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NEMATICIDAL AND QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF THE CHROMATOGRAPHIC FRACTIONS FROM THE LEAF AND STEM OF *Allamanda cathartica* (L).

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Abstract
The environmental pollution of synthetic nematicides is enormous, due to their residual effects and non-degradability. This study was conducted to investigate the possibility of a green alternative that will eventually 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. Isolates from the extracted plant materials were tested at various concentrations (0 mg/mL, 0.06 mg/mL, 0.1 mg/mL and 0.2mg/mL) against Meloidogyne incognita juveniles and eggs. The effect was determined through testing the inhibition of egg hatch and larval mortality. The result showed that egg hatching was totally inhibited among all the isolates at different concentrations except in the control which had 48.39% on day 5. The isolates showed strong nematicidal effect, with percentage mortality of 16.87% at the first hour of exposure which compared well with the commercial nematicide (oxamyl). However the leaves of A. cathartica proved more effective than the stem. Preliminary phytochemical screening revealed that the quantities of secondary metabolites are more in the leaves of A. carthatica 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 1733cm-^{1}) and phenols at (3650; 1115cm-^{1}). Aliphatic amines (3200; 1460 and 1600 cm-^{1}) and non-cyclic ethers (1310; 1300; 1240 and1000cm-^{1}) are present in isolate four. This study showed that the leaves of A. cathartica could serve as a bio-pesticide to reduce environmental pollution

Key words: *Extraction, n-hexane, Isolation, Oxamyl, Nematodes, 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 free-standing height of about 2meters 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. *A. cathartica* is notable for its medicinal properties; all the 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 anti cancer 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* is renowned for its vigorous growth and this has endeared it to farmland owners who use it as a groundcover or for hedges and screens (Barcellos, 2002). *A. cathartica* extracts has shown remarkable wound healing activity and it has been suggested for treating various wounds in humans (en.wikipedia.org). Studies of tissues treated with *Allamanda* extracts showed significant increase in collagen deposition, this property of *Allamanda* is attributed to the phyto-constituents in it (Patil and Kulkarni, 1996). The extracts of *Allamanda* has been indicated in snake bite treatment, because of its anti- haemorrhaging properties (Knowles, 1992). Plant parasitic nematodes are a major factor reducing crop yield all over the world. Severe crop losses do adversely affect the economy of a country, plunging the nation into food scarcity (Hafeez, 1986). Among a total of about 70 species of *Meloidogyne* identified so far (Luc *et al*., 1990), only four are widely distributed throughout the

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agricultural regions of the world, these include *Meloidogyne incognita*, *Meloidogyne arenaria*, *Meloidogyne javanica* and *Meloidogyne hapla*. *Meloidogyne incognita* is the most abundant and damaging in Nigeria, infecting about 100 plant species. Most nematodes are highly pathogenic causing serious yield losses in ornamentals, cereals, vegetables, fruits and tree crops. Synthetic nematicides have been used to combat the problem of plant parasitic nematodes, this approach no doubt minimised the losses caused by plant parasitic nematodes, but with pollution problems such as soil contamination, destruction of the ecosystem and adverse effect on the farmers health. 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. 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.*

Materials and Methods

Extraction of Plant Materials

The plant materials were collected from Ilorin town in Kwara state Nigeria. The plant was identified by comparing with herbarium samples deposited at the Dept of Plant Biology University of Ilorin Nigeria. The air dried leaves and stems were weighed (500mg each) and chopped into small pieces and then transferred separately into extraction bottles, two litres of n- hexane was poured into each of the extraction bottles; the bottles were then covered tightly and the materials were left to extract for five days each. After five days the extracts were decanted and filtered 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 lamp 254nm (short wave length) a pink fluorescence was observed in the two extracts, while under the 366nm, (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 isolating 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 U.V lamp (366nm), 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 leave extracts to ascertain their purity.

Isolate 1 (stem): This had a blue fluorescence under the UV lamp (366nm), 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 60^0C 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 respective isolates were weighed and each afforded 14, 16, 12 and 15 mg respectively. Infra-red (IR) spectra of the isolates were recorded on SHIMADZU 8400s FTIR (Fourier Transform) spectrophotometer at the Chemistry

Department, Redeemer's University Mowe, Lagos, Nigeria.

Nematicidal Assay

The four isolates were dissolved separately in 50 mL distilled water at 10mg, 5mg and 3mg, thus making 0.2mg/ml; 0.1mg/ml and 0.06mg/ml respectively. The experimental design was a 4x4x3 factorial experiment conducted in completely randomised design (CRD). Involving four treatments at four levels, while each concentration was replicated three times and ordinary distilled water served as control. A total of forty eight counting dishes were used in all. *Meloidogyne incognita* juveniles were assessed by chopping previously infected roots of *Celosia argentea* into tiny pieces, the roots were shaken with 0.8% sodium hypochlorite 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 nematicide (Oxamyl).

Statistical Analysis

All data collected were subjected to analysis of variance (ANOVA) and significant means separated with the Duncan's multiple range test (Gomez and Gomez, 1984).

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 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 100mL ethanol and stirred on the magnetic stirrer for 6hrs at 55^0C. 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 and Discussion

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. 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 treatment. 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 (0.2mg/mL) was significantly more effective than all the other levels of dosage application (Table 2). There was total egg hatch inhibition in oxamyl (a standard nematicide) as well as the isolates from *A. cathartica* throughout the duration of the experiment; the lowest (0.06mg/mL) level of application recorded a few hatches (0.26%) that are significantly different from the control which had 48.39% on day5 of the experiment (Table 3).

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. Several researchers have

established the toxicity of various plant secondary metabolites to plant parasitic nematodes. Wani and Bhat, 2012 stated 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 (Fabiyi *et al*., 2012). Investigations by Meher *et al.,* 1988 revealed that natural glycosides from the seeds of *Asparagus adescendens* are toxic to *M. incognita* at concentrations as low as 200 μg/mL. The Infra-red spectra of the various isolates revealed the presence of some functional groups from which the likely compounds present in *A. cathartica* could be inferred. 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 and1000cm^{-1}, C-H of alkanes at 2930; 2923 and 1460 cm^{-1}, C=O and C-H of aldehydes at 2855; 2851 and 1733cm^{-1} and O-H and C-O of phenols at 3650; 1115cm^{-1}

Conclusion

This research has demonstrated that the leaves and stem of *Allamanda cathartica* contain effective nematicidal principles. The bio-pesticide 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.

Table 1: The quantity of phytochemicals in the leaves and stems of *Allamanda cathartica*

Plant material	Alkaloids mg	Flavonoids mg	Saponins mg	Tannins mg
Stems	39	23	36	29
Leaves	88	82	71	59

Table 2: Effects of *Allamanda cathartica* isolates on *M. incognita* juvenile mortality

	Exposure Time of Juveniles to Isolate						
Treatments	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}
Oxml	17.08^{a}	28.15^{a}	45.00^{a}	66.05^{a}	100.00^{a}	100.00^{a}	100.00^{a}
S.EM	0.51	0.83	0.98	1.15	1.41	1.71	1.93
Treatment 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}
0.06	3.21^{c}	10.01^{c}	24.25^{c}	46.60^{c}	65.58^{c}	86.12^{c}	100.00^{a}
0.1	8.33^{b}	16.29^{b}	31.18^{b}	52.72^{b}	74.00^{b}	93.64^{b}	100.00^{a}
0.2	11.21^{a}	20.15^{a}	39.80^{a}	60.24^{a}	81.20^{a}	100.00^{a}	100.00^{a}
S.E.M	0.27	0.36	0.43	0.49	0.60	0.73	1.02

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

Table 3: Effects of *Allamanda cathartica* isolates on *M. incognita* egg hatch

	Exposure Time of Eggs to Treatment				
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
Oxml	0.00	0.00	0.00	0.00	0.00
S.E.M	0.00	0.00	0.00	0.00	0.00
	NS	NS	NS	NS	NS
Treatment Level (%)					
0	12.61^{b}	21.22^{b}	30.00^{b}	37.29^{c}	48.39^{c}
0.06	0.00^{a}	0.00^{a}	0.07^{a}	0.18^{b}	0.26^{b}
0.1	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
0.2	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
S.E.M	0.08	0.11	0.23	0.29	0.32

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

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