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Research Article
Nematocidal Effect of 1,2,4-triazole Derivatives Against *Bursaphelenchus xylophilus*

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Abstract

Background and Objective: Pine wood nematode, *Bursaphelenchus xylophilus*, infects pine trees and causes the pine wilt disease. This disease is one of the major threats to pine trees, thus causing global concern. The present study aimed to evaluate the potential nematocidal effect of some 1,2,4-triazole derivatives and to analyze their structure-activity relationships. **Materials and Methods:** Eight previously synthesized 1,2,4-triazole derivatives were selected for this study and designated as compounds 1 through 8. The nematocidal effects of 6 different concentrations (50, 100, 200, 300, 400 and 500 μM) of these compounds were tested against *Bursaphelenchus xylophilus*. **Results:** At concentrations of 50-500 μM and with 6 h of exposure, the all of the test compounds killed nematodes in a concentration-dependent manner. Among these compounds, compound 1 exhibited the most potent nematocidal activity at all tested concentrations and exhibited the lowest LD_{50}. The highest mortality rate of 100% was only observed by compound 1, followed by compound 2 with 85% mortality rate after exposure to 500 μM for 6 h. Under the same conditions, the lowest mortality rate of 33% was obtained by compound 4. The LD_{50} of 50 μM was recorded for compound 1, followed by compound 2 with 70 μM. The LD_{50} value of compound 4 was at least 10 times higher than that of compound 1. Analysis of their structure-activity relationships suggested that the highest nematocidal activity was due to the presence of a primary amine on the substituted phenyl ring. **Conclusion:** Monosubstituted forms of the phenyl ring displayed much better nematocidal activity compared to unsubstituted or di-substituted forms. Compound 1 showed the highest nematocidal activity of all tested compounds, a finding that corresponded to the presence of a primary amine on the phenyl ring. These results demonstrated for the first time, that compounds containing a 1,2,4-triazole ring could be of importance as nematocidal drugs for combatting the pine wilt disease, the potency of which is influenced by the specific substitution pattern on the phenyl ring.

Key words: Antioxidant, parasitic species, phenyl ring, pine wilt disease, synthetic drugs

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nematodes are nonsegmented roundworms that have a smooth outer wall. Nematodes occur almost anywhere, implying that they are highly diverse in their habitats. The majority of nematodes exists as free living organisms in the sea, in the fresh water and in the soil[1-3]. A small number of nematodes may be found as parasitic species of plants or animals[4,5]. It was reported that the plant parasitic species spend most of their life span in the soil or within plant roots. Plant parasitic nematodes can cause extensive agriculture destruction to a wide range of plants such as common vegetables, fruits, trees and ornamental plants worldwide[2,5-7]. Globally, it was estimated that crop losses due to plant parasitic nematodes was $358.24 billion per year[5]. In light of this, studies have focused on various plant extracts and fungal cultures as a source of nematocidal activities *in vitro* and in the soil that may act to reduce or eliminate the parasitic nematode populations[3,8,9]. Despite these efforts, most of these studies failed to reveal or commercially develop efficient natural nematocidal compounds.

Several synthetic compounds with nematocidal effects were synthesized and tested *in vitro* or in the soil, including ethylene dibromide, 1,3-dichloropropene, imidazothiazole, pyrimidine, benzimidazoles, diethylcarbamazine and many others[2,6,10]. Some of these synthetic compounds were found to be unsuitable as nematocidal compounds due to the lack of desirable characteristics such as solubility and mobility in the soil, long-term suppression of nematodes, low cost and other desirable properties. The negative impacts of various nematocidal compounds in the environment have long been recognized, such as groundwater contamination, atmospheric ozone depletion and others[1,6,8]. Besides, the wide use of nematocidal compounds could have significant health consequences, such as cancer, sterility and general toxicity[6]. Due to these undesirable characteristics, the uses of most nematocidal synthetic compounds have been prohibited or restricted. Thus, the need for safe and environment friendly nematocidal compounds with maximized efficacy, minimum groudwater and atmospheric contamination as well as minimum side effects and toxicity remains.

A number of compounds containing a 1,2,4-triazole ring were designed and synthesized by our laboratory and others. These synthetic 1,2,4-triazoles were found to possess diverse pharmacological properties, including anticonvulsant, antifungal, antimicrobial, antihypertensive, analgesic, antiviral, anti-inflammatory, antioxidant and antitumor activities as well as nonsteroidal inhibitors of 17b-hydroxysteroid dehydrogenase type 1[11-18]. Synthetic 1,2,4-triazole derivatives such as ribavirin (antiviral), rizatriptan (antimigraine), alprazolam (antianxiety), vorozole, letrozole and anastrozole (antitumoral) have been used as chemotherapeutic drugs to treat various human diseases[19-22]. However, no report had been published about the antinematode properties of 1,2,4-triazole derivatives. This reason prompted us to carry out this study to assess the potential nematocidal properties of compounds containing a 1,2,4-triazole ring, particularly against *Bursaphelenchus xylophilus* (*B. xylophilus*). *Bursaphelenchus xylophilus* is also known as a pine wood nematode. It infects healthy pine trees and causes the pine wilt disease, causing tremendous damage[9,23]. In the present study, eight 1,2,4-triazole containing compounds were tested for nematocidal activity and the relationship between the strength of that activity and the compound's structure was assessed.

MATERIALS AND METHODS

Chemical and the test compounds: All the chemical reagents utilized in this study were purchased from sigma-aldrich. Eight 1,2,4-triazole containing compounds were utilized during this study and designated as compounds 1-8. These compounds were previously synthesized in the laboratory using the microwave-assisted method[15]. The structures and purity of these compounds were characterized and confirmed by ^{1}H, ^{13}C NMR and mass spectral analyses and by elemental analysis. The official IUPAC chemical nomenclature was followed during the naming process of the test compounds.

Nematode culturing: The pine wood nematode, *B. xylophilus* was used during this experiment. The nematodes were obtained from laboratory stocks and cultured on potato dextrose agar in petri-dishes, colonized with the fungus *Botrytis cinerea* for 7 days at 25°C.

Bioassay of nematocidal activity: Different concentrations of the test compounds under investigation were dissolved in dimethyl sulfoxide (DMSO) and prepared in serial dilution (50, 100, 200, 300, 400 and 500 µM) in millipore water. The test was carried out in a 24-well microtiter plate (CellStar, TC, Germany). To each well of the plate, 900 µL of each concentration of the test compound were added, followed by the addition of 100 µL of water containing 250 nematodes

(a mixture of juvenile and adult nematodes approximately 1:10). A well containing only 1% of DMSO in millipore water and nematodes was used as a negative control. Plates were soft shacked on shaker plate (Heidolph type, titramax-1000, 230/240 V, 50/60 Hz), in the laboratory at normal room conditions. The assay was replicated at least three times.

The mortality of adult and juvenile nematodes was recorded after 6 h of exposure by microscope inspection (Nekon Japan, Model ECLIPSE-TS-100). Nematodes were identified as dead if their body were straight and did not move, even after mechanical prodding. The average percentage mortality was calculated at six different concentrations for each test compound and plotted with the concentration tested to obtain LD_{50} values. The LD_{50} is the lethal concentration of the test compound required to kill 50% of the nematodes within 6 h of exposure. The LD_{50} values were expressed as μM.

RESULTS AND DISCUSSION

Chemistry of 1 ,2,4-triazole derivatives: In present study, eight 1,2,4-triazole containing compounds were evaluated as nematocidal agents. The IUPAC names and molecular weights of these compounds were tabulated in Table 1. The molecular weights of the test compounds were estimated using electrospray ionisation-mass spectrometry. The test compounds were identified to have molecular weights in the range of 341-401 kDa.

***In vitro* evaluation of nematocidal activity:** All compounds were tested for their potential nematocidal effects against the pine wood nematode, *B. xylophilus* at 6 different concentrations, ranging from 50-500 μM. The results of nematocidal activities at different concentrations of these compounds were presented in Table 2. These data revealed that mortality gradually increased with increasing concentrations of the test compounds in a dose-dependent manner. The highest mortality rates for all examined compounds were obtained after 6 h of exposure at a concentration of 500 μM, the highest concentration tested. At concentration of 500 μM, the mortality rate of nematodes ranged from 34% for compound 4 to 100% for compound 1. The lowest mortalitiy rate of 4% was observed at the lowest concentration of 50 μM of compound 4. Thus, compound 1 had the strongest nematocidal activity, followed by compound 2, whereas compound 4 showed the weakest nematocidal activity. This finding was the first demonstation of antinematode activity for the 1,2,4-triazole derivatives. Moreover, this finding was not surprising, given that some 1,2,4-triazole derivatives were previously shown to display various biological activities such as antifungal, antimicrobial, antiviral, antioxidant and antitumor[12-18].

Lethal Dose 50% (LD_{50}) is a dose needed to the reduced number of nematodes by 50% was calculated. The LD_{50} values of the test compounds on survival of pine wood nematodes after 6 h of exposure were presented in Table 3. The test compounds were found to display varied nematocidal

Table 1: IUPAC chemical names for the test compounds 1-8, molecular formula and their Molecular Wieghts (MW)

Compounds	IUPAC name	Molecular formula	MW
1	4'-[3-(4-Methoxyphenyl)-1-methyl-1H-1,2,4-triazol-5-yl]biphenyl-3-amine	$C_{22}H_{20}N_4O$	356
2	5-(2'-Fluorobiphenyl-4-yl)-3-(4-methoxyphenyl)-1-methyl-1H-1,2,4-triazole	$C_{22}H_{18}FN_3O$	359
3	4'-[3-(4-Methoxyphenyl)-1-methyl-1H-1,2,4 -triazol-5-yl]biphenyl-3-carbonitrile	$C_{23}H_{18}N_4O$	366
4	5-(2',6'-Difluorobiphenyl-4-yl)-3-(4-methoxy phenyl)-1-methyl-1H-1,2,4-triazole	$C_{22}H_{17}F_2N_3O$	377
5	5-(3'-Fluoro-4'-methoxybiphenyl-4-yl)-3-(3-methoxyphenyl)-1-methyl-1H-1,2,4-triazole	$C_{23}H_{20}FN_3O_2$	389
6	3-Fluoro-4'-[3-(3-methoxyphenyl)-1-methyl-1H-1,2,4-triazol-5-yl]biphenyl-4-ol	$C_{22}H_{18}N_3O_2$	375
7	5-(Biphenyl-4-yl)-3-(3-methoxyphenyl)-1-methyl-1H-1,2,4-triazole	$C_{22}H_{19}N_3O$	341
8	5-(4',5'-Dimethoxybiphenyl-4-yl)-3-(3-methoxy phenyl)-1-methyl-1H-1,2,4-triazole	$C_{24}H_{23}N_3O_3$	401

Table 2: Nematocidal activity of different concentrations of 1, 2 ,4-triazole containing compounds against *Bursaphelenchus xylophilus* after 6 h of exposure

	Cocentration (μM) mortality rate±SD (%)					
Compounds	50	100	200	300	400	500
1	48.4±13.5	50.3±15.4	53.9±9.7	64.4±9.0	95.8±8.3	100.0±0.0
2	45.1±9.8	55.2±15.8	61.1±18.3	61.7±11.2	81.1±6.3	85.1±7.5
3	20.2±3.5	21.2±10.3	30.0±2.7	52.1±2.7	55.0±2.5	75.8±11.0
4	40.0±3.10	80.3±5.10	12.6±4.5	21.4±4.3	25.9±2.9	33.7±8.5
5	15.2±4.4	21.6±1.3	25.3±1.3	30.1±4.7	38.6±4.7	50.1±5.3
6	21.6±4.1	29.3±2.8	34.2±6.8	49.7±2.5	55.3±3.7	66.3±7.4
7	15.8±4.5	27.7±4.0	33.0±1.6	40.8±2.5	50.0±2.5	52.3±3.6
8	20.6±4.5	31.3±5.5	47.0±6.8	56.5±3.5	62.0±3.7	74.0±8.0
DMSO (1%)						8.0±6.50

Numbers represent Means±Standard Deviation of three independent experiments, DMSO: Dimethylsulfoxide was used as a negative control

Table 3: LD_{50} values of the tested 1,2,4-triazole containing compounds against *Bursaphelenchus xylophilus* after 6 h of exposure

Compounds	LD_{50} (μM)
1	50
2	70
3	282
4	>500
5	498
6	302
7	401
8	223

LD_{50}: The concentration of the test compound that killed up to 50% of the nematodes within 6 h of exposure

effects, ranging from weak, moderate and strong effects as determined by LD_{50}. The lowest LD_{50} value of 50 μM was observed for compound 1 against the pine wood nematodes. A low LD_{50} value is indicative of greater antinematode activity. Compound 2 had a LD_{50} value of 70 μM. This result suggested that the effectiveness of compound 2 was closely similar to compound 1. The LD_{50} values of 223, 283 and 302 μM were recorded for compounds 8, 3 and 6, respectively. Compounds 5, 7 and 4 had the highest LD_{50} values of 401, 498 and >500, respectively. Based on these data, it could be concluded that the weakest nematocidal effects were exhibited by compounds 4, 5 and 7.

Correlation between antinematode and antioxidant activities of the test compounds: There is no infomration about the mechanism of the test compounds against nematodes. However, these compounds have antioxidant activities as previously assayed in laboratory by measuring the scavenging activity against DPPH and by estimation of the reducing power using Fe^{3+} and Cu^{2+} reduction method[15]. To search for the possible correlation between the antioxidant and nematocidal activities among the test compounds, the results of their nematocidal activity was correlated with their antioxidant activities done by the previous study. In the previous study, compound 6 showed the best DPPH scavenging activity at 200 μM and reducing activities for copper and iron ions compared to compounds 1-5 and 7-8. By contrast, the present data revealed that the antinematode activity of compound 6 against *B. xylophilus* was weaker than that of compounds 1, 2, 8, 3. It was also found that compounds 1 and 2 exhibited antioxidant activity greater than that of compounds 3, 4, 5 and 7. Moreover, compounds 4, 5 and 7 showed the lowest antinematode and antioxidant activities. Overall, no clear correlation was detected between the antioxidant and nematocidal activities of the test compounds. Despite the absence of clear correlation, the presence of antioxidant and nematocidal activities in compounds 1 and 2 are very encouraging and may paved the way for possibility of utilization of these two compounds as nematocidal drug against *B. xylophilus*. In addition, antinematode drug that has antioxidant activity as independent mode of action can be seen as a way of reducing side-effects for human, while ensuring mutual protection against nematodes.

Relationship between the structure of the test compounds and their nematocidal activities: The chemical structures of the test compounds 1 through 8 were illustrated in Fig. 1. The nematocidal activity of compound 1, which has phenyl ring with a primary amine at the meta (or 3rd) position of the phenyl ring was much stronger than that of the other test compounds. It was also noted that replacement of the amine group with the carbonitrile group on the phenyl ring as in compound 3 , created a compound less potent than that with amine group. Similarly, compound 7 having an unsubstituted phenyl ring, showed lower nematocidal activity than that of compound 1. The nematocidal activity of compound 2 with monofluoro on the phenyl ring was nearly three times greater than that of compound 4 with difluoro on the phenyl ring. Besides, nematocidal activities of compounds 1, 2 and 3, which have monosubstitutions on the phenyl ring were found to be greater than that of compounds 4, 5, 6 and 8, which have disubstitutions, regardless of their position on the phenyl ring. Interestingly, the presence of the hydroxy group in the 4-position of the 3-fluorophenyl ring of compound 6 had a slight positive effect on the nematocidal activity in comparison with compound 5, which has a methoxy group in the 4-position of the 3-fluorophenyl ring. These findings suggest that an increase in the hydrophilic properties at the phenyl ring appear to enhance the nematocidal activity of 1,2,4-triazole derivatives.

In general, the investigation of the structure-activity relationship of the selected compounds revealed that the nematocidal activities of the test compounds were affected by the kind and number of the substituted groups. It can be concluded that the substitution with electron donor groups (NH_2 and OH) are important factors that influence the nematocidal activity of the test compounds and gave positive effects on their LD_{50} values. On other side, substitution with flouro was also another factor that gave a negative effect on the nematocidal activity of the test compounds, increasing their LD_{50} values. Therefore, a compound with 3-amine substituent attached to the phenyl ring has promising nematocidal activity.

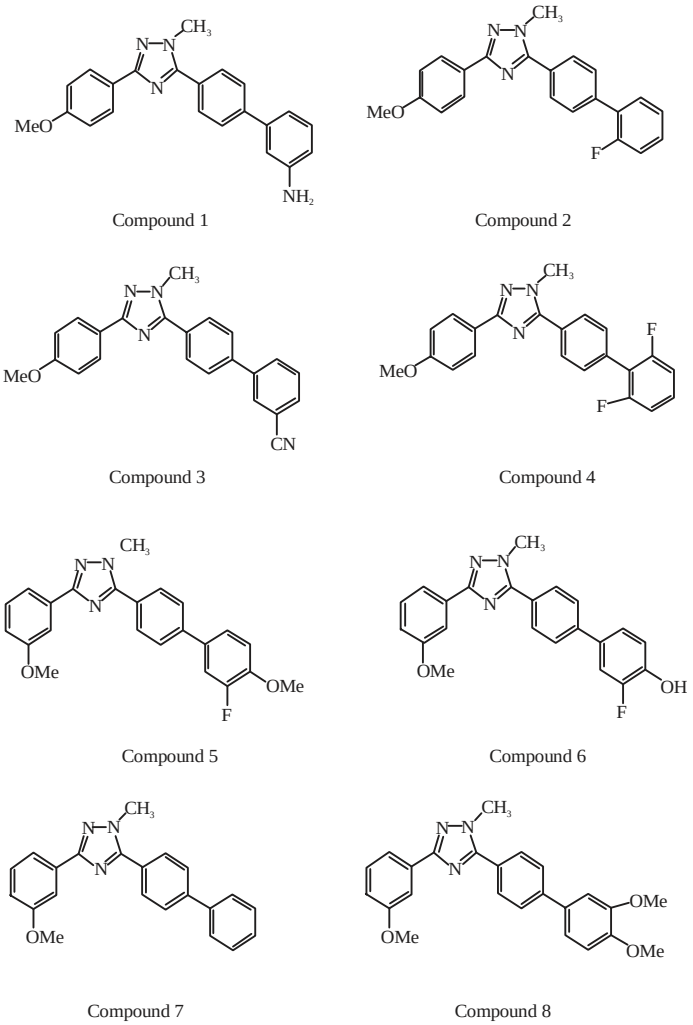

Fig. 1: The chemical structures for the test compounds 1-8

In order to identify a leading antinematode agent and to clarify the structure-nematocidal activity relationship, it is necessary to examine other similar 1,2,4-triazole containing compounds. In this context, the search for new classes of antinematode drugs from compounds containing a 1,2,4-triazole ring with improved efficacy for the pine wilt disease without incurring additional toxicity is ongoing.

CONCLUSION

In the present study, eight 1,2,4-triazole derivatives previously synthesized in our lab were designated as 1 through 8 and tested for the first time for their nematocidal activities against *B. xylophilus*, the pine wood nematode. The following conclusions can be drawn from this present study:

- 1,2,4-triazole derivatives have nematocidal activities
- The secondary group on the phenyl ring significantly affects efficacy
- A single primary amine at the position 3 of the phenyl ring appears to be the most effective form
- Among the test compounds, compounds 1 and 2 were the most potent in term of nematocidal activity
- The antinematode mechanism is unknown, though there is some evidence that antioxidant activity may be involved

Based on these findings, compounds 1 and 2 may have great potential to be explored as promising synthetic nematicides or be useful as lead molecules for designing new nematocidal agents with low side effects and high therapeutic capacities for controlling *B. xylophilus*. Decipher the possible mode of action of compounds 1 and 2 should be

investigated. From this study, further studies are underway at different stages of synthesis and testing different 1,2,4-triazole compounds as antinematode drugs.

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