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Antifungal activities of novel synthetic compounds against *Phomopsis azadirachtae*- the causative agent of die-back disease of neem

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ABSTRACT

Seven novel synthetic heterocyclic compounds containing naphthofurans were evaluated for their inhibitory effect on Phomopsis azadirachtae, the causative agent of destructive die-back disease of neem. Twigs of Azadirachta indica (Neem) infected with die-back were collected and were analyzed to determine the pathogen. Phomopsis azadirachtae the causal organism was isolated on malt extract agar from die-back infected neem twigs. They were identified by PCR based molecular methods. Phomopsis genus specific primers (5.8S r-DNA) were then used for the confirmation of P. azadirachtae – the causative agent of die-back of neem by Polymerase chain reaction (PCR). Studies revealed the amplification of expected 141bp DNA in P. azadirachtae isolated from the diseased trees confirming the causal organism of die-back of neem. Studies revealed a very effective in vitro control of P.azadirachtae mycelia growth at very significant concentration.

Key words: *Phomopsis azadirachtae*, *Azadirachta indica*, naphthofurans, die-back of neem, Polymerase chain reaction.

INTRODUCTION

Neem (*Azadirachta indica*) commonly called 'Indian Lilac' is one of the most versatile, multifarious trees of tropics, with immense potential to protect the environment while developing sustainable agriculture [1]. Neem tree is unique among the plant kingdom in terms of its heritage, myriads of chemical entities present in its various parts and economic significance to the mankind. Neem makes an important member among forest trees. More than 150 compounds have been isolated from different parts of this enigmatic tree [2,3]. Intensive search during the past decade for a safer insecticide has resulted in identification of neem as a better alternative to toxic pesticides due to its biodegradability, relatively low toxicity and abundance. Neem-based pest control has a natural advantage in the race to help farmers demonstrate that their production processes are clean and green [4]. It has been reported that more than 350 species of arthropods, 12 species of nematodes, 15 species of fungi are affected by neem [3,5]. The important quality of neem is that it has little or no toxicity to warm blooded animals including birds and human beings. Neem the ecofriendly native tree is now under great threat due to a destructive die-back disease [6]. The disease is not outright killer of the tree but very devastating in nature. The causal organism of the disease is a deuteromycetes fungus called '*Phomopsis azadirachtae*'. Die-back disease affects leaves, twigs and the inflorescence of neem trees of all ages and sizes [7]. It causes almost always 100% loss of fruit production in severely infected trees. This results in total loss of the seeds used for the extraction of several pesticidal active ingredients by the industries. The disease is spreading very rampantly in different parts of India [8]. Forest trees are effectively controlled by fungicidal applications, which is one of the effective means of disease control [9]. A ray of fungicides, especially systemic ones are known to suppress many fungal pathogens [10]. Fungicidal applications have effectively managed many die-back diseases [11]. Many plant diseases caused by Phomopsis spp. and other fungi have been controlled by chemicals including synthetic compounds [12, 13, 18]. Therefore in the present study, we have tested the efficacy of a few novel synthetic heterocyclic compounds against *P. azadirachtae*.

EXPERIMENTAL SECTION

Isolation of pathogen:

The healthy and die-back affected neem twigs were collected from diseased trees at Sri Jayachamarajendra College of Engineering (SJCE) campus, Mysore and were bought to laboratory. Both healthy and diseased twigs (with middle transition zone) were cut into 2-3 cms and washed with running tap water for an hour. Further they were trimmed to short segments of 1-1.5 cms, the diseased twigs having transition zone at centre. Segments were surface sterilized with 4% Sodium hypochlorite for 5 min. and rinsed 6-8 times in sterile distilled water. Segments were plated on MEA amended with 100 ppm chloramphenicol and the inoculated plates were incubated for 7 days at 26 ± 2^{o}C with 12 h photoperiod.

PCR-based molecular detection of the pathogen:

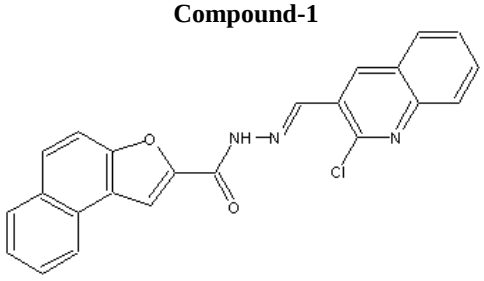
Nucleic acid (DNA) preparations were made from the pathogen isolates obtained from the diseased neem twigs of trees at SJCE campus, Mysore, separately, by following the procedures of [15] with slight modification[16]. Primers of 5.8S r-DNA of *Phomopsis* with conserved sequences of forward and reverse primers of 141bp DNA was used [16, 17]. DNA was also

isolated from *Fusarium verticillioides* and used as control. PCR was performed using Advanced Thermus 25 thermocycler.

Chemical Synthesis of compounds:

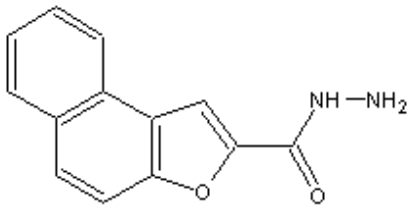
Naphthofurans possess a broad range of biological activities that are constituents of important natural products. These compounds from plant origin have been used for traditional medicines [14a]. Naphthofurans alone or coupled with nitrogen heterocycles do not occur in nature. Several synthetic compounds bearing this ring skeleton are associated with diverse biological activities such as antifungal, antibacterial, antiviral, antitumor and antihelminthic[14b]. The selected naphthofurans are synthesized and characterized as reported earlier [14c]. (The seven chemicals studied are shown in **Fig 1**).

Compound-1



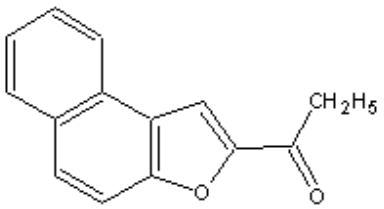
N-[(1*E*)-(2-chloroquinolin-3-yl)methylene]naphtho[2,1-*b*]furan-2-carbohydrazide

Compound-2



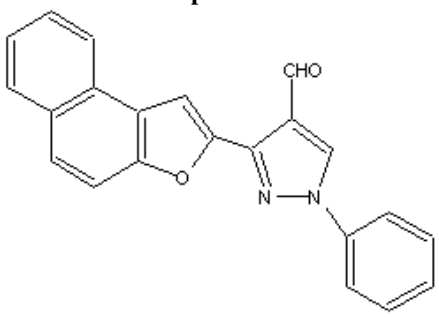
naphtho[2,1-*b*]furan-2-carbohydrazide

Compound-3



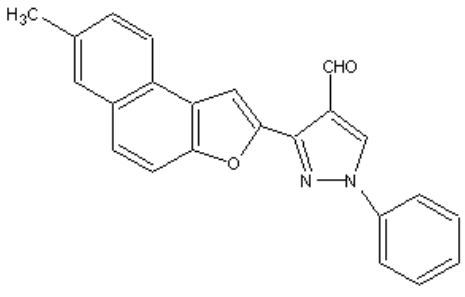
1-naphtho[2,1-*b*]furan-2-ylethanone

Compound-4



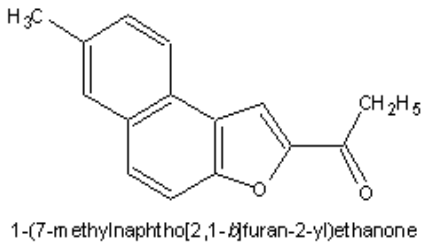
3-naphtho[2,1-*b*]furan-2-yl-1-phenyl-1*H*-pyrazole-4-carbaldehyde

Compound-5



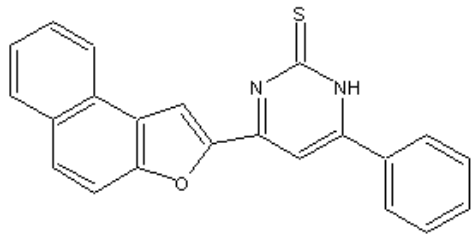
3-(7-methylnaphtho[2,1-*b*]furan-2-yl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde

Compound-6



1-(7-methylnaphtho[2,1-*b*]furan-2-yl)ethanone

Compound-7



4-naphtho[2,1-*b*]furan-2-yl-6-phenylpyrimidine-2(1 *H*)-thione

Fig 1: Structures of the synthesized compounds tested for antifungal activity

***In-vitro* antifungal activity of novel synthesized compounds against Phomopsis azadirachtae:**

Synthesized compounds containing naphthofurans were evaluated for their inhibitory effect on the mycelial growth of the *P. azadirachtae*. This was done by growing the fungus on MEA medium supplemented with the various concentrations of the chemicals by food poisoning technique [18]. The compounds were dissolved in DMSO (dimethyl sulphoxide) and incorporated in to the malt extract medium at different concentrations, i.e., 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm, 80 ppm, 90 ppm and 100 ppm. Medium without chemical and only with emulsifier served as control. The media were poured on to the sterile Petri plates (90 mm), and inoculated with mycelial discs from seven-day-old culture of *P. azadirachtae*. Plates were incubated for seven days at 26 ± 2^{o}C with 12 h photoperiod. All the treatments had four replications and the experiment was repeated thrice. Mean colony diameter was found out by measuring linear growth in three directions at right angles. The colony diameter was compared with the control as a measure of fungitoxicity. The per cent mycelial growth inhibition (PI) with respect to the control was computed from the formula

$$PI = \frac{(C-T)}{C} \times 100$$

RESULTS AND DISCUSSION

The pathogen was isolated from all the diseased neem twigs. The expected 141bp size of amplified DNA product was detected in the fungus isolated from diseased trees, confirming that the fungus is *P. azadirachtae* (**Fig. 2**).

The chemically synthesized compounds[16] were tested for their *in-vitro* antifungal activity [13] against Phomopsis azadirachtae. The compounds completely inhibited the mycelial growth of *P. azadirachtae*. However, slight varying level of effect was observed among the chemicals (**Table 1**). Progressive decrease in the colony diameter was observed with an increase in the concentration of all seven fungicides. All the compounds inhibited the fungal growth at 60 ppm except compounds (C-2 and C-3) which were effective at little higher concentration i.e., at 70 ppm. All the tested compounds showed 100% inhibition at 100 ppm. Compounds C-1 and C-2 showed almost 50% inhibition of mycelia growth at just 10 ppm. Compounds C-6 and C-7 showed almost complete inhibition of mycelia growth at 50 ppm, where C-7 proved to be most effective among the compounds tested.

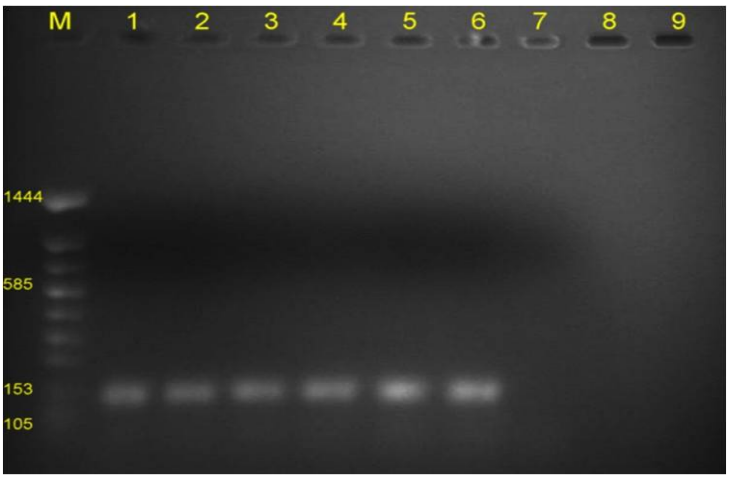


Fig 2: Agarose gel amplified products of 141bp of isolates of *P. azadirachtae* Lane M, 100bp DNA ladder: 1-6 *P.azadirachtae* and 7. *Fusarium moniliforme* (negative control)

Table 1: % Inhibition of *Phomopsis azadirachtae* by synthesized compounds (ppm)

Compound	% inhibition (ppm)						
	Control	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm
C-1	0.00	45.5±2.1	40.5±1.8	58.8±2.7	71.8±3.2	84.4±3.8	100±4.1
C-2	0.00	44.8±1.9	61.5±2.7	67.1±3.1	71.1±3.0	76±3.6	100±3.9
C-3	0.00	24.1± 0.9	39.3±1.7	47.7±2.2	49.3±2.3	73±3.2	100±4.3
C-4	0.00	17.4±0.07	38.5±1.6	60±2.7	72.6±3.1	83.3±3.9	100±3.8
C-5	0.00	13.3±0.5	60±2.7	69.6±3.1	73.3±3.2	83.3±4.0	100±3.6
C-6	0.00	15.2±0.6	57.7±2.6	68.8±2.8	75.5±3.4	89.6±4.1	100±4.2
C-7	0.00	14.4±0.65	60±2.7	66.6±3.1	75.5±3.3	90±4.2	100±4.3

[a] *Values are mean of three determinations, the ranges of which are less than 5% of the mean in all cases.*

The die-back is caused by *Phomopsis azadirachtae* and is systemic [19, 20] disease is spreading very alarmingly in Karnataka and Tamilnadu [8a, b]. Precise identification of a pathogen is must for the proper management of any plant disease. PCR-based method provides quick and reliable identification [21]. PCR method for identification of *P. azadirachtae* has been successfully employed [16, 22a]. The use of chemical fungicides is inevitable until the development of a better method of disease management [6]. Also chemical fungicides provide a cheaper and reliable source for the control of plant diseases. Norman Borlaug, father of the green revolution, argued for the use of synthetic chemical control methods though they can cause environmental hazardous effects [3]. Bavistin has been found to be very effective against *P.* azadirachtae [22b]. But it is a known fact that in the course of time the plant pathogenic fungi develop resistance against chemicals on continuous exposure [23] and identification of new chemicals for effective management of the plant diseases is a continuous need. Thus, in the present investigations seven novel compounds were screened *in vitro* for their antifungal activity against *P. azadirachtae*. *In vitro* screening helps to identify fungicides that are effective against plant pathogens by maintaining a protective barrier [24]. The antifungal activity of the chemicals observed proves their bioactive nature.

P. azadirachtae is seed-borne reducing the quality of neem seeds [20]. Seed treatment with the chemicals studied would help to overcome this problem. Thus all the seven compounds tested, which effectively controlled the growth of *P. azadirachtae* under *in vitro* conditions, can be considered for the effective control of die-back of neem.

CONCLUSION

To study the effect of synthetic compounds on the mycelial growth of *P. azadirachtae.* Plants are constantly threatened by a ray of pathogenic microorganisms present in the environment. The overall loss in crop yield worldwide is contributed significantly by plant pathogens including bacteria, fungi and viruses [25, 26, 27]. Plant fungicides which are formulated by synthetic ways are extensively used in agriculture. There are now more than 113 active ingredients registered as fungicides worldwide [28]. However, extensive use of chemicals causes severe long-term environmental pollution and are acutely toxic and even some prove carcinogenic towards humans and animals [29]. Further, pathogens with constant exposure to same chemicals become resistant to many of chemicals [30]. So, there is an obvious need to search for better alternative compounds that are non toxic to animals and are less pollutive environmentally for controlling plant diseases.

A lot of researchers have documented the antimicrobial activity of novel synthesized compounds against different fungal species [14a, 31, 32, 33, 34, 30, 35]. The present study has evaluated the effect of seven synthetic heterocyclic compounds on *P. azadirachtae* the causal agent of die-back of neem. Although all tested compounds inhibited the growth of the fungus at different concentrations, C-7 proved to be the most effective inhibiting the mycelial growth almost 100% at as low as 50 ppm. In the present study two chemicals C-6 and C-7 have shown promising results against *P. azadirachtae*. The result obtained confirms the antimicrobial activity of all synthetic compounds used in the present study. C-7 showed excellent fungitoxic activity against *P. azadirachtae* followed by C-6, C-1, C-4, C-5, C-2 and C-3 **(Fig 2).** Our results indicated the efficacy of C-7 and C-6 on the inhibition of the fungal mycelium. This study has to be envisaged by *in vivo* studies to fully understand the overall process when C-7 and C-6 are used for spraying on diseased neem trees.
Nagaraja *et al*., 2006 have documented the antifungal efficacy of naphthofuran derivatives against *Aspergillus niger*. This study indicated that synthetic heterocyclic compounds containing naphthofurans possess antifungal activity and can be effectively exploited as an ideal treatment for future plant disease management programmes. Overall effect of synthetic compounds on *P. azadirachtae* mycelial inhibition is as shown in **Fig 2**. Among the different concentration of synthetic compounds tested 100 ppm seems to be the most effective range, except for C-6 and C-7 where as low as 50 ppm was good enough for 100% mycelial growth inhibition.

The information obtained in the present study suggests that synthetic compounds containing naphthofurans show promising results in controlling the growth of *P. azadirachtae* under laboratory conditions. *In vivo* studies now need to be carried out on diseased neem trees, to further support the potential of synthetic compounds to control the pathogen growth over a wide range of environmental conditions.

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