

Original Research Article

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Isolation and Identification of Nematicidal Principle in *Lippia nodiflora* against Root Knot Nematode *Meloidogyne incognita*

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ABSTRACT

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The protein and non protein parts present in the leaves of *Lippia nodiflora* were separated and test their nematicidal activity against egg hatching and juvenile mortality of *Meloidogyne incognita*. The non protein part was found to inhibit the root knot nematode egg hatching under in vitro conditions. Leaf extraction was done with different solvent such as petroleum ether, chloroform methanol using soxhlet apparatus and the residue was discarded. Thin layer chromatography method was used to isolate and identify the nematicidal principle present in *Lippia nodiflora*. The identified compounds in non protein parts such as phenols, alkaloids and terpenoids were tested for their nematicidal activity on egg hatching and mortality of *M. incognita* juveniles. The nematicidal assay of these compounds revealed that the alkaloids inhibited egg hatching absolutely and caused juvenile mortality followed by phenols. The terpenoids did not show any marked inhibition on hatching of eggs and causing mortality of juveniles of *M. incognita*.

Introduction

The root knot nematode *Meloidogyne incognita* is a serious threat to the cultivation of agriculture and horticultural crops all over the world. Management of root knot nematodes through nematicides not only polluting the environment and also results in health hazards due to their residual effects. Natural plant products are considered as important source of biopesticide and an alternative to chemicals being used in the management of plant diseases. Recent studies have shown that the importance of plant

products as possible source of non-phytotoxic, systemic and easily biodegradable alternative pesticides. Information on possession of nematicidal and nematostatic properties by several plants and their parts is well documented. Many medicinal plants have the active principle which is used against several disease of human as well as plant. The preliminary studies have shown that *Lippia nodiflora* reduced the egg hatching and increased mortality of root knot nematode juveniles. However, the nature of specific compounds of *Lippia nodiflora* is responsible for nematicidal activity has not yet been

elucidated. The isolation and identification of nematicidal principle of the phytochemicals will be helpful in synthesizing novel bionematicide. Keeping this in view the present study was taken up to identify the nematicidal compounds present in *Lippianodiflora*.

Materials and Methods

Separation of the protein and non-protein part from the leaf extracts

The protein and non-protein part was separated from the aqueous leaf extract by the method given by Bradford (1976). A sample of 40g fresh leaves of *Lippianodiflora* was macerated with 100ml distilled water using pestle and mortar. The aqueous extract was filtered through a four play muslin cloth in order to remove plant debris and then centrifuged for five minutes at 4000 rpm under 4°C. Ammonium sulphate (52.3g) was added to the supernatant solution and it was kept overnight in a refrigerator followed by centrifugation at 10,000 rpm under 4°C for 20 minutes. The supernatant of the sample of non-protein part was transferred to separate flasks. The pellet, settled at the bottom was the protein part and it was dissolved in equal quantity of sterile distilled water and transferred to dialysis tubes, kept in a beaker filled with water. The water was changed every 12h for one day and stored in a refrigerator. This dialysis was done to discard the ammonium sulphate from protein part. Thereafter the resultant protein and non-protein part were taken for hatching study to check which part was inhibitory in nature.

Hatching study

One ml of protein part and 9ml of distilled water was pipette out into petri dishes. Likewise 1 ml of non-protein part and 9ml of distilled water was pipette out into petri dishes. Single egg mass was transferred into

each petri dish containing test compound. One egg mass taken in 10ml distilled water was served as control. Number of juveniles hatched was recorded every day up to seven days.

Leaf extraction by different solvent using Soxhlet apparatus

Fresh leaf material was cut into small pieces and ground well after drying in oven at 60°C for 48 hours. Leaf extraction was done with petroleum ether (40-60°C) from the leaf powder thimble in a filter paper and kept in soxhlet apparatus. The crude petroleum ether extract was collected and the residue was again used for chloroform extract. The crude petroleum ether extract was collected and the residue was again used for chloroform extract. The crude chloroform extract was collected and the residue was again used for methanol extract. Crude methanol extract was collected and the residue was discarded.

Identification of the nematicidal compound using Thin Layer Chromatography (Srivastava and Srivastava, 1991)

Preparation and activation of TLC plate

Thin layer chromatography method was used to isolate and identify the nematicidal principle in *Lippia nodiflora*. Silica Gel-G was used for preparing TLC plates (20x20 cm). Powdered silica gel weighing 40g was thoroughly mixed with 80ml of distilled water. The slurry was then poured into TLC applicator, which was adjusted for 0.5mm thickness and it was slowly moved o the clean glass plate. The glass plates were allowed to dry in open air for an hour. Then the glass plate with silica gel coating was heated in an oven at 110°C for 2 hours. Thus the TLC plate was activated. After activation of the TLC plate, sample (20µl) was spotted on the plate with the help of micropipette without disturbing the silica gel layer.

Separation of compounds

The solvent systems used were acetic acid: chloroform (1:9), methanol: chloroform (1:9) and chloroform: methanol (1:1). The solvent was poured into the TLC tank and then the TLC plate was kept into the tank so as to immerse (0.5cm) into solvent at the bottom. The tank was closed with a glass lid so as the chamber filled with the solvent vapours completely. Within two hours the solvent front reaches the end of the TLC plates. Then the plate was removed from the tank and kept in open air at room temperature so as to enable the solvent to get evaporated. Then the plates were sprayed with chromogenic reagent like Ten per cent Vanillin-Sulphuric acid, Dragendorff's reagent, Diazotized sulphailic acid solution, Folin-Ciocalteu reagent, Ethanolic Alumionium chloride, Methanolic KOH so as to identify the compounds present in the leaf extract based on the coloured spots and the R_f (Relative front= Distance moved by the solute from the origin/Distance moved by the solvent from the origin) values were recorded. To screen the saponins, a small amount of all the three crude extract was placed in test tubes and to it 2ml of water was added. The tubes were shaken vigorously and the formation of persistent foam was indicated the presence of saponins. A drop of methyl alcohol was added to enhance the further formation of foam. After development of the compounds, the corresponding place was scraped off. This scraped off material was eluted with methanol and recovered in a relatively pure form. This was used for egg hatching and mortality study to confirm the nematicidal action.

Hatching study

One egg mass was transferred into each petri dish containing eluted compound. One egg mass taken in 10ml distilled water was served as control. Number of juveniles hatched was recorded seven days after initiation.

Mortality test

The nematode, *M. incognita* inoculum required for the experiment was obtained from *Coleus* sp. Raised in glasshouse. The egg masses were collected from the pure culture and incubated for hatching at room temperature for seven days. Freshly hatched second stage juveniles were used for mortality test. Hundred juveniles were introduced in each petridish containing eluted compound and petridish with distilled water was served as control.

Results and Discussion

The protein and non-protein part of aqueous leaf extracts were tested for their influence on egg hatching under laboratory conditions. The protein part was found to have lesser inhibition effect compared to non-protein part (Table 1) as 343 juveniles were hatched from one egg mass in protein part, and it was only 5 in non protein part on 7th day of exposure.

Detection of active compounds by thin layer chromatography

The petroleum ether, chloroform and methanol extracts of *Lippia nodiflora* leaves were prepared and subjected to thin layer chromatography using different solvent systems and different chromogenic reagents. The few spots with different R_f values were observed in TLC plates on visual observation. The spots were identified based on the colour formation after spraying of different chromogenic reagents (Table 2).

Phenolic compounds

The phenolic compounds from leaf extracts were separated by TLC with acetic acid: chloroform (1:9) as solvent, and diazotized sulphanilic acid and folin-ciocalteu reagent as chromogenic reagent. Single circular spot with R_f value of 0.842 appeared as orange

colour when sprayed with diazotized sulphanic acid. Similarly spot with Rf values of 0.843 appeared as bluish when sprayed with folincioalteau reagent. Orange and bluish coloured spots indicated the presence of the phenolic compounds.

Terpenoid compounds

The solvent of methanol: chloroform (1:9) and 10 per cent vanillin sulphuric acid was used for isolation of terpenoid compounds from the leaf extract. Three spots were noticed which turned into greenish spot when sprayed with

10% vanillin sulphuric acid which indicated the presence of terpenoid compounds.

Alkaloid compounds

In order to separate alkaloids from leaf extracts, the solvent chloroform: methanol (1:1) and Dragendorffs reagent were used. Presence of three spots was noticed on visual observation. Single spot with Rf value of 0.892 turned into yellow colour when sprayed with Dragendorff’s reagent which indicated the presence of alkaloid compounds.

Table.1 Effect of protein and non protein part on egg hatching of *M. incognita*

Leaf extract	Number of juveniles at different days of intervals						
	1	2	3	4	5	6	7
Protein part	269	284	320	336	342	342	343
Non-protein part	-	-	4	4	5	5	5
Control	332	350	369	375	387	387	387

Table.2 Rf values of different spots on visual observation and compounds sprayed with different reagent in the isolation of nematicidal principles using TLC

Chromogenic reagent	Petroleum ether		chloroform		Methanol	
	Visual Obser vation	Colour of Nematicidal Compounds	Visual observation	Colour of Nematicidal Compounds	Visual observation	Colour of Nematicidal Compounds
Dragendorffs	0.892	Yellow	0.865	-	0.827	-
Folin-ciocalteau	0.843	Blue	0.784	-	0.746	-
Diazotized sulphanic acid	0.842	Orange	0.589	-	0.637	-
	0.705	-	-	-	-	-
Vanillin-Sulphuric acid	0.827	-	0.876	Green	0.892	-

Table.3 Effect of different compounds on inhibition of egg hatching of *M. incognita*

Compounds	Per cent inhibition
Phenols (Diazotized sulphanic acid)	92
Phenols (Folin-ciocalteau)	94
Alkaloids	100
Unsaturated terpenoids	6

Table.4 Influence of different compounds on juvenile mortality of *M. incognita*

Compounds	Mortality (%)		
	1 st day	2 nd day	3 rd day
Phenols (Diazotized Sulphanilic acid)	82	100	100
Phenols (Folin-ciocalteau)	85	100	100
Alkaloids	92	100	100
Unsaturated terpenoids	5	7	10
Control	-	-	8

Effectiveness of active compounds on egg hatching and juvenile mortality

The compounds such as phenol, alkaloids and unsaturated terpenoids were tested on egg hatching and juvenile mortality (Table 3 and 4). Among the compounds alkaloids eluted from Rf value 0.892 showed cent per cent inhibition of egg hatching and increased *M. incognita* juvenile mortality. Phenols eluted from Rf value of 0.842 (diazotized sulphanilic acid) and Rf value of 0.843 (folinciocalteau reagent) showed inhibition of egg hatching as well as increased juvenile mortality. The unsaturated terpenoids did not show any inhibition on egg hatching and juvenile mortality of root knot nematode.

Lippianodiflora is an evergreen plant having many medicinal properties. Earlier studies on alcoholic extracts of rhizome showed good anti-helminthic activity against *Ascaris lumbricoides* in human beings under in vitro condition (Raj, 1975). The preliminary studies made on *L. nodiflora* showed possession of nematicidal properties against eggs and juveniles of root knot nematode *Meloidogyne incognita*. The protein and non protein parts separated from the aqueous leaf extract tested further for their effect on hatching revealed that non protein part from leaves of *L. nodiflora* possessing nematicidal action. Thin layer Chromatography was used to identify and isolate the nematicidal

compound present in the non protein part of the leaves. Phenols at Rf value 0.843 (blue) and 0.842 (orange), alkaloids at 0.892 (yellow) and terpenoids at 0.876 (green) spots were identified. Terpenoids present in chloroform extract, whereas phenols and alkaloids present in petroleum ether extract of leaves. Alkaloids and phenols were found to inhibit the egg hatching and increase juvenile mortality when compared to terpenoids. In the present studies alkaloids and phenols were found to be responsible for nematicidal activity against root knot nematode *M. incognita* juveniles. Further studies on this line may throw light on feasibility of evolving commercial botanical pesticides against root knot nematode.

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