Original Research Article

Activity of Catambra extracts against *Meloidogyne* spp.

Aims: In this study we tested four Catambra commercial formulates (hydroalcoholic extracts) used as insecticides in Italy, against *Meloidogyne incognita* and *Meloidogyne javanica*.
Catambra plant (Ambrogios, Catalpa) a European patent belonging to Ambrogio srl, rich in an iridoid glycoside catalpol, is used to date as a mosquito deterrent, but no data exist on its biological activity against soil parasites as *Meloidogyne* sp. *Catalpa* species demonstrate various biological properties of pharmaceutical and agronomical interest, mainly due to their contents in glycosides.
Place and Duration of Study: All paralysis bioassays were performed in Pesticide Science Laboratory, of Aristotle University Thessaloniki, Greece, while chemical analysis in Department of Life and Environmental Sciences, University of Cagliari.
Material and Methods: The activity was tested in paralysis bioassays with freshly hatched second-stage juveniles (J2) immersed in test solutions for four time periods (12, 24, 48 and 96 hours). Catalpol contents of the extracts were measured by means of HPLC/MS and its activity was also tested against J2. The effect of exhaustive evaporation of hydroalcoholic extracts prior use in bioassays was also studied for nematicidal activity.
Results: Out of the four extracts tested without being evaporated first, two extracts (C-2 and C-4) exhibited activity and the $EC_{50/48h}$ on *M. incognita* were calculated at 640 and 728 µg/ml. On the other hand, *M. javanica* was proven sensitive only to the C-4 extract and the activity had an $EC_{50/48h}$ value at 500 µg/ml. When extracts were exhaustively evaporated before use, only the C-4 paralysed J2 and the EC_{50}/48 h values were 728 and 500 µg/ml against *M. incognita* and *M. javanica,* respectively. Interestingly, catalpol was not found to be nematicidal at the concentration range of 100 to 1000 µg/ml. The nematicidal activity of the extracts was therefore not correlated with their content in catalpol. This was confirmed by the chemical composition study, since the C-4 extract, exhibiting activity against both species, was of low contents in catalpol (21 µg/ml), and the C-3 extract that had the highest contents in catalpol (358 µg/ml) did not paralyse J2.
Conclusion: Catambra nematicidal activity is not based in its contents in catalpol, and further studies are conducted to delineate for activity among other constituent components.

Keywords: Catambra; catalpol; nematicidal activity; Meloidogyne spp.; bionematicides

1. INTRODUCTION

Plant parasitic nematodes are among the most destructive group of plant pathogens worldwide and their control is extremely challenging [1, 2]. Phytonematodes parasite a large

variety of crops worldwide and their impact on yield losses has been estimated to 100 billion US $ annually [3, 4]. Specifically, Root-knot nematodes (RKN) or *Meloidogyne* sp. is the most notorious genus [3]. Strategies to control these pests include chemical nematicides combined with cultural practices, crop rotation, and resistant cultivars. Considering that only few synthetic nematicides are still authorized [5], natural nematicides isolated from plants or microorganisms are gaining an increased interest as alternative less toxic and eco friendly biocontrol agents [1, 6]. The new European legislation asks from the members to adopt (by 1 January 2014) national action plans and take all necessary measures to promote low pesticide-input farming, including integrated pest management, and to ensure that professional users of pesticides shift towards a more environmentally-friendly use of all available crop protection measures, giving priority to low-risk, non-chemical alternatives wherever possible [7, 8]. This action gives a specific status to nonchemical and natural alternatives rather than conventional chemical pesticides, and requires them to be given priority wherever possible. As nematodes cause severe economic loss to many agricultural crops, a great demand for discovering environmentally acceptable substitutes for commercial nematicides exists, creating a great market opportunity for biorational products such as botanical nematicides. Recently, it has been reported that plant derived nematicidal compounds, such as furfural, (E)-2-decenal, (E,E)-2,4-decadienal, and 2-undecanone are greatly effective in controlling RKN both in vitro and in pot experiments [9-13]. Furthermore, according to the international bibliography, the control of nematodes on different vegetables has been often performed by botanical extracts [14-18]. Natural substances of plant origin that have been found to exhibit nematicidal activity are Plant Secondary Metabolites (PSMs) of various plants and belong to different chemical classes, such as aldehydes and ketones, alkaloids, glycosides, glucosinolates and isothiocyanates, limonoids, quassinoids and saponins, organic acids, phenolics and flavonoids, polyacetylenes and terpenes [1, 19].

Catalpa (Bignoniaceae) species exhibit a range of biological activities of interest in pharmacy and agriculture. *Catalpa ovata* stem bark may be a useful external medicine for treatment of atopic dermatitis [20] and enhancement T cell-mediated immune responses with anticancer properties. [21] *Catalpa bignonioides*, known for its contents in napthoquinone, catalponol, catalponone and catalpalactone [22] exhibits significant antioxidant activity [23]. D. Muñoz-Mingarro et al. [24] reported anti-inflammatory and antinociceptive actions in ethyl ether, butanolic and aqueous fractions of *Catalpa bignonioides* pod extract. In fact due to the interesting biological properties of the phenylethanoid glycosides, advances have been made so as to increase 10 times their production infecting *C. ovata* root cultures with four agropine strains of *Agrobacterium rhizogenes* [25].

Interestingly, soil treating with *C. bignonioides* has been found to act as biological soil disinfestations for 4 years against the nematode *Pratylenchus fallax* [26]. On the other hand, flavone and flavonol glycosides, as constituent compounds of *Vicia pannonica* Crantz var. purpurascens have been proven of considerate anthelmintic activity [27]. Nonetheless, to date no data exist on the activity of Catalpa extracts against *Meloidogyne* sp.

Catambra plant belonging to the Bignoniaceae family coming from northern America, has been developed and increased in Giovanni Ambrogio's nursery, and has been protected by a European patent 2006/011 (no. EU27931). This tree is infested by few pests of any consequence because it exhibits significant insecticidal activity. Thanks to the high contents of catalpol, catambra shows a repellent action against mosquitoes and in fact the tree is sold in the market as anti-mosquito. Catalpol is an iridoid glucoside (Figure 1), which is simply monoterpene with an attached glucose molecule and its pharmacological action has not been fully established. Chemical analysis carried out from the Experimental Centre for Floriculture confirms that Catambra contains four times a quantity of catalpol contained in other Catalpa species [28].

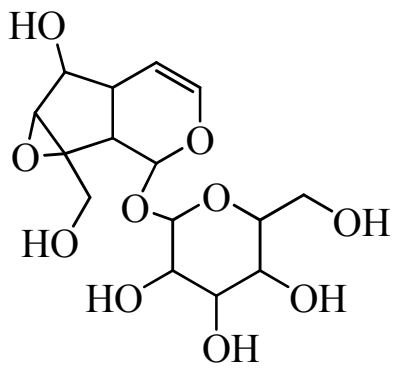

Figure 1. Structure of catalpol

~~Structure of catalpol~~

In this study, four commercial Catambra formulates (hydroalcoholic extracts), used as insecticides in Italy, were tested against *M. javanica* and *M. incognita*, in paralysis bioassays with 24 h second stage juveniles (J2) and we correlated the nematicidal activity with the extracts content in catalpol, determined by HPLC/MS analysis. To study for the activity of volatile and non volatile compounds in the extracts, the paralysis experiment was performed using a) the hydroalcoholic extracts and b) the dry yield of the extracts after exhaustive evaporation of the solvents.

2. MATERIAL AND METHODS

2.1. Hydroalcoholic Catambra Extracts

Four commercial Catambra formulates (hydroalcoholic extracts) were provided from Ambrogio Vivai Srl. Original Catambra is guaranteed by the European Patent and it is recognizable thanks to the anti-rip label on its foliage with a special control code and an indelible brand on its trunk [28]. The four Catambra formulates were hydroalcoholic extracts (coded as C-1 C-2, C-3, C-4), obtained from the Catambra plant and used in paralysis bioassays at concentrations of 62.5 to 1000 µg/ml, in order to evaluate their nematicidal activity.

2.2. Chemical composition analysis

Catalpol contents were measured on a HPLC-MS system, Varian tandem mass spectrometer (Palo Alto, CA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with a 1100 series diode array detector (Agilent, Waldbronn, Germany). The column used was a Phenomenex GEMINI 3 µm C18 (100 x 4.6 mm). The mobile phase consisted of acqueous 0.1% formic acid (A) and (B) methanol. The solvent gradient started at 20 % of A and 80 % of B, reaching 100 % of B at 10. The flow was 0.4 mL/min and the injection volume was 20 µL.Spectrum scan was from 200 to 600 nm. For the detector's analysis parameters set and for the HPLC data analysis, the software ChromQuest Version 4.0 Build 1120 and HP Chem, Version A.07.01 were used, respectively. LC-MS analysis was performed in electrospray ionization interface, in the positive ion mode, as follows: shield potential, 775 V; capillary potential, 55 V; needle voltage, 5700 V; nitrogen pressure, 54 mTorr; housing temperature, 56 ºC; drying gas temperature, 380 ºC; scan time 1; detector multiplier voltage, 1200 V; isolation width of m/z 1.6 for the quadrupole 1; scan 100-1500 amu.

2.3. Nematode motility bioassays

2.3.1. Nematode cultures

A population of *M. incognita* and *M. javanica* originally obtained from tomato roots collected from a greenhouse in Vasilika, Thessaloniki, Northern Greece, was reared on tomato, (*Solanum lycopersicum* L.) cv. Belladonna, a cultivar which is very susceptible to root-knot nematodes. Roots of inoculated plants, 40 days after inoculation, were uprooted, washed free of soil and cut into 2 cm pieces. Eggs were extracted with sodium hypochlorite according to the method of Hussey and Barker, 1973, [29]. Second-stage juveniles (J2) were allowed to hatch in modified Baermann funnels at 27 ^{o}C. All J2 hatching in the first 3 days were discarded and freshly hatched second-stage juveniles (J2) collected during the subsequent 24 hours were used for the bioassays, according to methods described by Ntalli et al., 2009, [30].

2.3.2. Effect of Catambra extracts and catalpol on J2 motility.

Effects of Catambra extracts on J2 motility were tested at the concentrations 62.5, 125, 250, 500, and 1000 μg/ml. Catambra formulates were directly and serially diluted in distilled water containing Tween-20 (0.6% v/v) to produce test solutions, the concentration of which was calculated according to the extract's yield in dry components, after exhaustive evaporation of the solvents under stream of nitrogen. Additionally, weighed amounts of the dried Catambra extracts, obtained after this exhaustive evaporation of the solvents, were diluted in distilled water containing Tween-20 (0.6% v/v) to prepare test solutions of the above concentrations. Preliminary trials showed that nematodes exposed at these concentrations of Tween-20 were not affected. Plain water as well as a mixture of water, and Tween-20 (0.3% v/v) (carrier control) served as controls. Nematicidal activity of commercially available catalpol was used at a concentration of 100, 250, 500, 750 and 1000 μg/ml. Catalpol stock solution was prepared in methanol, whereas distilled water containing Tween-20 (0.6% v/v) was used for further dilutions. Final concentrations of methanol and Tween-20 in each well never exceeded 1 and 0.3 % v/v, respectively. Distilled water as well as a mixture of water, methanol and Tween-20 (1 and 0.3 % v/v) (carrier control) served as controls. In all cases, working solutions were prepared containing double the test concentration and were then mixed in Cellstar® 96-well cell culture plates (Greiner bio-one) at a ratio of 1:1 v/v with suspensions of approximately 15 J2 added to each well. Multiwell plates were covered to prevent evaporation and were maintained in the dark at 27°C. Juveniles were observed with the aid of an inverted microscope (Euromex, Holland) at 40X after 12, 24, 48, 96 h and were ranked into two distinct categories: motile or paralysed. Each treatment was replicated 5 times as a split plot design, and each bioassay was performed twice.

2.4. Statistical analysis

The data of nematicidal activity are presented as percentages of paralyzed J2 in the microwell assays corrected by elimination of the natural death/paralysis in the water control according to the Scheider-Orelli's [31] formula: Corrected % = [(Mortality % in treatment − Mortality % in control)/(100 − Mortality % in control)] × 100, and they were analyzed by ANOVA combined over time. ANOVA analysis was indicated no significant interaction between treatment and time, therefore means were averaged over experiments. Corrected percentages of paralysed J2 treated with extracts were subjected to Probit analysis and the effective concentrations (EC_{50}) were calculated after transformation of concentrations in logarithmic scale (Figure 2). The mean value of the five replicates per Catambra extract concentration and five concentrations was used to calculate the EC_{50} value.

3. RESULTS AND DISCUSSION

The four Catambra extracts (coded as C-1 C-2, C-3, C-4) were found active against *M. incognita* and *M. javanica,* although in most tests paralysis percentage assessed at the highest exposed dose, was less than 42%. Additionally, in order to study for effects of volatile constituents on the extracts activity exhaustive evaporation was also tried before using the hydroalcoholic extracts in bioassays. According to the paralysis bioassays results (Table 1, Figure 2), only two out of the four Catambra extracts and in specific the extracts C-2 and C-4 were exhibited clear nematicidal activity. For these extracts a dose relationship was established and the EC_{50} values were calculated. The extract C-4 was found to exhibit nematicidal activity against both *Meloidogyne* species and the respective EC_{50} values were calculated at 728 and 500 μg/ml after 48h against *M. incognita* and *M. javanica*. Additionally, the extract C-2 exhibited an EC_{50} value of 640 μg/ml after 48 h of *M. incognita* J2 immersion in test solutions, but it was not found active against *M. javanica* (data not shown).

Table 1. **Paralysis activity (EC_{50} values)[a] of Catambra extracts against *Meloidogyne incognita* and *Meloidogyne javanica* calculated after immersion of J2s in test solutions of extracts for 12, 24, and 48 hours**

	Extracts							
	C-1		**C-2**		**C-3**		**C-4**	
	EC_{50} (μg/ml)							
	M.javanica	***M.incognita***	***M.javanica***	***M.incognita***	***M.javanica***	***M.incognita***	***M.javanica***	***M.incognita***
12h	n.c[b].	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
24h	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	**680**	**780**
48h	n.c.	n.c.	n.c.	**640**	n.c.	n.c.	**500**	**728**

[a] In cases where EC_{50} values have not been calculated because they were outside the test concentration range, they are estimated higher than the upper concentration level (1000 μg/ml).
[b] n.c.: not calculated

Finally, the rest of the Catambra extracts tested were not found to be nematicidal at the test concentration range used for the bioassay, since the percentage of paralyzed J2 was in all cases less than 42% at the highest tested dose. The activity of catalpol against both nematode species was tested in a dose response experiment, in the concentration range of 100 to 1000 μg/ml, catalpol did not exhibit considerable activity against *Meloidogyne* species and the percentage of paralyzed J2 did not exceed 37% after J2 immersion for 96 h at the highest dose (1000 μg/ml). This fact indicated that the nematicidal activity of the extracts C-2 and C-4 could not be attributed to their catalpol contents. This result was further sustained by the chemical composition analysis results, according to which the catalpol content in the extract C-4, exhibiting activity against both nematodes species, was rather low determined at 21 μg/ml (Table 2). On the contrary, the extract yielding the highest contents in cataplol amounted to 430 μg/ml was found to be the C-3, not of any nematicidal activity whatsoever (Table 1). Interestingly, exhaustive evaporation prior to the extracts' use in bioassays, led to loss of activity of extract C-2, while only the C-4 extract paralyzed J2 (Figure 2, c). The EC_{50} value calculated after 48 h of J2 immersion in extract C-4 test solutions was 950 and 874 μg/ml for *M. javanica* and *M. incognita*, respectively (Figure 2, c). This fact indicates that the nematicidal activity of extract C-2, on the contrary with extract C-4, is due to volatile components, but further analysis was out of the scope of the study

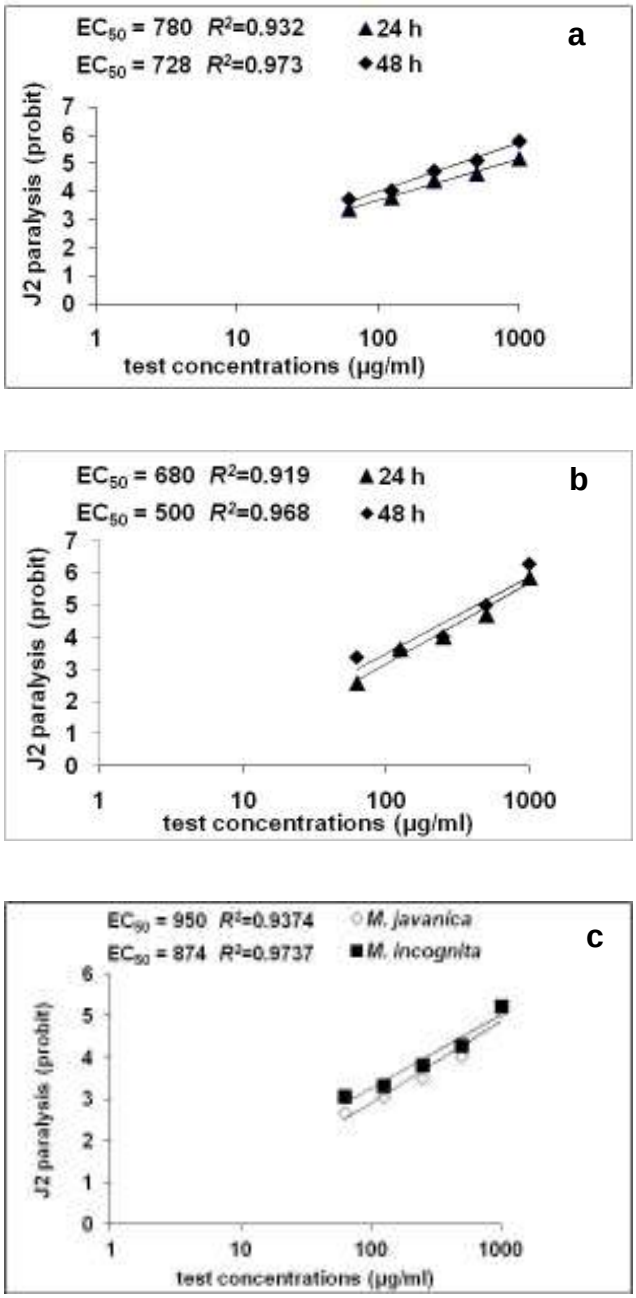

Figure 2. Dose–response curves of *M. incognita* (a) and *M. javanica* (b) paralysis following J2 immersion in solutions of extract C-4, for 24 h and 48 h and J2 immersion for 48 h in solution of extract C-4 after exhaustive evaporation of solvents (c). Each point represents the average percentage of paralyzed J2 of five replications per treatment (two experiments replications) after elimination of natural paralysis/death observed in the control.

Table 2. Catalpol contents in Catambra extracts according to LC/MS analysis.

Extract	Catalpol mg/ml
C-1	19
C-2	358
C-3	430
C-4	21

Nevertheless, the Catambra extracts activity against *Meloidogyne* sp. is significant compared to the concentration levels of other nematicidal botanical extracts, such as *Melia Azedarach* or essential oils from various plants [10, 30, 32, 33]. For this reason, it still remains to be delineated the activity among Catambra components. There was no clear trend of sensibility between the two nematode species, since at some cases *M. incognita* was found more sensitive, but in other cases the contrary occurred.

4. Conclusion

On the basis of the present results, Catambra extracts exhibit promising nematicidal activity against both species *M. incognita* and *M. javanica*. Since Catambra is already used as insecticide its use could be amplified for the control of other agricultural pests, such as nematodes. Nonetheless, the nematicidal activity of the extracts is not correlated to catalpol contents. In our ongoing studies, we focus on searching among other constituents compounds for activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ntalli N.G., Caboni P. Botanical Nematicides: A Review. J. Agric. Food Chem. 2012; 60: 9929−9940.
2. Ntalli N.G., Caboni P. Botanical nematicides in the mediterranean basin, Phytochem Rev. 2012; 1–9.
3. Mai W.F. Plant-Parasitic Nematodes: Their Threat to Agriculture; Sasser, J. N., Carter, C. C., Eds.; North Carolina State University Graphics: Raleigh, NC, 1985; Vol. I, pp 11−17.
4. Oka Y., Koltai H., Bar-Eyal M., Mor M., Sharon E., Chet I. New strategies for the control of plant-parasitic nematodes, Pest Manag Sci. 2000; 56: 983–988.
5. http://ec.europa.eu/sanco_pesticides/public/index.cfm.
6. Ntalli N. G., Menkissoglu-Spiroudi U. Pesticides of Botanical Origin: A Promising Tool in Plant Protection. Ch. 1, In Pesticides - Formulations, Effects, Fate, pp 3-14, Ed. Margarita Stoytcheva, 2011, InTech, Croatia.
7. European Commission 2009. Commission Directive 2009/128/EC of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. OJ No L 309, 24.11.2009, p.71.

8. European Commission 2009. Council Regulation (EC) no. 1107/2009 of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ No L 309, 24-11-2009, pp 50.
9. Caboni P., Aissani N., Cabras T., Falqui A., Marotta R., Liori B., Ntalli N.G., Sarais G., Sasanelli N., Tocco G. Potent Nematicidal Activity of Phthalaldehyde, Salicylaldehyde, and Cinnamic Aldehyde against *Meloidogyne incognita*. J. Agric. Food Chem. 2013; 61: 1794−1803.
10. Ntalli N. G., Vargiu S.; Menkissoglu-Spiroudi U., Caboni P. Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. J. Agric. Food Chem. 2010; 58: 11390−11394.
11. Cavoski I., Al. Chami Z., Bouzebboudja F., Sasanelli N., Simeone V., Mondelli D., Miano T., Sarais G., Ntalli N. G., Caboni P. *Melia azedarach* controls *Meloidogyne incognita* and triggers plant defense mechanisms on cucumber. Crop Prot. 2012; 35: 85−90.
12. Caboni P., Ntalli N. G., Aissani N., Cavoski I., Angioni A. Nematicidal activity of (E,E)-2,4-decadienal and (E)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*. J. Agric. Food Chem. 2012; 60: 1146−1151.
13. Ntalli N. G., Manconi F., Leonti M., Maxia, A., Caboni P. Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes. J. Agric. Food Chem. 2011; 59: 7098−7103.
14. Cetintas R., Yarba M. M. Nematicidal effects of five plant essential oils on the southern root-knot nematode, *Meloidogyne incognita* race 2. J. Anim. Vet. Adv. 2010; 9: 222−225.
15. Javed N., Gowen S. R., Inam-ul-Haq M., Abdullah K., Shahina F. Systemic and persistent effect of neem (*Azadirachta indica*) formulations against root-knot nematodes, *Meloidogyne javanica* and their storage life. Crop Prot. 2007; 26: 911−916.
16. Ladner D. C., Tchounwou P. B., Lawrence G. W. Evaluation of the effect of ecologic on root knot nematode, *Meloidogyne incognita*, and tomato plant, *Lycopersicon esculenum*. Int. J. Environ. Res. Public Health 2008; 5: 104−110.
17. Giannakou I. O. Efficacy of a formulated product containing *Quillaja saponaria* plant extract for the control of root-knot nematodes. Eur. J. Plant Pathol. 2011; 130: 587−596.
18. Maistrello L., Vaccari G., Sasanelli N. Effect of chestnut tannins on the root-knot nematode *Meloidogyne javanica*. Helminthologia 2010; 47: 48−57.
19. Chitwood D.J. Phytochemical based strategies for nematode control. Annu. Rev. Phytopathol. 2002; 40: 221-249.
20. Yang G., Choi CH., Lee K., Lee M., Ham I., Choi HY. Effects of *Catalpa ovata* stem bark on atopic dermatitis-like skin lesions in NC/Nga mice. J Ethnopharmacol. 2013; 145(2): 416-23.
21. Oh CH., Kim NS., Yang JH., Lee H., Yang S., Park S., So UK., Bae JB., Eun JS., Jeon H., Lim JP., Kwon J., Kim YS., Shin TY., Kim DK. Effects of isolated compounds from *Catalpa ovata* on the T cell-mediated immune responses and proliferation of leukemic cells. Arch Pharm Res. 2010; 33(4): 545-50.
22. Park B.M., Hong S.Su., Lee C., Lee M.S., Kang S.J., Shin Y.S., Jung J.-K., Hwang B.Y. Naphthoquinones from C*atalpa ovata* and their inhibitory effects on the production of nitric oxide, Arch Pharm Res. 2010; 33: 381-385.
23. Dvorská M., Zemlicka M., Muselík J., Karafiátová J., Suchý V. Antioxidant activity of *Catalpa bignonioides*. Fitoterapia. 2007; 78: 437-439.
24. Muñoz-Mingarro D., Acerob N., Llinaresc F., Pozuelod J.M., Galán de Merab A., Vicentenb J.A., Moralese L., Alguacile L.F., Péreze C. Biological activity of extracts from *Catalpa bignonioides* Walt. (Bignoniaceae). J Ethnopharmacol. 2003; 87:163-167.
25. Wysokińska H., Lisowska K., Floryanowicz-Czekalska K. Transformation of *Catalpa ovata* by *Agrobacterium rhizogenes* and phenylethanoid glycosides production in transformed root cultures. Z Naturforsch C. 2001; 56: 375-381.
26. Goud J.-K.C., Termorshuizen A.J., Blok W.J., Van Bruggen A.H.C. Long-term effect of biological soil disinfestation on verticillium. Plant Dis. 2004; 88(7): 688-694.

27. Kozan E., Anul SA., Tatli II. In vitro anthelmintic effect of *Vicia pannonica* var. purpurascens on trichostrongylosis in sheep. Exp Parasitol. 2013; 134(3): 299-303.
28. http://www.catambra.it/caratteristiche.htm
29. Hussey R.S., Barker K.R. A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. Plant Dis Reptr 1973; 57: 1025–1028.
30. Ntalli N. G., Menkissoglu-Spiroudi U., Giannakou I. O. Efficacy evaluation of a neem (*Azadirachta indica* A. Juss) formulation against root-knot nematodes *Meloidogyne incognita.* Crop Protection. 2009; 28: 489–494.
31. Puntener W. (1981) Manual for field trials. In Plant Protection. 2nd edn. Basle, Switzerland: Ciba-Geigy Limited. 205 pp.
32. Ntalli N.G., Ferrari F., Giannakou I., Menkissoglu-Spiroudi U. Phytochemistry and nematicidal activity of the essential oils from 8 Greek lamiaceae aromatic plants and 13 terpene components. J. Agric. Food Chem. 2010; 58: 7856-7863.
33. Ntalli N.G., Ferrari F., Giannakou I., Menkissoglu-Spiroudi U. Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. Pest Manag. Sci. 2011; 67: 341-351.