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ARTICLE

Nematicidal Carboxylic Acids and Aldehydes from *Melia azedarach* Fruits

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Melia azedarach is a species gaining scientific interest mostly concerning its range of biological activities against agricultural target pests. The nematicidal melia methanol extract (MME) obtained from the fruits, acting against the phytonematode *Meloidogyne incognita*, is herein reported to contain hexadecanoic, acetic, and hexanoic acids as well as furfural, 5-hydroxymethylfurfural, 5-methylfurfural, and furfurol. All compounds were tested individually for nematicidal activity against the nematode second-stage juveniles, in paralysis experiments. The nematicidal activity was studied both after nematodes' immersion in treatment solutions and after exposure to test substance vapors. Clear dose and time response relationships were established at the dose ranges of 31.2−500 and 1−100 μg/mL, concerning the aldehydes and carboxylic acids, respectively, implementing analogous predominance of nematicidal activity. Nevertheless, no synergistic effects were observed in respective mixture interaction bioassays among furfural, 5-hydroxymethylfurfural, 5-methylfurfural, and furfurol. Furfural was the most active bionematicidal compound reported herein for the first time as a natural constituent of *M. azedarach*.

KEYWORDS: GC-MS; *Melia azedarach*; furfural; furfurol; acetic acid; *Meloidogyne incognita*; fumigant

INTRODUCTION

Melia azedarach, commonly known as chinaberry, is a plant species of the Meliaceae family exhibiting a range of biological activities of practical agricultural and pharmaceutical use. Extracts as well as purified individual compounds obtained from various parts of *M. azedarach* are reported to exhibit insecticidal (*1*−*9*), antifungal (*10*, *11*), and nematicidal (*12*) properties of agronomical interest. Additionally, *M. azedarach* compounds are used to treat human and animal parasites or pathogens (*13*−*25*) and possess antioxidant properties (*26*). The increased interest in chinaberry's biorational uses can be in fact demonstrated by recent attempts of its large-scale production by in vitro propagation (*27*, *28*).

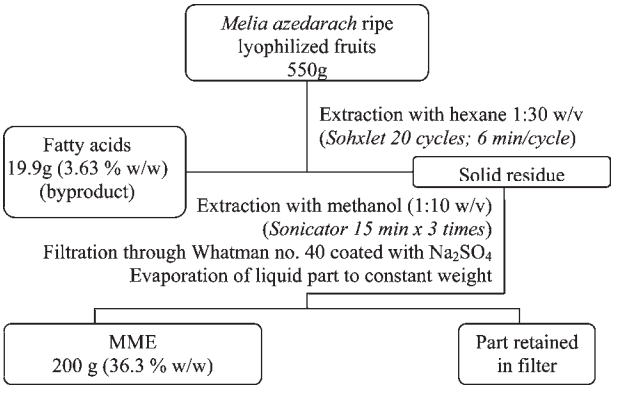
In our previous study we have reported the nematicidal activity of the melia methanol extract (MME) obtained from the ripe fruits of *M. azedarach* against *Meloidogyne incognita* second-stage juveniles (J2) (*12*). In the present investigation we report (1) the chemical characterization of the nematicidal MME by means of GC-MS and (2) the MME constituent components' individual and paired activity against J2, after immersion in test solutions or exposure to their vapors.

MATERIALS AND METHODS

Extraction and Chemical Analysis. *Chemicals*. Furfural, 5-hydroxymethylfurfural, and furfurol as well as acetic, butyric, hexanoic, decanoic, and hexadecanoic acids were purchased from Sigma Aldrich Greece. Nemathorin 150EC (ai fosthiazate 15%) was supplied from Hellafarm Co.

Extraction of Melia azedarach Fruits. Ripe fruits of *M. azedarach* were collected in Thessaloniki, Greece, in February 2007. A voucher specimen was deposited at the University of Biology in Thessaloniki, Greece, for species identification. The first steps of the matrix cleanup, resins and fats removal, and the extraction procedure up until the acquisition of MME are presented in **Chart 1**.

Chart 1. Extraction Procedure of MME from *M. azedarach* Fruits



GC-MS Analysis. A Trace GC Ultra gas chromatograph (Thermo Finnigan), coupled with a Trace DSQ mass spectrometry detector, a split−splitless injector, and an Xcalibur MS platform, was used. The column was a fused silica capillary Varian CP-WAX 57CB (60 m × 0.25 mm; film thickness = 0.25 mm) (Varian Inc.). The injector and the transfer line

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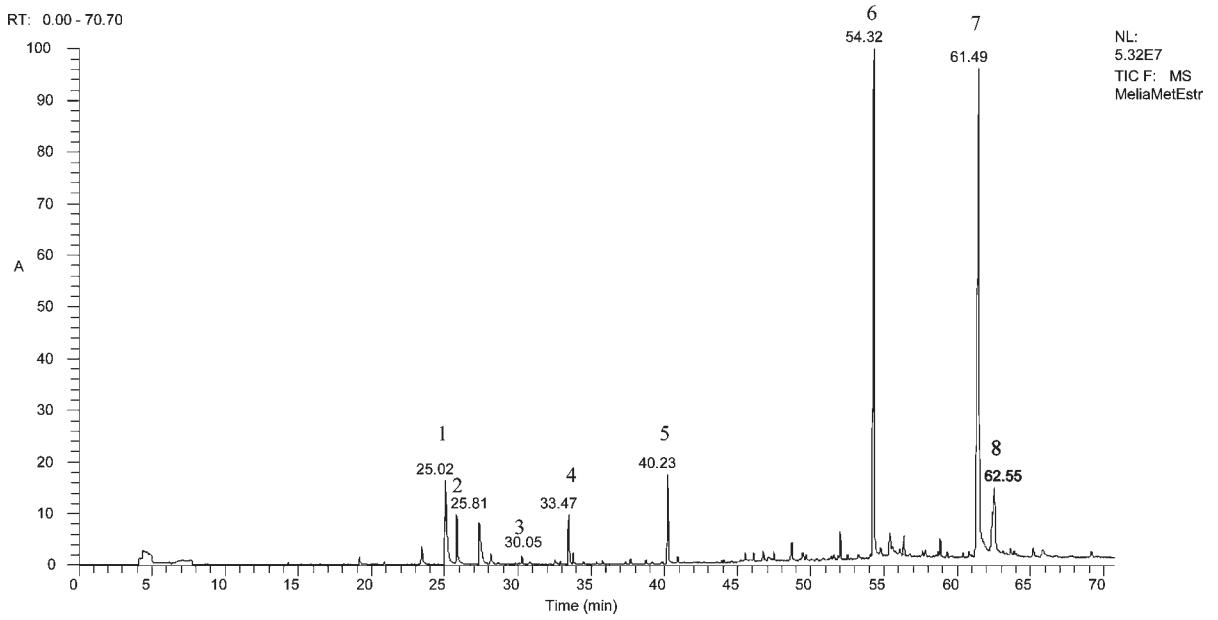

Figure 1. GC-MS chromatogram of MME. Peaks: (1) acetic acid; (2) furfural; (3) 5-methylfurfural; (4) furfurol; (5) hexanoic acid; (6) 5-hydroxymethylfurfural; (7) 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one; (8) hexadecanoic acid.

temperatures were set at 200 °C. The oven temperature was programmed as follows: 50 °C (hold for 1 min), raised to 220 °C (3 °C/min), and isothermally held for 30 min. Helium was used as carrier gas at 1 mL/min; 1 μL of MME in water at a concentration of 2500 μg/mL was injected in the splitless mode. MS conditions were as follows: ionization mode EI positive from 40 to 300 amu. The components of MME were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against the NIST98 library. Quantitative analysis of each component was carried out with the external standard method.

J2 Paralysis Bioassays. Freshly hatched J2 (24 h) were extracted from tomato roots infested with *M. incognita* according to the method of Hussey and Barker (*29*) and were used for the experiments. Nemathorin 150EC (ai fosthiazate 15%, Hellafarm Co.) and tap water as well as solvent carriers (used to surpass test substances insolubility problems, as described successively) served as bioassay controls for the paralysis correction. The bioassays were performed in Cellstar 96-well cell culture plates (Greiner bio-one), and each treatment was represented by 25 J2 juveniles per well. Plates were covered with plastic lids and were maintained in the dark at 28 °C. Juveniles were observed with the aid of an inverted microscope (Euromex) at 40× and were ranked into two distinct categories: motile or paralyzed. Moreover, after the last assessment J2 juveniles were moved to plain water, after washing in tap water through a 20 μm pore screen to remove excess test substance. The paralysis experiments were performed twice, and every treatment was replicated per experiment six times.

MME Constituent Compound Activity against J2 Immersed in Treatment Wells. Furfural, 5-hydroxymethylfurfural, furfurol, and acetic, hexanoic, and hexadecanoic acids were individually subjected to dose response experiments against J2 (31.2−500 μg/mL, aldehydes and alcohols; 1−100 μg/mL, carboxylic acids). The EC_{50} values were additionally calculated for the paired combinations of furfural, 5-hydroxymethylfurfural, and furfurol in order to study synergistic and antagonistic interactions. As part of a preliminary study on different molecular weight organic acids' nematicidal activity, butyric and decanoic acid EC_{50} values were calculated as well. Stock solutions of furfurol, 5-hydroxymethylfurfural, furfural, and hexadecanoic and hexanoic acids were prepared in ethanol and were successively diluted in distilled water containing the polysorbate surfactant 20 (Tween-20). Stock solutions of butyric and acetic acid were prepared in water and similarly were the dilutions made. Final concentrations of ethanol and Tween-20 in treatment wells never exceeded 1 and 0.3% v/v, respectively. Assessments were made 1 h and 1 day after the bioassay start.

MME Constituent Compound Fumigant Activity against J2. J2 were immersed in water, in wells adjacent to the treatment wells where the test solution was poured. For every treatment well four adjacent ones were used, and in each plate was only one treatment dose. Paralysis percentages recording the fumigant activity in the four adjacent to the treatment wells served as an experiment's treatment replication with 120 J2 instead of 30. Assessments were made 1 h and 1 day after the start of the experiments.

Table 1. Yield in Aldehydes, Alcohols, and Carboxylic Acids from *Melia azedarach* Dried Fruits

t_R (min)	compound	yield[a] (mg/kg, w/w)
25.02	acetic acid	2043
25.81	furfural	1131
30.05	5-methylfurfural	2.5
33.47	furfurol	811
40.23	hexanoic acid	1502
54.32	5-hydroxymethylfurfural	9652
61.49	2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	NC[b]
62.55	hexadecanoic acid	1245

[a] Expressed as dry fruit weight. [b] Not calculated.

Statistical Analysis. Because paralysis in solvent (DMSO, ethanol, Tween-20) was not significantly different from that observed in distilled water, the percentages of paralyzed J2 recorded in the microwell assays were corrected by eliminating the natural death/paralysis in the water control (0−5% of total number of J2) according to Schneider Orelli formula (*30*): corrected % = {(mortality % in treatment − mortality % in control)/(100 − mortality % in control)} × 100. They were analyzed (ANOVA) after being combined over time. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2 treated with tested compounds were subjected to nonlinear regression analysis using the log−logistic equation proposed by Seefeldt et al. (*31*): $y = C + (D - C)/\{1 + \exp[b(\log(x) - \log(EC_{50}))]\}$, where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the pure or paired substances' concentration required for 50% death/paralysis of nematodes after elimination of the control (natural death/paralysis). In the regression equation, the pure or paired substances' concentration was the independent variable (x) and the paralyzed J2 (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates per pure or paired substances' concentration and immersion (or exposure to vapors) period was used to calculate the EC_{50} value. Mean data values are presented with respective standard deviations (**Figure 2**).

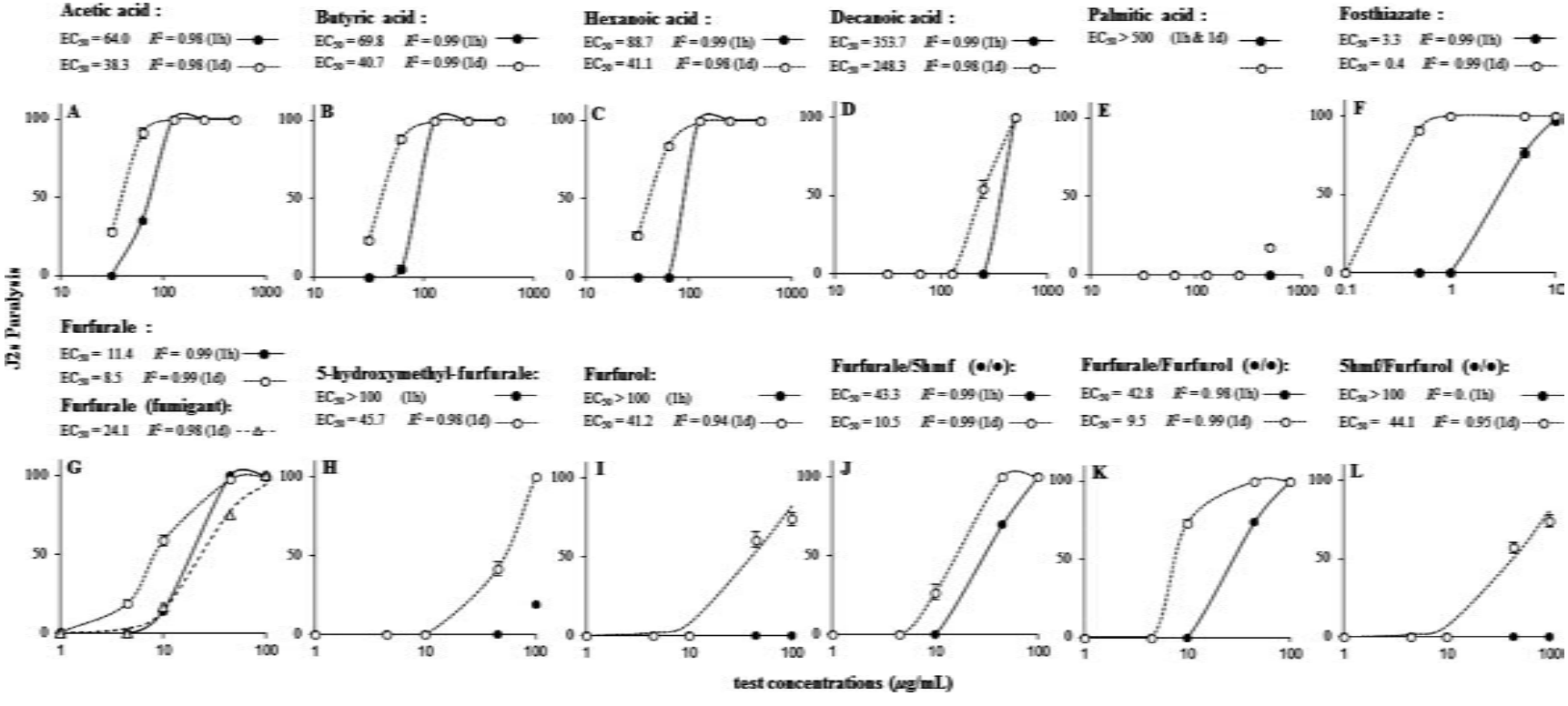

Figure 2. Regression curves of *M. incognita* paralysis following J2 immersion in solutions of pure organic acids (**A**−**E**), pure (**G**−**I**) and paired (**J**−**L**) aldehydes and alcohols, and fosthiazate (**F**) for 1 h and 1 day or exposure to vapors of furfural solutions (**G**) for 1 day. Each point represents the average % number of paralyzed J2 of six replications per treatment (two experiments replications) after elimination of natural paralysis/death observed in the control. Standard deviations of mean data values are presented as error bars.

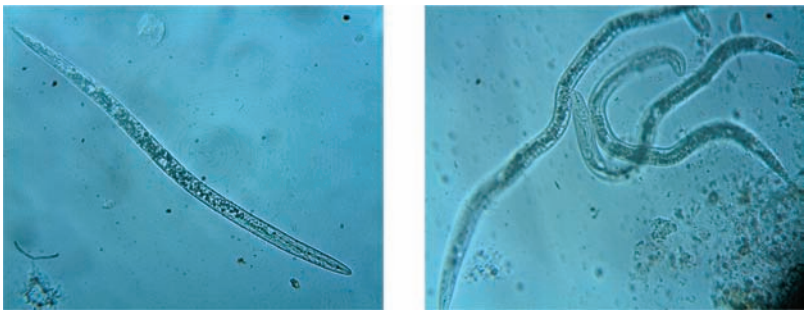

Figure 3. Straight (left) as well as curled, semicircular, and hook-shaped motionless−paralyzed J2 after 1 day of immersion in 10 μg/mL furfural and fosthiazate, respectively.

RESULTS AND DISCUSSION

According to the GC-MS analysis (**Figure 1**), MME afforded mainly aldehydes, alcohols, and carboxylic acids. Specifically, the compounds participating in MME chemical composition were furfural, 5-hydroxymethylfurfural, furfurol, acetic acid, hexanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, and hexadecanoic acid, and the relative yields expressed as dry material are presented in **Table 1**. When MME components were tested against *M. incognita*, clear dose response relationships were established and significant paralysis of J2 was evident as early as 1 h after the experiments had begun (**Figure 2**). The organic acids were found to have nematicidal effect at the dose range of 31.2−500 μg/mL in the decreasing order of acetic ($EC_{50/1\ h}$ = 64.0 μg/mL), butyric ($EC_{50/1\ h}$ = 69.8 μg/mL), hexanoic ($EC_{50/1\ h}$ = 88.7 μg/mL), and decanoic ($EC_{50/1\ h}$ = 353.7 μg/mL), revealing the linear relationship of C atom number and nematicidal activity. Hexadecanoic acid was not found to be nematicidal at the dose range used for the experiment ($EC_{50/1\ h}$ > 500 μg/mL), and the induced paralysis did not exceed 17% over control 1 day after the beginning of the experiment. Aldehydes and alcohols were found to have an even higher nematicidal effect than organic acids. As a result, to achieve better linearity the EC_{50} value calculation was performed at the dose range of 1−100 μg/mL. The EC_{50} value of furfural, 1 h after J2 immersion in test solutions, was calculated at 11.4 μg/mL, whereas such rapid activity was not evident in 5-hydroxymethylfurfural (paralysis 19% over control) or furfurol treatment wells. One day after the experiments' establishment, the EC_{50} values for furfural, 5-hydroxymethylfurfural, and furfurol were calculated at 8.5, 45.7, and 41.2 μg/mL, confirming once more the high nematicidal activity of furfural. Fosthiazate's EC_{50} values recorded 1 h and 1 day after the beginning of the experiments (3.3 and 0.4 μg/mL) were of levels similar to those of furfural. Interestingly, the paralyzed J2 employed different body shapes between the two cases (**Figure 3**). Also, no synergic action was revealed between furfural, 5-hydroxymethylfurfural, and furfurol because furfural tested individually achieved lower $EC_{50/1\ h}$ and $EC_{50/1\ day}$ values than those calculated for the paired mixtures (**Figure 2**). With the exception of hexadecanoic acid and 5-hydroxymethylfurfural, all other tested substances revealed a fumigant activity; that is, they paralyzed to some extent J2 immersed in tap water in adjacent treatment wells. With regard to the organic acids, this phenomenon was observed 1 day after the experiments had begun only at the highest test concentration of 500 μg/mL. Specifically, butyric acid vapors paralyzed all J2, whereas no other acid fumigant activity exceeded 30% (data not shown). Interestingly, furfural exhibited the highest fumigant nematicidal activity, and the $EC_{50/fumigant}$ value was calculated as 24.1 μg/mL, 1 day after the experiments' establishment (**Figure 2**). On the contrary, no fumigant activity was revealed 1 day post J2 exposure to vapors of 5-hydroxymethylfurfural and furfurol solutions (1−100 μg/mL). Furfural and organic acids are already known to possess high nematicidal fumigant activity against plant parasitic nematodes (*32*, *33*). *M. azedarach* content in furfural, reported herein for the first time, entails its innovative ecofriendly biorational use as a biofumigant. Because phytonematodes live in soil or within plant roots, the target of any chemical nematicide often resides a fair distance away from the site of application (*34*). This is why the activity in the vapor phase becomes of paramount importance for nematicides, because it enlarges their activity in adjacent nontreated soil layers. The emission of biocidal volatiles during decomposition of soil-incorporated tissues is called biofumigation. It represents an implementation of integrated management and biocontrol of crop nematodes, and *Brassica* species are maybe the most representative current example (*35*). Chinaberry, too, has demonstrated biofumigant properties when incorporated as pulverized fruits in *M. incognita* infested soil to be tested for its effect on nematode life cycle (*12*). In fact, an enhanced activity was observed after the amended soil's incubation period had been increased from 24 to 48 h, prior to J2 inoculation and further on tomato transplant. A better uniformity in soil of the nematicidal volatile substances contained in *M. azedarach* fruits, such as furfural, could possibly be an explanation for this enhanced activity. Interestingly, MME did not contain azadirachtin, and its purified limonoids have not been found to contribute to its nematicidal activity (*36*). Also, azadirachtin was not found to be active against nematodes as reported in our previous studies (*37*).

This is the first report with respect to the purification and chemical characterization of the main nematicidal and cytotoxic constituents of *M. azedarach* fruits. The results suggest that chinaberry has a substantial potential for use in crop protection. Further studies will be extended to evaluate the mode of action of the nematicidal principles as well as the practical challenge of their integration in phytonematode management practices.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; MEM, melia methanol extract.

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