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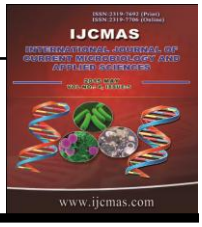

Original Research Article

Antifungal activity of *Viola canescens* against *Fusarium oxysporum* f. sp. *lycopersici*

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Keywords

Fusarium, *Viola canescens*, MIC, paper disc diffusion assay

A B S T R A C T

In an attempt to reduce the use of synthetic fungicides, extensive investigations into the possible exploitation of plant compounds as natural commercial products were performed. The present work describes the antifungal activity of plant extracts on the development of *Fusarium oxysporum* f. sp. *lycopersici*. The tests *in vitro* was carried out through paper disc diffusion assay by using acetone, ethanol, petroleum ether and water solvents. The tested plant *Viola canescens* had an intermediate level of antifungal activity. Highest antifungal activity was observed as 17.62 mm inhibition zone in case of 1000 mg/ml acetone extrat of *Viola canescens*. The other solvents were moderately effective. Minimum Inhibitory Concentration (MIC) was also determined by tube dilution method. Highest MIC showed by ethanol and Petroleum ether solvents as 100 mg/ml.

Introduction

Any plant is considered to be as drug yielding plant when its one or more organ contains substances that can be used for medicinal and therapeutic means or from which precursor molecules for drugs have been isolated (Sofowora, 1982, Medicinal Plant and Traditional Medicine in Africa).

All the plants have an extreme efficiency to synthesize aromatic substances, most of which are phenols or phenol's oxygen substituted derivatives (Geissman, 1963). Most of these aromatic compounds are secondary metabolites, out of which more then 12,000 have been isolated, their number estimated to be less then 10% of total (Schultes, 1978).

All these active chemical compounds are the most important part of plant's defense mechanism. In nature these secondary metabolites are specific and varies from species to genera. These secondary metabolites are not used by plants in normal primary metabolic need but they enhance the overall capacity of it to survive and prepare to face challenges by giving them ability to interact with their environment (Harborne JR, 1993). Table.1 represents major antimicrobial compounds from plants and their relative mechanism on pathogen-

Himalayas is a rich source of many drug yielding, aromatic, oil yielding plants, fiber, fodder yielding plants. Many of these plants

possess alkaloids, glycoside alkaloids, resins and other secondary metabolites for which they have been badly exploited to fulfill the daily drug need of common people.

In present study medicinal plant has been selected to know its antifungal activity. The description of this plant species is as follows;

Viola is a genus of flowering plants of family Violaceae. It is the largest genus in the family, containing between 525 and 600 species (Ning *et al*., 2012). It is a nearly prostrate herb found in the Himalayas, from Kashmir to NE India, at altitudes of 1500-2400 m. Basically it is native to India ,China and Bhutan. Commonly it is called as Himalayan white violet.

Viola canescens has pale violet, often almost white, flowers ranging 1-1.8 cm across, with a short blunt spur, and hairy sepals, on erect stalks 5-15 cm. long. Sepals are five in number, Petals are up to 1.5 cm long, and about 4 mm broad, obovate, obtuse, upper two are wedge-shaped, while two lateral ones are narrower and bearded at the base, marked with dark coloured streaks. Its Lower most petals is the shortest, patterned with dark coloured stripes. Usually its Leaves are ovate-heartshaped to kidney-shaped with a blunt tip. Leaves are thick and hairy. Leaf stalks are also covered with hairs oriented towards base Stipules are lance-shaped. Its Flowering time is March to June (M. Adnan and D. Hischer, 2010).

Many species contains antioxidants called anthocyanins. The chemical components found in *V. canescens* include methyl salicylate alkaloid violin, glycoside viola quercitrin, saponins, and glucosides (Rana *et al.*, 2010).

V. canescens is effective when used as carminative, demulcent, astringent, antipyretic, diaphoretic, and purgative properties. Plant is anticancerous in action and is used to treat nervous disorders (Ahmad *et al.,* 2012 ; Hussain *et al.,* 2011; M. Adnan and D. Hlscher, 2010).

Tomato (*Lycopersicon esculentum* Mill.), one of the most important vegetable in many countries has a worldwide economic and nutritive importance (Khoso, 1994). Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici,* one of a limiting factor in the production of tomato and accounts for yield losses annually. This study aimed to determine the influence of *Viola canescens* at different concentration, on the *in vitro* growth of *Fusarium oxysporum* f. sp. *lycopersici*.

Materials and Methods

Plant samples (whole plant) was air dried under shade at room temperature for 2 weeks, then ground to fine powder using a laboratory mill. Powders were packed in air tight bags, weighed and stored at 4 ºC.

Organic and water extracts were prepared for plant sample. 3.0 gram of organic extracts were prepared by extracting sample successively with acetone, ethanol and petroleum ether in a ratio of 1:5. The resultant extract was weighed and stored in airtight sample bottles. For the water extracts, plant powder of sample was soaked in distilled water. All the extracts were oven evaporated till complete dryness for 5 -7 days at 30 ±2 ºC. Plant extract was second time extracted with DMSO. 200 mg of each extract was weighed into a sterilized sample bottle and dissolved in DMSO (Sigma) to make a concentration of 200 mg/ml.

Microorganism used in this study *Fusarium oxysporum* f.sp. *lycopersici* was obtained from Microbial Type Culture Collection MTCC, Institute of microbial technology, Chandigarh. Fungus culture (MTCC 1755) was maintained in Potato sucrose agar medium for an optimum pH of 6.8.

The fungal spore suspension was prepared by the addition of a loopful of fungal spores in a 5 ml of sterile distilled water and 1 ml Tween 20. Then 0.1 ml fungal spore suspension was mixed well in aseptic conditions and spread evenly on the petridishes containing 20 ml of solidified potato sucrose agar.

Antimicrobial activity

In Paper disc method Some amount of Potato Sucrose Agar (PSA) was dispersed in petridishes and allowed to solidify. A micropipette will be used to introduce 0.1 ml. spores on agar medium and spread with glass rod spreader under sterile conditions. Sterilized discs (6 mm, Whatmann No. 1 filter paper) will be prepared by soaking in different concentrations of the extracts ie, 250, 500, 750, 1000 mg/ml for 6 hour. The discs will be then removed and allowed to dry. To assay for antifungal activity various discs impregnated with different concentrations of the extracts will be placed on the fungal spore or mycelium with the help of sterilized forceps. The petridishes incubated at 35˚ C for 48 h. Antifungal activity will be determined by measurement of the zone of inhibition around the discs after the period of incubation.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC is defined as the lowest concentration of antibiotics or plant extracts that did not show any growth of tested pathogens. The entire test sample of eight medicinal plants were dissolved in acetone, aqueous, ethanol and petroleum ether solvents to dilute the highest concentration (1000 mg/ml DMSO) to be tested and then serial dilutions were made to get 4 different concentrations of 1000, 100, 10 and 1 mg/ml in sterile test tubes containing standardized inoculums (Ramasamy *et al.,* 2014).

Each culture tube contains 2 ml culture medium, 1 ml of plant extrcats and 0.1 ml individual microorganism. These tubes were incubated at 37º C for 24 hours. The MIC determination for aqueous extracts were performrd in distilled water instead of DMSO. The growth of the organism for each dilution was observed and thus the MIC was evaluated.

Experiments were performed in the laboratory of L.S.M.G.P.G. college Pithoragarh.

Data analysis

Data from antifungal activity screening were analyzed using simple statistics from Microsoft Excel and recorded in appropriate tables as mean ± standard deviation of mean. Data from Minimum Inhibitory Concentration (MIC) were recorded in Table-3, as + for a positive test result and - for a negative test result.

Result and Discussion

Table -2 showed that the whole plant extract of *Viola canescens* was moderately effective against the tested pathogen *Fusarium oxysporum* f.sp. *lycopersici*. The acetone extract showed no inhibition zone at 250 mg/ml concentration. 500 mg/ml concentration was moderately effective with 8.1 mm inhibition zone. At 750 mg/ml and 1000 mg/ml the zone of inhibition were observed as 8.25 mm and 17.62 mm.

Table.1 Major classes of antimicrobial compounds from plants

Class	Subclass	Example(s)	Mechanism	Reference(s)
Phenolics	Simple phenols	Catechol	Substrate deprivation	Phillipson J D, 1987.
		Epicatechin	Membrane disruption	Toda *et al.*,1992.
	Phenolic acids	Cinnamic acid		Fernandez *et al.*, 1996.
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes	Duke J A,1985 .
	Flavonoids	Chrysin	Bind to adhesions	Perrett *et al.*,1995.
	Flavones		Complex with cell wall	
		Abyssinone	Inactivate enzymes	Brinkworth *et al.*,1971.
			Inhibit HIV reverse transcriptase	Ono *et al.*,1989.
	Flavonols	Totarol	-	Kubo *et al.*, 1993.
	Tannins	Ellagitannin	Bind to proteins	Schultz J C,1988.
			Bind to adhesions	Scalbert A, 1991.
			Enzyme inhibition	Haslam E,1996.
			Substrate deprivation	Brownlee *et al.*,
			Complex with cell wall	1990.
			Membrane disruption	
			Metal ion complexation	
	Coumarins	Warfarin	Interaction with eucaryotic DNA (antiviral activity)	Brandao *et al.*, 1997.
Terpenoids, essential oils		Capsaicin	Membrane disruption	Cichewicz R H, 1996.
Alkaloids		Berberine	Intercalate into cell wall and/or DNA	Atta-ur-Rahman M I, 1995.
		Piperine		
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption	Meyer *et al.*,1997.
		Fabatin	Form disulfide bridges	
Polyacetylenes		8S-Heptadeca-2(*Z*),9(*Z*)-diene-4,6-diyne-1,8-diol	-	

Table.2 In -vitro antifungal activity of *Viola canescens* whole plant extract at various concentrations against fungal pathogen *Fusarium oxysporum* f.sp. *lycopersici*

Plant Name	Concentration-s (mg/ml)	Mean Inhibition Zone in various solvents			
Viola canescens (After adding 30 µl extract)		Acetone	Aqueous	Ethanol	P.E.
	250	0 ± 0	0 ± 0	0 ± 0	10.12 ± 0.8
	500	8.12 ± 1.4	0 ± 0	0 ± 0	9.7 ± 0.2
	750	8.25 ± 1.3	0 ± 0	0 ± 0	12.37± 0.9
	1000	17.62 ± 1.4	0 ± 0	14.32 ± 1.3	14.75 ± 1.7

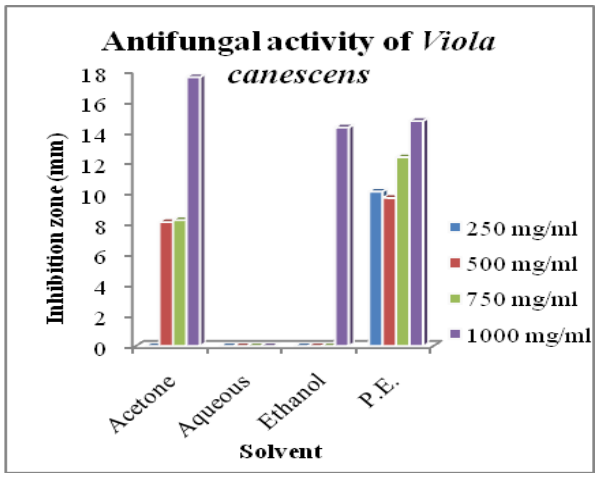
Results are the mean of four replications ± S.D

Table.3 MIC determination for various solvents of *Viola canescens* against *Fusarium oxysporum* f.sp. *lycopersici*

Plant Name	Concentration (mg/ml)	Acetone	Aqueous	Ethanol	P.E.
Viola canescens	1000	-	+	-	-
	100	+	+	-	-
	10	+	+	+	+
	1	+	+	+	+
MIC		1000	-	100	100

+ Growth ; - No growth

Graph.1 Antifungal activity of *Viola canescens*



Graph.2 MIC for *Viola canescens*

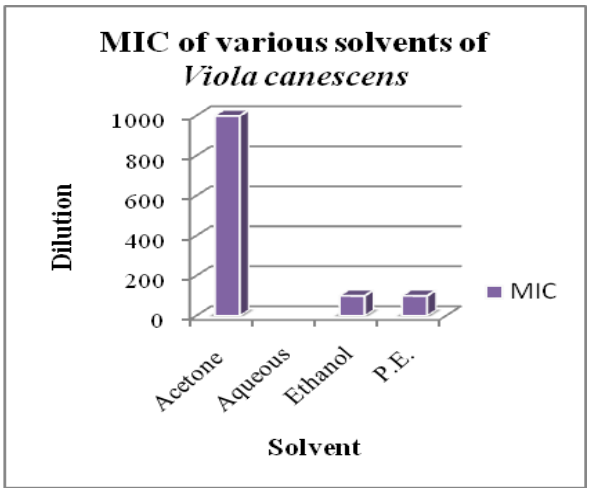


Figure.1 Antifungal activity of acetone extract of *Viola canescens*

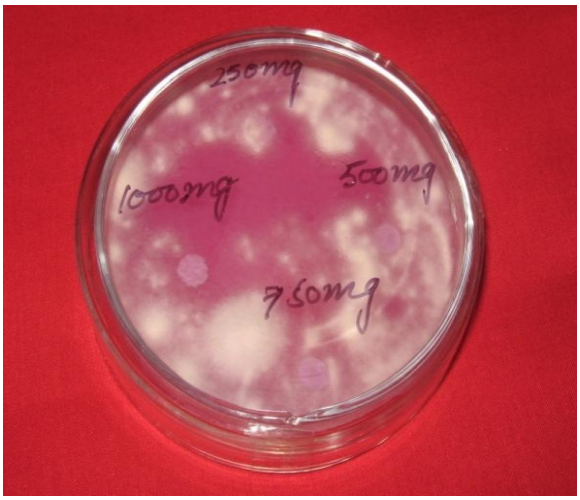


Figure.2 Antifungal activity of aqueous extract of *Viola canescens*

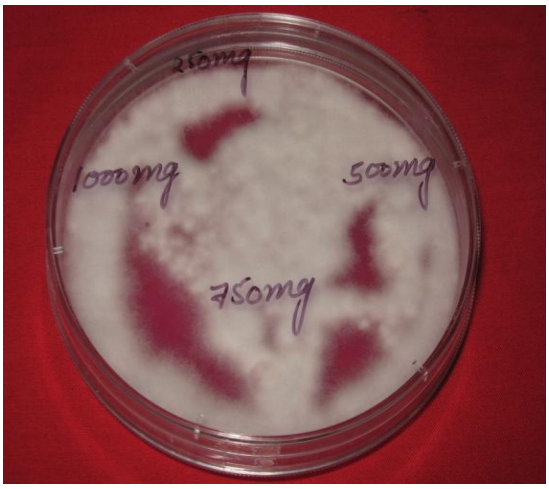


Figure.3 Antifungal activity of ethanol extract of *Viola canescens*

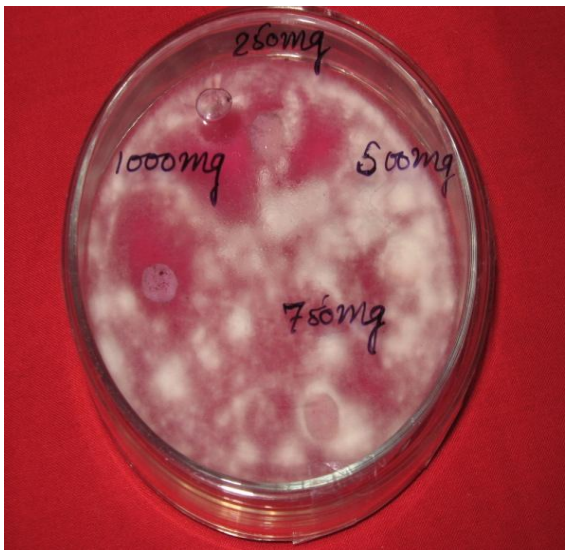


Figure.4 Antifungal activity of P.E. extract of *Viola canescens*

Aqueous extract of *V. canescens* showed no effectiveness against tested pathogen at any concentration. Its ethanol extract proved to be effective at only 1000 mg/ml concentration with inhibition zone of 14.32 mm. While petroleum ether extract showed 10.12 mm inhibition zone at 250 mg/ml concentration. 500 mg/ml concentration was effective with 9.7 mm inhibition zone. 12.37 mm inhibition zone was observed at 750 mg/ml concentration. And 1000 mg/ml concentration of *V. canescens* showed 14.75 mm. zone of inhibition.

MIC of acetone extract of *Viola canescens* was observed to be as 1000 mg/ml. Aqueous extract was not effective at any concentration with overall visible growth. Ethanol extract of *V. canescens* showed MIC at 100 mg/ml while similar 100 mg/ml

MIC was observed in case of petroleum ether extract of *V. canescens* (Table-3).

The use of phytochemicals derived from plants, with known antimicrobial properties, are of great significance to medicinal treatments (Nagesh and Shanthamma, 2009). *Viola canescens* was also found moderately effective as antifungal against exposed pathogen (Banaszczak WE 2005; Muhammad *et al.,* 2013; Dwarika Prasad, 2014).

Therefore, bio-pesticides (botanicals and bio-agents) made from *Viola canescens* may be used as an alternative to pesticides to minimize the wilt disease of the crops as they are environmental safe.

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