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Characterisation of an antinematicidal compound from *Leucaena leucocephala*

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Abstract. *Leucaena leucocephala* is a small tropical tree used for a variety of purposes in agriculture, land management and homeopathic medicine. Quercetin, a flavonoid, was isolated and characterised from extracts of leaves of *L. leucocephala* and its effects on egg hatching and juvenile mortality of *Meloidogyne incognita* were investigated at 0.8, 0.4 and 0.2% *in vitro*. The compound was highly toxic to eggs and juveniles of the nematode at the three rates tested.

Additional keywords: phenolic compound, root-knot nematode.

Introduction

Globally, the most important plant-parasitic nematodes are the root-knot nematodes, *Meloidogyne* spp., since they infect the majority of the economically important species of plants in the world (Trudgill and Blok 2001). This genus contains at least 80 species (Karssen 2002) reported to cause an estimated US$100 billion loss per year worldwide (Oka *et al*. 2000).

Over the last 30–40 years, agricultural chemicals have been the main control method for plant-parasitic nematodes in intensive agriculture (Talavera and Mizukubo 2005). However, increasing public and governmental concerns over the harmful effects of some chemical pesticides have led to their withdrawal or reduced use. Therefore, there is a need for alternative control methods to manage plant-parasitic nematodes with the aim of maintaining the current standards of quality and production.

Crude extracts of roots and leaves of *Leucaena leucocephala* have been found to be toxic to eggs and juveniles of *Meloidogyne incognita* (Adekunle and Akinlua 2007). Also, reduced populations of *M. incognita*, *Pratylenchus* spp., *Paratylenchus* spp. and *Hoplolaimus* spp. were recorded when okra varieties were planted in alleys of *L. leucocephala* (Adekunle 2008). *Leucaena leucocephala* is not a host of *M. incognita* and *M. javanica* (Stirling *et al*. 1992). In this study, we report the isolation and structural elucidation of a phenolic compound (flavonoid) – quercetin, an antinematicidal agent from the ethyl acetate fraction of a 20% aqueous methanol extract of leaves of *L. leucocephala* – and the toxicity of the isolated compound to *M. incognita*.

Materials and methods

Cultures of Meloidogyne incognita *and extraction of eggs*

Pure cultures of *M. incognita* obtained from nematode culture plots of Obafemi Awolowo University, Ile-Ife, Nigeria were maintained on *Celosia argentea* cv. TLV8 in the greenhouse. *M. incognita* eggs were extracted by shaking infected celosia root pieces with 0.5% sodium hypochlorite (Hussey and Barker 1973). Some of the extracted eggs were incubated using extraction trays to obtain second-stage juveniles (J_2).

Collection of plant materials and extraction

The leaves of *L. leucocephala* were collected from the Obafemi Awolowo University Teaching and Research Farm, Ile-Ife, Nigeria. The leaves were air-dried for 2 weeks and powdered. The powdered leaves (500 g) were extracted with 20% aqueous methanol (6 L) at room temperature for 24 h and filtered. The crude extract was concentrated *in vacuo* at 40°C and reduced to about one third of the original volume. This was extracted with *n*-hexane, dichloromethane, ethyl acetate and finally *n*-butanol. Four solvent fractions were obtained and concentrated to dryness *in vacuo*.

General

All thin-layer chromatography analyses were performed at room temperature using pre-coated plates (MERCK, silica gel 60 F_{254} 0.2 thickness). Nuclear magnetic resonance (NMR) ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were recorded on a Bruker UltraSheild spectrometer. The electron spray ionisation (ESI) mass spectrum was recorded on a Finnigan LCQ deca spectrometer.

Isolation of an antinematicidal agent from the ethyl acetate fraction of the crude extract

Column chromatography of the ethyl acetate fraction (10 g) on silica gel (70–230 mesh) using a mixture of hexane and ethyl acetate followed by an increasing gradient of methanol up to 20% afforded four fractions (A_1–D_1), as determined by analysis using a silica gel TLC plate and 9.5 : 0.5 CH_2Cl_2 : MeOH as the solvent

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system. Repeated purification of fraction D_1 (0.5 g) on a Sephadex LH 20 column using a mixture of CH_2Cl_2 : MeOH and EtOAc : MeOH led to isolation of compound 1 (7 mg) after analysis of the different fractions collected on a TLC plate using CH_2Cl_2 : MeOH (7 : 2) as the solvent system.

Egg-hatch and juvenile mortality tests

In total, 4 mL aliquots of 300 eggs or juveniles of *M. incognita* in water suspension were transferred separately into Petri dishes (60 mm diameter). Compound 1 was introduced into the Petri dishes at concentrations of 0.8%, 0.4% or 0.2%. Petri dishes containing nematode eggs or juveniles in water suspension without compound 1 served as controls. Petri dishes were incubated in the laboratory at ambient temperature. The numbers of hatched or immobilised juveniles were counted every 24 h for 14 days. Nematodes were confirmed as being dead when they remained immobile and failed to respond to touch when transferring them by picking brush into distilled water. Each treatment was replicated four times while the egg-hatch and juvenile mortality trials were performed twice.

Statistics

Egg hatch data were subjected to statistical analysis using the SAS statistical package (1985, SAS Institute Inc. Cary, NC, USA). Differences between means were tested using Fisher's least significant difference at $P = 0.05$.

Results

Identification of the antinematicidal compound

The isolated compound was identified based on the following spectral characteristics. ^{1}H NMR (300 MHz, MeOD): δ 6.20 (1H, d, J = 2.1 Hz, H-6), 6.41 (1H, d, 2.1 Hz, H-8) meta related ring A hydrogen atoms, 6.91 (1H, d, J = 8.4 Hz, H-5′), 7.66 (1H, dd, J = 2.1 and 8.4 Hz, H-6′), 7.75 (1H, d, J = 2.1 Hz, H-2′), showing meta related H-2′ and H-6′ in addition to ortho related H-5′ and H-6′ of ring B. ^{13}C NMR spectroscopic data are in good agreement with the literature (Harborne and Mabry 1982; Markham 1982). The molecular formula of compound 1 is $C_{15}H_{10}O_7$ from the interpretation of NMR spectra. This corresponds to a molecular weight of 302. The ESI mass spectrum (negative mode) showed a molecular ion $[M^+-H]$ corresponding to the molecular mass of the compound at m/z = 301.1 as the base peak. The structure of compound 1 is presented in Fig. 1 and identified as quercetin.

Egg-hatch and juvenile mortality tests

Toxicity of quercetin to eggs of *M. incognita* is presented in Table 1. The phenolic compound tested was highly toxic to eggs of the nematode at 0.8%, 0.4% and 0.2%, with only 22–28% of the eggs hatching into juveniles over a period of 14 days. Also, juveniles of *M. incognita* in 0.8% quercetin were all killed within 4 days while in 0.4% and 0.2% quercetin all juveniles were killed in 5 days (Fig. 2). In the water (control), however, only 73% of the juveniles were killed by 14 days.

Discussion

Egg hatching and the number of active living juveniles were reduced in the presence of quercetin isolated from

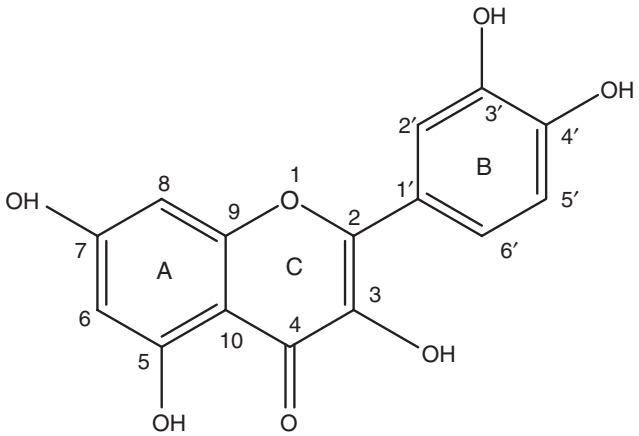

Fig. 1. Structure of quercetin – an antinematicidal agent extracted and purified from the leaves of *Leucaena leucocephala.*

Table 1. Toxicity of quercetin isolated from *Leucaena leucocephala* to eggs of *Meloidogyne incognita*

Each value is a mean of four replicates and two trials. Means followed by the same letter are not significantly different ($P < 0.05$) using Fisher's least significant difference test

Quercetin conc. (%)	Egg-hatch in 14 days (%)
0.8	22a
0.4	26ab
0.2	28b
Water (control)	96c
l.s.d. ($P = 0.05$)	5.07

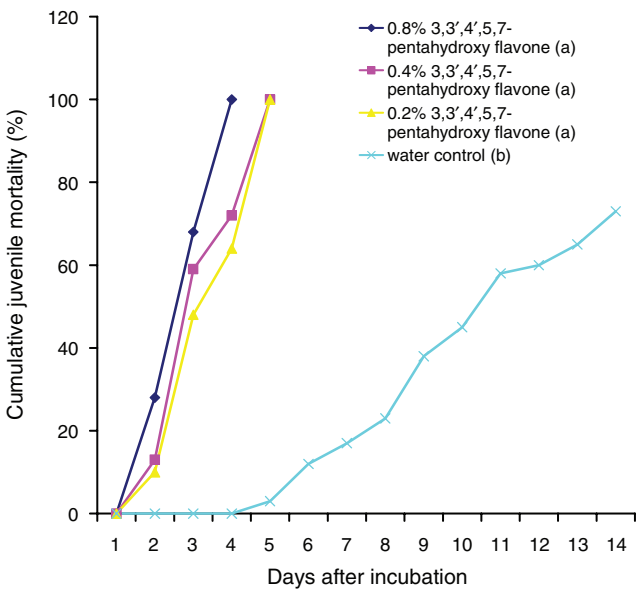

Fig. 2. Toxicity of quercetin isolated from *Leucaena leucocephala* to *Meloidogyne incognita* juveniles. Means with the same letters are not significantly different according to Fisher's least significant difference test ($P < 0.05$). Treatment means were compared over a 14-day period.

L. leucocephala, when compared with eggs and juveniles kept in water. The findings of the current study corroborate those of a recent study, which reported that crude extracts of *L. leucocephala* exhibited antinematicidal properties (Adekunle and Akinlua 2007).

The effect of quercetin on *Caenorhabditis elegans* was a reduction in the reactive oxygen species accumulation at thermal stress (Kampkotter *et al*. 2007) indicating that quercetin may act as an antioxidant as well as a modulator of cellular signalling processes to exert inhibitory effects on microorganisms. Several studies have demonstrated antithelminthic effects of extracts of *L. leucocephala* in animals (Bamualim *et al*. 1984; Pearce *et al*. 1984; Ademola *et al*. 2005) and crude extracts of its bark and roots have broad uses medicinally in Latin America (Duke 1983).

Given increasing public and governmental concerns over the use of synthetic nematicides, antinematicidal compounds of plant origin could be used for nematode management, complementing synthetic nematicides to reduce the quantities of synthetic chemicals used in agriculture. Testing the isolated compound on root-knot nematodes infecting plants under greenhouse conditions is recommended. The results of this study suggest that concerted efforts should be made to further cultivate *L. leucocephala* with the aim of extracting antinematicidal compounds from it for management of phytonematodes.

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