NEMATICIDAL ACTIVITY OF *STRYCHNOS NUXVOMICA* LEAF AND ITS CONSTITUENTS AGAINST THE ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

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Summary. Studies were undertaken on the nematicidal activity of *Strychnos nuxvomica* leaf extracts and its constituents on the second stage juveniles of the root-knot nematode, *Meloidogyne incognita*. Aqueous leaf extracts at 2% concentration caused 100% mortality of second stage juveniles of the nematode. The biological activity of the known phytochemicals from *S. nuxvomica*, evaluated by *in silico* analysis through prediction of activity spectra for substances (PASS) software, indicated that seven phytochemicals were expected to show nematicidal activity at various levels. *In silico* studies showed that the values of Pa (probability to be active) for brucine and strychnine were 0.986 and 0.785, respectively. Bioassay-guided fractionation of the leaf extract of *S. nuxvomica* indicated that the active fractions contained brucine and strychnine as major components. The nematicidal activity of brucine and strychnine was validated through *in vitro* bioassays and LD_{50} values were determined to be 665 ppm and 833 ppm, respectively.

Key words: Control, Pa, plant extracts.

Plant parasitic nematodes are serious threats to crop production, causing an estimated loss of US $125 billion/year worldwide (Chitwood, 2003). Root-knot nematodes, *Meloidogyne* spp., are the most damaging nematodes of crop production worldwide. The impact of this nematode genus is enhanced by its wide host range of more than 5,000 plant species and it causes severe economic losses in many agricultural and horticultural crops (Ntalli *et al*., 2010). It has been estimated that global losses due to *Meloidogyne incognita* (Kofoid *et* White) Chitw. amount to $78 billion (Chen *et al*., 2004). Hence the control of root-knot nematodes is necessary for sustainable crop production.

The management tactics used against root-knot nematodes include soil fumigation, soil pasteurization, rotation with non-host crops and use of resistant cultivars. However, due to environmental concerns caused by synthetic nematicides and the limited number of available resistant cultivars, alternative methods, such as biocontrol agents and natural products, are being exploited (Echeverrigaray *et al*., 2010).

Many compounds with nematicidal activity have been isolated from plants; these include alkaloids, diterpenes, glucosinolates, isothiocyanates, phenols, polyacetylenes, sesquiterpenes and thienyls (Gommers, 1981; Gommers and Bakker, 1988; Chitwood, 2002; Ferraz and de Freitas, 2004). Furthermore, *Strychnos nuxvomica* L., a member of the family Loganiaceae, has been reported to be a useful pesticide to kill vermin in fields (Anonymous, 1976). A recent publication (Arivoli and Tennyson, 2012) also reports on the larvicidal efficacy of *S. nuxvomica* leaf extract against the Filarial vector *Culex quinquefasciatus* Say (Diptera: Culicidae).

Therefore, a study was undertaken on the *in silico* analysis of the nematicidal efficacy of *S. nuxvomica* leaf extract and its constituent compounds. The predicted activity against *M. incognita* (J_2) was validated through *in vitro* bioassays.

MATERIALS AND METHODS

Preparation of solvent extracts. Dried and powdered leaves of *S. nuxvomica* (50 g) were added to 100 ml distilled water and boiled for 2-3 minutes. The decoction was filtered, again extracted twice with distilled water (80 ml × 2) and the combined extract was concentrated to 100 ml and used as a stock solution (50%). Dry leaf powder (500 g) of *S. nuxvomica* was also extracted successively with petroleum ether (60-80 °C), methanol and water. The extracts were filtered and concentrated to dryness, under reduced pressure. The residue so obtained was dissolved in ethanol and few drops of 0.2% Tween-20 were added and tested for nematicidal activity.

Nematode cultures. *Meloidogyne incognita* was originally isolated from black pepper (*Piper nigrum* L.) at the Indian Institute of Spices Research, Experimental Farm, Peruvannamuzhi, Calicut (Kerala) and maintained on tomato (*Solanum lycopersicum* L.) in a greenhouse. When large egg masses had formed, the roots were washed free of adhering soil and the egg masses were isolated and incubated in a conical flask containing sterile distilled water at room temperature for hatching. Emerging juveniles were collected and an aqueous nematode suspension (100 active juveniles in 200 µl dis-

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tilled water) was prepared by diluting the standard nematode suspension. Only freshly hatched second stage juveniles collected within 48 h were used in the study.

Bioassay of the extracts. The aqueous leaf extract (5%) was diluted to obtain final concentrations of 2%, 1%, 0.5% and 0.25%. The residues from petroleum ether and methanol extracts were suspended in ethanol, a few drops of 0.2% Tween-20 were added and diluted with water to give 0.5%, 0.25% and 0.13% solutions. One ml of the test solution was poured into each cavity block and 100 second stage juveniles of *M. incognita* were handpicked and added to the cavity blocks. The mortality of nematodes was determined after an incubation period of 24 h. Inactive juveniles that did not move after pricking the tail were considered to be dead. Ethanol containing 0.2% Tween-20 served as control. Each treatment was replicated six times. The mortality was calculated according to following formula

$$\text{Mortality (\%)} = D \times 100/N$$

where D = Number of dead nematodes and N = Total number of nematodes.

In silico *screening of nematicidal compounds*. From literature and web sources (De, 1997; Yin *et al*., 2003; http://ars-grin.gov/duke/) a list of the phytochemicals isolated from *S. nuxvomica* was compiled. The survey of literature indicated that, so far, no phytochemical with nematicidal activity has been reported from *S. nuxvomica.* Therefore the bioactivities of the known phytochemicals were predicted by *in silico* tools (http://ars-grin.gov/duke/,http://195.178.207.233/PASS/AP.html, Filimonov, 1995; http://chemspider.com) and seven compounds that were expected to exhibit nematicidal activity were shortlisted (Tables I and II).

Isolation and identification of nematicidal compounds. The crude aqueous extract of *S. nuxvomica* leaves exhibited 100% nematicidal activity at 2% concentration. Sequential extraction of leaf powder with petroleum ether, methanol and water revealed that the nematicidal activity of the methanol extract was greater than that of the other extracts. Hence, the residue (10 g) from the methanol extraction was subjected to column chromatography over silica gel (60-120 mesh). The column was eluted with petroleum ether-ethyl acetate mixtures of increasing polarity (9:1, 8:2, 7:3, 1:1) and finally with ethyl acetate. Individual fractions were collected, concentrated and based on TLC (thin layer chromatography) analysis, similar fractions were pooled and concentrated. The concentrates were bioassayed and the nematicidal activity of the fractions was tested in 1 ml suspension containing 100 second stage juveniles of *M. incognita* and a range of concentrations of the extracts in cavity blocks and the mortality of nematodes was determined after an incubation period of 24 h along with that in the control. Each treatment was replicated three times. Inactive juveniles that did not move after pricking the tail were considered to be dead. Percentage mortality was calculated.

Thin layer chromatography: Thin layer chromatography of the column-chromatographed fractions was performed on silica gel plates and developed with chloroform-methanol (95:5 and 9:10) as mobile phase. Sulphuric acid (10%) in methanol was used as a spray reagent and the plates were heated at 110 °C for 15-20 minutes. The chief components of the active fractions were identified as strychnine and brucine by co-TLC with the authentic standards purchased from Sigma Chemicals (De and Dutta, 1988).

In vitro *bioassay of nematicidal compounds*. To test the nematicidal activity, brucine and strychnine (Sigma) were dissolved in ethanol and brucine sulphate and strychnine hydrochloride (Hi-Media) were dissolved in water to yield 1000 ppm, 1500 ppm and 2000 ppm solutions. Few drops of 0.2% Tween-20 were added to get clear test solution. The efficacy of these compounds was tested against the second stage juveniles of *M. incognita* in six-well plates. One hundred juveniles were handpicked and added to each well containing 1 ml of test solution, incubated at 28 °C and replicated six times. The plates were covered with the lid, leaving a gap for aeration. Sterile distilled water with equivalent concentrations of ethanol and few drops of 0.2% Tween-20 was used as control. The mortality of the second stage juveniles was determined with the aid of a binocular microscope after 24 h of exposure. Inactive juveniles that did not move after pricking the tail were considered to be dead. Percentage mortality of second stage juveniles over the control was calculated.

Statistical analysis. All data were subjected to analysis of variance (ANOVA) and means compared according to Duncan´s multiple range test. LD_{50} values were worked out by probit procedure using SAS software version 9.2. Before analysis, data of mortality of nematodes were square root-transformed and those of percentages of nematode mortalities were arcsine transformed. All mean values were transformed back to the original units for presentation.

RESULTS AND DISCUSSION

Bioassay of leaf extracts and identification of nematicidal compounds. A positive and significant correlation (r^2 = 0.88) was found between the mortality of nematodes and concentration of leaf extracts. The aqueous leaf extract showed 100% mortality of the nematodes at 2% concentration, followed by 93% at 1%. The least mortality (5%) of the juveniles was recorded at 0.25% concentration and there was no mortality in the control (Fig. 1).

When the leaf powder was successively extracted with petroleum ether, methanol and water, the methanol

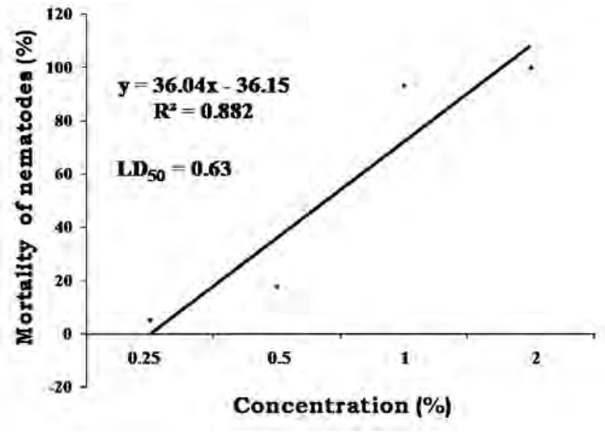

Fig. 1. Nematicidal activity of aqueous leaf extract of *Strychnos nuxvomica* against the second stage juveniles of *Meloidogyne incognita*.

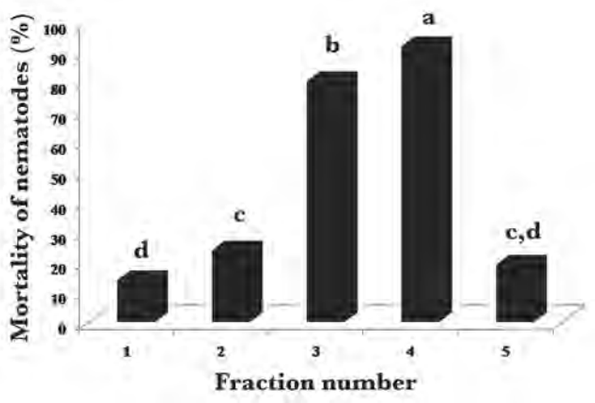

Fig. 2. Nematicidal activity of various fractions of *S. nuxvomica* leaf extract against the second stage juveniles of *M. incognita*. Fraction numbers: 1, eluted with hexane:ethyl acetate 9:1; 2, eluted with hexane:ethyl acetate 8:2; 3, eluted with hexane:ethyl acetate 7:3; 4, eluted with hexane:ethyl acetate 1:1; 5, eluted with ethyl acetate. Bars with different letters indicate significant differences according to Duncan's multiple range test at P = 0.05.

extract showed the greatest nematicidal activity. Bioassay guided fractionation of the methanol extract indicated that the fractions eluted with petroleum ether:ethyl acetate in the ratio 1:1 possessed greater nematicidal activity than other fractions (Fig. 2). There was a significant difference (DF = 4; F = 372.3; P <0.0001) in the mortality of nematodes among the fractions. A positive and significant correlation (r^2 = 0.98) was found between the mortality of nematodes and the concentration of fractions. The chief constituents in these fractions were identified as strychnine and brucine by co-TLC with the authentic standards (De and Dutta, 1988).

In silico *and* in vitro *analysis of nematicidal compounds*. Table I shows the list of predicted biological activities of the known phytochemicals from *S. nuxvomica*. Among the 33 compounds listed, only seven compounds were predicted to possess anthelmintic/nematicidal activity by PASS (Predicted Activity Spectra for Substances) (Table II), which included the chief constituents of the leaf, namely, brucine and strychnine. According to PASS, if the value of Pa (probability to be active) of a compound exceeds 0.5 on a scale of 0 to 1, the compound is expected to reveal the activity in experiments. Among the shortlisted compounds, brucine recorded the highest Pa value of 0.986, followed by brucine-N-oxide (0.859) and strychnine (0.785), indicating a high probability of these compounds possessing nematicidal activity. Most of the compounds isolated from *S. nuxvomica* were neurotoxic and the predicted mode of action was either acetylcholine or cholinergic antagonism (Table I).

In vitro, the constituents brucine, strychnine, brucine sulphate and strychnine hydrochloride showed 67-87%, 41-58%, 32-74% and 66-97% mortality of the nematodes, respectively (Table III). Among these four compounds, strychnine hydrochloride exhibited the greatest nematicidal activity (97%) at 2000 ppm concentration, followed by brucine (87% at 2000 ppm concentration), which was on par with that at 1500 ppm (85%).

Strychnine is reported to have higher animal toxicity than brucine (Anonymous, 1976) but, in the present study, brucine caused higher *in vitro* mortality (67-87%) of the nematodes than strychnine (41-58%) at all concentrations tested (Table III). This is in agreement with the *in silico* results, which also predicted the highest Pa value for brucine (Table II). Strychnine hydrochloride was more effective as a nematicide than its free base, strychnine but this was not true for brucine. Brucine caused higher nematicidal activity than its sulphate salt at all concentrations.

The greater nematicidal activity of strychnine hydrochloride at 2000 ppm could be due to its higher aqueous solubility. Thus, it is interesting to note that, although the solubility of brucine sulphate in water was greater than that of brucine, it was less nematicidal. A possible reason for this could be the difference in membrane permeability caused by the hydrochloride and sulphate moieties. LD_{50} values for brucine, strychnine, strychnine hydrochloride and brucine sulphate were estimated as 665 ppm, 833 ppm, 1505 ppm and 1446 ppm, respectively.

Many plant protection chemicals have been isolated from various plant parts, usually by aqueous or organic extraction methods (Nidiry *et al.*, 1993; Ferraz and De Freitas, 2004). The suppressive effect of phytochemicals on nematode populations has been well documented in several pathosystems (Chitwood, 2002). Zhao (1999) reported the nematicidal activity of the four alkaloids, aloperine, cytosine, N-methylcytisine and matrine on

Table I. Biological activity spectrum of phytochemicals present in *Strychnos nuxvomica* by PASS prediction.

S.N.	Phytochemical	Plant part	Predicted properties
1.	3-Methoxy icajine	Bark	Neurotoxic, acetyl choline receptor antagonist, acetyl choline antagonist, neurotransmitter antagonist
2.	4-Hydroxyl-3-methoxy strychnine	Leaf	Cholinergic antagonist, acetylcholine antagonist, acetylcholine muscarinic antagonist, neurotoxic
3.	4- Hydroxyl strychnine	Leaf	Neurotoxic, toxic, acetylcholine M2 receptor antagonist, acetylcholine antagonist, cholinergic antagonist, acetylcholine muscarinic antagonist
4.	α-Amyrin	Seed	Nephrotoxic, Neurotransmitter uptake inhibitor
5.	α-Colubrine	Seed	Neurotoxic, acetylcholine M2 receptor antagonist, acetylcholine antagonist, acetylcholine muscarinic antagonist, cholinergic antagonist
6.	**Arachidic acid**	Seed	Acetylesterase inhibitor, superoxide dismutase inhibitor, aminoacylase inhibitor, neurotoxic, **anthelmintic (nematicidal)**, nephrotoxic
7.	**Behenic acid**	Seed	Acetylesterase inhibitor, superoxide dismutase inhibitor, neurotoxic, **anthelmintic (nematicidal)**
8.	β-Colubrine	Fruit	Neurotoxic, acetylcholine M2 receptor antagonist, cholinergic antagonist
9.	**Brucine**	Bark, fruit, leaf, root, seed	Neurotoxic, Toxic, Acetylcholine M2 receptor antagonist, Cholinergic antagonist, Acetylcholine antagonist, **Anthelmintic (nematicidal)**
10.	**Brucine-N-oxide**	Fruit	Neurotoxic, cholinergic antagonist, **anthelmintic (nematicidal)**
11.	**Chlorogenic acid**	Seed	**Anthelmintic (nematicidal)**, neurotoxic
12.	C-Mavacurine	Root, bark	Acetylcholine antagonist
13.	Condilocarpine	Seed	Acetylcholine antagonist, cholinergic antagonist
14.	Cycloartenol	Seed	Nephrotoxic
15.	**Deoxyloganine**	Root	Nephrotoxic, **anthelmintic (nematicidal)**
16.	Diaboline	Seed	Neurotoxic
17.	Geissoschizine	Seed	Nontoxic and no destructive activity against nematodes
18.	Hydroxystrychnine	Fruit	Neurotoxic, acetylcholine antagonist, cholinergic antagonist, acetylcholine muscarinic antagonist
19.	Icajine	Fruit	Neurotoxic, acetylcholine antagonist, cholinergic antagonist, neurotransmitter antagonist
20.	Isostrychnine	Seed	Acetylcholine antagonist, cholinergic antagonist, neurotoxic,
21.	Loganinic acid	Seed	Neurotoxic, cholinergic antagonist, acetylcholine antagonist
22.	Loganin	Fruit	Nephrotoxic, neurotoxic
23.	N-Oxystrychnine	Leaf	Neurotoxic
24.	Nor-Macusine	Seed	Nontoxic and no destructive activity against nematodes
25.	Novacine	Fruit	Neurotoxic, acetylcholine antagonist, cholinergic antagonist, neurotransmitter antagonist
26.	O'- Methyl macusine	Seed	Neurotoxic, nephrotoxic
27.	Pseudobrucine	Fruit	Neurotoxic, neurotransmitter antagonist
28.	Pseudo strychnine	Fruit	Neurotoxic, acetylcholine muscarinic antagonist, cholinergic antagonist, acetylcholine antagonist
29.	Secologanin	Fruit	Nephrotoxic
30.	Strychnicine	Leaf	Neurotoxic, neurotransmitter antagonist
31.	**Strychnine**	Bark, Leaf, Root, Fruit	Neurotoxic, toxic, acetylcholine muscarinic antagonist, cholinergic antagonist, **anthelmintic (nematicidal)**
32.	Strychnine- N- oxide	Fruit	Neurotoxic
33.	Vomicine	Fruit	Neurotoxic, toxic, neurotransmitter antagonist

the pinewood nematode, *Bursaphlenchus xylophilus* (Steiner *et* Buhrer) Nickle. Chandravadana *et al.* (1994) reported nematicidal activity of the alkaloid, serpentine. These plant-derived compounds could be developed as nematicides themselves, or could serve as model compounds for the development of environmentally friendly synthetic derivatives.

It is noteworthy that the main components of *S. nuxvomica* leaf, namely brucine and strychnine, which exhibited nematicidal activity in this study, were reported to possess antifungal and antibacterial activity also (Chang *et al.*, 2000). The mode of action of leaf extracts and their components against the nematodes is not clear. These compounds are reported to be neurotoxins.

Table II. Nematicidal activity of compounds from *S. nuxvomica* by PASS prediction.

S. N.	Compound name	Predicted Activity	Pa*
1.	Arachidic acid	Anthelmintic (nematicidal)	0.315
2.	Behenic acid	Anthelmintic (nematicidal)	0.737
3.	Brucine	Anthelmintic (nematicidal)	0.986
4.	Brucine-N-oxide	Anthelmintic (nematicidal)	0.859
5.	Chlorogenic acid	Anthelmintic (nematicidal)	0.620
6.	Deoxyloganin	Anthelmintic (nematicidal)	0.349
7.	Strychnine	Anthelmintic (nematicidal)	0.785

*Pa = Probability to be active.

Table III. Nematicidal activity of compounds from *S. nuxvomica* against second stage juveniles of *Meloidogyne incognita*, after a 24 h incubation period.

Concentration (ppm)	% mortality of nematodes over control*			
	Strychnine	Strychnine hydrochloride	Brucine	Brucine sulphate
1000	41 (39.7)	66 (54.1)	67 (55.0)	32 (34.0)
1500	48 (42.7)	78 (62.5)	85 (67.6)	44 (41.2)
2000	58 (49.8)	97 (82.5)	87 (69.7)	74 (60.57)
Mean	48 (44.1)	80(66.4)	80 (66.1)	50 (45.2)
LD_{50}	1505 ppm	833 ppm	665 ppm	1446 ppm

*Mean of six replicates.
Arc sine value in parenthesis. CD (0.05): Treatments- 5.45; Control-3.57; Treatments × Control - 7.14

The involvement of leaf extracts and their components in interrupting the nematode nervous system is not clear. However, these compounds may disrupt the cell membrane of the nematode and change its permeability.

In conclusion, chemical-intensive management is being widely advocated for nematodes. In view of this and the ban on most of the recommended nematicides in Kerala, there is a need to identify suitable alternative methods for managing nematodes. The leaf extracts of *S. nuxvomica* and its components brucine and strychnine showed interesting nematicidal activity and offer promise as alternatives to synthetic nematicides in the control of *M. incognita.* However, further experiments are required to validate the nematicidal activity under field conditions.

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