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SHORT COMMUNICATION

ANTIFUNGAL PROPERTIES OF PLUMBAGIN

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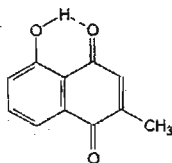
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Plumbagin is a constitutive metabolite of plants including *Plumbago zeylanica* L.,¹ *Plumbago indica*,² *Plumbago europaea* (Plumbaginaceae)³ and is also found to accumulate as a secondary stress metabolite. Plumbagin is among several phytoalexins accumulated during the incompatible interaction between the parasitic *Cuscuta reflexa* (Convolvulaceae) and the host *Ancistrocladus heyneanus* (Ancistrocladaceae).⁴ This leads to the degeneration of haustoria in the invading parasite and eventually its abortion.⁴ Plumbagin was shown to display antibacterial and antifungal properties.^{1,5,6} It also causes mortality of *Culex fatigans* mosquito larvae,⁷ inhibition of nematode larvae⁸ and exhibits cytotoxic,⁹ antitumour and radiomodifying properties.¹⁰

Ethnomedical claims on *P. zeylanica* are reported from many parts of the world.^{11,12} The plant is used in many Asian countries to increase digestive powers, promote appetite and treat dyspepsia, piles, anasarca, skin diseases and fever.¹³ Numerous pharmacological investigations have been carried out on *P. zeylanica* where plumbagin is an active constituent of roots of the plant.¹⁴ This plant is a rich source of naphthoquinones.¹⁵ The root of the plant is used as an abortifacient in several countries.¹⁶

It was earlier shown that the addition of an excess of cysteine which binds to the alpha, beta unsaturated carbonyls of plumbagin neutralised the fungitoxicity of the compound to *Pythium* sp.¹⁷ This paper reports some antifungal properties of plumbagin and examines the significance of chelating ability associated with plumbagin in its antifungal action.



Plumbagin

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Plumbagin

Plumbagin, isolated from *P. zeylanica* roots according to the method described by Guneharath *et al.*,¹⁵ was used in all the experiments. The orange crystals obtained were found to be identical to an authentic sample (m.p., m.m.p., IR and co-TLC). Antifungal activity of plumbagin against *Cladosporium cladosporioides*, *Alternaria tunis*, *Botrydiplochia theobromae*, *Aspergillus* sp., *Fusarium* sp., *Cercospora nicotinae* and two isolates of *Colletotrichum gloeosporioides* obtained from avocado and papaya was determined by Thin Layer Chromatography (TLC) bioassay.¹⁸ The activity was compared with a commercial fungicide, benlate [Du Pont; methyl-1-(butylcarbonyl)-2-benzamidazolecarbamate].

All the fungi were maintained on Cook's No. 2 agar medium at $28 \pm 2^\circ\text{C}$. Test solutions of plumbagin and benlate were prepared by dissolving 4 mg each, separately in 4 ml portions of distilled water. Eight TLC plates (silica gel 60 PF 254-366, 0.5 mm x 20 cm x 20 cm) were used and each plate was spotted with 10, 25, 50, 100 and 150 μl aliquots of plumbagin and 10, 25, 50 and 100 μl aliquots of Benlate (50% active ingredient). Plates were developed in dichloromethane and allowed to air-dry overnight. One plate each was carefully sprayed with a dense suspension of conidia of the above fungal species prepared by suspending mycelia, scraped from 5-6 day old colonies, in Czapek Dox nutrient solutions and filtering them through glass wool. The TLC plates were incubated in aluminum trays lined with moist paper to provide adequate moisture at $28 \pm 2^\circ\text{C}$ for 48 hours. The regions which lacked aerial mycelial growth were taken as inhibition areas and the diameter of the inhibition zones and their R_f values were measured and recorded.

Antifungal properties of plumbagin were further studied by spore germination assay¹⁹ using *C. gloeosporioides* (avocado isolate) as the test fungus. Mycelia scraped from 5-7 days old cultures were suspended in sterile distilled water (SDW) and the suspension was passed through glass wool to remove hyphae. The conidia in the filtrate were washed by repeated centrifugation (for 3 min. at 3000 rpm) followed by resuspension in SDW. The final concentration of conidia in the suspension was adjusted to 5×10^5 conidia ml^{-1} . A series of solutions of plumbagin was prepared in 10% ethanol in distilled water. Four aliquots (10 μl) from each solution were placed on clean glass slides (two drops per slide) taking care to ensure that the drops occupied a minimum area on the slide. Two slides were used per solution. Drops (10 μl) of suspension of conidia were pipetted on to each drop and mixed using a fine glass rod. The control slides contained 10% ethanol in SDW instead of plumbagin. The glass slides were then incubated to allow conidia to germinate inside a moist chamber for 6 hours at $26 \pm 2^\circ\text{C}$. Germination was stopped at the end of the incubation period by adding a drop of lactophenol. Two hundred randomly selected conidia were counted for germination under microscope in each drop and the percentage germination was determined.

To examine whether the action of plumbagin is fungistatic or fungicidal, the germination ability of conidia of *C. gloeosporioides* was tested after they had been exposed to plumbagin for a period of time. One ml portions of a suspension of conidia, prepared as described above, were applied to four small test tubes. One ml portions of a solution of plumbagin (2 mg/ml in 10% ethanol) were added to two tubes and one ml portions of 10% ethanol in SDW were added to the other two as controls. The tubes were allowed to stand at 26 ± 2 °C for 6 hours. The contents were centrifuged (3000 rpm for 5 min.) and the supernatant was discarded. The conidia were washed three times in SDW by centrifugation the suspension followed by resuspension of conidia in fresh SDW. The washed conidia were taken up in 1 ml SDW and tested for germination on glass slides as described above. The percentage germination was determined and compared against the control.

The significance of the chelating ability associated with plumbagin in its antifungal property was examined indirectly by first blocking its ligand by a chelator i.e. Fe^{3+} and then assaying its antifungal activity. The following solutions of plumbagin and/or $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were prepared in 40 ml portions of 2% solution of glucose in 10% ethanol:

0.004 g plumbagin (0.0212 m.mol) + 0.006 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.0229 m.mol)

0.002 g plumbagin(0.0105 m.mol) + 0.003 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.0114 m.mol)

0.002 g plumbagin (0.0105 m.mol) + 0.0015 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.007 m.mol)

0.004 g plumbagin (0.0212 m.mol)

0.002 g plumbagin (0.0105 m.mol)

0.006 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.0229 m.mol)

0.003 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.0114 m.mol)

Aliquots (5 μl) were first placed on clean slides (two slides per solution) and drops of conidia of *C. gloeosporioides* were pipetted into each drop of solution and mixed. The glass slides were incubated in moist chambers for 6 hours and the percentage germination of conidia in each solution was determined.

The results showed that plumbagin could inhibit the growth of all fungal pathogens tested in the study on TLC bioassay (Table 1). Benlate too was inhibitory on all fungi except *B. theobromae*. The size of the inhibition areas produced by the two substances, however, varied with the fungal species. Plumbagin showed greater toxicity against *C. cladosporioides* and *A. tunis* than benlate at similar concentrations. Both compounds exhibited comparable activity against *Fusarium* sp., *C. gloeosporioides* (papaya isolate) and *C. nicotinae*. On the other hand, benlate was more inhibitory against *C. gloeosporioides* (avocado isolate) and *Aspergillus* sp.

Table 1: Area of fungal growth inhibition caused by plumbagin and benlate on TLC bioassay.

Fungus		Inhibition area (cm ²) at concentrations (µg)				
		10	25	50	100	150
<i>Cladosporium cladosporioides</i>	plumbagin	3.8	12.5	19.6	28.2	38.4
	benlate	3.8	7.0	10.2	12.8	-
<i>Alternaria tunis</i>	plumbagin	4.5	6.2	10.2	12.5	19.6
	benlate	1.5	3.8	6.2	9.0	-
<i>Botrydiplodia theobromae</i>	plumbagin	0.8	2.5	7.0	12.5	19.6
	benlate	0.0	0.0	0.0	0.00	0.0
<i>Aspergillus</i> sp.	plumbagin	1.0	3.8	8.0	12.5	18.1
	benlate	3.5	8.0	12.5	19.6	19.6
<i>Fusarium</i> sp.	plumbagin	2.5	3.8	5.3	7.1	10.2
	benlate	1.0	3.4	4.5	7.1	-
<i>Colletotrichum gloeosporioides</i> (avocado isolate)	plumbagin	1.0	2.0	3.2	5.4	8.1
	benlate)	0.7	3.1	7.1	12.5	-
<i>C. gloeosporioides</i> (papaya isolate)	plumbagin	1.8	2.7	4.0	5.2	7.0
	benlate	1.6	2.0	2.3	4.9	-
<i>Cercospora nicotinae</i>	plumbagin	1.0	2.5	3.8	5.3	7.0
	benlate	1.0	2.0	3.1	4.5	-

On a germination assay conducted using conidia of *C. gloeosporioides* (avocado isolate), plumbagin solutions at and above $0.31 \times 10^2 \mu\text{g/ml}$ caused total inhibition and the amount required for 50% inhibition was $6.86 \mu\text{g/ml}$. Much higher concentrations of benlate (above $5 \times 10^1 \mu\text{g/ml}$) were required for total inhibition of conidia and the amount of benlate required for 50% inhibition was $0.44 \times 10^2 \mu\text{g/ml}$. These results further confirm the observations made in the TLC bioassay that plumbagin is more fungitoxic than benlate.

The experiment conducted to ascertain whether plumbagin's action is fungistatic or fungicidal using *C. gloeosporioides*, revealed that the exposure to plumbagin had not caused any permanent damage to the conidia and that the inhibitory effect is only temporary. Plumbagin is therefore only fungistatic.

Plumbagin is a naturally occurring bidentate chelating ligand. Its keto and the hydroxy functionalities are common binding moieties found in microbial iron scavengers called siderophores. Iron is known to form tris (ligand) complexes with

such bidentate chelating ligands.²⁰ It has been reported that iron (III) is involved in the inhibition of germination of *Colletotrichum musae*.^{21,22} Thus, chelating compounds such as 2,3-dihydroxybenzoic acid promote fungal germination through removing iron (III) from the spore by chelation.

In order to determine whether the chelating groups of plumbagin play a role in its antifungal activity, the effect of adding a mixture of iron (III) and plumbagin into the germinating medium of conidia of *C. gloeosporioides* was investigated. The results showed that at a concentration of 0.0003 g/40 ml (III) or less, iron had no effect on germination (Table 2). However, at double the concentration, iron (III) inhibited germination substantially. When a mixture of plumbagin and iron (1:1 ratio) was incorporated, the percentage germination increased closer to that of controls. On the other hand, when the ratio of 2:1 was maintained, the germination percentage was low, at 32%. When the same ratio was maintained at a different concentration of plumbagin and iron (III), the result was similar. This indicates that in both, the uncomplexed plumbagin molecules present in the medium may have caused inhibition. It appears that chelation of iron (III) to plumbagin probably blocks the sites of the molecules that are associated with the antifungal properties. Transitional metal ions Al, Fe, Co, Ni and Cu chelate with plumbagin.^{23,24} All the metal chelators show marginal antibacterial activity and the activity of metal chelators is less than that of the ligand against some microorganisms.²⁴ The significance of alpha and beta carbonyl groups of several compounds such as patulin, penicillic acid, parasorbic acid, tulipalin and plumbagin in their mode of action has been demonstrated by using cysteine which binds to these groups neutralising their activity. It has been suggested that the mode of action of these substances could be by the binding of alpha, beta unsaturated carbonyl groups to sulphhydryl groups of enzymes and other macromolecules.¹⁷

Table 2: Effect of iron (III) on the antifungal activity of plumbagin against *Colletotrichum gloeosporioides*

Treatment	Concentration mg/ml	%Germination
Plumbagin	0.05	37
	0.10	7
FeCl ₃ .6H ₂ O	0.075	70
	0.15	16
Plumbagin+FeCl ₃ +6H ₂ O	0.05+0.075(1:1)	57
	0.10+0.15 (1:1)	62
	0.1+0.075(2:1)	32
	0.05+0.038(2:1)	38
Control (2% glucose in 10% ethanol)		72

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