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Full Length Article

Antifungal Activity of Different Extracts of *Chenopodium album* against *Fusarium oxysporum* f. sp. *cepae*, the Cause of Onion Basal Rot

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Abstract

Antifungal potential of different parts of *Chenopodium album* L. was examined against *Fusarium oxysporum* Schlechtend. f. sp. *cepae* (Hans.) Snyder and Hansen, the cause of basal rot disease of onion (*Allium cepa* L.). In screening bioassays, the effect of different concentrations (0.5, 1.0, …3.0%) of methanolic leaf, stem, root and inflorescence extracts of *C. album* was investigated. Extracts of different parts of the test plant species showed variable antifungal activity. The highest antifungal activity was exhibited by inflorescence extract. Different concentrations of this extract suppressed fungal growth by 24–80%. Methanolic inflorescence extract was successively extracted with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The highest antifungal activity was shown by ethyl acetate fraction resulting in 68–100% reduction in fungal biomass. From ethyl acetate fraction, three unknown compounds viz. A, B and C were isolated through thin layer chromatography (TLC). TLC fraction A exhibited the highest antifungal activity with minimum inhibitory concentration (MIC) of 250 μg mL^{-1}. Thus, antifungal constituents of ethyl acetate fraction of methanolic inflorescence extract of *C. album* can be used as natural fungicides for the management of basal plate rot of onion.

Keywords: Basal plate rot; *Chenopodium album; Fusarium oxysporum;* Inflorescence extract; Onion

Introduction

As an important horticultural crop worldwide, onion (*Allium cepa* L.) is the second after tomato (Benítez *et al*., 2012). It has great economic importance, because of its medical and nutritional values (Nasri *et al.,* 2012). Onions can be used in almost each type of food either cooked foods or fresh salads. Onions pickled in vinegar are eaten as a snack. The mature bulbs contain starch, sugar, some protein, and vitamins A, B and C (Baloch, 1994). Onion is known for prevention or treatment of many diseases such as coronary heart diseases (Lanzotti, 2006), cancers (Shutenko *et al.,* 1999), cataract (Sanderson *et al.,* 1999) and diabetes (Sheela *et al.,* 1995). The characteristic sulfur containing secondary metabolites alliins (cysteine sulfoxides) cause taste and sharpness, and are criteria for the pharmaceutical quality (Bloem *et al.,* 2005). Another sulfur containing compound thiosulfinates exhibit antimicrobial properties (Peter, 2003).

A number of diseases generally caused by fungi and bacteria affect onions. Pink root [*Pyrenochaeta terrestris* (Hans.) Gorenz, Walker, and Larson], thrips (*Thrips tabaci* L.) and Fusarium basal rot (*Fusarium oxysporum* f. sp. *cepae*) are the economically important diseases of onion. Basal plate rot is a highly destructive disease of onion caused by *Fusarium oxysporum* Schlechtend. f. sp. *cepae* (Hans.) Snyder and Hansen and is found in many countries of the world (Bayraktar, 2010). It induces significant yield losses of the crop worldwide (Ozer and Koycu, 2004). Both infected seeds and soil are the chief source of pathogen inoculum. The fungus causes infection at the basal stem plate of the onion bulb and degrades it, finally destroys the whole plant (Cramer, 2000). Starting from tips of leaves, progressive wilting, yellowing, curving and eventually dying back symptoms appear on infected plants (Sumner, 1995). The disease is prevalent both in the field as well as during storage (Ozer *et al.,* 2003). The disease can effectively be controlled by seed treatment with benomyl and soil fumigation with metam sodium or methyl bromide (Jaworski *et al.,* 1978; Ozer and Koycu, 1998). However, the use of these synthetic agro-chemicals is becoming more restraining due to health and environmental issues (Rial-Otero *et al.,* 2005). Alternatively, use of natural plant products is gaining importance for the management of phytopathogens (Kanwal *et al.,* 2010; Riaz *et al.,* 2010; Jabeen *et al.,* 2011). The current research work was, therefore, undertaken to assess the potential of extracts of different parts of *C. album* to control basal rot disease of onion.

Materials and Methods

Isolation of Pathogen

Onion bulbs infected with basal plate rot disease were

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collected from field of district Mandi Bahaud-Din of province Punjab, Pakistan in 2009. Infected tissues of onion bulb were surface-sterilized with 1% solution of sodium hypochlorite for 1 min, rinsed several times in sterile distilled water. Two growth media viz. potato dextrose agar and malt extract agar were used for fungal isolations. Pieces of the surface sterilized onion tissues were plated on the two growth media in Petri plates and incubated at 23ºC in the dark for 7 days. The isolated fungal pathogen was subcultured using a single spore technique for culture purification. The pure culture was preserved at 4°C. The isolated fungal species was identified as *F. oxysporum* f. sp. *cepa* with reference to the literature (Leslie and Summerell, 2006).

Preparation of Methanolic Extracts

Leaves, stems, inflorescence and roots were collected from mature *C. album* plants. All the materials were thoroughly washed under tap water, dried in an electrical oven at 45ºC, thoroughly crushed and ground to fine powder. Two hundred grams of each of the four powdered plant parts were soaked in 2 L methanol in air tight glass jars separately for 7 days at room temperature. Afterwards extracts were obtained from soaked materials by filtering through muslin cloth followed by filter papers and preserved in plastic bottles. Filtrates were evaporated in rotary evaporator under vacuum to reduce the volume to 20 mL. Then 20 mL of the extracts was poured in open wide mouth pots and put in the air dried oven at 40ºC to completely evaporate the methanol. Finally, 26.63 g leaf, 18.67 g stem, 16.81 g inflorescence and 8.4 g root extract were obtained.

Bioassays with Methanolic Extracts

In vitro bioassays were carried out with methanolic extracts of different parts of *C. album*. Crude methanolic extract (8.4 g) of each of the four parts of *C. album* was dissolved in sterilized distilled water to prepare 14 mL of stock solution. Seventy six milliliter malt extract (ME) broth was autoclaved at 121ºC for 30 min in 250 mL conical flasks and cooled at room temperature. Chloromycetein at 50 mg 100 mL^{-1} of the medium was added to avoid bacterial contamination. Six concentrations viz. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g 100 mL^{-1} were prepared by adding 0.7, 1.3, 2, 2.7, 3.3 and 4.0 mL stock solution and 3.3, 2.7, 2.0, 1.3, 0.7 and 0 mL distilled water, respectively, to each flask to make total volume of the medium up to 80 mL. The 80 mL of each treatment was divided into four equal portions in 100 mL conical flasks to serve as replicates. For control treatment, 4 mL of distilled water was added to 76 mL of ME broth. Mycelial discs (5 mm) were removed from the tips of one-week old actively grown culture of *F. oxysporum*. f. sp. *cepa* using a sterilized cork borer and put in each conical flask. Flasks were incubated for ten days in an incubator maintained at 20ºC. Fungal harvest was taken by filtering the fungal mat through pre weighed Watman No. 1 filter papers followed by oven drying to gain dry biomass from each flask (Javaid and Sammad, 2012).

Fractionation of Methanolic Inflorescence Extract

Methanolic extracts of inflorescence of *C. album* was found highly effective in suppressing *in vitro* growth of *F. oxysporum* f. sp. *cepa*. This extract was selected for further fractionation through *n*-hexane followed by chloroform, ethyl acetate and *n*-butanol successively following the protocol of Iqbal and Javaid (2012).

Two and a half kilograms inflorescence of *C. album* were extracted with 7 L methanol twice for two weeks. Following filtration, methanol was evaporated under vacuum at 45ºC using a rotary evaporator to yield 210 g of crude methanolic extract. The crude methanolic extract was mixed with 1.0 L distilled water and the mixture was extracted with *n*-hexane in separating funnel several times to completely separate the *n*-hexane soluble fractions. The *n*-hexane phase was separated and evaporation was done on a rotary evaporator to get 5.3 g *n*-hexane fraction. The aqueous phase was submitted to further fractionation successively with 500 mL of each of chloroform, ethyl acetate and *n*-butanol, to yield chloroform (2.1 g), ethyl acetate (2.8 g) and *n*-butanol fraction (7.4 g). Finally the remaining aqueous extract was evaporated to give 2.3 g of this fraction.

Bioactivity of Different Fractions of Methanolic Inflorescence Extract

The bioactivity of different fractions of methanolic inflorescence extract was evaluated against target fungal species by liquid culture method in 10 mL test tubes. Weighed amount (1.2 g) of each of the five fractions of methanolic inflorescence extract was dissolved in 1 mL of dimethyl sulphoxide (DMSO) and added to 5 mL of malt extract broth. This stock solution (200 mg mL^{-1}) was serially double diluted by adding malt extract broth to prepare lower concentrations of 100, 50, …, 3.125 mg mL^{-1}. For control, 1 mL of DMSO was dissolved in 5 mL malt extract broth and serially double diluted to prepare control treatments corresponding to various extracts concentrations. Bioassays were conducted in 10 mL volume glass test tubes each containing 1 mL of medium. Test tubes were inoculated with one drop of conidia of *F. oxysporum* f. sp. *cepa*. There were three replicates of each treatment. Test tubes were incubated at room temperature for 7 days. Thereafter, fungal biomass in each test tube was filtered and weighed after drying at 60°C (Javaid and Saddique, 2012).

Separation of Antifungal Compounds through TLC

Ethyl acetate fraction was selected for separation of antifungal constituents as this fraction showed the best antifungal activity. Thin layer chromatography (TLC) was done for separation of different compounds present in this

fraction. Isolation and purification was done by performing preparative thin layer chromatography (PTLC). Ethyl acetate fraction of methanolic inflorescence extract yielded three TLC fractions namely A, B and C with R_f values of 0.46, 0.61 and 0.71, respectively, using solvent system $CHCl_3$: MeOH (8:2).

Appraisal of Minimum Inhibitory Concentration (MIC) of the Isolated Compounds

The MIC values of the isolated compounds and a reference synthetic fungicide mancozeb (80%WP, KSS) were tested in culture tubes by serial double dilution assay (Jabeen *et al*., 2011). The three isolated compounds of inflorescence ethyl acetate fraction were used for MIC's assays. Eight milligrams of each of the three isolated compounds and mancozeb were dissolved in 0.5 mL DMSO and 1.5 mL malt extract was added to prepare 4000 µg mL^{-1} concentration. Medium was serially double diluted with malt extract broth in culture tubes to prepare lower concentrations of 2000, 1000, …, 7.81 µg mL^{-1}. Control treatments were prepared by adding 0.5 mL DMSO in 1.5 mL malt extract broth and serially double diluted to prepare corresponding control treatments for each dilution. Seven days old fungal culture was added to double distilled water to make conidial plus mycelial suspension. One drop of the suspension was added to different concentrations of the growth medium in 5 mL volume glass tubes containing 1 mL of the medium each. Tubes were incubated at room temperature. The MIC was recorded after 24, 48 and 72 h.

Statistical Analysis

All the data were analyzed by ANOVA followed by Duncan's Multiple Range Test to delineate the treatment means.

Results and Discussion

Screening Bioassays

ANOVA for the effect of concentrations of methanolic extracts of stem, leaf, root and inflorescence on biomass of *F. oxysporum* is presented in Table 1. The effects of plant parts (P) and extract concentration (C) were significant while that of interactive effect of P × C was non-significant for the fungal biomass.

Inflorescence extract exhibited the highest activity against the target pathogen followed by extracts of leaves and roots. All the concentrations of methanolic inflorescence and leaf extracts significantly reduced fungal biomass by 20–66% and 24–80%, respectively. Similarly, various concentrations of the root extract reduced the fungal biomass by 8–58%. Methanolic stem extract was the least effective resulting in 7–53% decline in fungal biomass. The effect of the highest concentrations 2.5% and 3.0% was significant statistically (Fig. 1). These findings are in agreement with the results of Javaid and Amin (2009), who reported that alcoholic extracts of different parts of *C. album* were very effective in controlling the growth of *Macrophomina phaseolina*.

Table 1: Analysis of variance for the effect of different concentrations of methanol leaf, stem, inflorescence and root extracts of *Chenopodium album* on *in vitro* growth of *Fusarium oxysporum*

Sources of variation	df	SS	MS	F values
Treatments	27	440	0.016	7.7***
Plant parts (P)	3	0.035	0.011	5.5**
Conc. (C)	6	0.384	0.063	30.2***
P × C	18	0.021	0.001	0.55 ns
Error	84	0.178	0.002	
Total	111	4.433		

**, ***Significant at P≤0.01 and P≤0.001, respectively
ns: non-significant

Table 2: Analysis of variance for the effect of different fractions of methanol inflorescence extract of *Chenopodium album* on *in vitro* growth of *Fusarium oxysporum* f. sp. *Cepae*

Sources of variation	df	SS	MS	F values
Treatments	34	92	2.71	94*
Fractions (F)	4	27	6.87	239*
Conc. (C)	6	53	8.81	306*
F × C	24	12	0.50	17*
Error	70	2	0.03	
Total	104	180		

*, Significant at P≤0.001

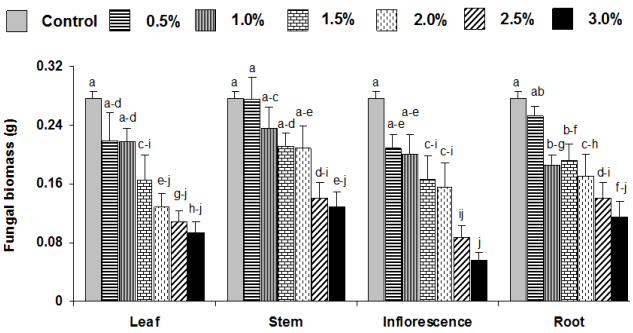

Fig. 1: Effect of different concentrations of methanol extract of leaf, stem, inflorescence and root of *Chenopodium album* on biomass of *Fusarium oxysporum*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P ≤ 0.05) as determined by Duncan's Multiple Range Test

Antifungal Activity of Different Fractions of Methanolic Inflorescence Extract

ANOVA for the effect of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fractions of methanolic inflorescence extracts of *C. album* on biomass of *F. oxysporum* is presented in Table 2. The effect of different fractions (F) and concentrations (C) was

Table 3: Effect of different concentrations of n-hexane, chloroform, ethyl acetate, n-butanol and aqueous fraction of methanolic inflorescence extract of *Chenopodium album*

Methanolic fraction	Conc. of DMSO (mL mL^{-1})	Extract conc. (mg mL^{-1})	Fungal biomass (mg)
Control	0.1666	0	0.33 m
	0.0833	0	1.6 f-h
	0.0416	0	1.8 d-g
	0.0208	0	1.9 c-f
	0.0104	0	2.26 b
	0.0052	0	2.4 b
	0.0025	0	2.93 a
n-hexane	0.1666	200	0 m
	0.0833	100	0.23 m
	0.0416	50	1.53 g-i
	0.0208	25	1.8 d-g
	0.0104	12.5	1.93 c-e
	0.0052	6.25	2.13 bc
	0.0025	3.125	3.13 a
Chloroform	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	0 m
	0.0104	12.5	0.86 l
	0.0052	6.25	1 kl
	0.0025	3.125	1.26 i-k
Ethyl acetate	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	0 m
	0.0104	12.5	0 m
	0.0052	6.25	0.83 l
	0.0025	3.125	0.93 l
n-butanol	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	1.03 k-l
	0.0104	12.5	1.13 j-l
	0.0052	6.25	1.66 e-h
	0.0025	3.125	2.1 b-d
Aqueous	0.1666	200	0 m
	0.0833	100	0.33 m
	0.0416	50	1.13 j-l
	0.0208	25	1.43 h-j
	0.0104	12.5	1.8 d-g
	0.0052	6.25	2.1 bc
	0.0025	3.125	3.1 a

In vertical column, values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test

significant at $P \leq 0.001$ as well as the interactive effect of F×C was also significant for the fungal biomass.

Data presented in Table 3 reveals that DMSO adversely affected the fungal growth. In control, the lowest fungal biomass was recorded with the highest concentration of DMSO. A gradual increase in fungal biomass was recorded with the decrease of DMSO concentration from 0.1666 to 0.0025 mL mL^{-1} of the growth medium. Among the various fractions of methanolic inflorescence extract, ethyl acetate fraction exhibited the best antifungal activity, where all the extract concentrations significantly reduced fungal biomass by 65–100% over corresponding control treatments and all except the two lower most concentrations completely inhibited the fungal growth. Similarly, all the concentrations of chloroform fraction suppressed the fungal biomass significantly by 57–100% and all except the three lower most concentrations (3.12–12.5 mg mL^{-1}) completely checked the fungal growth. The *n*-butanol fraction also exhibited pronounced antifungal activity. The three higher concentrations (50–200 mg mL^{-1}) of this fraction completely retarded the fungal growth while rest of the concentrations significantly reduced fungal biomass by 28–46% over corresponding controls. The effect of *n*-hexane and aqueous fractions was generally less pronounced where only the highest concentrations of 200 mg mL^{-1} completely controlled the fungal growth, while the lower concentrations showed variable effects (Table 3).

Table 4: Minimum Inhibitory Concentration (MIC) values of isolated compounds from *Chenopodium album* and synthetic fungicide Mancozeb against *Fusarium oxysporum* after 24, 48 and 72 h

Treatments	Concentrations µg mL^{-1}									
	4000	2000	1000	500	250	125	62.5	31.25	15.62	7.81
After 24 h incubation period										
Control	+	+	+	+	+	+	+	+	+	+
A	–	–	–	–	–	–	–	+	+	+
B	–	–	–	–	–	–	+	+	+	+
C	–	–	–	–	+	+	+	+	+	+
Mancozeb	–	–	–	–	–	–	–	–	–	–
After 48 h incubation period										
A	–	–	–	–	–	–	+	+	+	+
B	–	–	–	–	–	+	+	+	+	+
C	–	–	–	+	+	+	+	+	+	+
Mancozeb	–	–	–	–	–	–	–	–	–	–
After 72 h incubation period										
A	–	–	–	–	–	–	+	+	+	+
B	–	–	–	–		+	+	+	+	+
C	–	–	–	+	+	+	+	+	+	+
Mancozeb	–	–	–	–	–	–	–	–	–	–

\+ Fungal growth appear
– No fungal growth

MIC of the Isolated Compounds

Mancozeb was found highly effective against *F. oxysporum* f. sp. *cepae*. All the concentations completely inhibited the fungal growth after 24, 48 and 72 h. Isolated compounds A exhibited highest antifungal activity with MIC value of 62.5 µg mL^{-1}. Compounds B and C showed MIC values of 125 µg mL^{-1} and 500 µg mL^{-1}, respectively after 24 h. Compounds A was also found best in affectivity after 48 h of incubation with a MIC value of 125 µg mL^{-1}. Likewise, compounds B exhibited similar effect after 48 h with an MIC value of 250 µg mL^{-1}. Compounds C showed the least effectivity against test fungus with MIC of 1000 µg mL^{-1} after 48 h. After 72 h, all the isolated compounds showed antifungal activity trends similar to that after 48 h of incubation (Table 4). Earlier, Tahara *et al.* (1994) isolated a highly fungitoxic metabolic mucondialdehyde (trans-2, transe-4 hexadienedial) from the leaves of *C. album.*

Similarly, Lavaud *et al.* (2000) have identified three saponims viz. calenduloside E, chikusetsaponin IV and 3-o-[3-o (2-o-glycolyll)-glycoxylyl β-D-glucuronopyranosyl] oleanolic acid, from leaves of *C. album*, which could be responsible for antifungal activity against *F. oxysporum* f.sp. *cepa* (Stuardo and Martín, 2008). In addition, antifungal activity of inflorescence of *C. album* may also be attributed to its phenolic acid contents (Báidez *et al.*, 2006; Laghari *et al.*, 2011).

Conclusion

Ethyl acetate fraction of methanolic inflorescence extract of *C. album* has the potential to control *F. oxysporum* f. sp. *cepa*. Further studies are needed to identify these antifungal compounds using various spectroscopic techniques.

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