# CHEMICAL CONSTITUENTS FROM *MUCUNA ATERRIMA* WITH ACTIVITY AGAINST *MELOIDOGYNE INCOGNITA* AND *HETERODERA GLYCINES*

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## ABSTRACT

Barbosa, L. C. A., F. F. Barcelos, A. J. Demuner, and M. A. Santos. 1999. Chemical constituents from *Mucuna aterrima* with activity against *Meloidogyne incognita* and *Heterodera glycines*. Nematropica 29:81-88.

A phytochemical investigation of the stems and roots of *Mucuna aterrima* led to the isolation of the following constituents with nematicidal activity: $KNO_3$ + $NaNO_3$, a mixture of fatty acids, a mixture of triacylglycerols, β-sitosterol, stigmasterol, daucosterol, stigmasterol D-glycoside, allantoin. An *in vitro* bioassay was carried out, and at the concentration of 5 μg $ml^{-1}$, the percentage of mortality caused by the compounds varied from 2.1% to 74.4% in the case of *M. incognita* and 0% to 74.2% for *H. glycines*. The non-essential amino acid L-3,4-dihydroxyphenylalanine (L-Dopa), present at 6-9% in the seeds of *Mucuna spp.* was tested for the first time against the phytonematodes *M. incognita* and *H. glycines*, and the $LC_{50}$ was 21 μg $ml^{-1}$ and 0.17 μg $ml^{-1}$ respectively.

*Key words: Heterodera glycines*, L-Dopa, *Meloidogyne incognita, Mucuna aterrima*, nematicides, secondary metabolites.

## RESUMEN

Barbosa, L. C. A., F. F. Barcelos, A. J. Demuner y M. A. Santos. 1999. Compuestos químicos de *Mucuna aterrima* con actividad contra *Meloidogyne incognita* y *Heterodera glycines*. Nematrópica 29:81-88.

La investigación fitoquímica de tallos y raíces de *Mucuna aterrima* condujo al aislamiento de los siguientes compuestos, los que poseen actividad nematicida: $KNO_3$ + $NaNO_3$, una mezcla de ácidos grasos, y una de triacilgliceridos, β-sitosterol, estigmasterol, daucosterol, D-glicósido estigmasterol y alantoína. En un ensayo realizado *in vitro*, a la concentración de 5 μg $ml^{-1}$, el porcentaje de mortalidad causado por estos compuestos, varió desde 2.1 a 74.4% en el caso de *M. incognita* y de 0 a 74.2% para *H.glycines*. El aminoácido no esencial L-3,4-dihidroxifenilalanina (L-Dopa), presente en un 6-9% en las semillas de *Mucuna* spp., se evaluó por primera vez contra los fitonematodos *M. incognita* y *H. glycines*, el $LC_{50}$ fue 21 μg $ml^{-1}$ y 0.17 μg $ml^{-1}$ respectivamente.

*Palabras claves: Heterodera glycines*, L-Dopa, *Meloidogyne incognita*, metabolitos secundarios, *Mucuna aterrima*, nematicidas.

## INTRODUCTION

*Heterodera glycines* Ichinohe, is one of the most destructive parasites of soybean. In Brazil this parasite is found in cultivated areas in the states of Goias, Mato Grosso, Mato Grosso do Sul, Minas Gerais, São Paulo and Paraná. In heavily infested areas, the crop production can be reduced by 30 to 75% (Valle *et al.*, 1996). In the Northeast of Brazil, *Meloidogyne incognita* (Kofoid and White) Chitwood and *M. javanica* (Treub)

Chitwood cause more than 45% reduction in sugarcane production (Moura, 1991). In the state of Mato Grosso do Sul, the production of soybean was reduced by 10% in 1995 due to infestation by *Meloidogyne incognita* and *Heterodera glycines*.

Chemical nematicides are often used to control nematodes, but due to high costs, are integrated with other management practices. In Brazil, there are several plant species used by farmers as green manures, and also to reduce nematode population densities in cultivated areas (Santos and Ruano, 1987). The most widely used plant for this purpose is velvetbean or *Mucuna* spp. (Buckles, 1995; Miyasaka *et al.*, 1983). Although several investigations on the chemistry of this genus have been carried out (Bell *et al.*, 1971; Ding *et al.*, 1991; Goda *et al.*, 1987; Hasan *et al.*, 1980; Ishikura and Yoshitama, 1988; Yoshida and Akino, 1980), the only studies directed towards the isolation of the nematicidal constituents describe the identification of the aliphatic alcohol triacontan-1-ol and the ester triacontyl tetracosanoate (Nogueira *et al.*, 1996a; Nogueira *et al.*, 1996b).

Among all the compounds that have been isolated from *Mucuna* spp., L-Dopa is the one obtained in greatest amount. The content of this compound on the seeds of *Mucuna* spp. varies from 5.9 to 9.0% (Bell and Janzen, 1971). The concentration of L-Dopa in water culture solution of *Mucuna* sp. reaches 1 μg $ml^{-1}$. Since fresh leaves of *Mucuna* sp. contain as much as 1.0% of L-Dopa, and *Mucuna* produces 20 to 30 tons of fresh leaves and stems per hectare, about 200 to 300 kg of L-Dopa is produced and added to the soil per year (Fujii *et al.*, 1992).

Considering the information above, and the biological activities attributed to L-Dopa (Bell and Janzen, 1971; Fujii *et al.*, 1991; Rehr *et al.*, 1973), in this report we describe the isolation, identification, and biological activity of several constituents produced by *Mucuna aterrima* against the phytonematodes *M. incognita* and *H. glycines*.

## MATERIAL AND METHODS

*General experimental procedures*: Mass spectra were recorded under electron-impact (70 eV), using a G ZAB-E high resolution spectrometer. Infrared spectra were obtained on a Mattson Instruments FTIR 3000. NMR spectra were recorded with a Bruker DRX 400 (400 MHz) spectrometer, using tetramethylsilane (TMS) as internal standard. Flash chromatography was performed using Crosfield Sorbil C60 (40-60 μm).

*Plant material*: *Mucuna aterrima* was cultivated at the Campus of the Universidade Federal de Viçosa, from June to December 1994. The plant was harvested in December, and dried at room temperature.

*Extraction and isolation*: Dried stems (1820 g) of *M. aterrima* were ground and extracted with ethanol, in a Soxhlet apparatus, for 72 hours at 78.5°C. The extract was partially concentrated under reduced pressure in a rotary evaporator, resulting in the precipitation of a crystalline solid (fraction 1), that was removed by filtration. The filtrate was concentrated to yield 85 g of a green solid material. This material was fractionated on a silica gel column, eluting with hexane:ethyl acetate, of increasing polarity. The fractions obtained were combined into four groups, according to their similarities as analyzed by thin layer chromatography (TLC). All four fractions were then submitted to another fractionation on a silica gel column, eluting with a mixture of hexane:ethyl acetate of increasing polarity, resulting in the obtention of a mixture of fatty acids (fraction 2), triacylglycerides (fraction 3) and a mixture of β-sitosterol and stigmasterol (fraction 4).

The roots of *M. aterrima* (615 g), were powdered and successfully extracted in a

Soxhlet apparatus with hexane and ethanol. The hexane extract (6.7 g) was fractionated by silica gel column chromatography with hexane:diethyl ether, of increasing polarity. This resulted in the obtention of a mixture of fatty acids (fraction 5) and a mixture of β-sitosterol and stigmasterol (fraction 6).

The ethanolic root extract (30 g) was partially concentrated under reduced pressure, which resulted in the precipitation of a solid (fraction 7), which was removed by filtration. The filtrate was concentrated and fractioned by silica gel column chromatography, eluting with ethyl acetate:methanol (95:5). This procedure resulted in the isolation of allantoin (fraction 8), a mixture of daucosterol and stigmasterol D-glycoside (fraction 9) and an unknown alcohol (fraction 10).

*Gas chromatographic analysis*: A gas chromatographic analysis was carried out using a Shimadzu GC-14A equipped with a FID detector, a 30 m carbowax 20 M capillary column, i.d. 0.25 mm and the following temperature program: 150°C rising to 200°C (20 min.) at the rate of 5°C/min; injector and detector temperatures of 200°C and 220°C respectively. The carrier gas used was hydrogen at 0.9 ml/min.

*Transesterification of the triacylglycerols*: Fraction 3 (mixture of triacylglycerides, 20 mg) was dissolved in tetrahydrofuran (0.5 ml) in a test tube, and 0.5 M sodium methoxide in anhydrous methanol (1 ml) was added. The solution was maintained at 50°C/10 min, before addition of glacial acetic acid (50 µl), and water (3 ml). The methyl esters were extracted with hexane (2 × 5 ml), and the organic phase was dried over $Na_2SO_4$, containing 10% $NaHCO_3$. After filtration, the solvent was removed under reduced pressure and the residue was dissolved in hexane (0.5 ml), prior to the GC analysis.

*Esterification of the fatty acids (fractions 2 and 5)*: A sample of the fatty acid mixture (10 mg) was dissolved in boron trifluoride (14% $BF_3$) in methanol (0.5 ml). The solution was warmed at 60°C for 10 min, prior to the extraction with hexane (6 ml). The organic phase was washed with brine (2 × 5 ml), dried over $Na_2SO_4$, and concentrated under reduced pressure. The residue was dissolved in hexane (0.5 ml) for GC analysis.

*Bioassays*: For egg-hatching assays, dried and powdered stems and roots (10 g each) of *M. aterrima* were extracted with ethanol (130 ml), at room temperature for 7 days. After this period of time, the solvent was removed under reduced pressure to leave a residue. Continuous extraction of the stems and roots (10 g), were also carried out with ethanol, in a Soxhlet apparatus, for 60 hours at 80°C. For each extract, a 1% aqueous suspension containing 0.1% of the detergent Tween 20, was prepared.

The assays were carried out by adding the suspensions (3 ml) to a hatching chamber, made from Petri dishes (4.5 cm diam.) containing a 1 mm i.d. nylon screens covered with a facial tissue paper. Then 10 egg masses of *M. incognita* were added to each chamber and kept at 25°C in the dark. Hatched juveniles which passed through the filters were counted under a stereoscopic microscope at 48, 96 and 144 hours after exposure to the extracts. After each count, a new extract suspension (3 ml) was added to the Petri dishes. Each treatment was replicated 4 times. A control treatment, using a 0.1% Tween 20 aqueous solution, was included, and the data were subjected to a statistical analysis using the Scott-Knott test to determine the differences among treatments at 0.05 probability level (Scott and Knott, 1974).

A second bioassay was conducted using the "inverted tube" method developed by Kimura *et al.* (1981). A preliminary experiment was conducted with the compounds at the concentration of 50 µg $ml^{-1}$, against *M. incognita*. A further experiment with

*M. incognita* and *H. glycines* was carried out with the compounds at the concentration of 5 μg $ml^{-1}$.

According to this methodology, a methanolic solution of the tested compound, including the L-Dopa (0.1 ml at 5 μg $ml^{-1}$), was placed in a test tube (1.5 × 5 mm), containing a 0.1% aqueous solution of Tween 20 (1.9 ml). A suspension (0.5 ml) with approximately 400 *M. incognita* or *H. glycines*, J2 stage, was added to the tube. The tubes were then sealed with a filter paper (Whatman nº 1) and kept at 26°C for 48 hours. After this time, they were placed upside down in a Petri dish (4.5 cm diameter), containing a solution of streptomycin sulphate (2 ml, 1000 μg $ml^{-1}$), and allowed to stand in the dark, for another 48 hours at 26°C. The nematodes in the Petri dish were counted under a stereoscopic microscope. A control treatment using 0.1 ml of methanol was compared to each tested compound. The bioassay was replicated 6 times, in a completely randomized design. The activity against nematodes was expressed as percentage of mortality calculated in relation to the control experiment: Mortality (%) = [(B-A)/B] × 100, where A = number of living nematodes after treatment with chemicals, and B = number of living nematodes in the control.

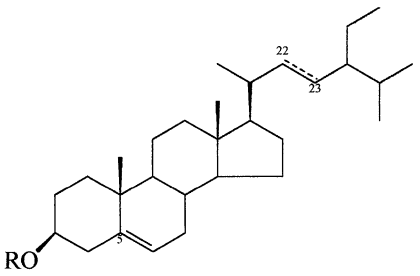
All data were subjected to a statistical analysis using the Scott-Knott test to determine the differences among treatments at 0.05 probability level (Scott and Knott, 1974).

## RESULTS AND DISCUSSION

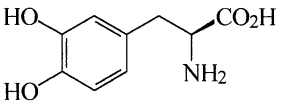
*Compound identification*: The infrared spectrum of fraction 1 (4.16 g), a solid that precipitated during the concentration of the ethanolic extract of the stems of *M. aterrima*, showed only a strong absorption maximum at 1 380 $cm^{-1}$ ($\nu_{as}$ N-O) and a weak band at 820 $cm^{-1}$. This suggested that the sample was an inorganic nitrate. The sample was dissolved in water and submitted to atomic absorption analysis, that revealed that it consisted of a mixture of $NaNO_3$ and $KNO_3$ (2:3). The infrared spectrum of fraction 7 (0.31 g), obtained from the root ethanolic extract, was identical to that of fraction 1, and the atomic absorption analysis showed it was a mixture of $NaNO_3$ + $KNO_3$ (1:2). The infrared spectrum of fractions 2 (4.10 g) and 5 (0.40 g) were similar, and showed the following major absorption bands: 3 600-2 500 $cm^{-1}$ (ν OH); 1 700 $cm^{-1}$ (ν C = O), 720 $cm^{-1}$ ($\delta_s$ $(CH_2)_n$, n > 4) typical of fatty acid. These fractions were esterified with $BF_3$/MeOH, and analyzed by gas chromatography, comparing with standards. This allowed for the identification of the following fatty acids: fraction 2: palmitic (C16:0) (70%), stearic (C18:0) (8%), oleic (C18:1) (17%), linoleic (C18:2) (5%); fraction 5: palmitic (1.9%), stearic (19%), icosanoic (C20:0) (1.9%), henicosanoic (C21:0) (1%), docosanoic (C22:0) (12.7%), tricosanoic (C23:0) (17.1%), tetracosanoic (C24:0) (35.2%). Two other compounds with retention time higher than tetracosanoic acid were observed, but not identified.

The infrared spectrum of fraction 3 (1.10 g), was typical of triacylglycerol (ν C = O, 1740 $cm^{-1}$). This fraction was hydrolyzed and methylated, with HCl/MeOH (Jham *et al.*, 1992), before carrying out GC analysis, showing the presence of the methyl esters of the following acids: myristic (C14:0) (1%), palmitic (48%), stearic (33%), oleic (14%) and linoleic (4%).

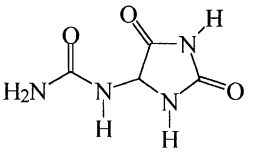
Fractions 4 (0.40 g) and 6 (1.42 g) presented the same infrared spectrum, showing a strong absorption maximum at 3420 $cm^{-1}$ (ν OH). The mass spectra showed a molecular ion at m/z 414 compatible with the formula $C_{29}H_{50}O$, for β-sitosterol (Fig. 1). A peak at m/z 412 was due to the molecular ion for stigmasterol ($C_{29}H_{48}O$).




β-sitosterol: R=H; 22,23-dihydro
stigmasterol: R=H; $\Delta^{22}$
daucosterol: R=glucose;22,23-dihydro
stigmasterol D-glycoside: R=glucose; $\Delta^{22}$




L-Dopa




allantoin

Fig. 1. Structure of some nematicidal organic constituents isolated from *M. aterrima.*

Comparison of these fractions, by TLC, with an authentic sample of β-sitosterol (containing c.a. 10% of stigmasterol), revealed that they had the same $R_f$. The $^1$H-NMR and the $^{13}$C-NMR spectra were also identical to those reported for a mixture of β-sitosterol and stigmasterol (Paula *et al.*, 1996), confirming the proposed structure for the compounds.

Fraction 8 (0.69 g), isolated from the root ethanolic extract, decomposed at 230°C. Its infrared spectrum showed major absorption maxima at 3 420, 3 320, 3 180 cm$^{-1}$ (ν N-H), 1 780, 1 710, 1 650, 1 600 cm$^{-1}$ (ν C = O), and was identical to the spectrum of allantoin (Pouchert, 1981). The structure of this compound was confirmed by comparison of its $^1$H-NMR and $^{13}$C-NMR spectra with those reported in the literature (Coxon *et al.*, 1977; Pouchert, 1981).

The infrared spectrum of fraction 9 (0.01 g) was identical to the one reported for a mixture of daucosterol + stigmasterol β-D-glycoside (9:1) (Paula *et al.*, 1998). This fraction was hydrolyzed with 0.2 M HCl in MeOH for 90 min at 60°C, and the aglycone was extracted with ethyl acetate (Iribarren and Pomilio, 1983). The infrared and $^1$H-NMR spectra, and the melting point data for the aglycone were identical to the one for β-sitosterol and stigmasterol (9:1). The structure of glucose was confirmed by analysis of its alditol acetates according to the procedure described by Englyst and Cummings (1984).

Fraction 10 was isolated as a white solid (mp, 75-77°C) in a very small amount (0.05 g) from the root ethanolic extract. Its infrared spectrum showed absorption maxima at 3 500 cm$^{-1}$ (ν OH), 2 920, 2 850, and 1 060 cm$^{-1}$ (ν C-O), typical of aliphatic alcohol. This compound when dissolved in $CDCl_3$ for the NMR spectrum, turned into a gel, even when the solution was very diluted. As a consequence of this, the $^1$H-NMR spectrum was not good, and the structure was not elucidated.

*Bioassay results*: The results of the egg-hatching tests carried out with the ethanolic root and stem extracts obtained at room temperature and at reflux (80°C), are shown in Table 1. The number of living juveniles present in all hatching chambers containing the extracts were significantly reduced, compared with the control. The cold root extract caused the higher percentage of hatching inhibition (64.3%).

Table 1. Total number of second stage juveniles (NJ) of *M. incognita*, race 3, hatched after 144 h incubation in the ethanolic extracts (hot and cold) obtained from the stems and roots of *M. aterrima*.

| Extracts | NJ[y] | Inhibition (%) |
|---|---|---|
| Control | 1289.6 A[z] | 0 |
| Stem (hot) | 718.0 B | 44.3 |
| Stem (cold) | 668.0 B | 48.2 |
| Root (hot) | 648.6 B | 49.7 |
| Root (cold) | 448.7 B | 64.3 |

[y]Average of 3 counts (4 replicates per count).
[z]Values in the same column followed by the same letters do not differ ($P < 0.05$) according to the Scott-Knott test.

In order to have enough material for chromatographic fractioning, a large scale extraction was carried out in a Soxhlet apparatus, as described in the experimental section. The extracts obtained from the roots and stems, were submitted to repeated column chromatography on silica gel, to lead to the separation of 10 fractions.

When tested at 50 μg $ml^{-1}$, using the "inverted tube" method, all the compounds (fractions) isolated from the stems and roots of *M. aterrima* caused >97% mortality of *M. incognita*. At the concentration of 5 μg $ml^{-1}$, the most active compounds against *M. incognita* were the nitrates (56.6% mortality), β-sitosterol + stigmasterol (9:1, 74.4%) and the unknown alcohol (69.7%) (Table 2).

The nitrates had no effect on *H. glycines*, and the mixture of fatty acid was the most active (74% mortality) fraction. The other compounds, caused mortality ranging from 45 to 55%.

Table 2. Nematicidal activities of compounds (5 μg $ml^{-1}$) isolated from *Mucuna aterrima* against *Meloidogyne incognita* and *Heterodera glycines*.

| | | M. incognita | | H. glycines | |
|---|---|---|---|---|---|
| Fraction | Composition | Living Nematodes[z] | Mortality (%) | Living Nematodes[z] | Mortality (%) |
| Control | — | 280.8 A | — | 309.5 A | — |
| 1 and 7 | $KNO_3 + NaNO_3$ | 121.8 C | 56.6 | 309.5 A | 0 |
| 2 and 5 | Fatty acids | 275.0 A | 2.1 | 80.0 C | 74.2 |
| 3 | Fatty esters | 157.5 B | 43.9 | 161.7 B | 47.8 |
| 4 and 6 | β-sitosterol + stigmasterol | 71.8 C | 74.4 | 138.3 B | 55.3 |
| 8 | Allantoin | 244.8 A | 12.8 | 150.7 B | 51.3 |
| 9 | Daucosterol + stigmasterol D-glycoside | 264.0 A | 6.0 | 171.7 B | 44.5 |
| 10 | Unknown alcohol | 85.2 C | 69.7 | 141.8 B | 54.2 |

[z]Values in the same column followed by the same letters do not differ ($P < 0.05$) according to Scott-Knott test.

The nematicidal activity of some fatty acids have already been reported for *M. incognita* and other phytonematodes, but not for *H. glycines* (Chitwood, 1993).

Against *M. incognita*, L-Dopa at the concentration of 50 μg $ml^{-1}$ caused 99.6% mortality, with $LC_{50}$ = 21mg $ml^{-1}$ (Fig. 2). Against *H. glycines* it was more toxic with $LC_{50}$ = 0.17 μg $ml^{-1}$. Although the toxic effect of L-Dopa against *Caenorhabditis elegans* is known (Kawaii *et al.*, 1993), to the best of our knowledge this is the first report on the nematicidal activity of L-Dopa against *M. incognita* and *H. glycines*.

Although the "inverted tube" method used in this work doesn't distinguish the nematostatic from the nematicidal effect of the chemicals, the results were expressed as mortality as suggested originally by Kimura *et al.* (1981). Further investigation on the mode of action of the chemicals isolated from *M. aterrima* against the phytonematodes studied should be carried out.

In summary, we have identified several chemical constituents produced by *Mucuna aterrima*, that present nematicidal activity, but we have no information about the nematotoxic mode of action of these chemicals. Some of the compounds were more active against the *M. incognita* (β-sitosterol + stigmasterol, unknown alcohol and $KNO_3$ + $NaNO_3$) while others were more toxic to *H. glycines* (fatty acids, allantoin, daucosterol + stigmasterol D-glycoside, L-Dopa).

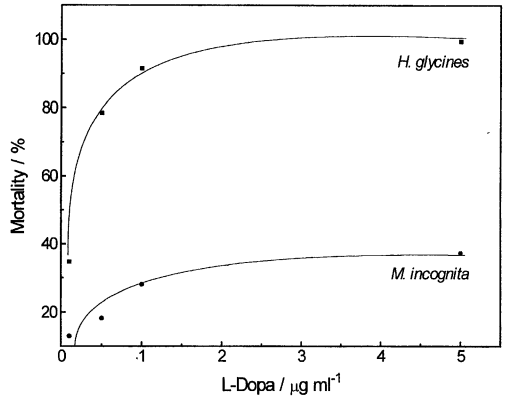



Fig. 2. Effect of L-Dopa on the phytonematodes *M. incognita* and *H. glycines*.

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