

Nematicidal and quantitative phytochemical analysis of *Allamanda cathartica* chromatographic fractions

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ABSTRACT

The environmental pollution of synthetic nematicides is enormous, due to their residual effects and non-degradability. This study investigated the possibility of a green alternative to replace the pollution problems of synthetic nematicides. The activity of plant secondary metabolites against plant parasitic nematodes was tested in-vitro using chromatographic isolates from the stem and leaves of *Allamanda cathartica*. Four isolates from the extracted plant materials were tested at various concentrations (0 mg/mL, 10 mg/mL, 20 mg/mL and 30 mg/mL) against *Meloidogyne incognita* juveniles and eggs via inhibition of egg hatch and larval mortality tests. The result showed that egg hatch was totally inhibited among all the isolates, few hatches were recorded in the 10mg/ml (0.26%) concentration while the control (0mg/ml) had 48.39% hatch on day 5. Isolate four showed strong nematicidal effect, with percentage mortality of 16.87 % after one hour of exposure comparable with the synthetic nematicide (carbofuran). A hundred percent juvenile mortality was achieved in all the isolates on day 2. However, the leaves of *A. cathartica* were more effective than the stem. Phytochemical screening revealed that the quantities of secondary metabolites were more in the leaves of *A. cathartica* than in the stems. Infra-red spectroscopic data of three of the isolates revealed the presence of alkanes (2930, 2923 and 1460 cm^{-1}) aldehydes (2855, 2851 and 1733 cm^{-1}) and phenols at (3650, 1115 cm^{-1}). Aliphatic amines (3200, 1460 and 1600 cm^{-1}) and non-cyclic ethers (1310, 1300, 1240 and 1000 cm^{-1}) are present in isolate four. This study showed that the leaves of *A. cathartica* could serve as a bio-nematicide to reduce environmental pollution.

Key words: Extraction, n-hexane, isolation, carbofuran, *Meloidogyne incognita* chromatography.

INTRODUCTION

Allamanda cathartica (L.) is an ornamental plant in the Apocynaceae family. It is an evergreen vine like woody shrub which may reach a freestanding height of about two metres or more. It is native to South and Central America (Garden guides, 2012). The all year round

production of bright flowers has made it a popular ornamental. *Allamanda cathartica* is notable for its medicinal properties because all parts of the plant contain allamandin, a toxic iridoid lactone (Barcellos, 2002). The leaves, roots and stems are used in the preparation of a powerful cathartic (Knowles, 1992) the

milky sap is also known to possess antibacterial and anticancer properties (Knowles, 1992). Gardeners exposed to the sap are known to develop rashes, itch and blisters (Knowles, 1992). It is mainly used in the treatment of malaria (Patil and Kulkarni, 1996). *Allamanda* spp is renowned for its vigorous growth and this has endeared it to farmland owners who use it either as a ground cover or for hedges and screens (Barcellos, 2002). *Allamanda cathartica* extracts has shown remarkable wound healing activity and it has been suggested for treating various wounds in humans (Nayak et al., 2006). Studies of tissues treated with *Allamanda* extracts showed significant increase in collagen deposition which is attributed to the phyto-constituents in it (Patil and Kulkarni, 1996). The leaf extracts of *A. cathartica* have been implicated in snake bite treatment because of its anti-haemorrhaging properties (Knowles, 1992). Globally plant parasitic nematodes are one of the major factors reducing crop yield all over the world. Severe crop losses do affect the economy of a country adversely, plunging the nation into food scarcity (Hafeez, 1986). Among a total of about 70 species of *Meloidogyne* identified so far (Luc et al., 1990; Makete et al., 2012), only four are widely distributed throughout the agricultural regions of the world, these include *Meloidogyne incognita*, *Meloidogyne arenaria*, *Meloidogyne javanica* and *Meloidogyne hapla* (Luc et al., 1990; Makete et al., 2012). *Meloidogyne incognita* is the most abundant and damaging phytonematode in Nigeria, infecting about 100 plant species (Makete et al., 2012). Most plant parasitic nematodes are highly pathogenic causing serious yield losses in ornamentals, cereals, vegetables, fruits and tree crops (Hussain et al., 2012).

Synthetic nematicides have been used to combat the problem of plant parasitic nematodes and this approach no doubt minimises the losses caused by plant

parasitic nematodes effectively (Fabiyi, 2016). However diverse pollution problems such as soil contamination, destruction of the ecosystem amongst others and adverse effect on the farmers health have been reported as banes to the use of synthetic nematicides. To tackle the problem of synthetic pesticide pollution, investigations are being made into the nematotoxic and nematicidal potential of plant metabolites. Several studies have established the importance of bio-nematicides as a good source of non toxic, biodegradable alternative to synthetic nematicides (Fabiyi et al., 2016). Therefore, considering the pollution problems of synthetic nematicides, the search for available and abundant sources of bio-nematicides is an important issue. In this context, this research was imperative to determine the nematicidal activity of the leaves and stem of *Allamanda cathartica* on *Meloidogyne incognita*.

MATERIALS AND METHODS

Extraction of Plant Materials

Allamanda cathartica plant materials were collected from Ilorin town (located on latitude 8°30' and 8°50'N and longitude 4°20' and 4°35'E of the equator) in Kwara state Nigeria. The plant was identified with herbarium samples deposited at the Deptment of Plant Biology University of Ilorin Nigeria. 500 mg each of air dried leaves and stems of *A. cathartica* were weighed and chopped into small pieces of 1-2 cm. The chopped materials were then transferred separately into extraction bottles and 20 litres of n-hexane was poured into each of the extraction bottles, the bottles were then covered tightly and the set ups were left to extract for five days. After five days the extracts were decanted and filtered with whatmans no 1 filter paper into two separately labelled flasks. Each extract was concentrated under reduced pressure using rotary evaporator.

Isolation of Chemical Constituents

The crude extracts were spotted on thin layer chromatographic plates (TLC) after which they were developed in the developing chamber using n-hexane. After some time the plates were removed and the solvent front marked. No visible coloured substance was observed on the TLC plate, but with the aid of the ultra-violet (UV) lamp 254 nm (short wave length) a pink fluorescence was observed in the two extracts also under the 366 nm (long wave length) a blue fluorescence was seen before spraying with vanillin spray reagent. After spraying, a dark purple colour component was seen in the middle of the chromatogram. These observations led to the isolation of the components using the technique of Preparative Thin Layer Chromatography (PTLC). The crude extracts (leaves and stems) were loaded on the PTLC plate with a micropipette, 150 mL of n-hexane was measured into the developing chamber. Thinning was done briefly to ensure that the plant components rise uniformly from the origin of the chromatographic plate. After development, a light yellow coloured line was observed at the solvent front of both plates. The plates gave a blue fluorescence under the UV lamp (366 nm), and the two plates also gave colour reactions when sprayed with vanillin reagent. Thin layer chromatography was carried out on the isolates obtained from the stem and leaf extracts to ascertain their purity.

Isolate 1 (stem): This had a blue fluorescence under the UV lamp (366 nm) but after spraying and heating there was no colour reaction observed.

Isolate 2 (stem): There was no fluorescence observed in this isolate when viewed under the UV lamps, that is long and short wave lengths. However, after spraying and heating at 60°C there was colour reaction on the TLC plate.

Isolate 3 (leaves): The fluorescence observed here were three in number under the UV before spraying; this fluorescence was closely knitted together. After spraying with vanillin spray reagent, a purple and green colour was observed close to the solvent front, while a purple colour was close to the origin. This was however considered for re-isolation on the PTLC plate.

Isolate 4 (Leaves): There was no fluorescence observed on the TLC plate under the UV lamps. After spraying, three spots were observed each with different colours. The procedure of isolation was repeated several times so as to have large quantity of isolated substances.

The isolates 1, 2, 3 & 4 were weighed and each was 500, 650, 530 and 712 mg respectively. Infra-red (IR) spectra of the isolates were recorded on SHIMADZU 8400s Fourier Transform Infra-red (FTIR) spectrophotometer at the Department of Chemical Sciences Redeemer's University Mowe, Lagos State, Nigeria.

Nematicidal Assay

Each of the four isolates was dissolved separately in 100 mL distilled water at 10 mg, 20 mg and 30 mg. The experimental design was a 5x4x3 factorial experiment conducted in completely randomised design involving five treatments at four levels, while each concentration was replicated three times and ordinary distilled water served as control. *Meloidogyne incognita* juveniles were assessed by chopping previously infected galled roots of *Celosia argentea* into tiny pieces of 1-2 cm. The chopped galled roots were shaken with 0.6 % sodium hypochlorite in a beaker for two minutes to digest the gelatinous matrix encasing the eggs. The recovered eggs were incubated for 24 hours at 27°C to hatch out the second-stage juveniles. The isolates were tested alongside a commercial synthetic nematicide (carbofuran) for juvenile

mortality and egg hatch, with 30 juveniles and 30 eggs in the counting dish for each test. The number of dead juveniles was counted after an hour of exposure, then every two hours until the 8th hour and later on daily basis for three days, while hatched eggs were counted daily for five days. All values obtained were converted to percentage of the total number of juveniles and egg.

Statistical Analysis

All data collected daily on percentage juvenile mortality and egg hatch were subjected to analysis of variance (ANOVA) and significant means separated with the new Duncan's multiple range tests (Gomez and Gomez, 1984). Alphabets assigned to denote significance.

Quantitative Analysis of Phytochemicals

The stems and leaves of *A. cathartica* were analysed for the quantity of phytochemicals contained in them.

Flavonoids: Twenty grams (20 g) each of the chopped pieces of *A. cathartica* stem and leaves were weighed separately into 2M hydrochloric acid; this was heated on the water bath for 35minutes. The extract was filtered with Whatman's no 1 filter paper and weighed.

Alkaloids: Twenty gram (20 g) of the plant material was weighed into a conical flask and 200 mL of 10% acetic acid in ethanol was added and allowed to stand for four hours. This was filtered and concentrated over water bath. Five drops of ammonium hydroxide was added to achieve precipitation while stirring on the magnetic stirrer. The precipitate was later filtered and this was taken as the crude alkaloid (Harborne, 1973).

Phenols: The plant materials were boiled with ether in a conical flask. 15 mL of the extract was taken into a 100 mL flask and 5 mL of distilled water was added. 5 mL of alcohol and 2 mL of ammonium hydroxide

was also added and the content of the flask was left for a while for a colour reaction to take place (Obadoni and Ochuko, 2001).

Saponins: Each of the plant material was soaked in 100 mL ethanol and stirred on the magnetic stirrer for 6hrs at 55⁰C. The mixture was filtered and the residue was extracted again with aqueous ethanol. The ethanol extract was concentrated on water bath. The extract was partitioned into diethyl ether in a separating funnel. The ether layer was discarded while the aqueous layer was recovered and 60 mL of n-butanol was added and later washed with 5% sodium chloride. The sample mixture was dried in the oven to a constant weight and the percentage saponin was calculated (Obadoni and Ochuko, 2001).

RESULTS

Table 1 shows the result of the quantitative phytochemical screening conducted on the leaves and stems of *A. cathartica*. The leaves had larger quantities of secondary metabolites than the stem; 88 mg of alkaloid was detected in the leaves as opposed to 39 mg in the stem. The activity of the various isolates on percentage mortality and percentage egg hatch is depicted in Tables 2 and 3. Isolate 4 was significantly ($p<0.05$) more effective than isolate 3 with a percentage mortality of 44.78% as opposed to 40.21% at four hours of exposure of juveniles to treatments. There was no significant difference observed between isolate 1&2 throughout the period of observation. The level of treatment application had a significant ($p<0.05$) effect on percentage mortality; however the third level of application (30mg/mL) was significantly more effective than all the other levels of dosage application (Table 2). There was total egg hatch inhibition in carbofuran (a standard nematicide) as well as the isolates from *A. cathartica* throughout the duration of the experiment. The lowest (10mg/mL) level of application recorded a few hatches (0.26%) that are significantly different

from the control which had 48.39% on day 5 of the experiment (Table 3). The highest juvenile mortality was recorded in day 2 from carbofuran (CBFN) which had 100% while isolate 4 had 97.07% (Table 2). In general leaf isolates are more effective than stem isolates. The spectral data of the isolates revealed the N-H stretch of amines and aliphatic amines at 3200, 1460 and

1600 cm^{-1} , C-O of non-cyclic ethers at 1310, 1300, 1240 and 1000 cm^{-1} , C-H of alkanes at 2930, 2923 and 1460 cm^{-1} , C=O and C-H of aldehydes at 2855, 2851 and 1733 cm^{-1} and O-H and C-O of phenols at 3650, 1115 cm^{-1} . These functional groups are associated with iridoid which is a major constituent of *A. cathartica*.

Table 1: Phytochemicals in Leaves and Stem of *Allamanda cathartica*

Plant Material	Alkaloids mg/kg	Flavonoids mg/kg	Saponins mg/kg	Tannins mg/kg
Stem	39	23	36	29
Leaves	88	82	71	59

Table 2: Effect of *Allamanda cathartica* isolate on *M. incognita* juvenile mortality

Treatments	Exposure time of juveniles to isolates						
	1hr	2hrs	4hrs	8hrs	Day1	Day2	Day3
Isolate1 (stem)	9.00 ^c	19.11 ^c	33.68 ^c	56.03 ^c	90.83 ^d	100.00 ^a	100.00 ^a
Isolate2 (stem)	8.75 ^c	18.97 ^c	34.01 ^c	55.73 ^c	91.13 ^d	100.00 ^a	100.00 ^a
Isolate3 (leaves)	12.15 ^b	23.08 ^b	40.21 ^b	60.45 ^b	92.23 ^c	100.00 ^a	100.00 ^a
Isolate4 (leaves)	16.87 ^a	27.89 ^a	44.78 ^a	65.00 ^a	97.07 ^b	100.00 ^a	100.00 ^a
CBFN	17.08 ^a	28.15 ^a	45.00 ^a	66.05 ^a	100.00 ^a	100.00 ^a	100.00 ^a
Level (mg/mL)							
0	0.00 ^d	0.00 ^d	0.00 ^d	0.00 ^d	0.00 ^d	2.16 ^d	3.71 ^b
10	3.21 ^c	10.01 ^c	24.25 ^c	46.60 ^c	65.58 ^c	86.12 ^c	100.00 ^a
20	8.33 ^b	16.29 ^b	31.18 ^b	52.72 ^b	74.00 ^b	93.64 ^b	100.00 ^a
30	11.21 ^a	20.15 ^a	39.80 ^a	60.24 ^a	81.20 ^a	100.00 ^a	100.00 ^a

Values with different alphabets along the same column are statistically different at $p < 0.05$

Table 3: Effect of *Allamanda cathartica* isolate on *M. incognita* egg hatch

Treatments	Exposure time of eggs to isolates				
Treatments	Day1	Day2	Day3	Day4	Day5
Isolate1 (stem)	0.00	0.00	0.00	0.00	0.00
Isolate2 (stem)	0.00	0.00	0.00	0.00	0.00
Isolate3 (leaves)	0.00	0.00	0.00	0.00	0.00
Isolate4 (leaves)	0.00	0.00	0.00	0.00	0.00
CBFN	0.00	0.00	0.00	0.00	0.00
Treatment Level (mg/mL)					
0	12.61 ^b	21.22 ^b	30.00 ^b	37.29 ^c	48.39 ^c
10	0.00 ^a	0.00 ^a	0.07 ^a	0.18 ^b	0.26 ^b
20	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
30	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a

Values with different alphabets along the same column are statistically different at $p < 0.05$

DISCUSSION

The presence of the different phytochemicals in various amounts in the stem and leaves of *A. cathartica* could be associated with the nematicidal activity observed in the various isolates. The significant nematicidal effect of the isolates from leaves over those of stem is also reflected in the quantity of plant metabolites present in the leaves. The functional groups revealed the presence of iridoid the toxic constituent of *A. cathartica*. The confirmation and presence of iridoid could be associated to the observed nematicidal activity in the leaf and stem of *A. cathartica*. Tiwari et al., (2003) established the antibacterial activity of the constituent of *A. cathartica*. Several researchers have established the toxicity of various plant secondary metabolites to plant parasitic nematodes. Wani and Bhat, (2012) reported a significant reduction in *M. incognita* population in soils amended with nimin and other natural oils. Products obtained from different plants have been used in the control of plant parasitic nematodes (Leela et al., 1992; Javed et al., 2008). Bio-pesticides such as heptadecan-4-olide and olen-12-en-3yl acetate from the leaves of *Alstonia boonei* and *Bridelia ferruginea* have been indicated to be nematicidal to the root-knot nematode *M. incognita* (Fabiya et al., 2012). Investigations by Meher et al., 1988 revealed that natural glycosides from the seeds of *Asparagus adscendens* are toxic to *M. incognita* at concentrations as low as 200 µg/mL under laboratory conditions.

CONCLUSION

This research has demonstrated that the leaves and stem of *Allamanda cathartica* contain effective nematicidal compounds. The biopesticide activity displayed by the leaves and stem of *A. cathartica* is directly proportional to the quantity of phytochemicals present in the plant. The nematicidal activity of *A. cathartica* is

being reported for the first time in literature. The plant could serve as a good alternative in the control of *M. incognita* and plant parasitic nematodes in general. A further investigation is suggested for the application of the isolates under field conditions.

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