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Nematicidal prenylated flavanones from *Phyllanthus niruri* ☆

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Abstract

Two prenylated flavanones have been isolated from the hexane extract of *Phyllanthus niruri* plant. The structure of these flavanones were established as 8-(3-Methyl-but-2-enyl)-2-phenyl chroman-4-one (**1**) and 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one (**2**) on the basis of spectral analysis. These were evaluated for nematicidal activity against root-knot, *Meloidogyne incognita*, and reniform, *Rotylenchulus reniformis*, nematodes. Compound **2** exhibited nematicidal activity at par with the standard carbofuran (LC_{50} 3.3 and 3.1 ppm, respectively) when tested against reniform nematode. The LC_{50} value against root-knot nematode was found to be 14.5 ppm. Compound **1** however, showed moderate activity against both the test nematodes.

Keywords: *Phyllanthus niruri*; Flavanones; *Meloidogyne incognita*; *Rotylenchulus reniformis*; Nematodes; Euphorbiaceae

1. Introduction

The genus *Phyllanthus* includes 500 temperate and tropical species many of which are used medicinally in different countries. *Phyllanthus niruri* L., (Syn. *P. fraternus* Webster), Euphorbiaceae, is a common kharif (rainy season) weed found in both cultivated fields and wastelands in India. It is an annual herb with height varying between 30 and 60 cm. Its roots, leaves, fruits, milky juice, and whole plants are used as medicine (Kirtikar and Basu, 1935). Fruits are useful for tubercular ulcers, wounds, sores, scabies and ring worm (Agharkar, 1991). The fresh root is believed to be an excellent remedy for jaundice, dropsy and genitourinary infections (Chopra et al., 1956). The infusion of the root and leaves is a good tonic and diuretic when taken cold in repeated doses (Ambasta, 1986; Satyavati et al., 1987). In different parts of India, specially, in Chhattisgarh state, it is used as a rich traditional medicine (Caius, 1986). *P. niruri* has shown clinical efficacy in viral Hepatitis B for which no effective specific therapy is available (Paranjpe, 2001). Fresh juice and powder of dried plant are used most frequently in Ayurvedic preparations (Sastry and Kavathekar, 1990). *P. niruri* is still widely used in herbal medicine in South America, remaining the most popular remedy for gallstones and kidney stones throughout the continent (Taylor, 2003). In Peruvian herbal medicine, it is also used for hepatitis, urinary infections, and as a diuretic (Devi, 1986). There are also reports of a host of other activities of different parts of *P. niruri* and its constituents (Taylor, 2003; Bagalkotkar et al., 2006). But no work has been reported on the potential of this plant in pest control.

A number of known lignans namely phyllanthin and hypophyllanthin, niranthin, nirtetralin, and phyltetralin have been reported from the leaves of this plant (Rastogi and Mehrotra, 1991). Other group of compounds reported to occur are flavanone glycosides (Gupta and Bahar Ahmed., 1984), lignans (Row et al., 1966), acyclic triterpene (Singh et al., 1989), phthalic acid bis-ester (Singh et al.,

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1986), glycoflavones (Chauhan et al., 1977). A new antioxidant flavone sulfonic acid has recently been reported from this plant (Thana et al., 2006).

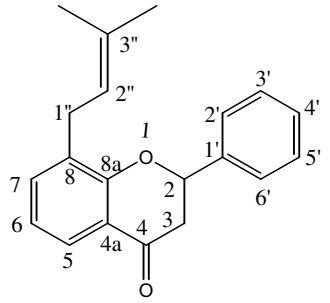
M. incognita, is the most economically important and widely distributed nematode throughout India and a considerable crop loss is caused by this nematode. *R. reniformis*, reniform nematode is one of the significant plant pest of tropics, semitropical and warmer areas of the temperate zone. It has been reported to be pathogenic in several vegetables, fruits, legumes, pulses, oilseeds, cereals and other commercial crops (Devakumar and Goswami, 1992; Gaur and Pankaj, 2006).

In our systematic search for pest control agents from Indian plants, the bioassay-guided fractionation of hexane extract of *P. niruri* plant led to the isolation of two new prenylated flavanones, namely, 8-(3-methyl-but-2-enyl)-2-phenyl-chroman-4-one (**1**) and 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one (**2**), which were evaluated for their nematicidal activity against root-knot and reniform nematodes.

2. Results and discussion

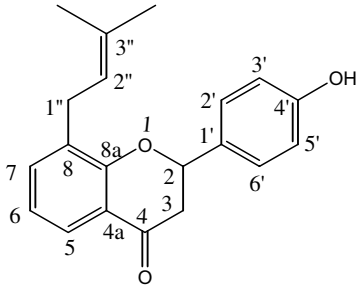
The hexane extract of *P. niruri* on preliminary evaluation, exhibited nematicidal activity as promising as the fungal filtrate of *Aspergillus niger*, a known natural nematicide. The hexane extract was therefore, further fractionated by column chromatography. The fraction, exhibiting maximum bioefficacy (eluted with 20% chloroform in hexane) yielded two compounds on rigorous column and preparative chromatography, which were characterized by detailed spectral analysis *viz.* ^{1}H NMR, ^{13}C NMR, HMBC, NOESY, COSY, IR, UV and mass spectra (see Fig. 1).

Compound **1**, obtained as yellow solid on crystallization from methanol and analyzed for $C_{20}H_{20}O_2$. In its mass spectrum a molecular ion peak $[M]^+$ appeared at *m/z* 292. Absorption at 1681 cm^{-1} in the IR spectrum showed the presence of carbonyl group in the molecule. This was supported by a signal at δ 198.3 for the carbonyl in its ^{13}C NMR spectrum. The ^{1}H NMR spectrum of **I** showed three signals in non-aromatic region placed at δ 5.42 (1H, dd, $J = 12.8$, 2.9 Hz), 3.26 (1H, dd, $J = 16.7$, 12.8 Hz) and 3.01 (1H, dd, $J = 16.7$, 2.9 Hz) characteristic of H-2, H-3$_{ax}$ and H-3$_{eq}$, respectively of a flavanone moiety (Mabry et al., 1970a, 1970b). The double-doublet at δ 5.42 is due to vicinal coupling of H-2 proton separately with the axial and equatorial protons at position 3, whereas the double-doublets at δ 3.26 and 3.01 are due to both vicinal and geminal coupling as evident from the *J* values. This coupled with the fact that it did not give any coloration with ferric chloride showed that compound **1** was a non-phenolic flavanone. The multiplet at δ 7.22–7.34, integrating for five protons was assigned to the protons of the phenyl ring attached at position 2. The triplet at δ 7.02 ($J =$ 7.8 Hz), integrating for one proton, was assigned to C–6H,



8-(3-methyl-but-2-enyl)-2-phenyl-chroman-4-one

1



2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one

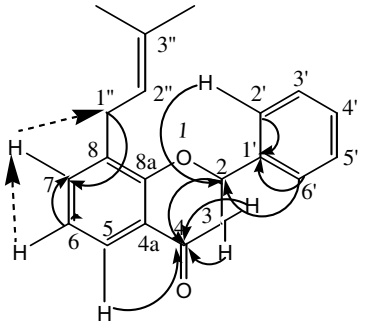
2

Fig. 1. Structures of prenylated flavanones (Shakil et al.).

whereas the C–5H and C–7H protons appeared as two separate double-doublets (1H, dd, $J = 7.8$, 1.6 Hz) at δ 7.94 and 7.55, respectively due to both *ortho*- and *meta*-coupling. In its mass spectrum, the loss of C_5H_9 (*m/z* 69), C_4H_7 (*m/z* 55) and C_3H_6 (*m/z* 42) indicated the presence of isopentenyl group in the molecule which was corroborated by a doublet at δ 3.22 ($J = 6.0$ Hz), integrating for two protons, due to the benzylic C–1′′H and a triplet at δ 5.24 ($J = 5.8$ Hz) for C-2′′ protons, whereas the two vinylic methyl groups appeared as two separate singlets at δ 1.71 and 1.81, respectively. The absence of *para* coupling indicates that isopentenyl group is either at C-5 or C-8 position. It was placed at C-8 as the proton on C-5 appeared at a very high δ value (7.94) because of the carbonyl group at C-4.

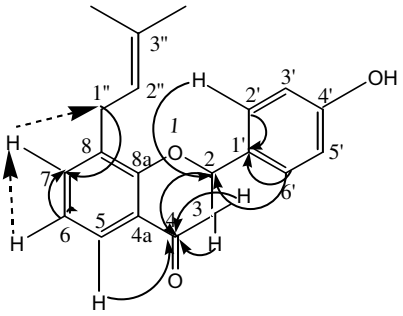
In its ^{13}C NMR spectrum, the two methylene carbons *i.e.* C-1′ and C-3 appeared at δ 26.8 and 46.4, respectively, C-2 carbon showed a signal at δ 74.8. The signals at δ 19.00 and 26.12 were due to the two vinylic methyl groups, C-3′′ at δ 135.20 and C-2′′ carbon appeared at δ 119.7.

The above proposed structure was further confirmed by two dimensional NMR spectroscopy of the compound. The signal at δ 3.22, assigned to H-1′′ protons, showed long range correlation with the carbon at δ 134.2 and was assigned to C-7 as it showed cross correlation with H-6 (δ 7.02) in its HMBC spectrum. This correlation was further evidenced by NOE correlation of H-1′′ protons with H-7 (δ 7.55) in its NOESY spectrum (Fig. 2).



8-(3-methyl-but-2-enyl)-2-phenyl-chroman-4-one

1



2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one

2

Significant NOESY (- - - ➤) and COSY & HMBC (——➤) correlations for **1** and **2**

Fig. 2. Significant HMBC and NOESY correlations for **1** and **2** (Shakil et al.).

Similarly, the signals at δ 7.94, 7.55 and 7.02 showed correlations with the signals at δ 126.2, 134.2 and 121.1, respectively in its COSY spectrum and were subsequently assigned to H-5, H-7 and H-6, respectively. The HMBC spectrum of **1** also showed long range correlation of signal at δ 7.94, assigned to H-5, with the signal at δ 198.34 and was assigned to C-4. The signal at δ 198.34 also showed correlations with the signals at δ 5.44 (H-2), 3.16 (H-$3_{ax.}$) and δ 2.98 (H-$3_{eq.}$). Thus on the basis of above spectral studies the structure of compound **1** was elucidated as 8-(3-methyl-but-2-enyl)-2-phenyl-chroman-4-one.

Compound **2**, obtained as viscous oil and analyzed for $C_{20}H_{20}O_3$. In its mass spectrum a molecular ion peak $[M]^+$ appeared at *m/z* 308. The spectral features of **2** showed resemblance to compound **1** except for an absorption at 3250 cm^{-1} in its IR spectrum indicating the presence of phenolic hydroxyl group in the molecule, which was supported by a signal for a quaternary carbon at δ 160.2 in its ^{13}C NMR spectrum. The ^{1} H NMR spectrum of **2** displayed two downfield signals as doublets (J = 8.0 Hz each) for aromatic protons at δ 6.66 and 7.20, integrating for two protons each, indicated the presence of a *para*-disubstituted benzene ring. Consequently, these signals were assigned to the C-3′ and C-5′ and C-2′ and C-6′ protons, respectively. Hence the signal at δ 160.2 in ^{13}C NMR was assigned to C-4′. The two methylene carbons *i.e.* CH_2Ph and C-3H appeared at δ 27.4 and 46.5, respectively. The C-2 carbon showed signal at δ 73.5 The signals at δ 19.00 and 26.12 were due to the two vinylic methyl groups and the signal at δ 136.1 was due to C-3′′ carbon. The C-2′′ carbon appeared at δ 119.9. The 2D NMR experiments with compound **2** showed long range correlation of signal at δ 3.23, assigned to H-1′′, with carbon at δ 134.8 and was assigned to C-7 as it also showed cross correlation with H-6 (δ 6.98) in its HMBC spectrum (Fig. 2). The NOESY spectrum further confirmed the correlation of H-1′′ protons with that of H-7 (δ 7.40). The COSY spectrum of compound **2** showed correlations of signals at δ 7.84, 7.40 and 6.98 with those at δ 126.8, 134.8 and 120.1, respectively and were subsequently assigned to H-5, H-7 and H-6, respectively. The signal at δ 7.84 also showed correlation with the signal at δ 197.6, assigned to C-4, in its HMBC spectrum. Similarly the protons in ring C were confirmed on the basis of its HMBC spectrum as the signal at δ 7.2, assigned to H-2′ & H-6′, showed correlation with the signal at δ 141.7 and was assigned to C-1′. The signal at δ 7.20 also showed correlation with the carbon at δ 73.5 which was assigned to C-2, the latter also showed long range correlation with carbon at δ 197.6, assigned to C-4. The H-$3_{ax.}$ and H-$3_{eq.}$ signals also showed correlations with the carbon at δ 197.6. Thus on the basis of above spectral data, the compound **2** was characterized as 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one. Although compounds **1** and **2** are not reported anywhere in literature from natural sources, a closely related tetrahydroxy prenylated flavanone has been reported by Gupta and Bahar Ahmed. (1984) from *P. niruri*. Gupta and Krishnamurti (1976) and Khan et al. (1986) have also reported the presence of 8-prenylated flavanone from *Milletia ovalifolia* and *Tephrosia falciformis*, respectively.

Nematicidal activity of 1 and 2. The above compounds were evaluated for efficacy against *Meloidogyne incognita* [(Kofoid and White) Chitwood] and *Rotylenchulus reniformis* (Linford *et* Oliveira) nematodes. Both the compounds showed dose-dependent nematicidal activity against the test nematodes (Table 1). The LC_{50} calculated after 72 h showed that compound **2** exhibited nematicidal activity much stronger than the standard bionematicide, *Aspergillus niger* (Van Tieghem) which has LC_{50} of 48 ppm after 72 h. This was then compared with another standard, carbofuran having LC_{50} 3.1 ppm. As evident from Table 1,

Table 1
Effect of prenylated flavanones **1** and **2** against root-knot (*Meloidogyne incognita*) and reniform (*Rotylenchulus reniformis*) nematodes

Compounds	LC_{50} (ppm) after 72 h			
	M. incognita	Std. error (mean)	*R. reniformis*	Std. error (mean)
1	70.9	0.14	102.9	0.60
2	14.5	0.96	3.3	1.13
A. niger			48.0	
Carbofuran			3.1	

compound **2** was found to be as effective as carbofuran (LC_{50} 3.3 ppm) when tested against reniform nematode. Compound **1**, however, was not found as effective. So far, the flavanones have been reported to have mostly pharmaceutical effects (Trikha et al., 2005), but no information is available as to their nematicidal nature, hence the importance of the present finding.

3. Experimental

3.1. General

Laboratory grade reagents and solvents were locally procured. TLC was run on precoated Merck silica gel $60F_{254}$ plates; the spots were detected either by UV light or by spraying with 5% alcoholic $FeCl_3$ solution. Melting points were determined in a sulphuric acid bath and are uncorrected. The IR spectra were recorded on either a Perkin–Elmer 2000 FT-IR or RXI FT-IR spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on a Bruker AC-300 Avance spectrometer at 300 and 75.5 MHz, respectively using TMS as internal standard. The chemical shifts values are on δ scale and the coupling constants (J) values are in Hertz. Mass spectra were determined on Jeol AX 505 W instrument at 70 eV. The elemental analysis was done on a Eurovector Elemental analyzer 3000 using sulphanilamide as standard with linear calibration. Optical rotations were measured with Bellingham-Stanley AD 220 polarimeter.

3.2. Plant material

Whole plant of *P. niruri* were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. A specimen No. 439 is also preserved in the herbarium of the Institute. It was shade dried and powdered.

3.3. Extraction and isolation

The shade dried and powdered plant of *P. niruri* (1 kg) was exhaustively extracted with 1.5 L of hexane for 3 h on a water bath at 50–60 °C. The contents were filtered through buchner funnel under vacuum. The solvent was removed under reduced pressure to obtain crude extract (15 g) which was used for column chromatography. Elution was started with hexane and polarity increased by subsequent addition of chloroform till pure chloroform. Further, methanol was used to increase the polarity of eluant. The fraction obtained after eluting with 20% chloroform in hexane showed two spots under UV-light (developing solvent 20% ethyl acetate in hexane). It was subjected to preparative chromatography to obtain the two compounds **1** and **2**. These were analysed and characterized by its IR, UV, ^1H NMR, ^{13}C NMR and mass spectroscopy data.

3.4. 8-(3-methyl-but-2-enyl)-2-phenyl-chroman-4-one (*1*)

Yellow solid (154 mg); mp 159–162 °C; $[\alpha]_D^{25} + 6.7°$ (c 0.16, $CHCl_3$); R_f 0.5, (20% ethyl acetate–hexane); UV (MeOH) λ_{max}: 255 sh, 293, 370 nm; IR Data (nujol) ν_{max}: 2924, 1681(C=O), 1620, 1430, 1304, 1200, 1096, 1038, 930, and 762 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.94 (1H, dd, $J = 7.8$, 1.6 Hz, H-5), 7.55 ((1H, dd, $J = 7.8$, 1.6 Hz, H-7), 7.22–7.34 (5H, m, H-2′, H-3′, H-4′, H-5′ and H-6′), 7.02 (1H, t, $J = 7.8$ Hz, H-6), 5.42 (1H, dd, = 12.8, 2.9 Hz, H-2), 5.24 (1H, t, $J = 5.8$ Hz, H-2″), 3.26 (1H, dd, $J = 16.7$, 12.8 Hz, H-3_{ax}), 3.22 (2H, d, $J = 6.0$ Hz, H-1″), 3.01 (1H, dd, $J = 16.7$, 2.9 Hz, H-3_{eq}), 1.81 (3H, s, vinylic methyl), 1.71 (3H, s, vinylic methyl); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 198.34 (C-4), 160.4 (C-8a), 140.9 (C-1′), 135.2 (C-3′), 134.2 (C-7), 129.6 (C-4a), 128.7 (C-3′ & C-5′), 127.3 (C-2′, C-4′ & C-6′), 126.2 (C-5), 123.3 (C-8), 121.1 (C-6), 119.7 (C-2″), 74.8 (C-2), 46.4 (C-3), 26.8 (C-1″), 26.12 (vinylic carbon), 19.00 (vinylic carbon); Mass Spectral Data, EIMS, *m/z* (% rel. int.): 292 ($[M]^+$, 5%), 277 (5%), 237(10%), 223(100%), 215 (10%), 201 (5%), 177(5%), 163(15%), 120(30%), 69 (30%), 55 (60%), 42 (75%); anal. C 82.01%, H 6.78%, calcd. for $C_{20}H_{20}O_2$ C 82.19%, H 6.84%.

3.5. 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one (*2*)

Viscous oil (234 mg); $[\alpha]_D^{25} + 10.4°$ (c 0.36, $CHCl_3$); R_f 0.38, (20% ethyl acetate–hexane); UV (MeOH) λ_{max}: 260 sh, 284, 368 nm; IR Data (nujol) ν_{max}: 3450, 3250, (OH), 2920, 1671(C=O), 1610, 1513, 1400, 1314, 1190, 1096, 1038 and 970 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.84 (1H, dd, $J = 7.8$, 1.6 Hz, H-5), 7.40 ((1H, dd, $J = 7.8$, 1.6 Hz, H-7), 7.20 (2H, d, $J = 8.0$ Hz, H-2′ and H-6′), 6.98 (1H, t, $J = 7.8$ Hz, H-6), 6.66 (2H, d, $J = 8.0$ Hz, H-3′ and H-5′) 5.44 (1H, dd, J = 12.8, 2.9 Hz, H-2), 5.28 (1H, t, $J = 5.8$ Hz, H-2″), 3.16 (1H, dd, $J = 16.7$, 12.8 Hz, H-3_{ax}), 3.23 (2H, d, $J = 6.0$ Hz, H-1″), 2.98 (1H, dd, $J = 16.7$, 2.9 Hz, H-3_{eq}), 1.84 (3H, s, vinylic methyl), 1.72 (3H, s, vinylic methyl); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 197.60 (C-4), 162.4 (C-8a), 160.2 (C-4′), 141.7 (C-1′), 136.1 (C-3″), 134.8 (C-7), 130.4 (C-4a), 129.5 (C-2′ and C-6′), 126.8 (C-5), 123.3 (C-8), 120.1 (C-6), 119.9 (C-2″), 116.8 (C-3′ & C-5′), 73.5 (C-2), 46.5 (C-3), 27.4 (C-1″), 26.12 (vinylic carbon), 19.00 (vinylic carbon); Mass Spectral Data, EIMS, *m/z* (% rel. int.): 308 ($[M]^+$, 5%), 293 (5%), 253(20%), 239(100%), 216 (10%), 202 (5%), 178(5%), 164(15%), 121(30%), 69 (25%), 55 (55%), 42 (40%); anal. C 77.78%, H 6.46%, calcd. for $C_{20}H_{20}O_3$ C 77.90%, H 6.49%.

3.6. Nematicidal bioassay

The prenylated flavanones (**1** and **2**) were tested for their efficacy against the root-knot nematodes (*M. incognita*) and reniform nematode, *R. reniformis*.

M. incognita: The above compounds (15 mg each) were weighed separately and dissolved in ethanol (0.5 mL) and the volume was made upto 5 mL by 0.5 % emulsified water (5 mL of tween 80 in 1 L of distilled water) to get a stock solution of 3000 ppm. The second stage juveniles (J2s) were isolated from the single egg mass culture (Brinjal cv. Pusa Purple Long) maintained at Division of Nematology, Indian Agricultural Research Institute, New Delhi (India). Later, they were multiplied and maintained on tomato (cv. Pusa Ruby). The egg masses were collected from the roots and kept in 10 cm diameter Petri plates which contained double distilled sterile water (DDSW) for hatching of eggs in BOD incubator at 25 °C for 24 h. Fifty freshly hatched second stage juveniles (J2s) of *M. incognita* were placed in all the Petri dishes (in triplicate) containing both the compounds. Number of dead juveniles in 1 mL suspension in each Petri dish was counted after 72 h, and LC_{50} was calculated by using a Basic LC_{50} program version 1.1 (Trevors, 1986). The nematodes were considered dead if they did not move at all when probed with a fine needle (Cayrol et al., 1989).

R. reniformis: The nematodes isolated originally from the a single egg mass from castor roots, were cultured, multiplied and maintained on cowpea plants raised in 30 cm earthen pots containing sterilized soil–sand mixture (2:1 ratio). The egg masses were collected and kept on two layers of tissue paper supported by aluminum wire gauge in 10 cm diameter Petri plates filled with fresh water. After 72 h the average population of *R. reniformis* that emerged in the suspension was determined by counting pre-adults of *R. reniformis* in 1 mL aliquots in triplicates under stereoscopic binocular microscope. Test solutions of 1500, 750, 500, 250 and 125 ppm were prepared by serial dilutions of the stock solutions with 0.5% emulsified water. Suspension of the juvenile nematodes was diluted with water to 100 mL to get approx. 50 juveniles mL^{-1}. To 1 mL of this nematode suspension in petriplates, an equal volume of the test solutions was added separately to obtain the desired test concentrations of 750, 500, 250, 125, and 62.5 ppm, respectively. Juveniles of the nematodes kept in water with ethanol served as the control. After 72 h of exposure, the suspension in three petriplates for each treatment was observed under stereoscopic binocular microscope for determining juvenile immobility. Revival test was also performed for each treatment. Test solvents were decanted and distilled water was added to all the petriplates to count the number of revived juveniles after 72 h. The revived juveniles were counted and deducted from the number of immobile juveniles obtained in the previous reading taken after 72 h. Juvenile nematodes found immobile after the revival test were considered as dead. The corrected percent mortality was calculated using the Abbot's formula: Corrected mortality $(\%) = ((T - C)/(100 - C)) \times 100$, where T is the percent mortality in treatment and C is the percent mortality in control. LC_{50} values (ppm) were calculated by using a basic LC_{50} program version 1.1 as described (Trevors, 1986). A bionematicide, *Aspergillus niger*, and carbofuran were kept as standards which are used for plant parasitic nematodes (*M. incognita* and *R. reniformis*).

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