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A Study On The Antimicrobial Activity Of New Substituted 1,3,4-Oxadiazole Analogues

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Research Article

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ABSTRACT

Substituted 1,3,4-oxadiazole derivatives such as 2-(chloromethyl)-5-heptadecyl-1,3,4-oxadiazole (4a), 2-benzyl-5-heptadecyl-1,3,4-oxadiazole (4b), 2-heptadecyl-5-phenyl-1,3,4-oxadiazole (4c), 2-heptadecyl-5-styryl-1,3,4-oxadiazole (4d), 2-(5-heptadecyl-1,3,4-oxadiazol-2-yl)phenol (4e), 2-heptadecyl-5-o-tolyl-1,3,4-oxadiazole (4f), 2-heptadecyl-5-(4-nitrophenyl)-1,3,4-oxadiazole (4g) and 4-(5-heptadecyl-1,3,4-oxadiazol-2-yl)benzenamine (4h) were subjected to antibacterial and antifungal activity assay by cup diffusion and poisoned food technique against *Escherichia coli*, *Staphylococcus aureus* and three strains of seed borne toxigenic *Fusarium verticilloides* isolated from maize (*Zea mays* L.) and paddy (*Oryza sativa* L.). *F. verticilloides* was confirmed by species specific primers VERT 1 and VERT 2 and fumonisin producing ability was confirmed by VERTF-1 and VERTF-2 using polymerase chain reaction. All the compounds were tested at different concentration (25 to 100 ppm). The results revealed that all eight oxadiazoles completely inhibited all the strains of toxigenic *F. verticilloides* at 100 ppm indicating Minimal Inhibitory Concentration (MIC). 4b recorded highly significant antibacterial activity against *E. coli* where as it was 4f against *Staph. aureus*. The study is successful in reporting the antimicrobial activity of the oxadiazoles and its possible application in management of seed borne fumonisin producing fungi and also bacterial infection in human.

INTRODUCTION

India is an agriculture based country where in agriculture continues to play a dominant role and it contributes about 26% Gross Domestic Product (GDP). Yield of any crop depends on the quality of seeds. Seed is the most important input for increased crop production which is need of the hour. Pathogen free healthy seed is urgently needed for desired plant populations and good harvest because several phytopathogens are seed-borne which can cause enormous crop losses[1]. In this connection seed health, the productivity of the crop, management practices and supplementary inputs exert profound influences. Contamination of Rice (*Oryza sativa* L.), Sorghum (*Sorghum vulgare* Pers.) and Maize (*Zea mays* L.) grains during storage by fungi is a major concern[2]. These fungi are not only involved in biodeterioration of grains but some are also known to produce mycotoxins which cause obvious reduction in crop, animal live stock production and diseases in humans. However, the serious problem with storage of seeds is that, it is highly susceptible to many fungi associated with grain mould disease such as species of *Fusarium*, *Aspergillus*, *Drechslera*, *Rhizopus*, *Pencillium*, *Alternaria*, *Curvularia*, and *Cladosporium*[3].

Fusarium verticillioides (Sacc) Nirenberg is a fungal pathogen with a wide range of plant hosts such as maize, sorghum, rice etc., along with plant diseases. *F. verticillioides* infection also results in food safety problems for humans and animals as the fungus produces mycotoxins which cannot easily be removed or detoxified from grains. Currently, fumonisins are considered as the most agriculturally significant environmental toxins produced by *F. verticillioides* and other *Fusarium* species that grow in grains during storage[4].

Fumonisin producing *F. verticillioides* was identified based on micromorphological characteristics and PCR using two sets of primers. One set of primers is *F. verticillioides* species specific, which selectively amplify the intergenic space region of rDNA. The other set of primers is specific to fumonisin producing *F. verticillioides*[4]. Forty three maize samples collected from different regions

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of Karnataka and Andhra Pradesh, were subjected for detection of *F. verticillioides*. Identification of *F. verticillioides* was done based on morphological characters and further confirmed by PCR.

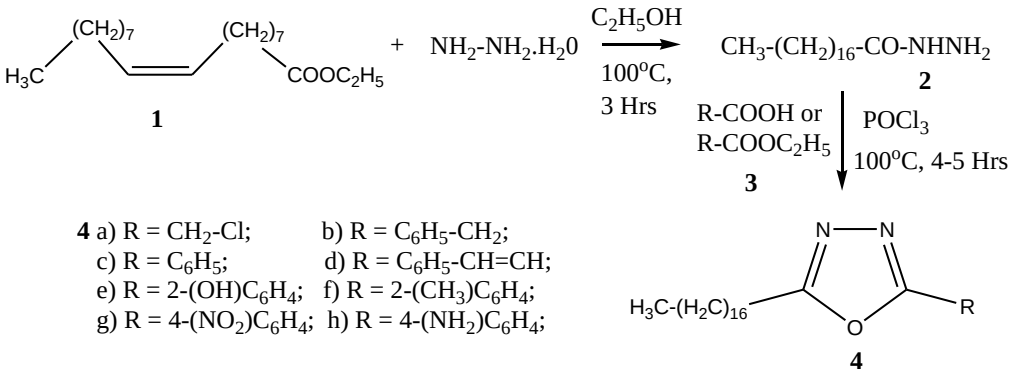
Human body requires fats to function properly; fatty acids serve as the components of more complex membrane lipids and as major components of stored fat in the body. Chemically, fatty acids and their esters are used in the synthesis of oxadiazoles, the utility of these adducts for structural investigations of natural and artificial fatty acids are now well established. Five membered nitrogen heterocycles have been successfully tested against several diseases and therefore received special attention in pharmaceutical chemistry due to their diverse medicinal potential.

Among the heterocycles; oxadiazoles continuously draws interest for development of newer drug moiety. They have demonstrated a broad spectrum of biological properties in both pharmaceutical and agrochemical fields. Substituted 1,3,4-oxadiazole derivatives have known to exhibit enormous biological activities such as CNS depressant, anti-inflammatory activity, as muscle relaxant, anticonvulsant, antitumour, herbicidal and HIV properties. They also show a remarkable analgesic, anti-convulsant, diuretic, hypnotic and sedative properties[5]. They also known to exhibit promising antimicrobial activities[6]. Prompted by the varied applications of 1,3,4-oxadiazoles, the present study has been undertaken to prove its efficacy against seed borne toxigenic fungi and human pathogenic *E. coli* and *Staph. aureus*.

MATERIALS AND METHODS

1,3,4-Oxadiazoles

Stearic acid hydrazide (**2**) was prepared by the reaction of ethyl oleate and hydrazine hydrate in absolute alcohol under reflux conditions. Then a mixture of hydrazide (**2**), suitable aliphatic or aromatic acids or ethyl oleate (**3**) and phosphorous oxychloride were refluxed on water bath for 4-5 hrs. After the completion of the reaction; the mixture was poured into ice cold water, and was neutralized with sodium bicarbonate solution. The resulting 1,3,4-oxadiazoles (**4**) were crystallized from 80% ethyl alcohol (Scheme-1)[7].



Scheme 1

Collection and screening of seed samples for *Fusarium verticillioides* incidence

Seed samples via, 15 samples of paddy (Sonam, Mili long, Mandya vijaya, Cross jaya, B1R, 64, 1001, Tanu, Bangla Rice, B2R, All dode, IR-64, Raja muddi, TN, BR1), six samples of Maize (Kaveri, All rounder) and five samples of sorghum (CHS-5 hybrids) were collected from in and around Mysore. The seed samples were subjected to surface sterilization with 2% sodium hypochlorite and analysed for *Fusarium verticillioides* incidence by Standard Blotter Method (SBM). Ten seeds of maize and 25 seeds of paddy and sorghum per plate were subjected to SBM and incubated for 7 days at 24±2°C under 12 hrs alternating cycles of light and darkness. Fungal growth after incubation was examined under stereo and binocular microscope. Habit and morphological characters of conidia were observed under microscope. *F. verticillioides* were isolated and pure cultured on Czapex Dox Agar (CDA) slants and maintained at 4°C for further use.

Molecular detection of *F. Verticillioides*

DNA extraction

Two week old cultures of the mycelial mat of F. verticillioides (100 mg) were ground in liquid nitrogen. The total genomic DNA was isolated using DNeasy plant Minikit (Qiagen, Germany) as per manufacture's instruction. DNA stock solution was diluted with nuclease free water (Qiagen, Germany) and the concentration was estimated at 260/280 nm (Hitachi, Japan). Fungal DNA was stored at – 80°C until further use.

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PCR amplification

Species specific primers VERT1 (5-GTCAGAATCCATGCCAGAACG-3) and VERT2 (5-CACCCGCAGCAATCCTCAG-3) was used in the present study to detect *F. verticillioides*. Using as reference the IGS sequence of isolate A0999 (EMBL accession no. AJ575185). Primer VERT-1 was reported within the 28S rDNA, 256 bp upstream of the IGS region and VERT-2 was located at position +501. VERTF1 (5-GCGGGAATTCAAAAGTGGCC-3) and VERTF2 (5-GAGGGCGCGAAACGGATCGG-3) located at positions +642 and +1014 helped to differentiate toxigenic and non-toxigenic *F. verticillioides*.

Test fungi

The seed samples were subjected to Standard Blotter Method[8] and incubated in alternative cycles of dark and light. On the seventh day of incubation, samples were screened for seed mycoflora and the associated fungi were identified based on growth characteristic, mycelial morphology of *Fusarium verticillioides*, which were frequently associated in higher percentage in maize. The cultures were maintained on CDA medium by frequent subculturing. Human pathogenic *Escherichia coli* and *Staphylococcus aureus* was obtained from the Government Medical College, Mysore, Karnataka which served as test pathogen.

Antifungal activity

Different concentrations (10ppm to 100ppm) of oxadiazoles 4a, 4b, 4c, 4d, 4e, 4f, 4g and 4h was prepared with CDA medium. 15ml of the medium was poured onto each Petri plate and allowed to solidify. Five mm disc of 7-day-old culture of the test fungi were placed at the center of the Petri plates and incubated at 25±2 ºC for seven days. After incubation the colony diameter was measured in millimeters. For each treatment triplicates were maintained. CDA medium without the aqueous extract served as control. The fungitoxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the formula;

$$\% \text{ inhibition} = dc - dt/dc \times 100,$$

Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment (Singh and Tripathi, 1999).

Antibacterial activity

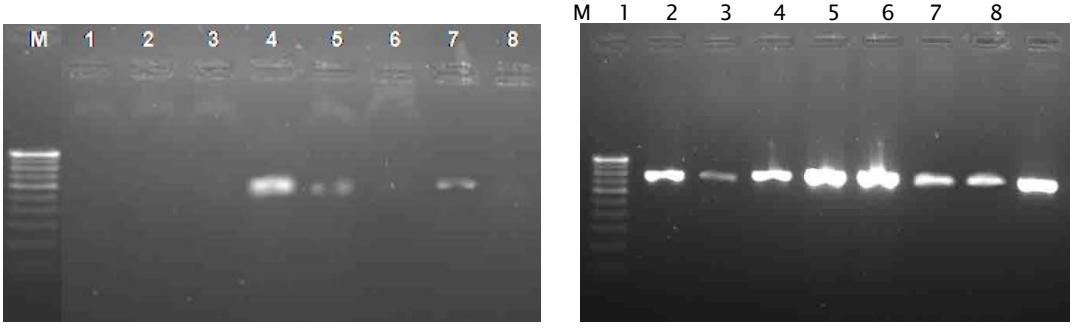
All the derivatives at concentration of 100ppm were subjected to cup diffusion method against *Escherichia coli* and *Staphylococcus aureus.* Cups were made in nutrient agar plate using cork borer (5 *mm*) and inoculums containing 10^6 CFU/ml of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension. Then 50µl of all the derivatives was placed separately in the cups of inoculated plates, the treatments also included 50µl of sterilized distilled water, which served as control. All the plates were incubated for 24 h at 37°C and zone of inhibition if any around the wells was measured in *mm* (millimeter).

RESULTS

DNA extraction and PCR amplification of *F.verticillioides*

DNA from *F. verticillioides* isolated from different Paddy and maize samples were subjected to PCR using the primer pair VERT-1 and VERT-2. A 800 bp (Fig-1) single fragment was amplified for all the cases. DNA from other *Fusarium* species viz., *F. oxysporum, F. chlamydosporum, F. thapsin,* and *F. proliferatum* did not record amplification during PCR assay.

Figure 1: PCR amplification using primers VERTF1/VERTF2 and DNA from *F. verticillioides*



Fumonisin ***F. verticillioides***

VERTF-1 and VERTF-2 primers differentiated non toxigenic and toxigenic *Fusarium verticillioides* and helped to differentiate it from other *Fusarium* species.

Antifungal activity

The percent inhibition for substituted 1,3,4-oxadiazole derivatives (**4a-4h**) at 25 to 100ppm recorded complete inhibition of mycelial growth of all the strains of *F. verticillioides* (Table-1). All the compounds recorded significant increase in antifungal activity with increase in concentration from 25 to 100 ppm. **4a** recorded complete inhibition of the mycelia growth of all the strains at 100 ppm concentration indicating MIC value. Even though inhibitory activity was varied among strains in 25 to 75 ppm concentration, all the strains were found completely susceptible at 100 ppm concentration.

Table 1: Antifungal activity of 1,3,4-Oxadiazoles against test fungi

Sl No.	1,3,4-Oxadiazoles	Test fungi	Percent inhibition of mycelial growth at different concentration in ppm			
			25	50	75	100
1	4a	*F.v.* 1	62.01±2.34	74.21±9.23	90.43±4.25	100±0.00
		F.v. 2	59.00±8.42	75.63±5.96	92.34±2.21	100±0.00
		F.v. 3	57.47±7.61	68.04±7.36	89.84±5.01	100±0.00
2	4b	*F.v.* 1	59.36±3.04	69.71±1.31	84.54±3.72	100±0.00
		F.v. 2	72.96±6.24	82.34±3.52	91.75±9.12	100±0.00
		F.v. 3	63.42±7.51	75.72±6.04	90.85±8.2	100±0.00
3	4c	*F.v.* 1	74.51±1.01	81.62±5.22	92.47±7.83	100±0.00
		F.v. 2	62.86±2.85	71.05±6.14	89.43±2.40	100±0.00
		F.v. 3	55.49±3.92	64.62±7.24	93.66±1.05	100±0.00
4	4d	*F.v.* 1	65.32±5.04	79.36±7.04	94.13±5.40	100±0.00
		F.v. 2	73.84±2.35	81.16±3.21	90.57±2.21	100±0.00
		F.v. 3	77.57±4.25	89.46±6.32	93.63±2.34	100±0.00
5	4e	*F.v.* 1	68.46±2.34	82.31±0.08	90.04±4.25	100±0.00
		F.v. 2	57.35±8.42	81.25±0.02	88.79±2.21	100±0.00
		F.v. 3	64.31±7.61	80.76±0.25	89.96±5.01	100±0.00
6	4f	*F.v.* 1	67.31±4.43	75.15±5.34	91.35±3.45	100±0.00
		F.v. 2	69.25±6.21	81.54±2.36	92.21±4.58	100±0.00
		F.v. 3	71.22±2.04	83.44±5.76	96.34±2.36	100±0.00
7	4g	*F.v.* 1	57.21±3.95	71.25±3.21	89.43±2.42	100±0.00
		F.v. 2	61.39±7.11	79.54±5.62	89.84±5.01	100±0.00
		F.v. 3	65.37±5.79	82.36±2.10	94.29±5.54	100±0.00
8	4h	*F.v.* 1	59.34±5.76	65.21±2.13	90.32±2.21	100±0.00
		F.v. 2	67.19±3.54	75.32±2.61	92.54±4.48	100±0.00
		F.v. 3	64.47±8.31	77.32±5.73	94.55±5.01	100±0.00

Results are means of four replicates ± Standard Error

At 25 and 50 ppm concentration F.v. 3 was found highly susceptible to compound **4d**, whereas at 75 ppm it as found susceptible to compound **4f**. It was interesting to note that at 100 ppm concentration all the strains were completed inhibited by all the derivatives tested in the present study. It was also noted that the concentration 100 ppm is the Minimal Inhibitory Concentration for all the derivatives.

Comparative evaluation of derivatives with fungicides generally employed for the seed treatment such as azoxystrobilurin, pyraclostrobin and kreoxim methyl revealed that the complete inhibition of the mycelial growth was observed at 2000 ppm concentration (Table-2). The inhibitory activity of all the derivatives were highly significant compared to all the fungicides tested at 500 and 1000 ppm concentration indicating the derivatives were highly effective even at low concentration compared to the fungicides.

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Table 2: Antifungal activity different fungicides at different concentration

Fungicides	Test fungi	Percentage of inhibition at different concentration of fungicides (g L^{-1})		
		2000 ppm	1000 ppm	500 ppm
Azoxystrobilurin	*F. verticillioides*	100±0.00	51.21±0.29	21.76±0.15
Pyraclostrobin	*F. verticillioides*	100±0.00	60.61±0.43	35.29±0.25
Kreoxim methyl	*F. verticillioides*	100±0.00	59.18±0.52	28.23±0.25

Antibacterial activity

Inhibitory activity of all the derivatives against *E. coli* and *S. aureus* were presented in table-3. *E. coli* was found to be slightly resistant to **4d** but was found highly susceptible to **4b**, the inhibitory activity varied among other derivatives. *S. aureus* was highly sensitive to compound **4f** and least for **4e**. Comparative evaluation of inhibitory activity of all the derivatives with Gentamicin and Streptomycin revealed that, against *E. coli* all derivatives recorded highly significant inhibitory activity compared to gentamicin except compounds **4d** and **4e**. Whereas against *S. aureus* both the antibiotics were highly effective compared to the derivatives tested in the present study.

Table 3: Zone of inhibition (mm) of 1,3,4-Oxadiazoles at 10 µg/ml against *E. coli* and *S. Aureus*

Sl. No.	1,3,4-Oxadiazoles	*E. coli*	*S. aureus*
1	4a	20±0.12	17±0.11
2	4b	22±0.15	19±0.13
3	4c	15±0.12	17±0.19
4	4d	10±0.23	15±0.11
5	4e	12±0.15	11±0.15
6	4f	20±0.09	20±0.05
7	4g	20±0.05	14±0.22
8	4h	16±0.15	15±0.13
9	Gentamicin	14.62±0.10	22.37±0.10
10	Streptomycin	10.62±0.10	22.75±0.15

Results are means of four replicates ± Standard Error

CONCLUSION

The present study employed the proper detection of the toxigenic *F. verticillioides*. In this connection the strains of *F. verticillioides* isolated from paddy, sorghum and maize were initially identified using stereo and binocular microscope. Synthesised oxadiazole derivatives tested in the present study were found highly effective even compared to generally employed fungicides to mange *Fusarium* contamination in seeds. It is interesting to note that MIC 100 ppm for all 1,3,4-oxadiazole derivatives draw the attention for further study related to development of seed treatment strategies to manage toxigenic fungi in particular and fungi associated with seed biodeteriation. Furher all the derivatives are highly effective in significantly inhibiting *E. coli* even compared to gentamicin antibiotic. Further study on its usage is subject to toxicological studies. As these derivatives have already proven biological activities, it is necessary further studies on these derivatives as a seed protectant will solve the problem of pesticide toxicity, as these are highly effective at concentration far less than the already employed fungicides.

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