarium oxysporum f.sp lycopersici*

Table 1. Effect of aqueous extracts of the used Umbillefera plants at different concentration on the radial growth of *Fusarium oxysporum f.sp lycopersici*

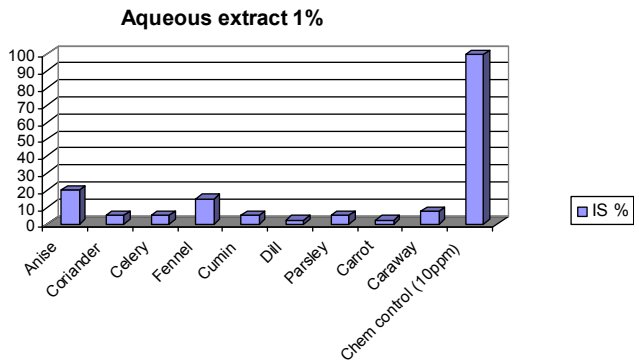
Concentration of aqueous extract	Diameter of radial growth (mm) mean								
	Anise	Coriander	Celery	Fennel	Cumin	Dill	Parsley	Carrot	Caraway
1%	46	24	44	38	50	50	46	23	30
Percent inhibition	14.81%	55.55%	18.51%	29.62%	7.40%	7.40%	14.81%	57.40%	44.44%
2%	43	22	44	34	45	50	46	22	30
Percent inhibition	20.37%	59.25%	18.51%	37.00%	16.66%	7.40%	14.81%	59.25%	44.44%
4%	35	22	43	30	44	36	42	18	29
Percent inhibition	35.18%	59.25%	20.37%	44.44%	18.51%	33.33%	22.22%	66.66%	46.29%
8%	7	16	29	22	43	31	23	18	29
Percent inhibition	87%	70.37%	46.29%	29.25%	20.37%	42.59%	57.40%	66.66%	46.29%
Chemical control	20								
Bavistin 10 ppm	62.96%								
Negative control	54-								

Table 2. Effect of aqueous extracts of the used Umbillefera plants at different concentration on the dry weight of *Fusarium oxysporum f.sp lycopersici*

Concentration of aqueous extracts	Dry weight of fungi (g) mean								
	Anise	Coriander	Celery	Fennel	Cumin	Dill	Parsley	Carrot	Caraway
1%	2.19	2.23	2.38	2.23	2.36	2.32	2.10	2.24	2.39
Percent inhibition	8.75%	7.08%	0.83%	7.08%	1.66%	3.33%	12.50%	6.66%	0.41%
2%	2.18	1.57	2.16	1.94	2.15	1.71	1.97	1.93	2.30
Percent inhibition	9.16%	34.58%	10.00%	19.16%	14.41%	28.75%	17.91%	19.58%	4.16%
4%	1.81	1.28	2.16	1.75	1.94	1.49	1.87	1.53	2.26
Percent inhibition	24.58%	46.66%	10.00%	27.08%	19.16%	37.91%	22.08%	36.25%	5.83%
8%	1.49	0.89	1.96	1.56	1.93	1.28	1.80	1.23	2.26
Percent inhibition	37.91%	62.91%	18.33%	35.00%	19.58%	46.66%	25.00%	48.15%	5.83%
Chemical control	0.12								
Bavistin 10 ppm	95%								
Negative control	2.4-								

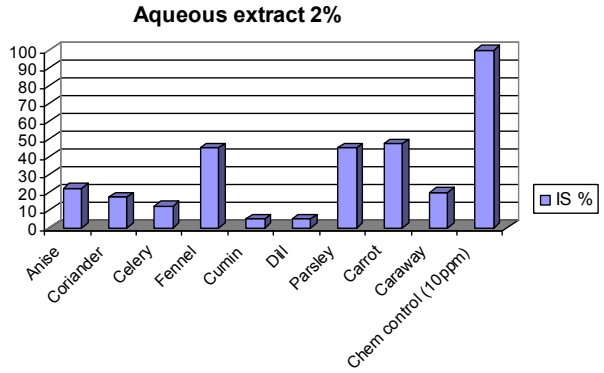
Table 3. Effect of aqueous extracts of the tested Umbillefera plants at different concentration on the sporulation of *Fusarium oxysporum f.sp lycopersici*

Concentration of aqueous extract	Number of spores (spores/ml)								
	Anise	Coriander	Celery	Fennel	Cumin	Dill	Parsley	Carrot	Caraway
1%	$3.2x10^5$	$3.8x10^5$	$3.8x10^5$	$3.4x10^5$	$3.8x10^5$	$3.9x10^5$	$3.8x10^5$	$3.9x10^5$	$3.7x10^5$
Percent inhibition	20%	5%	5%	15%	5%	2.5%	5%	2.5%	7.5%
2%	$3.1x10^5$	$3.3x10^5$	$3.5x10^5$	$2.2x10^5$	$3.8x10^5$	$3.8x10^5$	$2.2x10^5$	$2.1x10^5$	$3.2x10^5$
Percent inhibition	22.5%	17.5%	12.5%	45%	5%	5%	45%	47.5%	20%
4%	$2.6x10^5$	$3.1x10^5$	$3.5x10^5$	$1.6x10^5$	$3.6x10^5$	$3.2x10^5$	$1.9x10^5$	$1.7x10^5$	$2.7x10^5$
Percent inhibition	35%	22.5%	12.5%	60%	10%	20%	52.%	57.5%	32.5%
8%	$2.4x0^5$	$3.1x10^5$	$3.1x10^5$	$1.1x10^5$	$3.4x10^5$	$3.0x10^5$	$1.8x10^5$	$1.4x10^5$	$1.1x10^5$
Percent inhibition	40%	22.5%	22.5%	72.5%	15%	25%	55%	65%	72.5%
Chemical control	$9.9 x10^2$								
Bavistin 10 ppm	99.7%								
Negative control	$4.0x10^5$-								



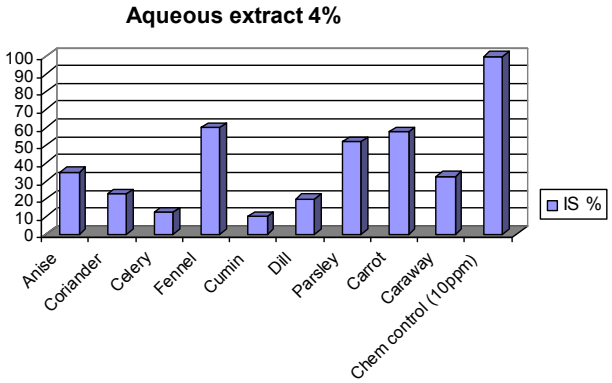
IS%= inhibitory activity to the sporulation

Figure 14. Effect of aqueous extracts of the tested Umbillefera plants at concentration 1% on the sporulation of *Fusarium oxysporum f.sp lycopersici*



IS%= inhibitory activity to the sporulation

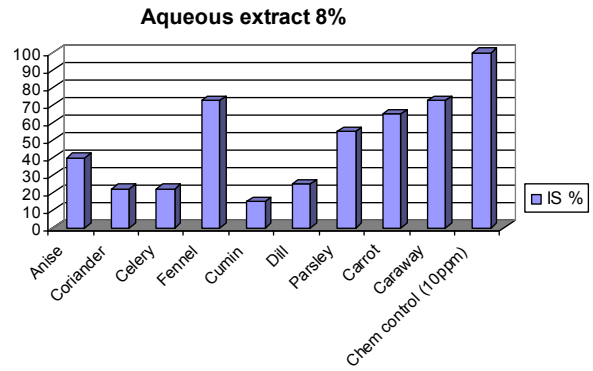
Figure 15. Effect of aqueous extracts of the tested Umbillefera plants at concentration 2% on the sporulation of *Fusarium oxysporum f.sp lycopersici*



IS%= inhibitory activity to the sporulation

Figure 16. Effect of aqueous extracts of the tested Umbillefera plants at concentration 4% on the sporulation of *Fusarium oxysporum f.sp lycopersici*

All treatments inhibited radial growth. The inhibition ranged from the lowest inhibitory percent 7.40% by cumin and dill at 1% to the high of 57.40% with the same concentration of carrot. Aqueous extract of Anise was highly effective on inhibiting the radial growth 87% when the concentration was 8% (Table 1). The biomass production response of *Fusarium oxysporum* toward different concentrations was variable. All treatments inhibited biomass production. The inhibition ranged from the lowest inhibitory percent 0.41% by aqueous caraway extract at 1% to the high of 12.5% with the same concentration of parsley. Aqueous extract of coriander is highly effective 62.91 on inhibiting the biomass production when the concentration was 8% (Table 2). All treatments inhibited sporulation. The inhibition ranged from the lowest inhibitory percent 5% by coriander celery, cumin and parsley extracts at 1% to the high of 20% with the same concentration of anise. Aqueous extract of fennel and caraway were highly effective 72.5% on inhibiting the sporulation when the concentration was 8% (table 3).



IS%= inhibitory activity to the sporulation

Figure 17. Effect of aqueous extracts of the tested Umbillefera plants at concentration 8% on the sporulation of *Fusarium oxysporum f.sp lycopersici*

DISCUSION

The difficulties in controlling *Fusarium oxysporum fsp lycopersici* have promoted scientists to search for biological alternatives (ARICI *et al.,* 2013). A number of different chemical and synthetic compounds have been used against phytopathogens. The use of these chemicals without careful judgment has led to development of fungicide resistance and more importantly, environmental pollution, representing a potential risk to animal and human health (Shukla and Dwivedi, 2012). Plant extracts have been widely investigated for the control of disease of agricultural crops (ARICI *et al.,* 2013). It is interesting to note that great efforts have recently been made for the development of biocontrol agents and biofungicide from medicinal plants as environmentally safe alternatives (Boonsang *et al.,*2014). Plants have ability to synthesize aromatic secondary metabolites, like phenols and phenolic acids compounds show antimicrobial effect and serves as plant defence mechanisms against pathogenic microorganisms (Gurjar *et al.,* 2012*)*. So in present study aqueous extracts from Cumin, Coriander, Anise, Caraway, Carrot, Parsley, Dill, Fennel and Celery were used as a natural fungicides. Our results indicate that the radial growth of FOFL was inhibited by aqueous extracts of Carrot at 1%, 2% and 4% were 57%, 59% and 67% respectively and aqueous extracts of Anise at 8% was 87% inhibition of radial growth. Carrot and Anise were show more than inhibition of radial growth than chemical fungicides (Bavistin at 10 ppm). Reduction of fungal dry weight of aqueous extract of Parsley at 1% was 12.5% and aqueous extracts of Coriander at 2%, 4% and 8% were 34.6% , 46.6% and 62.9% respectively then less than chemical fungicides (Bavistin at 10 ppm). Inhibition of fungal sporulation of Aise at 1% aqueous extract was 20%, at 2% Carrot aqueous extract was 47.5% and Fennel aqueous extract

4% and 8% were 60% and 72.5% respectively. All used plant aqueous extracts at different concentrations (1%, 2%, 4% and 8%) were showed the inhibition of fungal spores of *Fusarium oxysporum* less than chemical fungicides (Bavistin at 10 ppm). The ultimate aim of our study has been the development of alternative control strategies to reduce dependency on synthetic fungicides and our results clearly demonstrated the relative efficacy of plant extracts as a viable option for natural pesticides.

Conclusions and Recomindations

In present study aqueous extracts from Cumin, Coriander, Anise, Caraway, Carrot, Parsley, Dill, Fennel and Celery showed that significant antagonistic effect against the *Fusarium oxysporum* fsp lycopersici. In conclusion, aqueous extracts showed antifungal activites against phytopathogenic fungi in vitro and they have the potential to be used as antifungal agents for the inhibition of *Fusarium oxysporum* radial growth, fungal biomass and sporulation. However, for the development of aqueous extracts as potential antifungal agents, further studies are required to evaluate phytotoxicity of aqueous extracts for application on tomato plants for control Fusarium wilt. If they inhibit the growth of pathogen in the field conditions, they can be used as alternative control measures and an environmentally safe to chemical control. Further studies are needed to confirm and shed more light on the identification of active ingredient in plants, mode of actions, and stable formulations in the field , in addition to more field trials.

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