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A PTEROCARPAN COMPOUND FROM CULTURE FILTRATE OF *FUSARIUM OXYSPORUM* F.SP.*CICERIS* AND WILT RESISTANCE IN CHICKPEA

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ABSTRACT

A new pterocarpan derivative (**1**), a phytotoxin, has been isolated from the culture filtrate of *Fusarium oxysporum* that caused leaves and wilting to chickpea seedlings. The structure of the compound **1** was elucidated based on modern one- and two-dimensional NMR spectroscopic techniques.

Key words: *Fusarium oxysporum* f.sp. *ciceris*, phytotoxins, culture filtrate, wilt, Chickpea

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* Schlect. Fr. f.sp. c*iceris* (Padwick) Matuo & Sato (FOC) is the most important disease of chickpea, produced 10-50% yield losses in dry areas of Pakistan during the last several years, while in irrigated belts of Punjab, farmers have shifted to other crops only due to wilt disease (Haq and Jamil, 1992). The best way to overcome the yield losses due to disease is the use of cultivars resistant to all the prevalent races of the pathogen. Seven races of FOC have been identified based on their interactions with a set of chickpea varieties (Haware and Nene, 1982 and Trapero-Casas and Jimenez-Diaz, 1985). Two distinct groups have been identified in FOC by using genetic fingerprinting and random amplified polymorphic DNA techniques (Kelly *et al.*, 1994).

Pathogenic fungi may often damage their host plants by producing phytotoxins, which cause various symptoms including necrosis, chlorosis, wilting, water soaking, and eventually death of plants (Scheffer, 1983). In several host-plant interactions phytotoxins have been reported as pathogenecity or virulence factor (Khan, *et al.*, 1998; Yoder 1980). The phytotoxins, which have been categorized as virulence or pathogenecity factor could be used in identifying resistant cells in tissue culture or in screening and breeding for disease resistance (Vidhyasekaran *et al*., 1990; Bajwa *et al.*, 2000). Various species of *Fusarium* are reported to be toxigenic (McLean, 1996). Two novel phytotoxic compounds Phenylacetic acid and methyl p-hydroxyphenyl acetate were isolated from culture filtrates of *F. oxysporum* isolate were phytotoxic to pea, rapeseed, lettuce, garden cress, and barley (Kachlicki, and Jedryczka, 1997). Fusaric acid and dehydro fusaric acid and their corresponding ester compounds are the important phytotoxins of *Fusarium* sp. (Capasso *et al*., 1996). Naphthazarin phytotoxins are produced by *F. solani* in xylem fluid of scaffold roots from blight-diseased trees (Nemec, 1995). *Fusarium moniliforme* is reported to produce fumonisin B_1 (FB_1) and other related compounds such as FB_2, FB_3 and FB_4 (Abbas, 1992). The phytotoxin of FOC has been reported in impairment of most of the electrolytes such as phosphates, total phenols, protein, carbohydrates, K^+ and Ca^{+2} were significantly higher in toxin induced tissues than in control of both the cultivars. The susceptible cultivars released higher amount of phosphates, carbohydrates, K^+ and Ca^{+2} ions from toxin treated tissues than the resistant cultivars (Khan *et al.*, 2001).

The present studies deals with the purification and characterization of phytotoxins of *F. oxysporum* f. sp. *ciceris* so as to utilize it for chickpea screening program and also in identifying resistant cells in tissue culture.

MATERIALS AND METHODS

Fungal isolates:

A virulent, wilt causing isolate (2012) of *Fusarium oxysporum* f. sp. *ciceris (*Khan *et al.*, 2002) was used in this study, which was isolated from diseased chickpea material collected from Thal area of Punjab, Pakistan.

Production of culture filtrates:

Liquid czapek dox was prepared, distributed in 100 ml aliquots in one liter roux bottles, and after autoclaving at 121°C for 15 min and cooling, inoculated with two 2mm disc of 7 days old culture of FOC isolate. Roux bottles were incubated at 25°C in dark without shaking and culture filtrates were harvested after 14 days by filtering through muslin cloth and phytotoxicity was measured by cut seedling method against 12 days old cuttings of susceptible chickpea variety (Aug-424) as described by Huang and Hartman (1998).

Extraction of phytotoxicity:

The culture filtrates from 2.0 liter czapek dox medium was produced, its pH was adjusted to 3.0 with 2 M sulphuric acid (H_2SO_4) and extracted in half the volume of ethyl acetate three times. The ethyl acetate phases (upper part) were combined and dried over anhydrous sodium sulphate. They were filtered through Whatman filter paper no.1 to eliminate the sodium sulphate powder and the filtrate was evaporated to dryness at 30°C using a vacuum evaporator. The residues were dissolved in 10 ml ethanol. The toxicity of ethyl acetate extracts (30 µl) dissolved in 5.0 ml distilled water was tested in three replicates as described earlier.

Purification of phytotoxin:

The concentrated organic phase was subjected to column chromatography (silica gel was used as adsorbent) and further fractionated by 200 ml of the following solvents n-hexane, benzene, toluene, chloroform, diethyl ether, ethyl acetate, acetonitrile, acetone, and methanol. The fractions obtained were concentrated on rotary evaporator and then dissolved in 5 ml ethanol. The phytotoxicity of these fractions (50 µl) was determined by cut seedling method. The fraction(s) found toxic to chickpea cuttings were further subjected to thin layer chromatography (0.5 mm thick plates of silica gel GF_{254}, Merck), directed by cut seedling assay of different bands appeared on the TLC plates after developing them in either of the following solvent systems chloroform: methanol (9:1) and benzene: acetone: acetic acid (35:5:1).

RESULTS AND DISCUSSION

A virulent wilt causing FOC isolate 2012 was used in the present study. Two main groups have been recognized (Kelly *et al*., 1994 and Khan *et al*., 2002) based on the symptoms produced by FOC isolates i.e. yellowing and wilt. Wilt causing FOC isolates have been reported to be widespread in Pakistan while the yellowing causing isolates have been reported to be prevalent in Thal area of Pakistan (Khan *et al*., 2002) where 90% chickpea crop is sown. Jeminez Diaz *et al*., (1990) have reported that yellowing causing isolates belonging to race "0" were mainly found in Mediterranean region. That is the reason we used wilt causing isolate in the present studies.

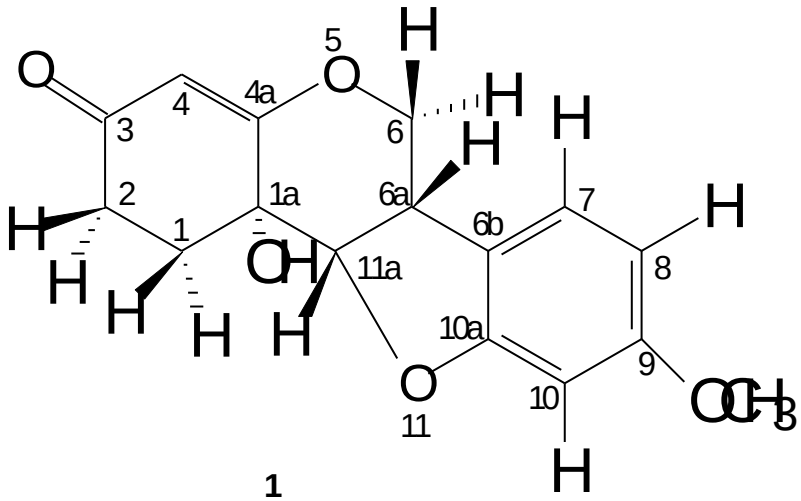
A total of 1.8 liter culture filtrate was harvested from 2.0 liter minimal medium and it was toxic during phytotoxicity assay. It produced leaves burning/yellowing initially after 48 hrs, and finally caused wilting to chickpea cuttings of Aug-424 when incubated up to 4 days. The ethyl acetate extract (30 µl diluted with 5.0 ml of distilled water) also caused wilting to chickpea cuttings (var. Aug-424) at this concentration which was equivalent to 5.4 ml of original culture filtrates, after four days incubation (**Fig. 1**). The control treatments remained healthy. So ethyl acetate phase was further subjected to purification step.

Phytotoxicity assay of the fractions obtained after solvent fractionation through column chromatography revealed that Ethyl acetate and diethyl ether phases were toxic to chickpea cuttings at 50 µl concentration, so these were further continued for thin layer chromatography to purify the phytotoxic compounds. The developed TLC plates were visualized under UV light and the bands fluorescing on TLC plate were scratched and dissolved in 2.0 ml distilled water and their toxicity was measured, which revealed that the compound at Rf value 0.26 (in benzene: acetone: acetic acid; 35:5:1) caused leaves burning and wilting to cuttings of chickpea (**Fig. 2**). No symptoms were observed on control cuttings.

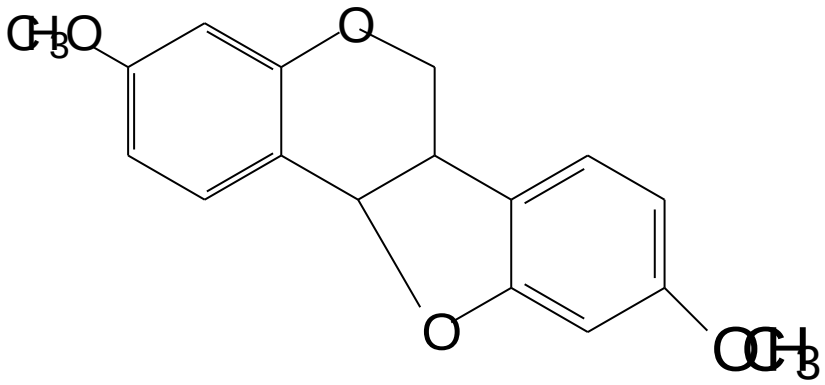
The culture filtrate of FOC inhibited the synthesis of chlorophyll 'a' and 'b' in chickpea leaves and was also found to inhibit the root length of germinating seeds of resistant and susceptible chickpea varieties (to be published elsewhere). Inhibition was more prominent in susceptible variety (Aug-424) as compared to resistant one (CM-98). The symptoms produced by the FOC toxins i.e. leaves burning/yellowing might be due to the result of chlorophyll degradation. The wilting has been related with the blockage of conducting vessels of the chickpea plants by the action of metabolic products or cell wall degrading enzymes produced by the vascular wilt pathogens (Beckman, 1964) but the leaching out of vital electrolytes from chickpea tissues by the FOC toxins (Khan *et.al*. 2001) might be the real factor causing wilting to the chickpea plants.

Elucidation of Structure:

The EIMS of compound **1** showed molecular ion peak at *m/z* 288. In the FAB-MS, the $[M+H]^+$ appeared at *m/z* 289 conforming to molecular ion peak. The HR EIMS exhibited M^+ at *m/z* 288.0975 corresponding to molecular formula $C_{16}H_{16}H_5$ (*calcd* 288.00) with nine degree of unsaturation. A prominent IR absorption band was observed at 1619 cm^{-1} indicating the presence of a conjugated carbonyl group. A broad IR absorption at 3239 cm^{-1} confirms the presence of hydroxy group in the molecule. The UV spectrum exhibited absorptions at 259.4 (4.26), 203 (4.47) and 191.6 (4.86). The optical rotation of this compound was found to be – 198.19.



1



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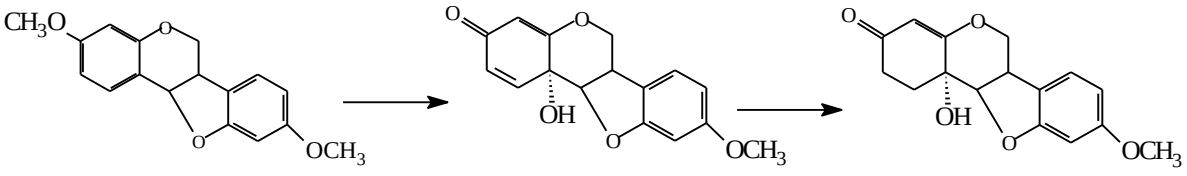


2 **Dienone** **1**

The ^{1}H-NMR spectrum of **1** showed a downfield singlet resonated at δ 5.20 attributed to H-4 methine of cyclohexenone moiety (ring-A). The spectrum contains two readily identifiable methylene protons adjacent to electronegative oxygen resonated at δ 4.66 (*dd*, $J_{6\beta,6a\beta}$ = 4 Hz, $J_{6\beta,6a\alpha}$ = 11 Hz H-6β) and 4.20 (*d*, $J_{6a,6\beta}$ = 11 Hz H-6α) in the pyran ring. Furthermore, the spectrum contains two sets of methylene proton in ring-A. Two multiplets at

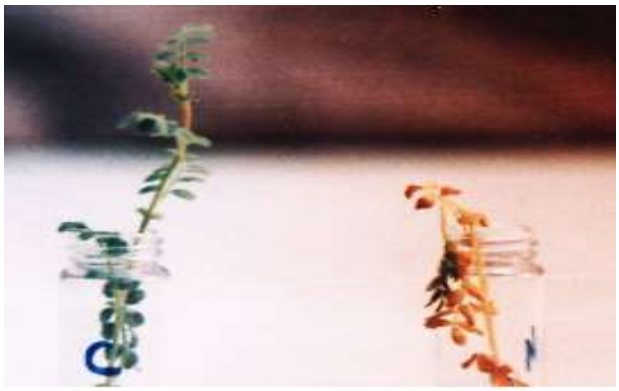
δ 2.22 and 1.93 were ascribed to H-1β and α methylene protons, respectively. The remaining two protons resonated at δ 2.70 (m) and 2.47 (*dd*, *J*= 4, 11 Hz) were assigned to H-2β and α, respectively. The spectrum also exhibited characteristic signals for two methine protons resonated at δ 3.8 (*dd*, $J_{6a\beta,6\beta}$ = 4Hz, $J_{6a\beta,11a\beta}$ = 10 Hz H-6aβ) and 4.98 (*d*, $J_{11a\beta,6a\beta}$ = 10 Hz H-11aβ) which were assigned to cis-ring junction. A 3H singlet resonated at δ 3.67 was designated to methoxy methyl. The three aromatic methine protons resonated at δ 7.22, 6.47 and 6.35 were assigned to H-7, H-8 and H-10, respectively. A hydroxy group was placed unambiguously at C-1a position with concomitant formation of enone-system in the ring-A. The propsed mechanism for the formation of compound **1** involves an intermediate dienone 3 (Scheme-1), which through reduction yields the compound **1**.

Table 1. ^{1}H and ^{13}C-NMR assignments of 1.

Carbon No.	^{13}C-NMR δ (ppm)	^{1}H-NMR δ (ppm), *J* in Hz
1	31.9	1.93 Hα *m*
		2.22 Hβ *m*
1a	67.9	*
2	32.0	2.47 Hα (*dd* 4,13)
		2.70 Hβ *m*
3	199.4	*
4	109.1	5.20 *s*
5	172.1	*
6	67.3	4.20 Hα (*d* 11)
		4.66 Hβ (*dd* 4,11)
6a	39.5	3.8 Hβ (*dd* 4,10)
6b	119.1	*
7	124.4	7.22 (*d*, 8)
8	107.5	6.46 (*dd* 2,8)
9	160.3	*
10	95.8	6.35 (*d* 2)
10a	161.2	*
11a	82.6	4.98 (*d*, 10)
-OCH_3	55.5	3.67 *s*

Control **C. Filtrates**

Fig. 1. Effect of culture filtrates of FOC Isolate (2012) on chickpea cutting.



Control **Toxin**

Fig.2. Effect of purified phytotoxin of *Fusarium oxysporum* f. sp. *ciceris* on chickpea cuttings.

The ^{1}H-NMR assignments were confirmed by the *Correlation Spectroscopy* and *HOHAHA* (100 ms) experiments. The Cosy-45° exhibited the presence of three isolated spin systems in the molecule. The H-6α (δ 4.20) showed coupling with H-6β resonated at δ 4.66, which in turn exhibited vicinal interaction with H-6aβ. The H-6aβ also displayed homonuclear correlation with H-11aβ resonated at δ 4.98. The spectrum contains cross-peaks

between H-1α and β (δ 2.70 and 2.47, geminally-coupled protons) which extended their interaction with mutually coupled H-2α and β (δ 2.47 and 2.70). The aromatic protons resonating at δ 7.22 and 6.47 showed vicinal couplings. H-8 (δ 6.46) also displayed meta-coupling (J = 2 Hz) with H-10 (δ 6.35.) The distortion of spin network in the aromatic ring suggested the placement of methoxy group at C-9 position, that was further supported by the HMBC experiment and literature cited.

The ^{13}C-NMR spectra (broad-band decoupled and DEPT) of **1** exhibited resonances for all sixteen carbon atoms in the molecule comprising of one methyl, three methylene, six methine and six quaternary carbons (Table 1). The heteronuclear connectivities were established by HMQC and HMBC experiments. The presence of α, β-unsaturated carbonyl moiety in the molecule in comparison with pterocarpin **2** was evident by the downfield signals at δ 199.4 and 172.1 attributed to C-3 and C-4a, respectively. The H-4 (δ 5.20) showed long-range heteronuclear correlation ($^3J_{C\text{-}H}$ and $^4J_{C\text{-}H}$) with hydroxyl bearing C-1a (δ 67.9), C-2 (δ 32.0) methylene and C-4a (δ 172.1) β-position to carbonyl carbon. The H-1α (δ 1.93) displayed $^3J_{C\text{-}H}$ interaction with C-3 carbonyl (δ 199.4) and C-4a (δ 172.1) suggesting the presence of carbonyl moiety in ring-A. The H-2α (δ 2.47) also presented $^2J_{C\text{-}H}$ heteronulear correlation with carbonyl carbon resonated at δ 199.4. various heteronuclear connectivities are shown on the structure-**1**.

The 1a, α-hydroxypterocarpinone (**1**) is proposed to be an oxidative product of pterocarpin (**2**), a metabolite having 6a,11a-dihydro-6H-benzofurobenzopyran nucleus.

1a,α-Hydroxypterocarpinone; Reddish brown solid; [α] ($_D$ = - 198.19° (*c* 0.333, $CHCl_3$); IR ν_{max} 3239, 2875,1619, 1450, 1141 cm^{-1}; λ_{max} (log ε) 259.4 (4.26), 203 (4.47), 191.6 (4.86); ^{1}H-NMR, δ (DMSO-d_6, 300 MHz), Table-1; ^{13}C-NMR, δ ($CDCl_3$, 100 MHz), see Table-1; EIMS *m/z* (rel. Int. %), 288 M$^+$ (47.56), 195 (10), 161 (100) and 69 (13).

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