

Fungicidal compounds from a marine Ascidian-associated fungus *Trichoderma harzianum*

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Abstract

A marine Ascidian-associated fungus, *Trichoderma harzianum*, (NIO/BCC2000-51) was assessed for its *in vitro* antagonistic activity against ten fungal phytopathogens and three food-infesting fungi. Fractionation of the cell-free culture filtrate of *T. harzianum* (NIO/BCC2000-51) showed its ethyl acetate (EA) fraction to be significantly active against fungal phytopathogens; *Macrophomina* sp, *Pyricularia oryzae*, *Sclerotium rolfsii*, *Penicillium piceum*, *Trichoderma koningii*, *T. longibrachiatum* and a food-infesting fungal pathogen *Penicillium griseofulvum*. Bioassay guided chemical investigation of the ethyl acetate fraction yielded five compounds (**1-5**); two α -pyrone derivatives, a phenol, a sesquiterpene and an unsaturated hydroxy fatty acid as methyl ester. Of the five isolated secondary metabolites, compounds **2** and **5** are being reported here for the first time from *T. harzianum*. Compounds **1** and **4** inhibited the growth of *Sclerotium rolfsii* causing sclerotium wilt or rot disease in tropical plants. Compound **2** and **5** were significantly effective in inhibiting the growth of *Macrophomina* sp. causing charcoal rot in cereals. Compound **3** showed moderate activity against plant pathogen *Penicillium piceum* and food pathogen *P. griseofulvum*. *T. harzianum* of the present investigation did not show antagonism towards all the three *Aspergillus* sp. tested besides being ineffective against *T. atroviride* of *Anacardium occidentale* and tomato fungus *Chalara* sp. Though compounds **1**, **3** and **4** are known metabolites of *T. harzianum*, to the best of our knowledge this is the first report of their antagonistic activity against the pathogens tested.

Key words: Marine fungi, *Trichoderma harzianum*, secondary metabolites, phytopathogens, food-infesting pathogens

1. Introduction

Marine microorganisms have evolved novel physiological and chemical capabilities to withstand harsh conditions prevailing in the marine environment. These adaptations have provided marine microbes with the ability to produce secondary metabolites not found in the terrestrial microorganisms. Marine derived fungi are potential source of biologically active and structurally diverse secondary metabolites with biotechnological applications.

Plant diseases or phytopathogens play a significant role in the destruction of agricultural resources causing tremendous loss to the economy of the region. Biological control which is the use of specific organisms that interfere with plant pathogens and pests, is a nature friendly ecological approach currently in use to combat with the problems caused by pesticides (Parani et al. 2011). *Trichoderma* spp. has received significant attention (Papavizas 1985; Elad 2000; Howell 2003; Roberts et al. 2010) and is presently marketed as biopesticides and biofertilizers due to their ability to protect plants, promote vegetative growth and control phytopathogenic agents under different agricultural conditions.

Seeking fungi suitable for biological control of soil borne pathogens, a strain of *T. harzianum*, isolated from a marine ascidian was found to show antagonistic activities against several phytopathogenic fungi and food pathogens. During the last few years several new compounds are reported from marine derived *Trichoderma* sp. however, none or very few are tested for antifungal activity. In the present investigation our focus is on the identification of active constituents of ascidian-associated fungus *T. harzianum* whose ethyl acetate fraction showed significant activity against several phytopathogens (*Macrophomia*, *Pyricularia oryzae*, *Sclerotium rolfsii*, *Penicillium piceum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*) and food infesting fungal pathogens (*Penicillium griseofulvum*). The compounds from its culture filtrate was subjected to bioassay guided isolation, HPLC purification and identification using spectroscopic techniques. The bioactivity of the crude as well as pure compounds against fungal pathogens have also been evaluated and reported.

2. Materials and methods

2.1 Isolation of fungal strains from marine invertebrate

A marine unidentified Ascidian (Fig. 1) from Kanyakumari (8°04'48"N and 77°34'12"E), South Indian coast was collected in sterile polyethene bags and transported to the laboratory in a deep freezer. At the laboratory, the sample was thawed to room temperature and rinsed using sterile sea water (SSW) to remove adherent particles and detritus material. The SSW was decanted off and the sample was first rinsed in methanol (70%) for 60-120s for surface sterilization followed by a repeated rinse with SSW. Finally, the sample was cut aseptically into small pieces (1x1 cm) and placed on Sabarauds Dextrose agar (SDA), prepared in seawater. The plate was incubated overnight at 27°C and examined everyday till visible growth of fungal hyphae was evident on the plate. After the fast growing cultures (Fig. 2) were isolated from the plates the portion of the agar was aseptically scooped off (Fig. 3) in order that the fast growing cultures do not overgrow on the plates. The plates were observed up to one month time and the slow growing ones which grew later were collected and all cultures preserved on Sabarauds Dextrose Agar (SDA) slants for enumeration and identification at Agarkar Research Institute, Pune, India. The endophytic fungal isolates (Fig. 4) were deposited at the Bioorganic Culture Centre (BCC), NIO, India [Accession code: NIO/BCC200047-54]. All the isolates were grown in small quantities (100ml) in the lab to test the cell-free culture filtrates for antifungal activity.

2.2. Antifungal assay

Thirteen fungal pathogens (10 phyto and 3 food-infesting) used in this study for screening are listed in Table 1. The 10 phytopathogens [generously provided by ICAR (Indian Council of Agriculture Research), Goa] were isolated from diseased parts (leaves, shoots, twigs) of economically important fruit trees from an orchard at Goa. The 3 food-infesting pathogens were isolated in-house from contaminated food using sterile needles, grown in mycological agar and subcultured for purification. All fungal cultures were identified at Agharkar Research Institute (ARI, Pune). Crude organic extracts were tested for antifungal activity against 13 fungal pathogens by paper disc diffusion assay as

described earlier (Devi et al. 2011). Briefly, Whatman (GF/C) filter paper discs of 6 mm diameter were prepared, sterilized for 15 minutes under 15 lb/m² pressure. About 200µg of the crude extract and 50µg of the pure compounds were loaded onto sterile paper discs and allowed to air-dry on the laminar air flow. Positive and negative control discs were also prepared using ketoconazole (25µg/disc) and ethyl acetate respectively. All the discs were placed in mycological agar plates seeded with the different fungal pathogens and incubated for 24-48 hours at room temperature (27°C). The zone of growth inhibition formed around each disc was measured in millimeters and the activity was scored using symbols expressed as inactive (-), mildly active (+), moderately active (++), significantly active (+++) and strongly active (++++). Each treatment consisted of three replicates and the experiment was repeated twice.

Small scale laboratory fermentation of fungi followed by preliminary screening for antifungal led to the selection of *T. harzianum* (NIO/BCC2000-51) for further study. Fractionation of its culture filtrate by sequential extraction with organic solvents of increasing polarity (petroleum ether, ethyl acetate, and butanol) followed by screening led us to concentrate on the ethyl acetate fraction of *T. harzianum* for chemical investigation.

2.3. Mass fermentation of *T. harzianum* and isolation of potential fungal inhibitors

Trichoderma harzianum (NIO/BCC2000-51) which was maintained on Sabarauds Dextrose Agar (SDA) slants at 4°C was inoculated into 100ml Sabarauds Dextrose Broth (SDB) medium (glucose 40g/l, peptone 10g/l) prepared in seawater. The flasks were incubated for 7 days on a shaker (100rpm) and this was used as inoculum to inoculate five, 5L Erlenmeyer flasks each containing 2L of the same sabarauds medium. The stationary culture was incubated for 45 days at 27°C. At the end of the incubation period, the culture was filtered under vacuum through Whatman No.1 filter paper to separate the mycelial mat from the fermentation medium. The filtered culture broth was extracted repeatedly with ethyl acetate, washed thrice with distilled water and the combined organic layer was dried over anhydrous sodium sulfate and later vacuum evaporated for chemical analysis. All solvents used for extraction, isolation and purification were of HPLC grade.

The crude EA extract was subjected to silica gel column chromatography and eluted using petroleum ether with increasing gradient of ethyl acetate. Fractions showing similar thin layer chromatography (TLC) profiles were combined and further purified by using high performance liquid chromatography (HPLC). The pure compounds thus obtained were subjected to Nuclear Magnetic Resonance (NMR) analysis.

2.4. General experimental procedures

TLC was performed either on silica gel (Kieselgel 60, HF₂₅₄, Merck) or on a reverse phase (KC-18F silica gel A, 0.20 mm, Whatman, Clifton, NY, USA) plates. Compounds were visualized as fluorescent spots under a dual wavelength (254 or 306 nm) UV lamp.

Separation on high performance liquid chromatography (HPLC) was performed using a gradient of methanol: water (0.1%TFA) as eluent. HPLC instrument of Thermo Supelco was used with a reverse phase C-18 column, 25cm x 4.6mm, 5 μ m; flow rate: 1.5ml.min⁻¹; UV detection at 210 and 254nm.

¹H NMR spectra were recorded on a 400MHz Bruker (Kalsruhe, Germany) instrument at 20°C in CDCl₃ or CD₃OD and chemical shifts were recorded as δ values. Signals arising from the solvent (CDCl₃ or CD₃OD) were used as internal standard (δ =7.26 or δ 3.30 ppm respectively). For the new compounds, carbon multiplicities were determined by DEPT and connectivity by 2D NMR experiments.

3. Results and Discussion

Fungicides are largely used to check fungal diseases in plants. The increasing awareness of hazards caused by synthetic fungicides, emergence of highly resistant fungal pathogens and insensitivity to existing drugs emphasized the need to look for chemicals against fungal diseases from newer sources which are more effective and ecofriendly. Marine Ascidian-associated fungi, *Trichoderma harzianum* (NIO/BCC2000-51) was selected for detailed chemical characterization after finding its crude EA extract to show significantly strong activity against fungal pathogens.

Species of genus *Trichoderma* are well documented as fungal biocontrol agents capable of growing on varying substrata (Brewer et al. 1987). Marine derived *T. harzianum* (NIO/BCC2000-51) was grown in sabarauds medium and fermented for 45 days on a shaker. Separation of the metabolites of the ethyl acetate extract (535mg) on a glass column followed by HPLC purification led to the isolation and identification of fungicides belonging to four different class of compounds (Fig. 5) which included two pyrone derivatives (**1-2**), a phenol (**3**), a sesquiterpene (**4**) and a di-unsaturated hydroxy fatty acid as its methyl ester (**5**). They were tested for activity against several fungal pathogens (Table 1) and activity demonstrated was tabulated in Table 2. The zone of growth inhibition formed around each disc, loaded with crude EA extract, pure compounds (**1-5**) and standard (ketoconazole), on some of the Petri plates are shown in Fig. 6 (A-F). Representative plate (Fig. 6A) is also shown, wherein pathogens like *Helminthosporium* sp., *A. niger* gr., *T. atroviride*, *A. oryzae*, *Chalara*, and *A. flavus* were neither sensitive to the crude EA extract or the pure compounds, while in some other cases (Fig. 6B) crude EA extract showed better activity when compared with the standard.

Compound **1** (7.0mg), identified as 6-*n*-pentyl- α -pyrone (6PAP), possessed a coconut-like aroma and had an $R_f=0.57$ when developed on normal TLC using petroleum ether: diethyl ether (4.5:5.5 v/v). It was detected at 210nm and had a retention time of 16.2 minutes when purified on HPLC using a Reverse Phase (RP) C-18 column when eluted with (60:40%) acetonitrile:water at 0 minutes gradually increasing to 100% acetonitrile after 20 minutes. Its ^1H NMR data is tabulated in Table 3 and is comparable with those reported by Benoni et al. (1990) and Cooney and Lauren, (1999). Antifungal screening of this compound showed plant pathogen, *Sclerotium rolfsii*, to be sensitive to 6PAP (**1**, Table 2) with an inhibition zone of 10-15mm (Fig 6E) at a concentration as low as $50 \mu\text{g}\cdot\text{disc}^{-1}$ corroborating with the reports of Evidente et al. (2003). *Sclerotium rolfsii* is a soil borne fungal pathogen causing sclerotium wilt or rot disease in tropic and subtropical plants where high temperature exists during the rainy season.

6PAP (**1**) was first identified by Collins and Halim from the culture broth of *T. viride* (Collins and Halim 1972). It is known to possess antifungal activity, phytotoxic properties, used as a flavoring agent (Reino et al. 2008) and is antityrosinase active (Li et al. 2007). Generally, it is stated that although 6PAP shows phytotoxic effects, the reduction in the activity after prolonged duration in the experimental conditions is mainly due to the hydroxylation of the side chains to give alcohol and finally propionic acid (Poole and Whitaker 1997). These metabolites formed, are less toxic than the parent compound and hence, represent a detoxification mechanism (Hanson 2005).

Compound **2** (1.6mg), 5-(2-pyron-6-yl) pentan-5-ol, was isolated as a colorless oily liquid and is reported here for the first time as a natural product from *Trichoderma harzianum*. It showed an R_f=0.88, when developed on RP TLC plate using 100% acetonitrile. It was detected on HPLC at 210 nm and isolated on a C-18, RP column using acetonitrile:water (40:60%) at 0 minutes followed by a gradient to 100% acetonitrile in 15 minutes. The retention time of elution of compound **2** was 5.00 minutes. Its ¹H NMR spectral data in CDCl₃ (deuterated chloroform) as shown in Fig. 7 and detailed in Table 3, was well in agreement with that reported in the literature for biotransformation studies (Cooney and Lauren 1999). The NMR experiments (COSY, HMBC, HSQC) were also recorded for confirmation of its structure. The presence of a secondary alcohol in this compound (**2**) made it suitable for the application of Mosher's method (Dale and Mosher 1973) for determining its absolute configuration which was established as *S* and is being reported here for the first time. This compound (**2**) showed (Fig 6D) moderate activity (6-10 mm growth inhibition) against plant pathogen *Pyricularia oryzae* causing rice blast disease and highly significant activity (11-15mm growth inhibition) against *Macrophomina* sp. (Fig. 6C) causing charcoal rot on cereals. Etebarian (2006), suggests fungicidal effect of metabolites produced by strains of *T. harzianum* and *T. virens* that caused inhibition of mycelia growth of 100% on *Marcophomina phaseolina*.

Compound **2** could be a biotransformation product of 6PAP as part of fungal strategy to eliminate 6PAP that would otherwise be toxic to its growth. Although, 6PAP

biotransformation by *Trichoderma* sp. has not been reported, it has been known to occur in other fungi (i.e. *Penicillium*, *Scelerotinia*, *Fusarium*, *Botrytis* (Cooney and Lauren, 1999).

Compound **3** (3.0mg), tyrosol [2-(4-hydroxyphenyl) ethanol], had an Rf=0.79 when developed on RP TLC using 100% acetonitrile. It was purified on HPLC using a C-18 RP column, using a gradient of acetonitrile:water (40:60%) at 0 minutes and gradually increasing to 100% acetonitrile after 15 minutes. The compound was detected at 210nm and eluted at 1.5 minutes. The ¹H-NMR spectral data in CDCl₃ (Table 3) was comparable with those reported by Turner and Aldridge (1983). When screened for fungicidal activity against test pathogens, it showed activity against plant pathogen *Penicillium piceum* and against food pathogen *Penicillium griseofulvum* with a growth inhibition zone of 6-10mm (Fig.6 F) for both the pathogens respectively (Table 2).

Tyrosol is a phenolic compound with antioxidant properties, also known to possess antifungal activity (Slininger et al. 2004) and quorum sensing properties (Chen et al. 2004). Recently, tyrosol has been reported to be effective in stroke therapy due to its neuroprotective effect in rats (Bu et al. 2007). Specian et al. (2012) report antibacterial activity against several human pathogens for the compound from the endophytic fungus *Diaporthe helianthi* isolated from *Luchea divarticata* but it had no effect against phytopathogenic fungi in the applied test system.

Compound **4** (25.3mg), cyclonerodiol is a sesquiterpene alcohol which showed an Rf=0.47 when developed on RP TLC plate and developed in 100% acetonitrile. Purification was carried out on HPLC using again a C-18 RP column using a gradient system of 40:60% (acetonitrile:water) during the start of the elution and then changing the gradient to 100% acetonitrile at the end of 15 minutes. The compound was detected at 210nm and the retention time was 9.7 minutes. The ¹H NMR of the compound is given in Table 3 and was comparable with those reported by Cane and Shiao (1978) and Yue et al. (2000). When screened for activity, cyclonerodiol was found to be active against plant pathogen *Sclerotium rolfsii* exhibiting an inhibition zone of 11-15mm (Fig. 6E) and mildly active against *T. longibrachiatum* and *T. koningii* (1-5mm zone)

respectively. Cyclonerodiol has also been isolated from several fungi including *Trichoderma koningi* as a plant growth regulatory active constituent (Cutler et al. 1991b). It is also reported to show antimicrobial activity towards *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Sporobolomyces salmonicolor* and *Rhodotorulla rubra* (Berg et al. 2004).

Compound **5** (4.0mg), 13-hydroxy-9(Z),11(E)-octadecadienoic acid, isolated as its methyl ester, is being reported here from *Trichoderma harzianum* for the first time (Fig. 8). It had an R_f=0.61 on a normal phase TLC when developed using petroleum ether : diethylether (4.5:5.5v/v). This compound was purified on a silica gel open glass column (40cm x 15mm) in petroleum ether with an increasing gradient of diethyl ether and detected at 230nm. Chisholm and Hopkins (1960) reported this compound from the seeds of a common terrestrial milkweed plant, *Asclepias syriaca*. Compound **5** showed significant activity (Table 2) against food pathogen *Penicillium griseofulvum* exhibiting an inhibition zone of 11-15mm (Fig. 6C) and also mildly active against plant pathogen *Macrophomina* sp. with an inhibition zone of 6-10mm (Fig. 6F). Acid corresponding to compound **5** arises by lipoxygenase catalysed oxidation of linoleic acid followed by reduction of the resulting hydroperoxide. It is known to be highly inhibitory to the germination of conidia of rice blast fungus *Magnaportha grisea* (Ohta et al. 1991).

In conclusion, the level of fungal disease and crop loss on a naturally infected field warrants efforts to screen as many extracts with a view to find a suitable solution to fungal infection. Isolation of individual components of the ethyl acetate extract of the marine ascidian-associated *T. harzianum* indicated the presence of 6-*n*-pentyl- α -pyrone (**1**), 5-(2-pyrone-6-yl) pentan-5-ol (**2**), 2-(4-hydroxyphenyl) ethanol (**3**), cyclonerodiol (**4**) and 13-hydroxy-9(Z),11(E)-octadecadienoic acid (**5**) (Fig. 5). Noticeable among them was the ability of **1** and **4** to inhibit growth of *Sclerotium rolfsii* causing sclerotium wilt or rot disease in tropical plants (Fig. 6E). Compound **2** and **5** was also significantly effective in inhibiting the growth of *Macrophomina* sp. causing charcoal rot in cereals (Fig. 6C). Compound **3** showed moderate activity against plant pathogen *P. piceum* and food pathogen *P. griseofulvum*. Fermentation for longer periods (45 days), probably

induced stress in the culture either for nutrients or toxicity due to its own byproducts and hence, can influence production of several different novel compounds. This study further shows that marine invertebrate-associated microorganism like fungi have biotechnological application in agriculture as antifungal agents.

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Captions to figures

Fig. 1. Marine Ascidian collected from Kanyakumari (South Indian coast)

Fig. 2. Fast growing cultures that developed on plates at the end of 48 h.

Fig. 3. Portion of agar aseptically scooped off to avoid fast growing cultures to overgrow on the plates.

Fig. 4. The endophytic fungal cultures isolated from Marine Ascidian.

Fig.5. Chemical structure of secondary metabolites isolated from *Trichoderma harzianum*. **1**, [6-*n*-pentyl- α -pyrone]; **2**, [5-(2-pyrone-6-yl)pentan-5-ol]; **3**, [2-(hydroxyphenyl) ethanol]; **4**, [cyclonerodiol]; **5**, [13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid methyl ester].

Fig. 6. Growth inhibition formed around discs loaded with crude EA extract, pure compounds (**1-5**) and standard (Ketoconazole).

Fig.7. ¹H NMR spectra of Compound 2

Fig.8. ¹H NMR spectra of Compound 5



Fig. 1 Marine Ascidian



Fig. 2 Fast growing cultures

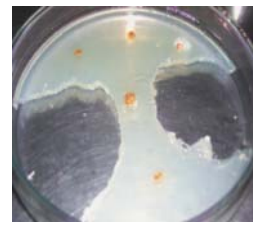


Fig. 3 Agar aseptically scooped off

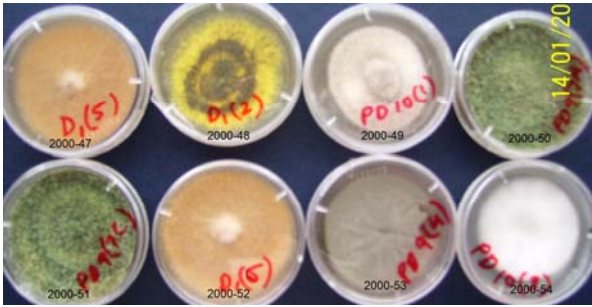


Fig. 4. Endophytic fungal cultures isolated from Ascidian

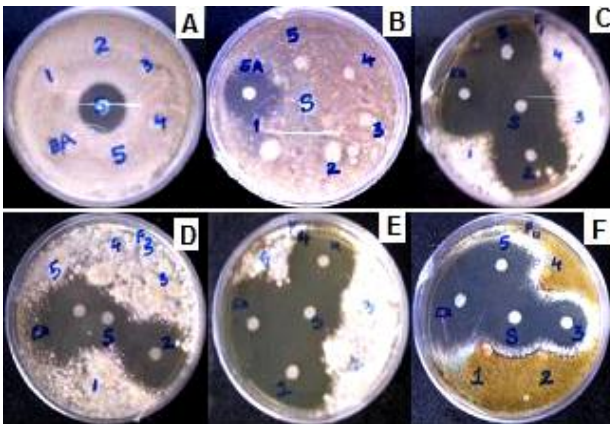


Fig. 6 Growth inhibition zones around discs loaded with crude EA extract, pure compounds (1-5) and standard Ketoconazole (s).

Fig. 5.

Fig.5. Chemical structure of secondary metabolites isolated from *Trichoderma harzianum*. **1**, [6-*n*-pentyl- α -pyrone]; **2**, [5-(2-pyron-6-yl)pentan-5-ol]; **3**, [2-(hydroxyphenyl) ethanol]; **4**, [cyclonerodiol]; **5**, [13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid methyl ester].

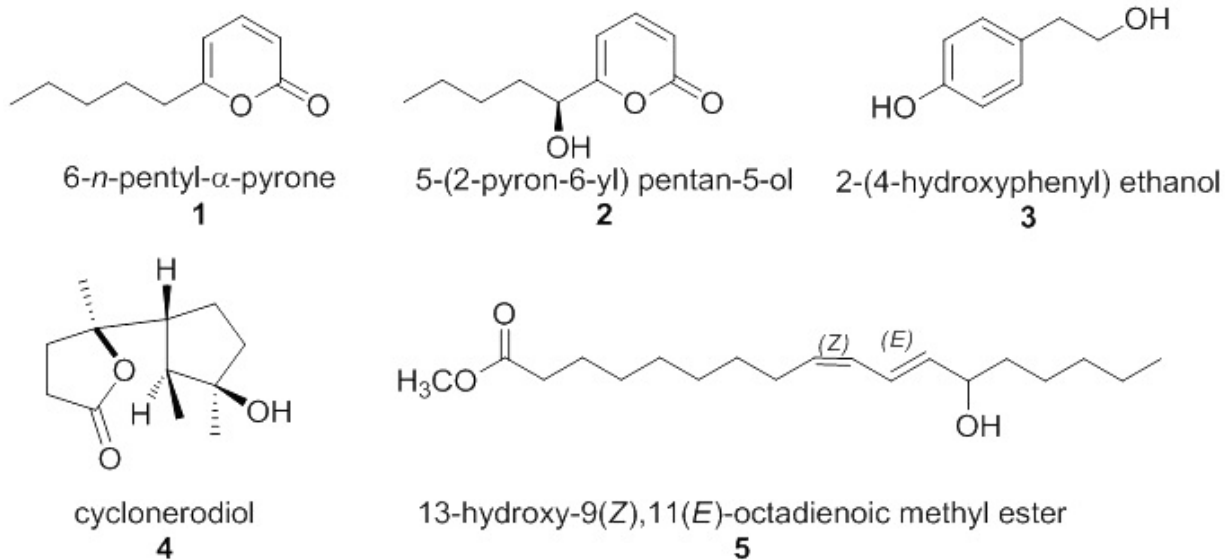


Fig.7. ^1H NMR spectra of Compound 2

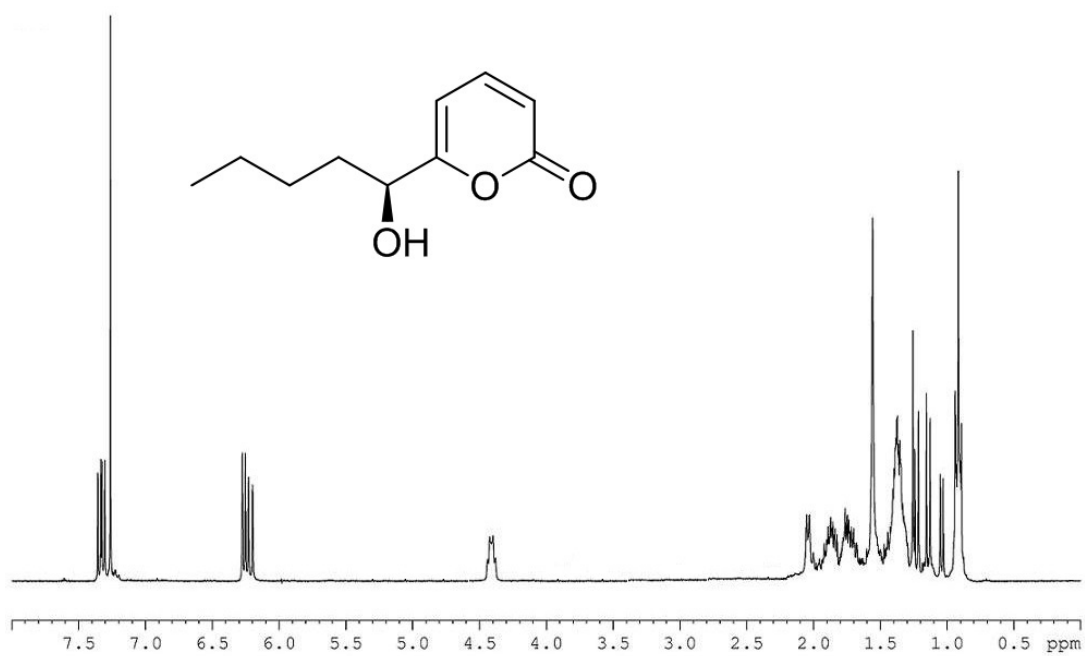


Fig.8. ^1H NMR spectra of Compound 5

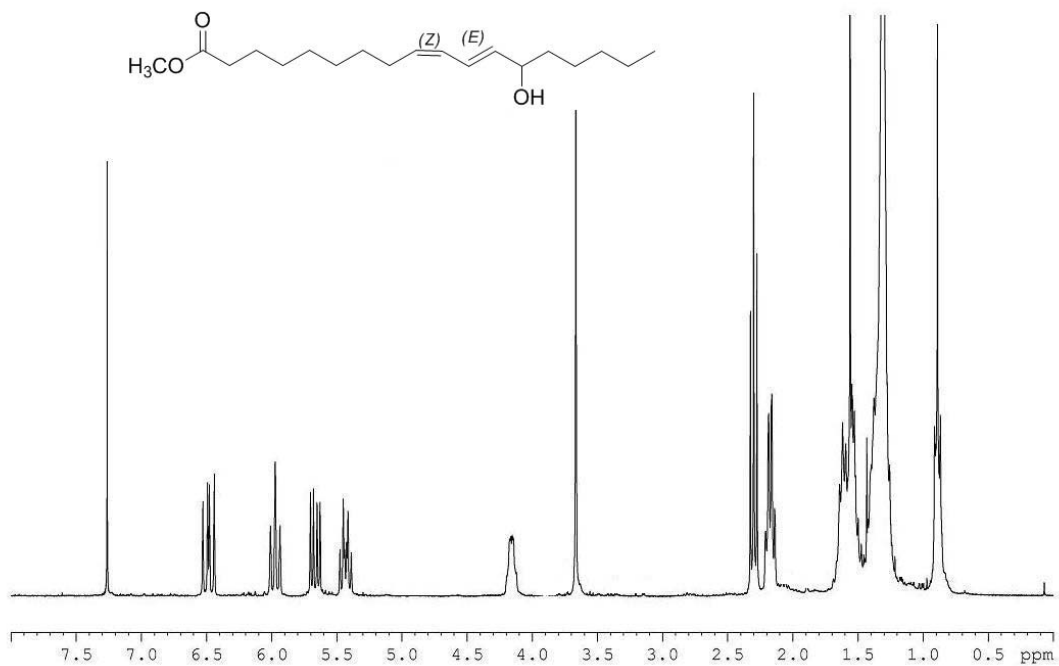


Table 1. List of fungal pathogens used in antifungal screening

Sr. No.	Fungal Pathogens	Source	Accession no.
	Plant pathogens		
1.	<i>Macrophomina</i> sp.	<i>Psidium guajava</i>	BCC-PP001
2.	<i>Helminthosporium</i> sp.	<i>Zea mays</i>	BCC-PP002
3.	<i>Pyricularia oryzae</i>	<i>Oryza sativa</i>	BCC-PP003
4.	<i>Sclerotium rolfsii</i>	<i>Solanum tuberosum</i>	BCC-PP004
5.	<i>Aspergillus niger</i> gr.	<i>Archus Sapodilla</i>	BCC-PP005
6.	<i>Penicillium piceum</i>	<i>Archus Sapodilla</i>	BCC-PP007
7.	<i>Trichoderma koningii</i>	<i>Mangifera indica</i>	BCC-PP009
8.	<i>Trichoderma longibrachiatum</i>	<i>Mangifera indica</i>	BCC-PP010
9.	<i>Trichoderma atroviride</i>	<i>Anacardium occidentale</i>	BCC-PP011
10.	<i>Aspergillus oryzae</i>	<i>Triticum aestivum</i>	BCC-PP014
	Food pathogens		
1.	<i>Penicillium griseofulvum</i>	Indian Bread	BCC-FP001
2.	<i>Chalara</i> sp.	<i>Solanum lycopersicum</i>	BCC-FP002
3.	<i>Aspergillus flavus</i>	<i>Daucus carota</i>	BCC-FP003

Table 2. Antifungal activity of crude Ethyl acetate (EA) fraction and pure compounds of *T. harzianum* against phyto and food pathogens

Test Fungi	Compounds						Std
	EA-Frac	1	2	3	4	5	
<i>Macrophomina</i> sp.	++++	-	+++	-	-	++	+++
<i>Helminthosporium</i> sp.	-	-	-	-	-	-	++
<i>Pyricularia oryzae</i> .	+++	-	++	-	-	-	++
<i>Sclerotium rolfsii</i>	++++	+++	-	-	+++	-	+++
<i>Aspergillus niger</i> gr	-	-	-	-	-	-	+++
<i>Penicillium piceum</i>	+++	-	-	++	-	-	-
<i>Trichoderma koningii</i>	++	-	-	-	+	-	-
<i>Trichoderma longibrachiatum</i>	++	-	-	-	+	-	+++
<i>Trichoderma atroviride</i>	-	-	-	-	-	-	+++
<i>Aspergillus oryzae</i>	-	-	-	-	-	-	++++
Food pathogens							
<i>Penicillium griseofulvum</i>	+++	-	-	++	-	+++	++++
<i>Chalara</i> sp.	-	-	-	-	-	-	++++
<i>Aspergillus flavus</i>	-	-	-	-	-	-	++

- = No activity, + = 1-5mm zone of inhibition (mild activity), ++ = 6-10 mm zone of inhibition (moderate activity), +++ = 11-15 mm zone of inhibition (good activity), ++++ = 16-25 mm zone of inhibition (strong activity)

Table 3. ¹H-NMR assignments for compounds **1-5**

Carbon	¹ H NMR (ppm, <i>J</i> in Hz) Comp 1	¹ H NMR (ppm, <i>J</i> in Hz) Comp 2	¹ H NMR (ppm, <i>J</i> in Hz) Comp 3	¹ H NMR (ppm, <i>J</i> in Hz) Comp 4	¹ H NMR (ppm, <i>J</i> in Hz) Comp 5
1				0.91(d,6.6)	
2			7.01(t)	1.45 (m)	2.3(t)
3	6.16 (d, 9.4)	6.2 (d,9.4)	7.01(t)		1.6 (m)
4	7.25 (m,9.4,6.5)	7.32 (1H,dd,9.4,6.6)		1.39-1.51(m)	1.51 (m)
5	5.98 (d, 6.5)	6.26 (1H, d, 6.6)	6.70 (t)	1.39-1.51(m)	1.51 (m)
6			6.70 (t)	1.98 (m)	1.51 (m)
7					2.18 (m)
8				1.40 (m)	8.4 (m)
9				1.90-2.01 (m)	5.98 (t)
10				5.06 (t,8.0)	6.5 (m)
11					5.66 (m)
12				1.62 (s)	4.13 (bm)
13				1.12 (s)	
14				1.09 (s)	
15				1.55 (s)	
13-17					1.35
18					0.9 (t)
1'	2.5 (t, 7.3)	4.4 (1H,dd,7.8,7.7)	2.77 (t)		
2'a 2'b	1.68 (t)	1.85(1H,m) 1.74(1H,m)	3.72(t)		
3'	1.32 (m)	1.25-1.48 (d)			
4'	1.32 (m)	1.3-1.5(w)			
5'	0.90 (t, 3H)	0.91(3H,t,7.0)			
OH			2.9 (brs)		
3 OH				3.1-3.25 (bs)	
7 OH				3.1-3.25 (bs)	
OCH ₃					3.68

^a in CDCl₃, ^b in CD₃OD

Data are reported as chemical shifts (δ), multiplicity (s=singlet, br=broad, d=doublet, t=triplet, m=multiplet), coupling constant (*J* in Hz).