ORGANIC SUBSTANCES FOR NEMATODE CONTROL IN COFFEE PLANTS

ALEXANDRO DA SILVA NUNES

2008

ALEXANDRO DA SILVA NUNES

ORGANIC SUBSTANCES FOR NEMATODE CONTROL IN COFFEE PLANTS

Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agroquímica, para a obtenção do título de "Mestre"

Orientador

Prof. Dr. Denilson Ferreira de Oliveira

LAVRAS
MINAS GERAIS – BRASIL
2008

Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca Central da UFLA

Nunes, Alexandro da Silva.
Organic substances for nematode control in coffee plants / Alexandro da Silva Nunes. -- Lavras : UFLA, 2008.
45p. : il.

Dissertação (Mestrado) – Universidade Federal de Lavras, 2008.
Orientador: Denilson Ferreira de Oliveira.
Bibliografia.

1. Nematicida. 2. *Meloidogyne exigua*. 3. *Staphylococcus aureus*. 4. *Acinetobacter jonhsonii*. 5. Chalcona. 6. Acil-hidrazona. 7. Cafeeiro. I. Universidade Federal de Lavras. II. Título.

CDD – 572.2

ALEXANDRO DA SILVA NUNES

ORGANIC SUBSTANCES FOR NEMATODE CONTROL IN COFFEE PLANTS

Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agroquímica, para a obtenção do título de "Mestre"

APROVADA em 31 de julho de 2008

Prof. Dr. Vicente Paulo Campos UFLA

Prof. Dr. Teodorico de Castro Ramalho UFLA

Prof. Dr. Jonas Leal Neto UFLA

Prof. Dr. Denilson Ferreira de Oliveira
UFLA
(Orientador)

LAVRAS
MINAS GERAIS – BRASIL

A meus pais Raimundo de Paula Nunes e Adir Lopes da Silva Nunes, pela educação e incentivo. A meus irmãos Alessandra, Alan e Anderson pela força.

DEDICO

AGRADECIMENTOS

Ao Departamento de Química da Universidade Federal de Lavras (UFLA) e à coordenadoria de aperfeiçoamento de pessoal de nível superior (CAPES), pela concessão da bolsa de estudo;

Ao orientador, professor Dr. Denilson Ferreira de Oliveira, pelos conhecimentos transmitidos que tornaram possível meu crescimento profissional;

Ao co-orientador professor Dr. Vicente Paulo Campos, pelos conhecimentos transmitidos e pelo apoio;

Aos professores do Departamento de Química, pelos conhecimentos transmitidos;

Aos colegas de curso e aos colegas de trabalho, cuja convivência tornaram melhor a realização deste trabalho.

Muito Obrigado!

INDEX

LIST OF ACRONIMS

$^{2,3}J_{CH}$	constant coupling between carbon and hydrogen two or three bonds distant
AcOEt	ethyl acetate
AcOH	acetic acid
C18	octadecil group
CC	column chromatography
TLC	thin layer chromatography
$CDCl_3$	deuterated chloroform
HPLC	high performance liquid chromatography
D	dublet
DAD	diode array detector
Dd	double dublet
DMSO-d_6	hexadeuterated dimethylsulfoxide
ESI-MS	electrospray ionization-mass spectrometry
H_2O	water
Hex	hexane
J	coupling constant
M	multiplet
m/z	relative mass/charge
MeOH	methanol
$NMR^{13}C$	carbon thirteen nuclear magnetic resonance
RMN ^1H	hydrogen nuclear magnetic resonance
NMR^1H	
S	singlet
Bs	broad singlet
UV-Vis	ultra violet- visible
δ	chemical shift

RESUMO

NUNES, Alexandro da Silva**. Substâncias Orgânicas para o Controle de Nematóides em Cafeeiros.** 2008. 45p. Dissertação (Mestrado em Agroquímica) – Universidade Federal de Lavras, Lavras, MG.*

Com vistas a contribuir para o desenvolvimento de novos métodos de controle do nematóide *Meloidogyne exigua* Goeldi, 1887, que é amplamente disseminado pelos cafezais brasileiros, dois projetos foram realizados. No primeiro deles, as bactérias *Acinetobacter jonhsonii* e *Staphylococcus aureus*, previamente isoladas de tomateiro (*Lycopersicon esculentum* Mill.) e de planta de pimentão (*Capsicum annum* L.), respectivamente, foram cultivadas em meio líquido e, após remoção das células bacterianas, os sobrenadantes foram liofilizados. Por apresentarem atividade *in vitro* contra juvenis do segundo estádio (J2) de *M. exigua*, as frações solúveis em diclorometano dos resíduos das liofilizações foram submetidas a fracionamentos por cromatografia em coluna e por cromatografia líquida de alta eficiência, o que resultou na purificação da uracila, diidrouracila e da 9H-purina. Todas as substâncias isoladas se mostraram ativas *in vitro* contra J2 de *M. exigua*, sendo que a mais ativa, diidrouracila, apresentou CL_{50} de 204 μg/mL contra tal nematóide, enquanto a CL_{50} do nematicida comercial Aldicarbe foi de 30μg/mL. Quanto ao segundo projeto, consistiu na avaliação inicial de 149 chalconas e análogos, obtidos por reações de condensação com aldeídos, quanto à atividade *in vitro* contra J2 de *M. exigua*. O melhor resultado foi obtido com a 2,4,5-trimetoxi-4'-nitrochalcona, que apresentou CL_{50} de 171μg/mL contra J2, enquanto uma CL_{50} de 24μg/mL foi observada para o nematicida comercial Aldicarbe nas mesmas condições. Quando um experimento com mudas de café inoculadas com *M. exigua* foi realizado, observou-se que a referida chalcona era tão eficiente quanto o Aldicarbe quando empregada em concentração 14 vezes maior que a do nematicida comercial. Embora menos ativas que o Aldicarbe, a diidrouracila e a 2,4,5-trimetoxi-4'-nitrochalcona apresentam potencial para emprego no controle de *M. exigua*, uma vez que podem ser facilmente obtidas a baixo custo, possuem menor toxicidade que vários nematicidas comerciais atualmente em uso e podem servir de base para a obtenção de moléculas modificadas que apresentem maiores atividades contra o mencionado nematóide.

*Orientador: Prof. Denilson Ferreira Oliveira – UFLA.

ABSTRACT

NUNES, Alexandro da Silva. **Organic Substances for Nematode Control in Coffee Plants**. 2008. 45 p. Dissertation (Master in Agroquímica) – Federal University of Lavras, Lavras, MG.*

Two projects were carried out in order to contribute to the development of new methods to control the nematode *Meloidogyne exigua* Goeldi, 1887, which is widespread through Brazilian coffee plantations. In the first one, the bacteria *Acinetobacter jonhsonii* and *Staphylococcus aureus*, previously isolated from tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annum* L.), respectively, were cultivated in liquid medium to afford metabolites that were separated from bacterial cells and freeze-dried. As the dichloromethane soluble metabolites presented nematicidal activity during an *in vitro* assay with second stage *M. exigua* juveniles (J2), they underwent fractionation by column chromatography and high performance liquid chromatography to afford uracil, dihydrouracil and 9H-purine. All substances were active against J2, being dihydrouracil the most efficient. Such substance presented LC_{50} of 204 μg/mL against the nematode, while a LC_{50} of 30μg/mL was observed for the commercial nematicide Aldicarb. Regarding the second project, 149 chalcones and analogues, prepared by aldolic condensations, were submitted to *in vitro* assays with *M. exigua* J2. The most active substance was 2,4,5-trimethoxy-4'-nitrochalcone, which presented LC_{50} of 171μg/mL against J2, while a LC_{50} of 24μg/mL was observed for the commercial nematicide Aldicarbe under the same conditions. When an experiment with *M. exigua* inoculated coffee plants was carried out, the above-mentioned chalcone was as efficient as Aldicarb when used at a concentration 14 times greater than the nematicide. Although less active than Aldicarb, both dihydrouracil and 2,4,5-trimethoxy-4'-nitrochalcone present potential for *M. exigua* control, since such structures can be easily obtained at low cots. Furthermore, they are less toxic than the commercially available nematicides and can be used as leading molecules for structural modifications aimed to increase the activity against the nematodes.

*Advisor: Prof. Denilson Ferreira Oliveira – UFLA

ARTICLE 1

Bacterial Metabolites with Nematicidal Properties

(Prepared in accordance with the European Journal of Plant Pathology)

Helvécio M. dos S. Junior[1]; Alexandro S. Nunes[1]; Denilson F. Oliveira[1]; Vicente P. Campos[2]; Juliana R. C. Silva[3], Rodrigo César da Silva[1].

[1]Universidade Federal de Lavras, Departamento de Química, CEP 37200-000, Caixa Postal, 3037, Lavras, MG, Brasil, e-mail: denilson@ufla.br; [2]Universidade Federal de Lavras, Departamento de Fitopatologia, CEP 37200-000, Caixa Postal 3037, Lavras, MG, Brasil; [3]Campos Carregal Pesquisa e Tecnologia Agrícola LTDA, Rua CL3, Quadra C, Lote 23 – Residencial Lausane, CEP 75907-454, Rio Verde, GO, Brasil.

(Accepted for publication in / /)

Author for correspondence: Denilson F. Oliveira

Abstract

To contribute to the development of new methods to control the nematode *Meloidogyne exigua* Goeldi, 1887, which is widespread through Brazilian coffee plantations, the bacteria *Acinetobacter jonhsonii* and *Staphylococcus aureus*, previously isolated from tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annum* L.), respectively, were cultivated in liquid medium to afford metabolites that were separated from bacterial cells and freeze-dried. As the dichloromethane soluble metabolites presented nematicidal activity during an *in vitro* assay with second stage *M. exigua* juveniles (J2), they underwent fractionation by column chromatography and high performance liquid chromatography to afford uracil, dihydrouracil and 9H-purine. All structures were active against J2, dihydrouracil being the most efficient. Such substance presented LC_{50} of 204μg/mL against the nematode, while a LC_{50} of 30μg/mL was observed for the commercial nematicide Aldicarb. Although less active than Aldicarb, dihydrouracil presents potential for *M. exigua* control, since such structure can be easily obtained at low costs. Furthermore, it is less toxic than the commercial available nematicides and can be used as a leading molecule for structural modifications aimed to increase the activity against the nematode.

Keywords: nematicide, uracil, *Meloidogyne exigua*, *Acinetobacter johnsonii*, *Staphylococcus aureus*.

Introduction

Plant-parasitic nematodes are a constant source of problems for Brazilian farmers due to the negative impact these animals cause to several crops in Brazil (Empresa Brasileira de Pesquisa Agropecuária - Embrapa, 2004). An example is coffee, whose production may be largely affected by *Meloidogyne exigua* Goeldi, 1887 (Campos & Villain, 2005), particularly in the State of Minas Gerais, which accounts for about 50% of the Brazilian production of such commodity (Ministério da Agricultura, Pecuária e Abastecimento - Mapa, 2008). The most effective methods to carry out such nematode control in coffee plantations are based on the use of synthetic nematicides, which increase production costs and contaminate humans and the environment with toxic substances (Chitwood, 2002). To circumvent such problem, in a previous work several rhizobacteria strains were studied in order to select those able to produce substances active against *M. exigua* (Oliveira et al., 2007). Then, the most active crude metabolites underwent a fractionation guided by *in vitro* assays with *M. exigua* second stage juveniles, which resulted in the isolation of common amino acids (results not published) that were probably formed in the culture medium by hydrolysis of proteins or peptides that should be used as nutrients by rhizobacteria. Although the low nematicidal properties of amino acids preclude such substances from being used commercially to control *M. exigua*, they are sufficiently active to cause false positives in a screening program aimed to detect bacterial metabolites with nematicidal activities. As a consequence, in a preliminary work they were removed from crude bacterial metabolites by solvent extraction before the screening process. To continue such work, the most active amino acid free crude metabolites, produced by *Staphylococcus aureus* (Rosenbach 1884) *and Acinetobacter johnsonii* (Brisou & Prévot 1954), were submitted to fractionations guided by *in vitro* assays with *M. exigua* in order to isolate and identify those substances with nematicidal properties.

Materials and methods

Crude metabolite production

This work was carried out with *Acinetobacter johnsonii* Brisou & Prévot (Strain 10JC) and *Staphylococcus aureus* Rosenbach (Strain 18JC), isolated from tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annum* L.), respectively. All microorganisms are on deposit in the Departament of Phytopathology-Federal University of Lavras. Each one was grown in 2.5 L of tryptic soy broth (TSB, Merck) during seven days, at 28º C, under constant stirring (100 rpm), without light incidence. After bacterial cell removal by centrifugation (10,000 g, 15 min), the supernatant liquids were freeze-dried and washed with dichloromethane (4 x 1.0 L). The resulting dichloromethane fractions were combined and concentrated to dryness in a rotatory evaporator. An aliquot (0.5%) of each final residue was dissolved in 2.0 mL of an aqueous 0.01g/mL Tween 80 solution and submitted to the *in vitro* assay.

In vitro assay

The test was performed as described by Amaral et al., (2002). Briefly, *Meloidogyne exigua* Goeldi (Heteroderidae) eggs were extracted from coffee (*Coffea arabica* L.) roots infected with the nematode in accordance with the Hussey and Barker (1973) technique, modified by Boneti and Ferraz (1981). Less than two-day old nematode second-stage juveniles (J2) hatched from the eggs were collected and employed in the *in vitro* assays. Into each 300μL well of a polypropylene 96-well plate, were poured 20μL of an aqueous suspension containing approximately 25 J2 and 100μL of the samples dissolved in aqueous Tween 80 or water. To evaluate dichloromethane soluble crude metabolites, 30μL of Pentabiótico 3000mg/mL (produced by Fort Dodge, Brazil) suspension were also poured into each well to prevent bacterial growth. After 48h at 28^{o}C, one drop of an aqueous 1.0 M NaOH solution was added and J2 that changed

their body shape from straight to curled or hook-shaped within 3 min were considered to be alive, whereas the nematodes not responding to the addition of NaOH were considered dead. This experiment was performed with four replicates per treatment, employing aqueous 0.01g/mL Tween 80 solution or water and Aldicarb (50μg/mL) as negative and positive control, respectively. For the Aldicarb solution preparation, 8.6 g of Temik 150 (150g of Aldicarb/kg), from Rhône-Poulenc AgroBrasil Ltda, were suspended in water and filtered through a filter paper. The resulting solution was diluted with water to the desired concentration. All values of dead J2 were converted to percentage before analysis of variance (ANOVA) and means separation according to the Scott and Knott (1974) test ($P \leq 0.05$), which were done by the use of SISVAR software (Ferreira 2000).

Fractionation of bacterial metabolites

Dichloromethane soluble crude metabolites, obtained as described above, were eluted through a silica gel column (3x15cm; 0.040-0.063 mm, Merck) with hexane (400mL), hexane/ethyl acetate (50:50, 400mL), ethyl acetate (400mL), methanol (800mL), distilled water (1600mL) and 0.1 M hydrochloric acid (1600mL). All resulting fractions were concentrated in a rotatory evaporator and freeze-dried. Only those fractions eluted with methanol were submitted to high performance liquid chromatography (HPLC) analyses with a Gemini Si-C18 column (4.6 mm x 250 mm x 5 μm, Phenomenex) and a diode array detector (DAD) set at 190-400 nm. A gradient elution at 1.0 mL/min was employed: water (10 min), water/methanol (0 to 100% methanol, 10-40 min) and methanol (40-50 min).

The methanol eluted fraction from *A. johnsonii* was fractionated on an HPLC machine equipped with a semi-preparative Gemini Si-C18 column (21.2 mm diameter), employing water (0-10 min), water/methanol (0% - 100%, 10-40

min) and methanol (40-50 min), at a flow of 15.0 mL/min, as mobile phases. All eluents contained 0.1% (v/v) acetic acid. All fractions were concentrated in a rotatory evaporator, freeze dried and analyzed by HPLC. Only fraction 2 (uracil: 3.3 mg) was submitted to the identification process.

Analogously, the methanol eluted fraction of *S. aureus* underwent fractionation by HPLC to afford seven new fractions, among which numbers 1 and 3 were further purified by elution through the larger Gemini column with an aqueous 0.1% (v/v) acetic acid solution at a flow rate of 2.3 mL/min and 13.0mL/min, respectively. Dihydrouracil (19.1mg) and uracil (1.2mg) were obtained as white solids from fraction 1 while fraction 3 afforded 9H-purine (3.2mg).

Identification of isolated substances

To obtain mass spectra, about 0.5 mg of each substance was dissolved in 0.5 mL of water/methanol (1:1) and 20 μL of the resulting solution were directly introduced (5.0 μL/min) into an Agilent 1100 LC/MS Trap spectrometer equipped with an electrospray ionization interface. Probe and cone were maintained at ±3.5 kV and ±25 V, respectively. Nitrogen at 250 ^{o}C was used as nebulizer (200 L/h) and gas drier (20 L/h), while selected ions underwent fragmentation by collisions with helium at 6 x 10^{-6} bar. Substances were also introduced into a Shimadzu spectrometer model PQ5050 through a probe, which was heated from 60 °C to 300 º C during 20 min. Ionization was carried out by electron impact at 70 eV.

Substances were also dissolved in 0.8 mL of hexadeutered dimethylsulfoxide (DMSO-d_6) to obtain hydrogen (^{1}H) and carbon thirteen (^{13}C) nuclear magnetic resonance (NMR) spectra in a Varian INOVA-500 spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz). Solvent peak was used as a reference.

Determination of LC_{50}

Aqueous Aldicarb and dihydrouracil solutions were prepared and submitted to the *in vitro* assay with *M. exigua* J2 as described above, using water as control. The final concentrations inside the wells were 8, 17, 25 and 33μg/mL for Aldicarb and 171, 192, 213 and 233 μg/mL for Dihydrouracil. Average values of dead J2 were converted to percentage, corrected {correct value = 100 [(value - control value)/control value]} and submitted to a probit analyses on POLO-PC software (LeOra Software, 1987).

Results and discussion

According to Bunch et al. (2003), amino acids present low solubility in liquids like dichloromethane. Thus, such solvent was the choice to eliminate those substances from crude bacterial metabolites, since they could afford false positives during the *in vitro* assay with *M. exigua* second stage juveniles (results not published). When submitted to the mentioned assay, both dichloromethane soluble fractions presented activity against the nematode (Table 1).

Table 1. Effect on *Meloidogyne exigua* second stage juveniles (J2) by dichloromethane soluble metabolites produced by *Staphylococcus aureus* and *Acinetobacter johnsonii.*

Dichloromethane soluble metabolite	Dead J2 (%)*
Acinetobacter johnsonii	60c
Staphylococcus aureus	51b
Tween 80 (0.01 g/mL)	19a
Aldicarb (50 μg/mL)	83e

* Means followed by different letters differ significantly ($P \leq 0.05$).

After the fractionation by elution through a silica gel column, all dichloromethane soluble metabolites afforded six fractions (Table 2), among which those of methanol presented the largest amounts. Thus, they were used in the following step, which comprised HPLC analyses. The other fractions were stored for future work.

Table 2. Fractions obtained after elution through a silica gel column of dichloromethane soluble metabolites produced by *Staphylococcus aureus* and *Acinetobacter johnsonii*.

Fractions	Amount (mg)	
	S. aureus	*A. johnsonii*
Hexane	1.5	4.6
Hexane/ethyl acetate (50:50)	1.9	7.5
Ethyl acetate	-	10.1
Methanol	108.0	129.0
Water	-	-
0.1 M hydrochloric acid	-	-

"-" Less than 0.1 mg

The methanol fractions (Table 2) were still very complex according to the HPLC analyses (Figure 1). Thus, during the preparative HPLC process, the main goal was the purification of substances present in larger amounts that could be easily separated from other substances. Such process resulted in the isolation of four substances, which underwent NMR and mass spectrometry analyses to elucidate their chemical structures. Interpretation and comparison of data with those reported in the literature allowed the identification of such substances as uracil, dihydrouracil and 9H-purine (Figure 2). One of the isolated metabolites produced by *Acinetobacter johnsonii* was identical to the isolated substance from *Staphylococcus aureus*.

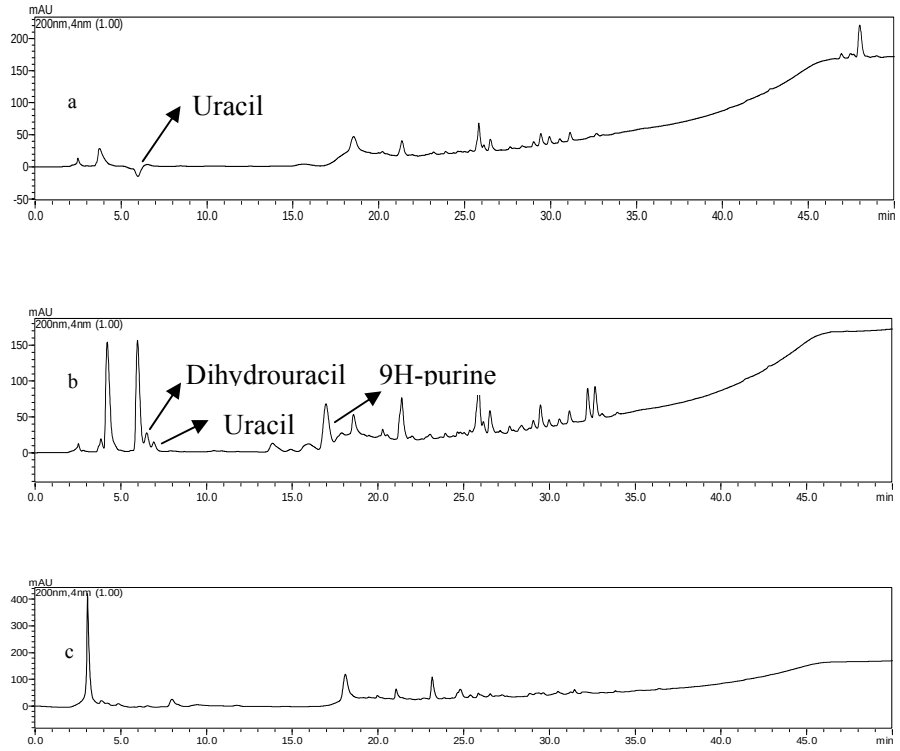
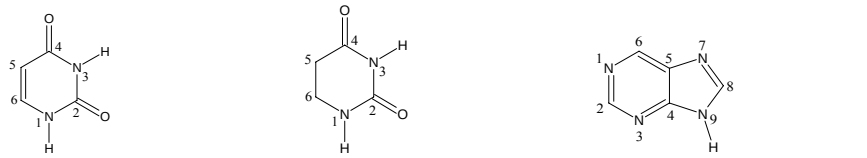


Figure 1. Chromatograms obtained during the HPLC analyses of dichloromethane soluble metabolites eluted with methanol through silica gel columns (Table 2), which were produced by: a) *Acinetobacter johnsonii*; b) *Staphylococcus aureus* c) culture medium without exposition to the microorganisms.



Uracil: produced by *A. johnsonii* and *S. aureus*

Dihydrouracil: produced by *S. aureus*

9H-purine: produced by *S. aureus*

Figure 2. Metabolites produced by *Staphylococcus aureus* and *Acinetobacter johnsonii*.

Chemical shifts observed (Table 3) during the NMR analyses are in perfect agreement with those published for dihydrouracil (Roberts et al., 1978), uracil (Roberts et al., 1978) and 9H-purine (Therese et al., 1975). Such results were corroborated by the mass spectrometry analyses, since 9H-purine afforded peaks at *m/z* (mass/charge) 121 and 119 uma during the positive and negative mode analyses, respectively, performed by direct introduction of the substance into the electrospray ionization interface. Regarding dihydrouracil and uracil, peaks at m/z 114 and 112 uma were respectively obtained after the electron impact ionization process.

Table 3. Hydrogen (^{1}H) and carbon thirteen (^{13}C) chemical shifts (ppm), number of hydrogens, multiplicity (d: dublet; s: singlet; t: triplet; dt: duple triplet) and coupling constant (J) in Hz of dihydrouracil, uracil and 9H-purine.

Position*	Uracil		Dihydrouracil		9H-purine	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1				7.39 (NH; br.s)		
2	151.5		154.0		151.9	9.06 (1H; s)
3				9.84 (NH; br.s)		
4	164.3		171.1		154.8	
5	100.2	5,45 (1H; d, J=7.6)	30.4	2.35 (2H; t, J=6,7)	130.5	
6	142.2	7.40 (1H; d, J=7.6)	35,4	3.11 (2H; dt, J=2.4/6.7)	145.4	9.11 (1H; s)
7						
8					146.4	8.59 (1H; s)
9						

* See Figure 2.

When submitted to the *in vitro* assay with *M. exigua* J2, all the isolated substances significantly increased the nematode mortality (Table 4). Particularly for dihydrouracil, which was the most active structure, a CL_{50} of 204 μg/mL was obtained while the commercial nematicide Aldicarb presented a CL_{50} of 30 μg/mL.

Table 4. Effect of isolated bacterial metabolites at 500 µg/mL on *Meloidogyne exigua* second stage juveniles (J2).

Substance	Dead J2 (%)
Dihydrouracil	100d
Uracil	38c
9H-purine	21b
Tween 80 (0.01 g/mL)	2a

Means followed by different letters differ significantly ($P \leq 0.05$).

Although biological properties have already been described for dihydrouracil (Roberts et al., 1978), uracil (Roberts et al., 1978) and 9H-purine (Therese et al., 1975), their nematicidal properties have been demonstrated for the first time. Further studies involving the bacteria used in this work should permit evaluation of their potential to be used directly to control nematodes or to produce metabolites with nematicidal properties.

Acknowledgements

The authors gratefully acknowledge CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support and fellowships.

Reference

AMARAL, D.R.; OLIVEIRA, D.F.; CAMPOS; CARVALHO, D.A. Efeito de alguns extratos vegetais na motilidade, mortalidade e patogenicidade de *Meloidogyne exigua* do cafeeiro. **Nematologia Brasileira**, Brasília, v. 26, n. 1 p. 43-48, out. 2002.

BUNCH, A. S. R.; MEUNIER, D.; RODIERB, C. L. C.; RAULINA, F.; VIDAL-MADJARC, C. Extraction of organic molecules of exobiological interest for in situ analysis of the Martian soil. **Journal of Chromatography A**, v. 99, n. 1/2, p. 165-174, May 2003.

CAMPOS, V. P.; VILLAIN, L. Nematode parasites of coffee and cocoa. In: LUC, M.; SIKORA, R. A. ; BRIDGE, J. (Ed.) **Plant parasitic nematodes in subtropical and tropical agriculture**. Wallingford UK: CAB Internacional, 2005. p. 529-579.

CHITWOOD, D. J. Phytochemical based strategies for nematode control. **Annual Review of Phytopathology**, Palo Alto, v. 40, p. 221-249, Sept. 2002.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. **Roberto Rodrigues lança oficialmente genoma do café na Embrapa**, 2004. Disponível em: < http://www.embrapa.br/imprensa/noticias/2004/agosto/bn.2004-11-25.2936405122/>. Acesso em: 21 jun. 2008.

FERREIRA, D. F. Análises estatísticas por meio do Sisvar para Windows versão 4.0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45., 2000, São Carlos. **Anais...** São Carlos: Universidade Federal de São Carlos, 2000. pg. 255-258.

LEORA SOFTWARE. **Polo PC** : a use'r to guide probiti logic analisy. , Berkely, CA: Leora Software, 1987. p. 22

MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO. Informe estatístico do café. Disponível em: <http://www.agricultura.gov.br/pls/portal/docs/PAGE/MAPA/SERVICOS/PORTAL_AGRONEGOCIO_CAFE/PORT_AGRO_CAFE_REL_ESTATISTICAS/INFORME%20CAF%C9%20-%20MAIO-08_0.PDF> Acesso em : 28 jun. 2008.

OLIVEIRA, D. F.; CAMPOS, V. P.; AMARAL, D. R.; NUNES, A. S.; PANTALEÃO, J. A.; COSTA, D.A. Selection of rhizobacteria able to produce metabolites active against *Meloidogyne exigua*. **European Journal of Plant Pathology,** Netherland**,** v. 119, n. 4, p. 477-479, Dec. 2007.

ROBERTS, J. L.; POULTER, C. D. 2',3',5'-Tri-*O*-benzolyl[4^{13}C]uridine. AnEfficient, regiospecific syntesis of the pyrimidine ring.**Journal of Organic Chemistry**, v. 43, n. 8, p. 1547-1555, Apr.1978.

THERESE M.; PUGMIRE, C. R. J.; GRANT, D. M.; PANZICA, R. P.; TOWNSEND, L. B. Carbon- 13 magnetic resonance. Quantitative determination of the tautomeric populations of certain purines. **Journal of the American Chemical Society**, v. 97, n. 16, p. 4636-4642, Aug. 1975.

ARTICLE 2

Evaluation of the Nematicidal Activity by Chalcones and Analogues

(Prepared in accordance with the Anais da Academia Brasileira de Ciências style).

Alexandro S. Nunes[1], Denilson F. Oliveira[1], Vicente P. Campos[2], Alan Rodrigues Teixeira Machado[1], Rafael César Russo Chagas[1], Helvécio M. dos S. Junior[1], Alessandra Mascarello[3], Louise D. Chiaradia[3], Ricardo J. Nunes[3] and Rosendo A. Yunes[3].

[1]Universidade Federal de Lavras, Departamento de Química, CEP 37200-000, Caixa Postal 3037, Lavras, MG, Brasil, e-mail: denilson@ufla.br; [2]Universidade Federal de Lavras, Departamento de Fitopatologia, CEP 37200-000, Caixa Postal 3037, Lavras, MG, Brasil; [3]Universidade Federal de Santa Catarina, Departamento de Química, CEP 88040970, Laboratório de Estrutura e Atividade, n. 309 , Santa Catarina, Brasil.

(Accepted for publication in / /)

Author for correspondence: Denilson F. Oliveira

Abstract

To contribute to the development of new methods to control plant parasitic nematodes, 149 chalcones and analogues were synthesized and screened for efficacy against *Meloidogyne exigua* Goeldi, which is widespread through Brazilian coffee plantations. The best results were obtained with 2,4,5-trimethoxy-4'-nitrochalcone, which presented LC_{50} of 171μg/mL against *M. exigua* second stage juveniles, while under the same conditions a LC_{50} of 24 μg/mL was observed for the commercial nematicide Aldicarb. When submitted to an assay with *M. exigua* inoculated coffee plants, the chalcone was as efficient as Aldicarb only when used at a concentration about 14 times higher than that employed for the nematicide. Nevertheless, chalcones still present potential for nematode control since they can be prepared from inexpensive reagents and seem less toxic to human beings and the environment than the commercial nematicides presently available.

Keywords: chalcone, acylhydrazone, nematicide.

Introduction

Losses of around 10% of the entire agricultural world production are caused by plant parasitic nematodes (Zacheo, 1993), which concerns Brazilian farmers and government due to the negative impacts such animals cause to several important crops in Brazil (Embrapa, 2004). An example is coffee, whose production can be largely affected by *Meloidogyne exigua* Goeldi, 1887 (Campos & Villain, 2005), particularly in the State of Minas Gerais, which is responsible for about 50 % of the Brazilian production of such commodity (Mapa, 2008). Once that nematode is established in coffee plantations, the most efficient methods to carry out its control are based on the use of synthetic nematicides, which increase cost production and contaminate human beings and the environment with harmful substances (Chitwood, 2002). As a consequence, less toxic alternatives to control nematodes have been searched for among natural products and analogues, resulting in promising structures such as chalcones, which are flavonoid precursors (Rao et al., 2004). Some of them have presented activity against *Globodera rostochiensis* Wollenweber and *Globodera pallida* Stone (González et al., 1998). Nevertheless, studies aimed to select and use such structures to control nematodes are scarce.

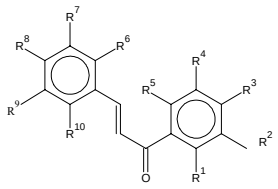
Chalcones can be prepared in a number of ways, the simplest being the aldolic condensation between benzaldehydes and acetophenones (Mc Murry, 1997). Such a method allows the fast synthesis of several chalcones by the use of common and inexpensive reagents. Thus, in order to contribute to the development of new products to control plant parasitic nematodes in coffee plants, the above-mentioned method was used to prepare the structures which were subsequently screened for efficacy against *M. exigua*. Analogously, acyl hydrazides and benzaldehydes condensations were carried out to afford acyl hydrazones, which are structurally similar to chalcones.

Material and Methods

Synthesis of chalcones

Chalcones (Table 1) were synthesized in accordance with the method described elsewhere (Chiardia, et al., 2008; Boeck et al., 2005; Mc Murry, 1997). Briefly, the aldehyde and the acetophenone were dissolved in a methanolic 50% (g/mL) KOH solution and the reaction medium was kept under stirring, at room temperature, during 24 h. Acidification with an aqueous 10 % (g/mL) hydrochloride acid caused precipitation of the product, which was separated by filtration and purified.

Table 1. Synthesized chalcones.



Structure	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}
1	H	H	OCH_3	H	H	H	H	H		
2	H	NO_2	H	H	H	H	H	H		
3	OH	H	H	H	H	H	H	H		
4	H	H	Br	H	H	H	H	H		
5	H	OCH_3	OCH_3	H	H	H	H	H		
6	H	H	NO_2	H	H	H	H	H		
7	H	H	H	H	H	H	H	H		
8	H	OCH_3	OCH_3	OCH	H	H	H	H		

"...continue..."

"TABLE 1, Cont."

9	OH	H	OCH_3	H	OCH_3	H	H	H		
10	OCH_3	H	H	OCH_3	H	H	H	H		
11	OCH_3	H	OCH_3	H	H	H	H	H		
12	H	OH	H	H	H	H	H	H		
13	H	H	Br	H	H	H	OCH_3	OCH_3	H	OCH_3
14	OCH_3	H	OCH_3	H	H	H	OCH_3	OCH_3	H	OCH_3
15	H	H	H	H	H	H	OCH_3	OCH_3	H	OCH_3
16	H	H	OCH_3	H	H	H	OCH_3	OCH_3	H	OCH_3
17	H	OCH_3	OCH_3	H	H	H	OCH_3	OCH_3	H	OCH_3
18	H	H	NO_2	H	H	H	OCH_3	OCH_3	H	OCH_3
19	H	NO_2	H	H	H	H	OCH_3	OCH_3	H	OCH_3
20	H	OH	H	H	H	H	OCH_3	OCH_3	H	OCH_3
21	OCH_3	H	H	OCH_3	H	H	OCH_3	OCH_3	H	OCH_3
22	OH	H	H	H	H	H	OCH_3	OCH_3	H	OCH_3
23	H	H		H	H	H	OCH_3	OCH_3	H	OCH_3
24	OH	Br	OCH_3	H	OCH_3	H	H	H		
25	OH	Br	OCH_3	H	OCH_3	OCH_3	H	H	H	H
26	OH	Br	OCH_3	H	OCH_3	H	H		H	H
27	OH	Br	OCH_3	H	OCH_3	H	H	Br	H	H
28	OH	Br	OCH_3	H	OCH_3	H	H	CH_3	H	H
29	OH	Br	OCH_3	H	OCH_3	H	NO_2	H	H	H
30	OH	Br	OCH_3	H	OCH_3	H	H	H	H	H
31	OH	Br	OCH_3	H	OCH_3	H	H	OCH_3	H	H
32	OH	Br	OCH_3	H	OCH_3	H	H	OH	H	H
33	OH	Br	OCH_3	H	OCH_3	H	H	H	H	H
34	OH	Br	OCH_3	H	OCH_3	H	H			H
35	OCH_3	H	OCH_3	OCH_3	H	H	OCH_3	OCH_3		H
36	OCH_3	H	OCH_3	OCH_3	H	H	H			H
37	OCH_3	H	OCH_3	OCH_3	H	H	H			H
38	OCH_3	H	OCH_3	OCH_3	H	H	OCH_3	H	H	H
39	OCH_3	H	OCH_3	OCH_3	H	H	H		H	H
40	OCH_3	H	OCH_3	OCH_3	H	Cl	H	H	H	H
41	OCH_3	H	OCH_3	OCH_3	H	H	H	H		
42	OCH_3	H	OCH_3	OCH_3	H	H	H	CH_3	H	H
43	OCH_3	H	OCH_3	OCH_3	H	H	H	H	H	H
44	OCH_3	H	OCH_3	OCH_3	H	H	OCH_3	H	H	H
45	OCH_3	H	OCH_3	OCH_3	H	OCH_3	H	H	H	H
46	OCH_3	H	OCH_3	OCH_3	H	H	H	NO_2	H	H
47	OCH_3	H	OCH_3	OCH_3	H	H	H	H	H	H
48	OCH_3	H	OCH_3	OCH_3	H	H	H	H	OCH_3	H
49	H	OCH_3	OCH_3	OCH_3	H	H	H	Cl	Cl	H

"...continue..."

"TABLE 1, Cont."

50	H	OCH_3	OCH_3	OCH_3	H	H	H	Cl	H	H
51	H	OCH_3	OCH_3	OCH_3	H	H	H	CH_3	H	H
52	H	OCH_3	OCH_3	OCH_3	H	Cl	H	H	H	Cl
53	H	OCH_3	OCH_3	OCH_3	H	H	H	Br	H	H
54	H	OCH_3	OCH_3	OCH_3	H	H	H	NO_2	H	H
55	H	OCH_3	OCH_3	OCH_3	H	H	H		H	H
56	H	OCH_3	OCH_3	OCH_3	H	H	H	F	H	H
57	H	OCH_3	OCH_3	OCH_3	H	H	H		H	H
58	H	OCH_3	OCH_3	OCH_3	H	H	H	H	NO_2	H
59	H	OCH_3	OCH_3	OCH_3	H	H	H	OCH_3	H	H
60	H	OCH_3	OCH_3	OCH_3	H	Cl	H	H	H	H
61	H	OCH_3	OCH_3	OCH_3	H	H	OCH_3	OCH_3	OCH_3	H
62	H	OCH_3	OCH_3	OCH_3	H	H	OCH_3	OCH_3	OCH_3	H
63	H	OCH_3	OCH_3	OCH_3	H	H	H			H
64	H	H	Br	H	H	H	H			H
65	H	H	H	H	H	H	H			H
66	OH	H	H	H	H	H	H			H
67	H	H	OCH_3	H	H	H	H			H
68	H	H	NO_2	H	H	H	H			H
69	H	NO_2	H	H	H	H	H			H
70	H	OCH_3	OH	H	H	H	H			H
71	H	OCH_3	OCH_3	H	H	H	H			H
72	OCH_3	H	H	OCH_3	H	H	H			H
73	H			H	H	H	H	Cl	H	H
74	H			H	H	H	H	H	Cl	H
75	H			H	H	H	H	H	Cl	H
76	H			H	H	H	H	Cl	Cl	H
77	H			H	H	H	H	NO_2	H	H
78	H			H	H	H	H	H	NO_2	H
79	H			H	H	H	H	Br	H	H

"...continue..."

"TABLE 1, Cont."

80	H		H	H	H	H	OCH_3	H	H
81	H		H	H	H	OCH_3	OCH_3	H	OCH_3
82	H		H	H	OCH_3	H	OCH_3	H	OCH_3
83	H		H	H	H	OCH_3	OCH_3	OCH_3	H
84	H		H	H	H	H		H	H
85	H		H	H	H	H	CH_3	H	H
86	H		H	H	H	H		H	H
87	H		H	H	H	H			H
88	H		H	H	H	H	H		
89	H	H	H	H	H	H	H	H	
90	H	H	H	H	H	H	H	CH_3	H	H
91	H	H	Br	H	H	H	H			H
92	H	H	NO_2	H	H	H	H			H
93	OCH_3	H	H	OCH_3	H	H	H			H
94	OH	H	H	H	H	H	H			H
95	H	OCH_3	OH	H	H	H	H			H
96	H	H	OCH_3	H	H	H	H			H
97	H	OCH_3	OCH_3	H	H	H	H			H
98	H	NO_2	H	H	H	H	H			H
99	H	OCH_3	OCH_3	OCH_3	H	H	H			H
100	OCH_3	H	OCH_3	H	H	H	H		OCH_3	H
101	H		H	H	H	H	H	NO_2	H
102	H		H	H	H	H	NO_2	H	H

"...continue..."

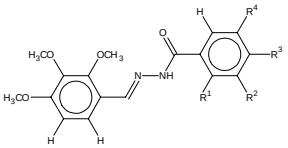
"TABLE 1, Cont."

103	H		H	H	H	H	Cl	H	H
104	H		H	H	H	H	Cl	Cl	H
105	H		H	H	H	H	H	Cl	H
106	H		H	H	H	H	H	H	H
107	H		H	H	H	H	CH_3	H	H
108	H		H	H	H	H	OCH_3	H	H
109	H		H	H	OCH_3	H	H	H	OCH_3
110	H		H	H	H	OCH_3	OCH_3	H	OCH_3
111	H		H	H	H	H		H	H
112	H		H	H	H	H		H	H
113	H		H	H	H	H	F	H	H
114	H		H	H	H	H	Br	H	H
115	H		H	H	H	H			H
116	H		H	H	H	H			H
117	H		H	H	H	H	H		
118	H		H	H	H	H	COOH	H	H
119	H		H	H	H	OCH_3	H	H	OCH_3
120	H		H	H	H	OCH_3	H	H	OCH_3
121	H		H	H	OCH_3	H	OCH_3	H	OCH_3
122	H		H	H	H	H		OCH_3	H
123	OCH_3	H	OCH_3	H	OCH_3	H	H	H	Cl	H
124	OCH_3	H	OCH_3	H	OCH_3	H	H	H	NO_2	H
125	OCH_3	H	OCH_3	H	OCH_3	H	H	H	Cl	H
126	OH	H	OCH_3	H	OCH_3	H	H	H	Cl	H
127	OH	H	OCH_3	H	OCH_3	H	H	H	NO_2	H
128	H	H	H	H	H	H	H	H	H	H

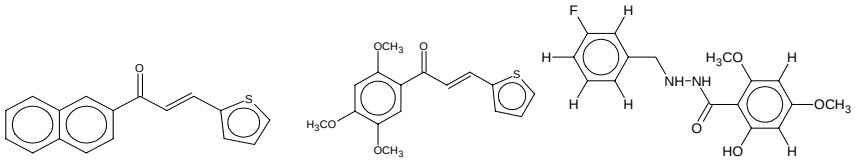
Synthesis of chalcone analogues

As described by Boeck et al., (2006) and Troeberg et al., (2000), acyl hydrazides and benzaldehydes were dissolved in methanol and heated under reflux for 3-4 hours. Crystals formed were filtered off and recrystallized from ethanol to afford pure acyl hydrazones (Figure 1; Table 2).

Table 2. Acyl hydrazones synthesized.



Estructure	R^1	R^2	R^3	R^4
129	H			H
130	Cl	H	H	H
131	OCH_3	H	H	OCH_3
132	F	H	H	H
133	H	OCH_3	OCH_3	OCH_3
134	OCH_3	H	OCH_3	OCH_3
135	H	H	F	H
136	H	H		H
137	H	H	H	H
138	H	H	Br	H
139	H	H	NO_2	H
140	H	H	OCH_3	H
141	H	Br	OH	OCH_3
142	H	I	OH	OCH_3
143	H	H	OH	OCH_3
144			H	H
145	H			H
146	H	H	Cl	H



147 148 149

Figure 1. Chalcones prepared from 2-thiophenecarboxaldehyde and acyl hydrazones synthesized from a fluorinated benzaldehyde.

In vitro* screening against *Meloidogyne exigua

M. exigua eggs were extracted from nematode infected coffee (*Coffea arabica* L.) roots in accordance with the Hussey and Barker (1973) technique, modified by Boneti and Ferraz (1981). Second-stage juveniles (J2) of the nematode were hatched from the the eggs, collected and used. Only two-day old J2 were employed in the *in vitro* assays, which were carried out in accordance with the method described by Amaral et al. (2002). Into each 300 μl well of a polypropylene 96-well plate, were poured 20 μL of an aqueous suspension containing approximately 25 J2 and 100 μL of the sample dissolved in aqueous 0.01 g/mL Tween 80 solution. After 24h at 28^{o} C, one drop of an aqueous 1.0 M NaOH solution was added and J2 that changed their body shape from straight to curled or hook-shaped within 3 min were considered to be alive, whereas nematodes not responding to NaOH addition were considered dead. This experiment was performed with four replicates per treatment, with 0.01 g/mL Tween 80 and the commercial nematicide Aldicarb at 50μg/mL as negative and positive control, respectively. For the Aldicarb solution preparation, 8.6g of Temik 150 (150g of Aldicarb/kg), from Rhône-Poulenc AgroBrasil Ltda, were suspended in water and filtered through a filter paper. The resulting solution was diluted with water to the desired concentration. All values of dead J2 were

converted to percentage to be submitted to variance analysis (ANOVA). Means were separated according to the Scott-Knott test ($P \leq 0.05$). Statistical analyses were done using SISVAR software (Ferreira, 2000).

Determination of LC_{50}

Solutions of Aldicarb and chalcone 18 at different concentrations were prepared and submitted to the *in vitro* assay with *M. exigua* J2 as described above, using water (for Aldicarb) and aqueous Tween 80 (for chalcone) as controls. The final concentrations inside the wells were 50, 40, 30, 20 and 10 μg/mL for Aldicarb and 120, 150, 180, 210 and 240μg/mL for chalcone 18. Average values of dead J2 were converted to percentage, corrected {correct value = 100 [(value - control value)/control value]} and submitted to probit analysess on POLO-PC software (LeOra Software, 1987).

Assay with *Meloidogyne exigua* inoculated coffee plants

A mixture of soil and sand at a ratio of 1:1 (v/v) was disinfested with methyl bromide (150 mL/m^3) and poured into 3 L pots. In each pot, a six-month old coffee plant (*Coffea arabica* L. cv. Catucaí Amarelo) (Rubiaceae) was planted. After 20 days in a greenhouse, each plant was inoculated with 10,000 *M. exigua* eggs suspended in 10 mL of water, through four 1.0 cm large and 4.0 cm deep equidistant cylindrical holes in the substrate, around the plant. Just after such inoculation, each treatment, comprising 100 mL of an aqueous 0.01 g/mL Tween 80 solution containing chalcone 18 (1,796, 3,591 and 7,182 μg/mL) or Aldicarb (500μg/mL), was also added through the same holes. After 90 days in a greenhouse, plant aerial parts were removed and roots were gently washed with water for weighting and gall counting. Then, eggs were extracted from roots as described elsewhere (Boneti & Ferraz, 1981) and counted in the resulting suspension by the use of an inverted microscope. This experiment was

carried out in a randomized six block design, employing 0.01g/mL Tween 80 and water as controls. Except for the conversion of values to percentage, statistical analyses were performed as described for the *in vitro* assay.

Results and Discussion

Although most of the substances studied (Tables 1 and 2; Figure 1) afforded values statistically superior to the one observed for the negative control (aqueous 0.01g/mL Tween 80 solution) during the *in vitro* assay with *M. exigua* J2, only chalcone 18 (2,4,5-trimethoxy-4'-nitrochalcone), presented potential for further studies, since it caused 100% of J2 deaths while values for the other active substances were always below 30%. When submitted to the same assay with different concentrations, LC_{50} of 171μg/mL and 30μg/mL were obtained for the chalcone 18 and Aldicarb, respectively.

Since the recommended amount of Aldicarb to be used in the *M. exigua* inoculated coffee plant assay was 21 times higher than the corresponding LC_{50} (Oliveira et al, 2007), chalcone 18 LC_{50} was also multiplied by 21 to achieve the value to be used in such experiment. Additionally, twice and half of the calculated concentration were also employed for chalcone 18. No statistical difference could be observed for root mass, but the nematode population was significantly reduced by the most concentrated solution (Figure 4).

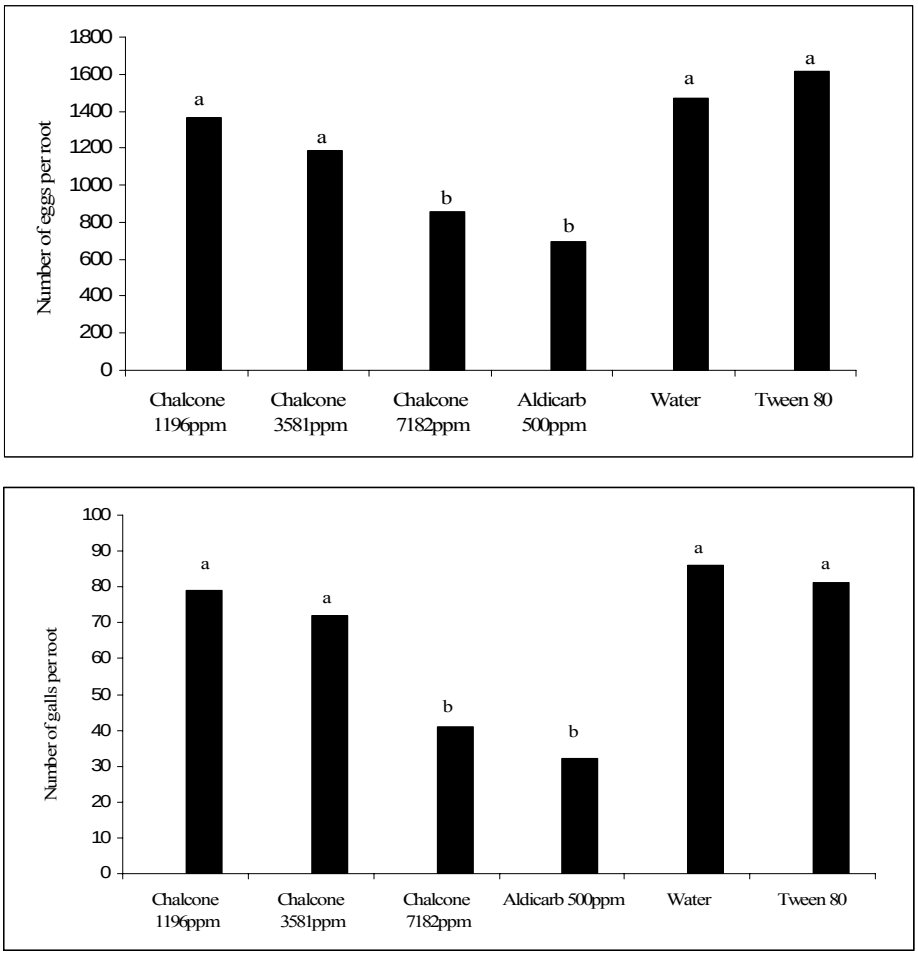


Figure 4. Effect of 2,4,5-trimethoxy-4'-nitrochalcone (chalcone 18) and Aldicarb on *Meloidogyne exigua* in coffee plants (*Coffea arabica* L. cv. Catucaí amarelo): galls per root x treatment (lower panel) and eggs per root x treatment (upper panel). In each graphic, columns with the same letters do not differ significantly ($P \leq 0.05$).

Although a concentration around 14 times higher than that employed for Aldicarb was necessary for chalcone 18 to achieve the same level of *M. exigua* control observed for such nematicide (Figure 4), the chalcone still presents

potential for commercial use in nematode control, since it can be easily prepared from inexpensive reagents. Furthermore, chalcones do not appear as toxic as Aldicarb and other commercial nematicides to human beings and the environment, since in experiments with albino rats some structures have presented LD_{50} around 1048 mg/kg (Alonso & Navarro, 1993). Thus, further evaluation of chalcone 18 as a nematicide and screening of other similar structures for efficacy against plant parasitic nematodes should be carried out in the future.

Acknowledgements

The authors gratefully acknowledge CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior), for fellowships.

Reference

ALONSO, J.; NAVARRO, E. **Estudio toxicológico de una chalcona sintética**. Report to the Canary Islands, Spain: Report to the Instituto Universitario de Bio-Orgánica, 1993. 13 p.

AMARAL, D.R.; OLIVEIRA, D.F.; CAMPOS; CARVALHO, D.A. Efeito de alguns extratos vegetais na motilidade, mortalidade e patogenicidade de *Meloidogyne exigua* do cafeeiro. **Nematologia Brasileira**, Brasília, v. 26, n. 1 p. 43-48, out. 2002.

BOECK, P.; FALCÃO, C. A. B.; LEAL, P. C.; YUNES, R. A.; CECHINEL F. V.; TORRES-SANTOS, E. C.; BERGMANN, B. Synthesis os chalcone analogues with increased antileishmanial activity. **Bioorganic & Medicinal Chemistry**, v. 14, n. 5, p. 1538-1545, Mar. 2006.

BOECK, P.; LEAL, P. C.; CECHINEL F. V.; YUNES, R. A. ; LOPES, S.; SORTINO, M.; ESCALANTE, A.; FURLAN, R. L.; ZACCHINO, S. Antifungal activity and studies on mode of action of novel xanthoxyline-derived chalcones. **Archiv Der Pharmacology**, Alemanha, v. 338, n. 2/3, p. 87-95, 2005.

BONETI, J. I. S.; FERRAZ, S. Modificação do método de Hussey e Barker para extração de ovos de *Meloidogyne exígua* de cafeeiro. **Fiopatologia Brasileira,** Brasília, v. 6, n. 3, p.553, out. 1981.

CAMPOS, V. P.; VILLAIN, L. Nematode parasites of coffee and cocoa. In: LUC, M., SIKORA, R. A. ; BRIDGE, J.(Ed.) **Plant parasitic nematodes in subtropical and tropical agriculture**. Wallingford UK: CAB Internacional, 2005. p. 529-579.

CHIARDIA, L. D.; SANTOS, A. R.; VITOR, C. E.; VIEIRA, A. A.; LEAL, P. C.; CALIXTO, J. B.; NUNES, R.J.; YUNES, R. A. . Synthesis and pharmacological activity of chalcones derived from 2,4,6-trimethoxyacetophenone in RAW 264.7 cells stimulated by LPS: quantitative structure-activity relationships. **Bioorganic & Medicinal Chemistry**, v. 16, n. 2, p. 658-667, Jan. 2008.

CHITWOOD, D. J. Phytochemical based strategies for nematode control. **Annual Review of Phytopathology**, Palo Alto, v. 40, p. 221-249, Sept. 2002.

EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA. **Roberto Rodrigues lança oficialmente genoma do café na Embrapa**, 2004. Disponível em: < http://www.embrapa.br/imprensa/noticias/2004/agosto/bn.2004-11-25.2936405122/>. Acesso em: 21 jun. 2008.

FERREIRA, D. F. Análises estatísticas por meio do Sisvar para Windows versão 4.0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45., 2000, São Carlos. **Anais...** São Carlos: Universidade Federal de São Carlos, 2000. pg. 255-258.

GONZÁLEZ, J. A.; BRAUN, E. A. Effect of (E)-Chalcone on Potato-Cyst Nematodes (*Globodera pallida* and *Globodera rostochiensis*) **Journal Agriculture Food Chemistry**, v. 46, n. 3, p. 1163 - 1165, Mar. 1998.

LEORA SOFTWARE. **Polo PC** : a use'r to guide probiti logic analisy. , Berkely, CA: Leora Software, 1987. p. 22

MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO. Informe estatístico do café. Disponível em: <http://www.agricultura.gov.br/pls/portal/docs/PAGE/MAPA/SERVICOS/PORTAL_AGRONEGOCIO_CAFE/PORT_AGRO_CAFE_REL_ESTATISTICAS/INFORME%20CAF%C9%20-%20MAIO-08_0.PDF> Acesso em : 28 jun. 2008.

MCMURRY, J. **Química Orgânica.** 4. ed. Rio de Janeiro: LTC, 1997. v. 2, cap. 23, p. 262-267.

OLIVEIRA, D. F.; CAMPOS, V. P.; AMARAL, D. R.; NUNES, A. S.; PANTALEÃO, J. A.; COSTA, D.A. Selection of rhizobacteria able to produce metabolites active against *Meloidogyne exigua.* **European Journal of Plant Pathology,** Netherland**,** v. 119, n. 4, p. 477-479, Dec. 2007.

RAO, Y. K.; FANG, S. H.; TZENG, Y. M. Differential effects of synthesized 2'-oxygenated chalcone derivatives : modulation of human cell cycle phase distribution. **Bioorganic & Medicinal Chemistry**, v. 12, n. 10, p. 2679-2686, May 2004.

TROEBERG, L.; CHEN, X.; FLAHERTY, T. M.; MORTY, R. E.; CHENG, M.; HUA, H.; SPRINGER, C.; MCKERROW, J. H.; KENYON, G. L.; LONSDALE-ECCLES, J. D.; COETZER, T. H. T.; COHEN, F. E. Chalcone, acyl hidrazide and related amides kill cultured *Trypanosoma brucei*. **Molecular Medicine**, v. 6, n. 8, p. 660-669, Aug. 2000.

ZACHEO, G. “Introduction”. In: KHAN, W. W. (Ed.). “**Nematode Interactions**”. Londres: Chapman and Hall,1993. p. 1-25.

ANNEXES

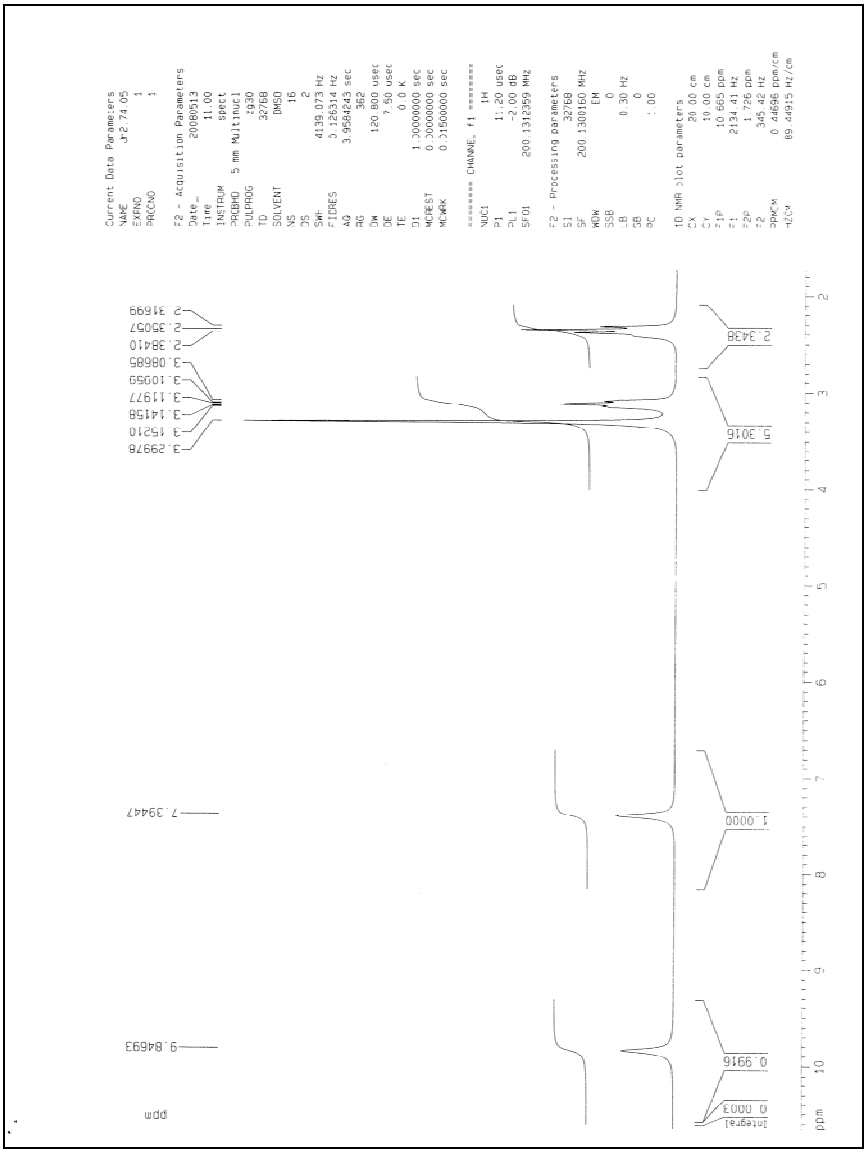


FIGURE 1: ^{1}H NMR Spectrum of dihydrouracil in DMSO-d_6, 200MHz

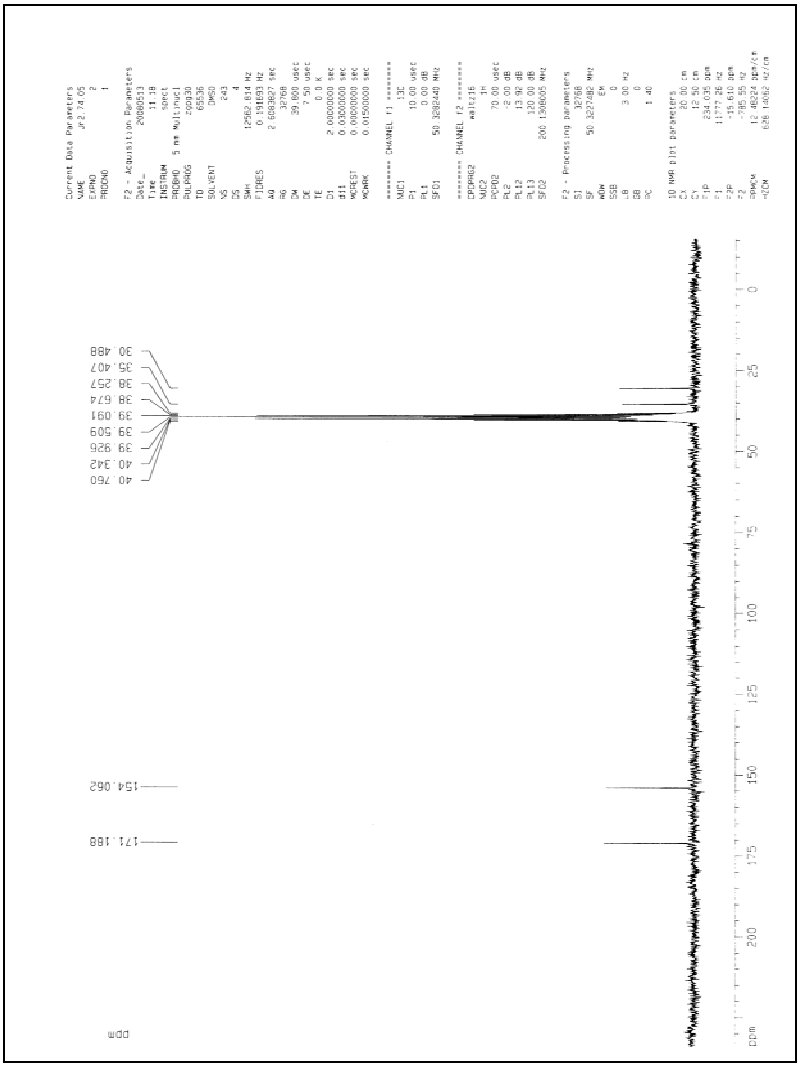


FIGURE 2: ^{13}C NMR Spectrum of dihydrouracil in DMSO-d_6, 50MHz.

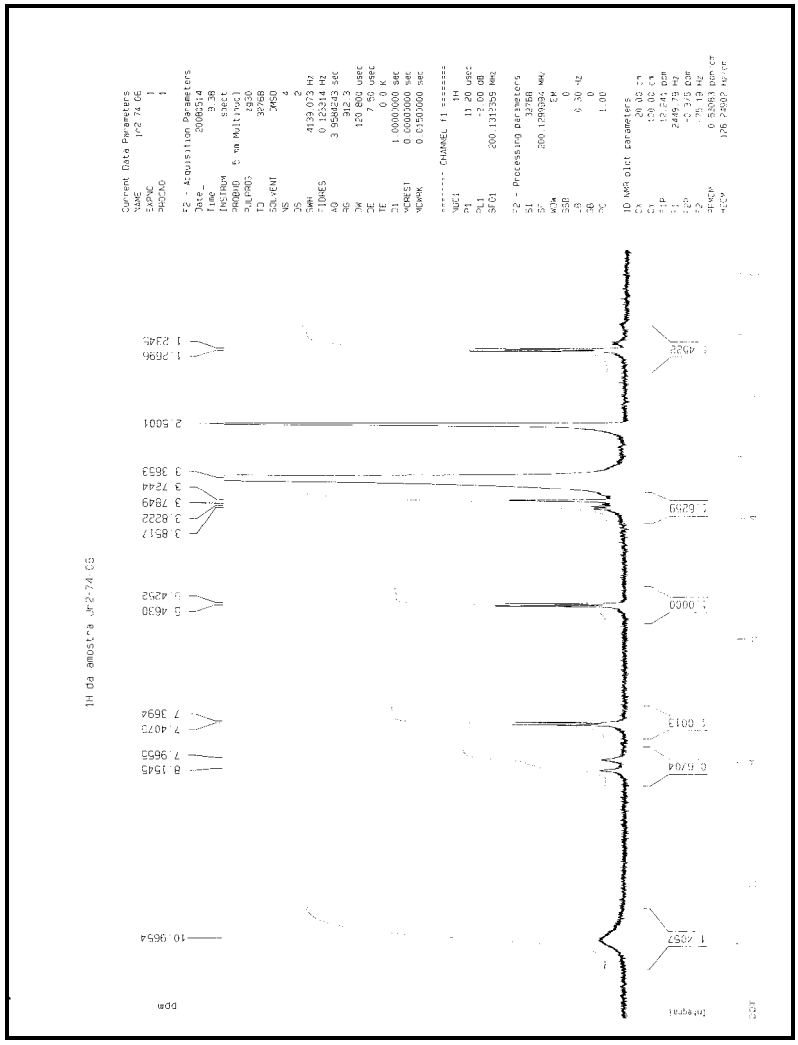


FIGURE 3: ^{1}H NMR Spectrum of uracil (produced by *Staphylococcus aureus*) in DMSO-d_6, 200MHz

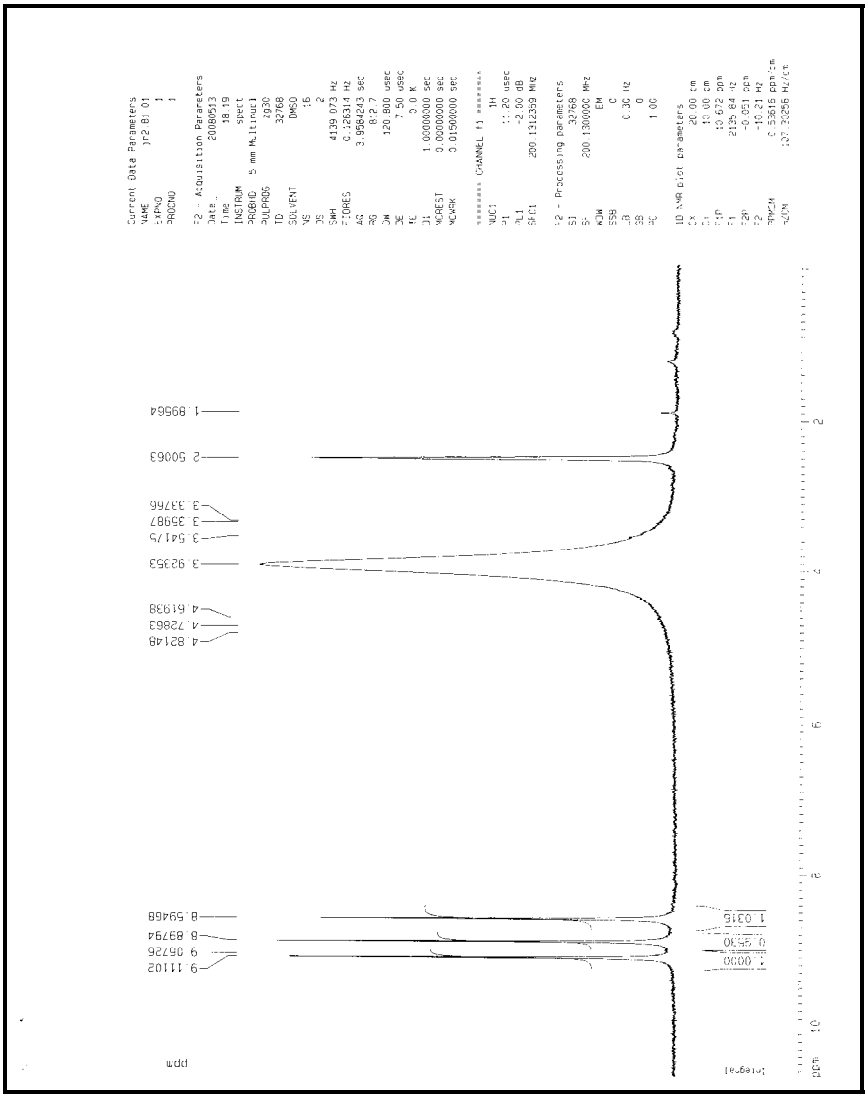


FIGURE 4: ^{1}H NMR Spectrum of 9H-purine in DMSO-d_6, 200MHz

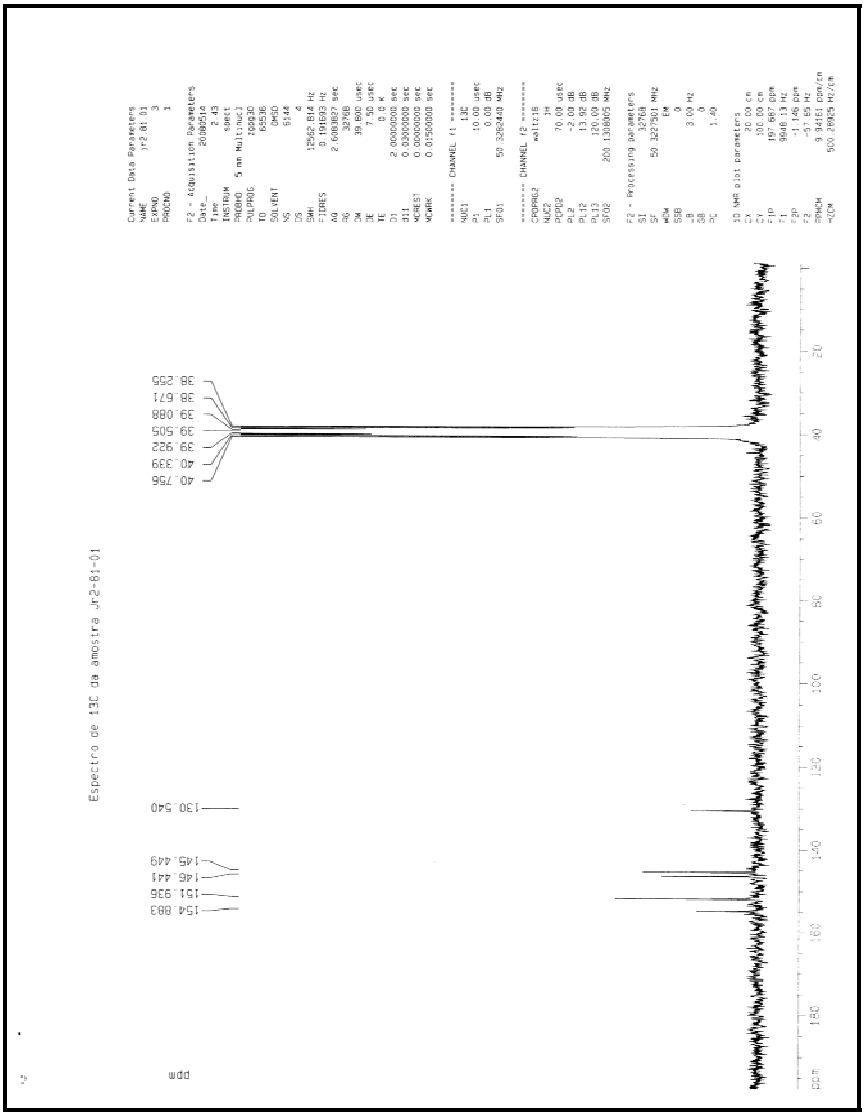


FIGURE 5: ^{13}C NMR Spectrum of 9H-purine in DMSO-d_6, 50MHz

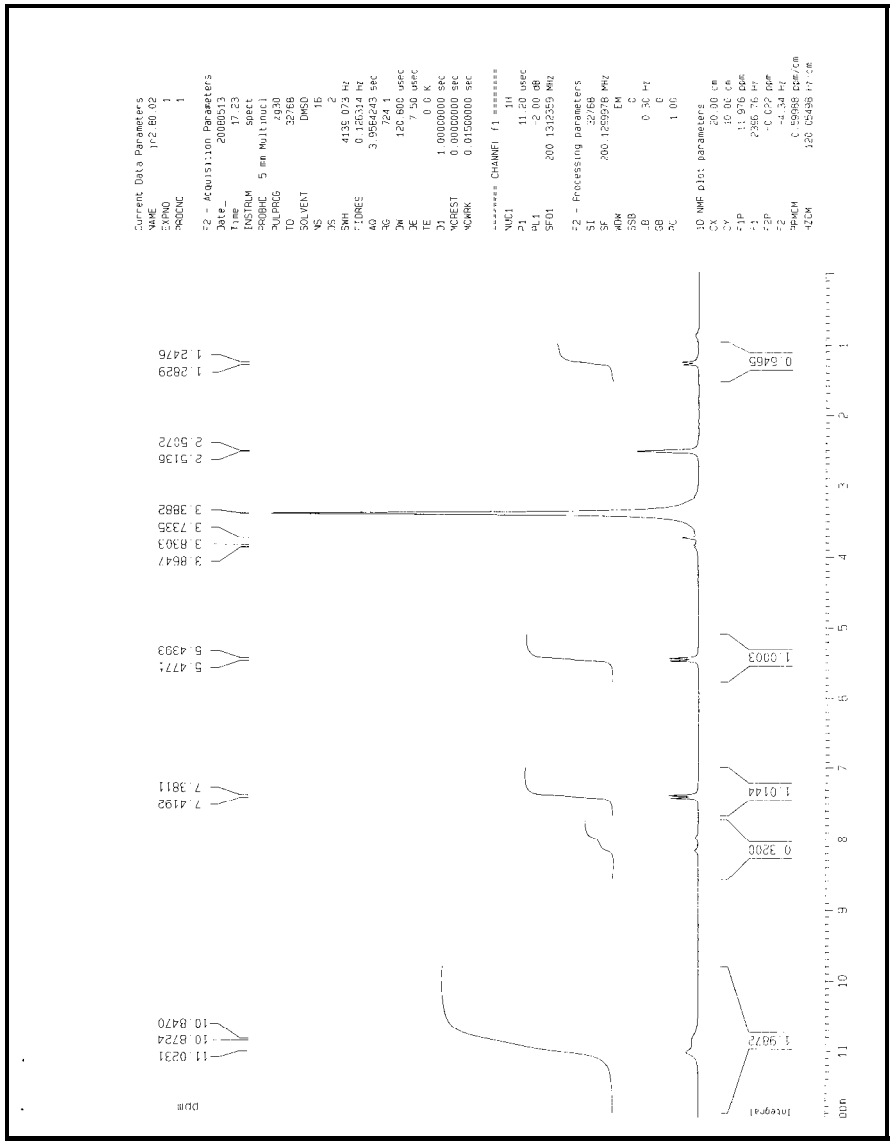


FIGURE 6:^{1}H NMR Spectrum of uracil (produced by *Acinetobacter johnsonii*) em DMSO-d_6, 200MHz

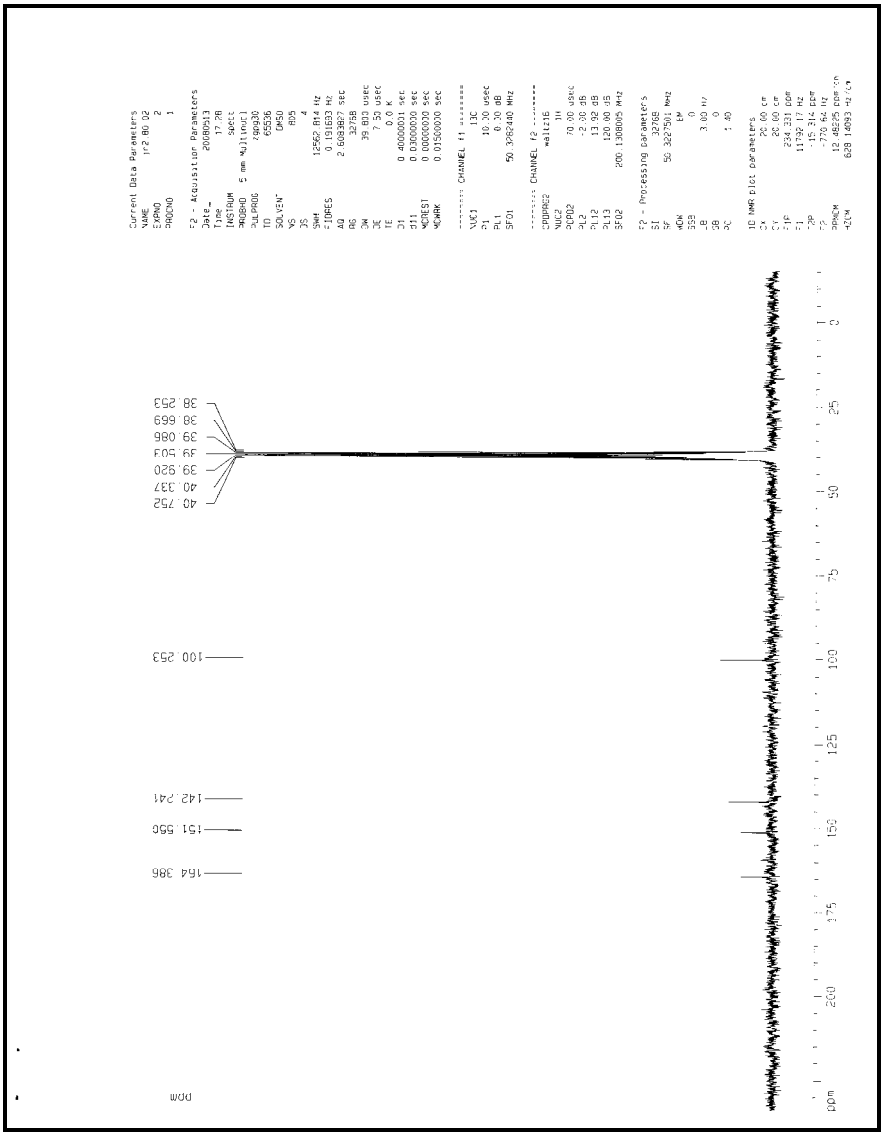


FIGURE 7: ^{13}C RMN Spectrum of uracil (produced by *Acinetobacter johnsonii*) in DMSO-d_6, 200MHz

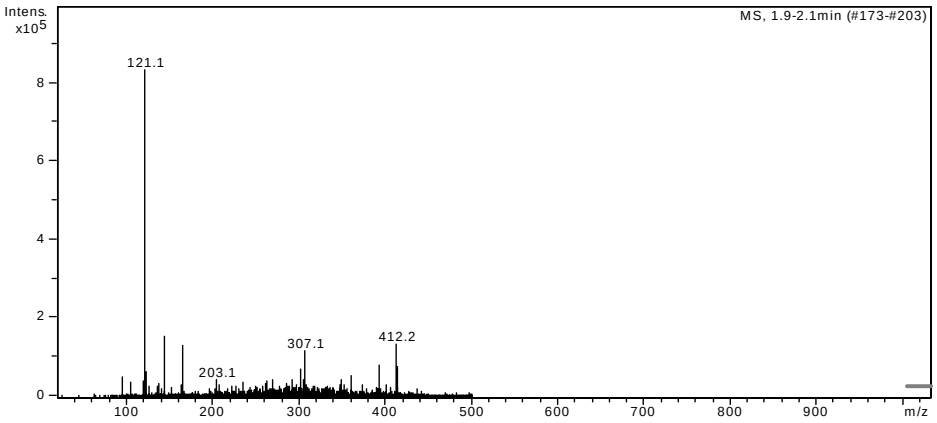


Figure 8. Mass spectrum (EI-MS) of 9H-purine, mode position

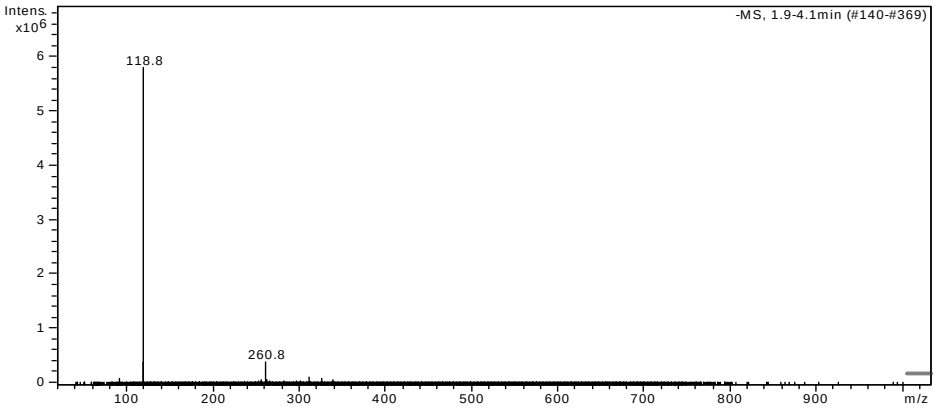


Figure 9. Mass spectrum (EI-MS) of 9H-purine, mode negative

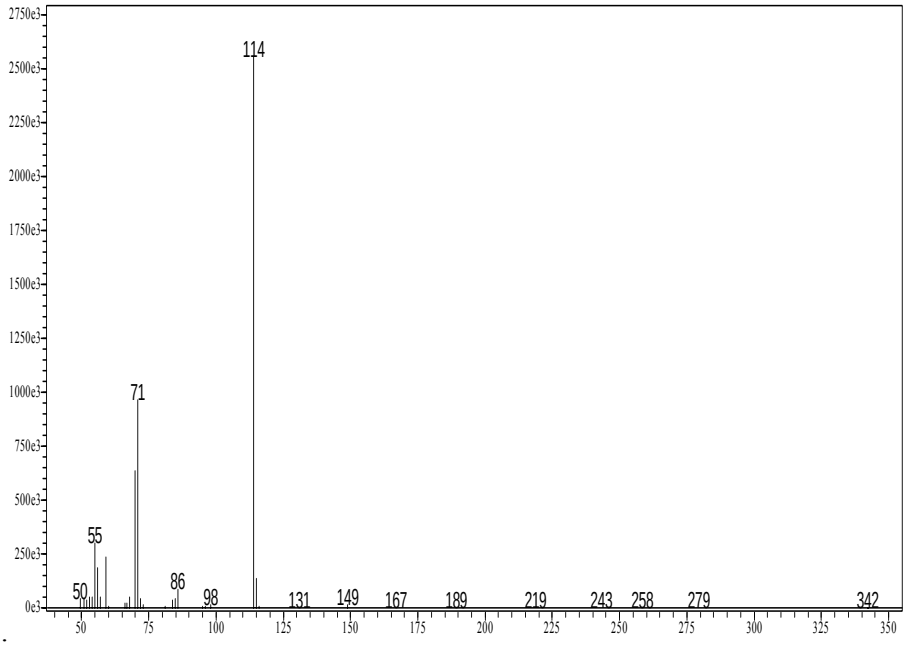


Figure 10. Mass spectrum (GC-MS) of uracil.

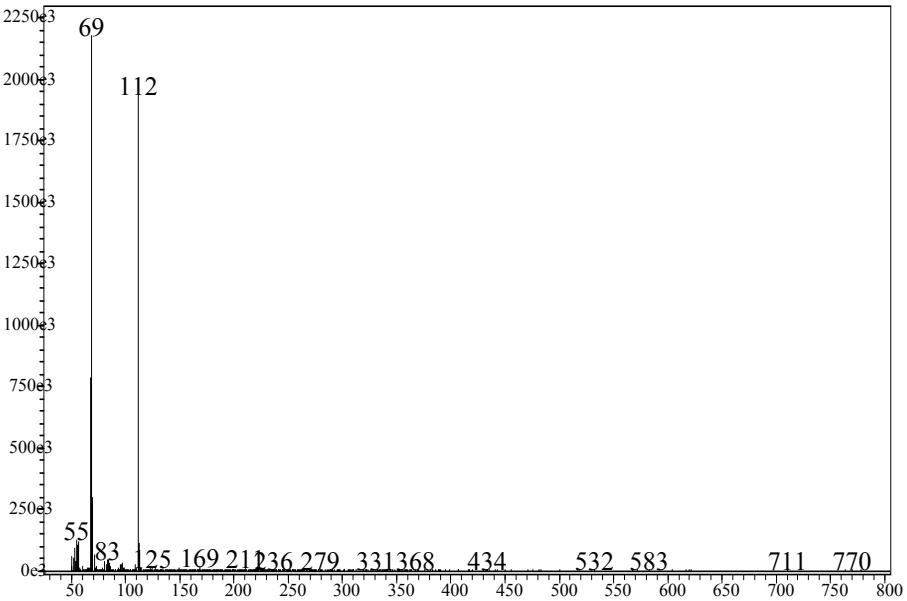


Figure 11. Mass spectrum (GC-MS) of dihydrouracil.